UNIVERSITAT DE BARCELONA

The impact of soluble epoxide hydrolase inhibition and epoxyeicosatrienoic acids on early-life microglia activation and neuroinflammatory responses in the brain

Clara Bartra i Cabré

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The impact of soluble epoxide hydrolase inhibition and epoxyeicosatrienoic acids on early-life microglia activation and neuroinflammatory responses in the brain

Dissertation presented by Clara Bartra i Cabré to apply for the doctoral degree at the University of Barcelona

Doctoral student

Clara Bartra i Cabré

Director

Coral Sanfeliu Pujol

Tutor

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Statement of originality

With this statement, I hereby declare that the submitted PhD thesis is my own original work and includes material for four scientific articles that have been or will be published in international peer-reviewed journals. All the assistance received in preparing this thesis and sources have been acknowledged.

Clara Bartra i Cabré

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive cognitive decline and is the most common cause of dementia worldwide. AD has a complex multifactorial aetiology influenced by both genetic and environmental factors that may contribute to its onset. Several biological processes are implicated in the pathogenesis of AD, acting as potential triggers for cognitive impairment.

Neuroinflammation, primarily driven by microglia, has been identified as one of the neuropathological characteristics of AD, although it is also believed to be one of its triggers. Concurrently, some oxylipins including the epoxyeicosatrienoic acids (EETs) derived from arachidonic acid, present beneficial anti-inflammatory properties. Since EETs are rapidly metabolized by the soluble epoxide hydrolase (sEH) into less bioactive metabolites, the inhibition of this enzyme has been proposed as a therapeutic pharmacological strategy for AD treatment.

A first study was conducted to assess the effects of maternal administration of the sEH inhibitor (sEHI) TPPU during pregnancy and lactation on their wild-type and 5XFAD offspring. Interestingly, TPPU prevented cognitive and behavioural changes in offspring with a 5XFAD genotype. Additionally, TPPU also induced neuroprotective epigenetic changes, mitigated p-tau levels, and reduced neuroinflammatory markers, thus highlighting the potential of sEHI as a promising therapeutic approach for AD and suggesting beneficial effects of EETs. In a subsequent study, we wanted to study the role of EETs and other oxylipins in the neurodevelopmental outcome of children exposed to low or high levels of environmental mercury (Hg) contamination. The primary contaminant form of Hg is methylmercury, which has neurotoxic effects during brain development. Prenatal exposure to methylmercury, as indicated by Hg levels in cord blood, induces changes in PUFA metabolites that can be associated to postnatal neurodevelopment in children.

Subsequently, we wondered whether the observed benefits of sEHI could be attributed to their impact on microglia. In the third study, we explored their anti-inflammatory effect on microglia following an inflammatory insult. To simulate an AD-like inflammatory environment, we utilized monomeric C-reactive protein (mCRP), which has been previously linked to the AD pathology. mCRP induced a pro-inflammatory phenotype in microglia, activating key inflammatory pathways and the release of pro-inflammatory cytokines. Successfully, newly-synthesized sEHI effectively countered microglial pro-inflammatory mechanisms, confirming their direct pharmaceutical benefits through the modulation of microglial activity.

To investigate more deeply into therapies against aberrant microglial inflammatory responses, in the last study, we investigated whether a nutraceutical treatment could mitigate the activation of microglia induced by our inflammatory agent mCRP. Resveratrol successfully countered the activation of microglia due to its antioxidant and anti-inflammatory properties, highlighting how dietary interventions with nutraceuticals can directly benefit the brain.

In summary, we have confirmed the anti-inflammatory and neuroprotective effects of sEHI, highlighting them as promising pharmacological agents. Our findings demonstrate that PUFA-derived oxylipins, specially EETs, can shape our health outcomes already from prenatal stages. Additionally, oxylipins may influence our susceptibility and resilience to both external and internal pathological insults. By understanding how microglia responds in front of endogenous inflammatory stimulus and the direct impact of sEHI in microglia activation, we can provide valuable insights for future therapies targeting neurodegenerative diseases with a neuroinflammatory basis. Given the complex aetiology of AD, a combinatory treatment strategy tackling several pathological aspects of the disease, along with healthy lifestyle interventions from an early age, particularly focusing on diet, would represent a significant step towards improving the overall outcome of AD.

Resum

La neuroinflamació, modulada principalment per la micròglia, s'ha identificat com un contribuent important en la malaltia d'Alzheimer (AD), convertint-la en una diana terapèutica prometedora. Per altra banda, les oxilipines, especialment els àcids epoxieicosatrienoics (EET), presenten propietats antiinflamatòries beneficioses. Tot i així, com que els EET es metabolitzen ràpidament per l'epòxid hidrolasa soluble (sEH), s'ha proposat inhibir aquest enzim com a estratègia terapèutica per al tractament de l'AD.

D'entrada, en un primer estudi, vam avaluar els efectes de l'administració materna de l'inhibidor de la sEH (sEHI) TPPU durant l'embaràs i la lactància en les cries de fenotip de tipus salvatge o 5XFAD. El TPPU va protegir els ratolins 5XFAD contra el deteriorament cognitiu i va reduir la p-tau i la reactivitat microglial. En un estudi posterior, vam investigar el paper de les oxilipines en el neurodesenvolupament d'infants exposats a nivells baixos o alts de mercuri (Hg) ambiental. Vam trobar que l'exposició prenatal al metilmercuri, mesurada pels nivells de Hg en la sang del cordó umbilical, indueix canvis en els metabòlits dels PUFA que s'associen amb el neurodesenvolupament postnatal dels nens.

En el tercer estudi, vam investigar si els beneficis dels sEHI eren deguts al seu efecte sobre la micròglia. Utilitzant la proteïna C-reactiva monomèrica per simular un entorn inflamatori com en l'AD, vam induir un fenotip proinflamatori a la micròglia. Els sEHI van contrarestar efectivament aquest efecte, confirmant el seu impacte positiu en la micròglia.

Finalment, a l'últim estudi, vam analitzar el resveratrol contra les respostes inflamatòries aberrants de la micròglia. Amb èxit, va aconseguir contrastar l'activació de la microglia gràcies a les seves propietats antioxidants i antiinflamatòries, destacant així com les intervencions dietètiques amb nutracèutics poden ser beneficiosos pel cervell.

Breument, podem confirmar els efectes antiinflamatoris i neuroprotectors dels sEHI, revelant una estratègia farmacològica prometedora. Els nostres resultats demostren que les oxilipines, especialment els EETs, poden modelar la nostra salut ja des d'etapes primerenques de la vida. L'impacte dels sEHI en l'activació de la micròglia és rellevant per a futures teràpies de malalties neuroinflamatòries. La combinació de tractaments i intervencions en l'estil de vida podrien millorar el desenvolupament de l'AD.

Thesis structure

The present doctoral dissertation is presented in the following chapters:

Chapter 1: Introduction

Provides background knowledge and summarizes the existing research of the topic in which this thesis is framed, with special attention to neuroinflammation in an Alzheimer's disease context.

Chapter 2: Hypothesis and objectives

Contains the initial hypothesis and the specific points that were addressed during the development of this thesis.

Chapter 3: Publications

It is divided into four sections, each one of which is based on a separate but related scientific investigations.

These sections present evidence that soluble epoxide hydrolase inhibitors, and hence epoxyeicosatrienoic acids, known for their anti-inflammatory properties, can impact neurodevelopment outcomes from early stages of life. Furthermore, inhibition of the soluble epoxide hydrolase is proposed as a potential treatment against neuroinflammation, achieved through specific modulation of microglial responses. Additionally, resveratrol is suggested as an anti-inflammatory nutraceutical.

Chapter 4: General discussion

By synthesizing the various research studies and their results, a deeper exploration of the dissertation topic is conducted.

Chapter 5: Conclusions

A set of concluding statements based on the findings collected.

Below, a list of the four scientific papers that form the research output of the present dissertation:

Publication 1

Bartra C, Irisarri A, Villoslada A, Corpas R, Aguirre S, García-Lara E, Suñol C, Pallàs M, Griñán-Ferré C, Sanfeliu C. Neuroprotective Epigenetic Changes Induced by Maternal Treatment with an Inhibitor of Soluble Epoxide Hydrolase Prevents Early Alzheimer's Disease Neurodegeneration. *Int J Mol Sci*. **2022**. 23(23):15151. doi: 10.3390/ijms232315151.

Publication 2

Bartra C, Llop S, Kuligowski J, Albiach-Delgado A, Suñol C, Rodríguez-Farré E, Ballester F, Soler-Blasco R, Jora B, Jarné-Ferrer J, Griñán-Ferré C, Vázquez S, Pallàs M, Sanfeliu C. High levels of anti-inflammatory epoxyeicosatrienoic acids may improve neurodevelopment in children prenatally exposed to mercury.

Pending for submission.

Publication 3

Bartra C, Vuraić K, Yuan Y, Codony S, Valdés-Quiroz H, Casal C, Slevin M, Griñan-Ferré C, Pallàs M, Morisseau C, Hammock B, Vázquez S, Suñol C, Sanfeliu C. Microglial pro-inflammatory mechanisms induced by monomeric C-reactive protein are counteracted by soluble epoxide hydrolase inhibitors.

Publication Submitted to Frontiers in Immunology.

Publication 4

Bartra C, Yuan Y, Vuraić K, Valdés-Quiroz H, Garcia-Baucells P, Slevin M, Pastorello Y, Suñol C, Sanfeliu C. Resveratrol Activates Antioxidant Protective Mechanisms in Cellular Models of Alzheimer's Disease Inflammation. *Antioxidants*. **2024**. *13(2):* 177. doi: 10.3390/antiox13020177

Table of contents

Abbreviations

AD	Alzheimer's disease
ALA	Alpha-linoleic acid
APOE	Apolipoprotein E
APP	Amyloid protein precursor
ARA	Arachidonic acid
Aβ	Amyloid beta
BBB	Brain-blood barrier
CNS	Central nervous system
COX	Cyclooxygenase
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CYP450	Cytochrome P450 system
DHA	Docosahexaenoic acid
DiHETs	Dihydroxyeicosatrienoic acids
DLB	Dementia with Lewy body
EETs	Epoxyeicosatrienoic acids
EOAD	Early-onset AD
EPA	Eicosapentaenoic acid
FTD	Frontotemporal dementia
GWAS	Genome-wide association studies
Hg	Mercury
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LA	Linoleic acid
LBD	Lewy body dementia
LOAD	Late-onset AD
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MCI	Mild cognitive impairment
mCRP	Monomeric C-reactive protein
NFT	Neurofibrillary tangles
NF-κB	Nuclear factor-κB

NLRP3	NLR family pyrin domain containing 3
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
PL	Phospholipase
PRRs	Pattern recognition receptors
PSEN1	Presenilin-1
PSEN2	Presenilin-2
p-tau	Hyperphosporilated tau
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RV	Resveratrol
sEH	Soluble epoxide hydrolase
sEHI	Soluble epoxide hydrolase inhibitors
TNF	Tumour necrosis factor
VaD	Vascular dementia
ω-3	Omega-3
ω-6	Omega-6

Chapter 1. Introduction

1.1. Dementia and types of dementia

Dementia is a clinical syndrome marked by a progressive and non-reversible decline in cognitive and behavioural abilities, leading to difficulties in performing basic daily living activities and ultimately resulting in an inability to live independently [1–3]. In dementia, there is an interplay between genetics and environmental factors [4].

Dementia can be divided into several types, each with its own characteristics and causes. However, all types of dementia share the same characteristics of cognitive impairment and cognitive decline, accompanied by behavioural and psychological symptoms of dementia (BPSD) [2,5]. The most common BPSD among different dementias are aberrant motor behaviour, agitation or aggression, and irritability; all with an influence of psychosocial and physical environment [2].

Dementias also share a common neuropathology, encompassing neuroinflammation, gliosis, synaptic loss, neurodegeneration, brain atrophy, and alterations in blood-brain barrier (BBB) permeability [6]. Notably, neuroinflammation stands out as one of the most significant neuropathological aspect, directly contributing to the pathogenesis and the progression of different dementias [3,4,6].

It is important to notice that dementia is not a specific disease but an umbrella term that includes several disorders. The most prevalent type of dementia is Alzheimer's disease (AD), but we must not overlook other dementias such as vascular dementia (VaD), frontotemporal dementia (FTD), Lewy body dementia (LBD), Parkinson's disease with dementia, Creutzfeldt-Jakob disease, Wernicke-Korsakoff Syndrome, normal pressure hydrocephalus, Huntington's disease, or mixed dementia [2,4,7–11]. In these work we will be focusing in Alzheimer's disease, but a small summary of the most common dementias is described as follows. Since they share common features, its description can give insight to better understand neurodegenerative diseases.

1.1.1. Vascular dementia (VaD)

VaD is the second most common dementia worldwide. Its cognitive impairment comes from cardiovascular and cerebrovascular pathologies, meaning that the main contributors to VaD are cortical and subcortical infarcts, lacunes, ischemic lesions, and large or small brain haemorrhages [12,13]. Since genetics, cardiometabolic risk factors, and age, are key determinants of this disease, its progression pattern is likely unpredictable [12,14].

Its main symptoms are deficits in attention and information processing, alterations in executive function and psychomotor slowing [12,14,15].

In VaD, neurovascular damage can lead to alterations in cerebrovascular fluid flow and in brain-blood barrier (BBB) permeability. These affectations can lead to tissue hypoxia and an increased oxidative stress, which triggers nuclear factor-κB (NF-κB) activation [4,15], leading to a neuroinflammatory state.

1.1.2. Frontotemporal dementia (FTD)

FTD includes neurodegenerative disorders characterized by the degeneration of frontal and temporal lobes. Additionally, FTD is usually associated with the deposition of aggregates either of the microtubule-associated protein tau, the TAR DNA-binding protein, or less commonly of the fused-in-sarcoma protein [16,17].

Its clinical features stand out for presenting behavioural and personality changes, impairment in social interactions, and/or language disturbances [17,18]; characteristics that might appear individually but converge at the final stages of the disease. Furthermore, some FTD patients also present motor neurons dysfunction, which resembles to amyotrophic lateral sclerosis symptoms [19].

In FTD, there is cortical inflammation, an increased activation and dystrophy of microglia, and an increase in astrogliosis [16]. Neuroinflammation and immune-mediated mechanisms, along with gene alterations might drive the progression of FTD [16–19].

1.1.3. Lewy Body dementia (LBD)

LBD encompasses dementia with Lewy body (DLB) and Parkinson's disease with dementia. These two pathologies share the same neuropathological feature of presenting aggregates of α-synuclein protein in the brain, concretely in both cortical and subcortical structures [20]. Anomalous α-synuclein proteins are phosphorylated, truncated, and nitrated, which leads to the formation of oligomers that aggregate into ubiquinated fibrils [21]. These aggregates trigger dysfunction and loss of dopaminergic and cholinergic neurons, presented in patients as memory dysfunction, visual hallucinations, depression, problem-solving difficulties, gait disturbances, and tremor and stiffness [21], this last characteristic shared with motor Parkinsonism [22].

Interestingly, LBD, particularly in DLB, is frequently accompanied with concomitant AD pathology, with hyperphosporilated tau (p-tau) and amyloid beta (Aβ) presence [20,22,23].

There is a tight connection between LBD, α-synuclein aggregates and neuroinflammation. In the pre-clinical and prodromal stages of LBD, brain inflammation escalates due to increased activation of astrocytes and microglial cells, with a high activation of the transcription factor NF-κβ. This inflammatory stage persists, albeit to a lesser extent [23], in later stages of the disease, but also accompanied with immune senescence [22]. The distinctive microglial activation in LBD is directly linked to greater cognitive dysfunction, contrasting with Parkinson's disease, which presents fewer cognitive deficits [24].

1.2. Alzheimer's disease (AD)

1.2.1. Definition and history

AD is a neurodegenerative disorder characterized by a progressive decline in cognitive functions, specifically memory loss. As the most prevalent cause of dementia worldwide, it is mainly influenced by both genetic and age-related factors [25,26].

It was not until 1907 that the German psychiatrist Aloysius “Alöis” Alzheimer described the disease that would now carry his name. One of his patients, a 51-year-old woman named Auguste Deter, had exhibited memory impairments along with other behavioural alterations. After her death, Alzheimer examined her brain under a microscope and, for the first time, observed and described the neuropathology hallmarks of the disease: neuritic plaques, neurofibrillary tangles (NFT), and amyloid angiopathy [27,28].

1.2.2. Epidemiology

In 2023, Gustavsson et al. estimated that at least 22 % of individuals aged 50 or above worldwide were within the AD continuum [29], taking into consideration people at all stages of AD. As a matter of fact, if dementia develops in 25-45 % of the elderly above 85 years old, AD is responsible for at least half of these cases. Additionally, it is anticipated that AD will reach nearly 24 million cases by the year 2050, carrying significant implications for patients, the healthcare system, and their caregivers [26].

1.2.3. Clinical features and diagnosis

AD has a complex multifactorial aetiology and manifests as a clinical continuum [27]. Preclinical AD denotes the asymptomatic phase, lasting over 10 years [30], and during which patients display the neuropathology of AD without evident symptoms. This asymptomatic phase can only be detected through biomarkers such as imaging and cerebrospinal fluid (CSF) analysis. Following this phase, individuals may enter a stage of mild cognitive impairment (MCI) and/or mild behavioural impairment, where they may exhibit memory impairment and alterations in various cognitive domains, with subtle alterations in language, episodic memory, executive memory, and executive function. Over time, the disease progresses to more recognizable symptoms commonly associated with AD. Genetic or environmental contribution will define the progression or not from one phase to another [31,32].

Due to the considerable variability in symptoms among individuals and the ambiguity of their presentation, the current diagnosis is performed based on a combination of psychological and cognitive tests [33], neuroimaging tests, and molecular biomarkers.

For structural imaging MRI is used to asses one of the possible signs of AD: temporal lobe atrophy. Following that, fluorodeoxyglucose-PET is performed to determinate areas of altered metabolisms and neurodegeneration in the brain [34]. Via PET scanning, imaging biomarkers can detect the presence of Aβ and plaques in the brain, allowing the determination of Aβ pathology.

Additional support to diagnose AD can be assessed using CSF biomarkers, already detected at early stages of the disease. The decrease in CSF levels of Aβ42, which forecasts the amyloid status, along with the increase in levels of tau isoforms, indicating the presence of NFT and neurodegeneration, are widely recognized as the most commonly employed biomarkers (figure 1). A decrease in CSF levels of C-reactive protein (CRP) can also be observed, possibly due to its entrance in the brain parenchyma and association with Aβ plaques [31,35,36].

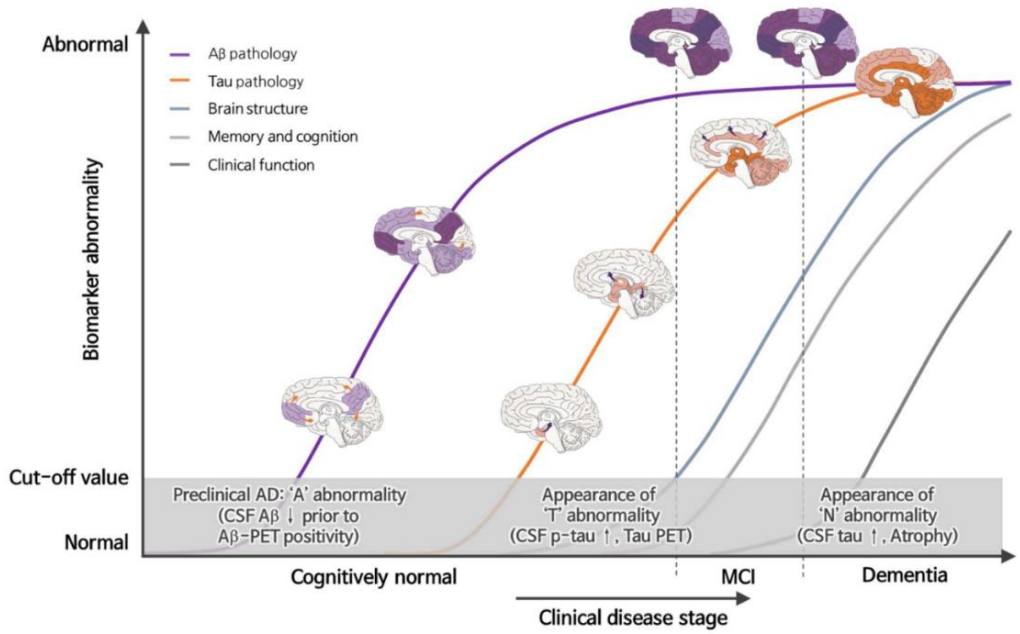

Figure 1. Biomarkers and alterations in the spatial and temporal distribution of Aβ and NFT deposition throughout the progression of the disease. In the preclinical stage, there is a decrease in CSF Aβ42 and Aβ-PET positivity (referred to as 'A' abnormalities) preceded by the clinical stages of the disease, with an increase in CSF p-Tau and Tau PET (referred to as 'T' abnormalities) and hippocampal atrophy (referred to as 'N' abnormalities). Image taken from the article authored by Mankhong et al, 2022 [37].

Additionally, levels in CSF of other biomarkers for axonal degeneration, synaptic degeneration, glial activation and α-Synuclein pathology, can also be assessed to detect pathological changes commonly found in patients with AD [38].

The development of robust blood biomarkers for AD diagnosis is still being advancing, with promising progress achieved in plasma detection of p-tau [39,40].

1.2.4. Neuropathology

1.2.4.1. Brain atrophy

In AD, the brain experiences progressive cortical atrophy, resulting in a gradual shrinking of specific regions of the brain cortex. This atrophy is more noticeable in the frontal, parietal, and temporal lobes. The hippocampus, vital for memory function, is particularly susceptible and often presents significant shrinkage, occasionally spreading to the adjacent amygdala. Moreover, ventricles are frequently enlarged [41].

1.2.4.2. Aβ and tau

As mentioned before, Aβ plaque deposition and NFT are the main neuropathological markers of AD. An abnormal processing of APP leads to increased production of Aβ40 or Aβ42, with the latter being the most abundant form in AD [42]. Moreover, alterations in tau protein, such as truncations or hyperphosphorylation, can lead to a loss of affinity to microtubules. This triggers the self-assembly of tau into paired helical and straight filaments, which results in the formation of NFT [43,44].

Aβ associate to form insoluble fibrils, subsequently leading to the formation of amyloid plaques [45]. Since Aβ has an adhesive nature, it results in its association with various proteins, forming senile plaques. Overproduction of Aβ and/or deficiency in its clearance leads to neurotoxicity and neurodegenerative processes [46]. Additionally, this peptide can interact with the membrane receptor $p75^{NGF}$, which activates the inflammatory pathway NF-κB [47]. Moreover, Aβ has been proposed to trigger the production of nitric oxide (NO) and reactive oxygen species (ROS) [46].

Tau phosphorylation plays a crucial role in synaptic plasticity. However, under conditions of oxidative stress, levels of phosphorylated tau may be altered and this same alteration increases oxidative stress [44]. The oligomeric form of tau is considered the most neurotoxic. These oligomers, with β-sheet conformation, are the ones promoting fibrils formation [43]. Hyperphosphorylation of tau leads to the dissociation from microtubules, resulting in cytoskeleton instability, synaptic loss, and neuronal atrophy [43,48].

For the past 30 years it has been believed that Aβ drives the formation of abnormal tau aggregates. Subsequently, this mediated neuronal injury and neurodegeneration, ultimately leading to cognitive impairment [49]. However, increasing evidence suggests that neurodegeneration and tauopathy may precede amyloid pathology [27]. In fact, it is believed that the interplay between Aβ and tau, alongside with neurodegeneration is complex, but its relationship may define AD presentation.

1.2.4.3. Other pathological mechanisms of AD

Various biological processes are implicated in the pathogenesis of AD, potentially serving as triggers for cognitive impairment.

Insulin malfunctioning has been linked not only to cognitive decline in AD but also to tau phosphorylation and Aβ homeostasis disturbances [50–52].

Dysfunction in mitochondrial-associated membranes, crucial for mitochondrial function [53], cholesterol and phospholipid metabolism [54], and calcium homeostasis [55], may initiate the pathology and exacerbate increased levels of Aβ due to the abundant presence of γ-secretase in these membranes [56,57].

Cerebrovascular changes, which increase hand in hand with disease progression, accelerate Aβ production and alter its clearance [58,59]. Oxidative stress and radical formation, noticeable in early AD stages, induce damage to biomolecules such as proteins, lipids, DNA and RNA damage [60].

Glucose metabolism impairment is directly related to increased tau phosphorylation [61,62]. General brain metabolism is also decreased in several regions in the brain, leading to an impaired energy supply that causes ion dysregulation [63].

Synapse loss, inversely proportional to NFT and Aβ burden, contributes to cognitive decline, alongside with deficiencies in neurotransmitters such as acetylcholine and glutamate [64–66].

All these mentioned interconnected events may collectively contribute to the manifestation and the progression of AD.

1.2.5. AD subtypes

Since age is the principal risk of developing AD [67], it is often used as a way of classifying AD subtypes: early-onset AD (EOAD) and late-onset AD (LOAD). Nonetheless, in this work, the classification of AD subtypes would be performed according to the aetiology, whether it is caused by autosomal dominant genetic forms or if it is due to a combination of genetic traits and environmental factors.

1.2.5.1. Familial AD

Familial AD is associated with classical Mendelian patterns of inheritance and it always presents as an EOAD [26,68]. Familial AD has an heritability of 92-100 % and accounts for just 1 to 6 % of individuals under the age of 60 [26,69].

The autosomal dominant forms of familial AD are caused by rare mutations in amyloid protein precursor (APP), presenilin-1 (PSEN1), and presenilin-2 (PSEN2) [70]. Mutations in PSEN1 are the most common ones, with over 300 distinct variants identified (ww.alzforum.org/mutations. 21/03/2024).

The Aβ peptide comes from the sequential cleavage of a precursor protein called APP. The amyloidogenic processing of APP involves the protease activity of α-, β-, and γ-secretases. This last one secretase is composed of four transmembrane proteins, including PSEN1 and PSEN2, which constitute the catalytic form of the complex [71,72]. Mutations in these three proteins can contribute to the aggregation of Aβ peptides in the brain, either by increasing the expression of Aβ or by promoting the formation of Aβ peptides, which have a higher propensity to clump together [70].

The primary impairment in familial AD lies in anterograde episode memory. Generally, people suffering from familial AD experience a more aggressive form of the disease, with increased pathological load and shorter life expectancy [73].

1.2.5.2. Sporadic AD

About 95 % of AD is considered as sporadic AD [74]. It arises from small genetic alterations, either common or rare variants, in combination with environmental factors. Although familial AD can manifest as EOAD, it typically presents as LOAD, being prevalent in people older than 85 years old and accounting for 50 % of the individuals [26]. LOAD usually has a long preclinical phase.

Epigenetics might also be involved in the aetiology of sporadic AD. There is evidence that DNA methylation and hydroxymethylation have potential roles in the development of AD. Additionally, epigenetic is altered during aging, and as mentioned before, ageing is the main risk for developing AD [75]. Moreover, both genetic and environmental risk factors for AD could act trough the DNA methylations in the CpGs sites [73].

1.2.5.2.1. Genetic predisposition

1.2.5.2.1.1. Apolipoprotein E (APOE)

APOE is a lipid-binding protein primarily synthetized by astrocytes and microglia [76]. This protein plays a crucial role in transporting and maintaining cholesterol homeostasis in the brain. Furthermore, APOE is actively involved in the clearance of inflammatory and pathogenic molecules, thereby directly influencing the brain's innate immune response.

The APOE protein is encoded on chromosome 19. It exists in three isoforms: ε2, ε3, ε4. Among these isoforms, the APOEε4 allele is the most significant genetic risk factor for sporadic AD, and it is associated with an earlier onset of the disease, neuropathology (Aβ deposition), and cognitive impairment. In addition, the risk of AD increases accordingly with the number of ε4 alleles presented [77,78]. Conversely, carriers of the ε2 alleles present a lower risk of AD and usually exhibit less severe pathology and cognitive impairment [79].

There are several proposed mechanisms and pathways through which APOEε4 contributes to AD neuropathology. It is believed that APOEε4 may decrease Aβ phagocytosis and clearance, which leads to an increased deposition of these peptide. Moreover, APOEε4 could participate in the formation of NFT by promoting tau hyperphosphorylation. In addition, APOEε4 may enhance the presence of pro-inflammatory phenotypes in microglia and astrocytes, consequently affecting BBB integrity [77].

1.2.5.2.1.2. Genes and risk loci

During the last few years, and thanks to numerous genome-wide association studies (GWAS), several genes and loci have been described as genetic risk factors of AD. Despite most of these genes giving just a small contribution to the increase in AD risk, when considered together, their combined effects can have a significant role in the development of the disease [68,74,80].

The most significant loci described by GWAS are summarized as follows (figure 2). Other than the APOE gene, CLU, ABCA7 and SORL1 are other genes involved in cholesterol metabolism that are implicated in the pathogenesis of AD [81–85]. Additional genes identified can be related to immune response dysregulation in AD, such as CR1, CD33, MS4A, CLU, ABCA7, EPHA1 and TREM2 [81–88]. Regarding endocytosis and synaptic function, BIN1, PICALM, CD2AP, EPHA1 and SORL1 have been identified to alter APP processing and neurotransmission [81–85].

It should be noted that there are gene mutations occurring de novo in the PSEN1 gene, previously associated only with familial AD. These mutations should also be considered as potential causes of sporadic AD with early-onset [70].

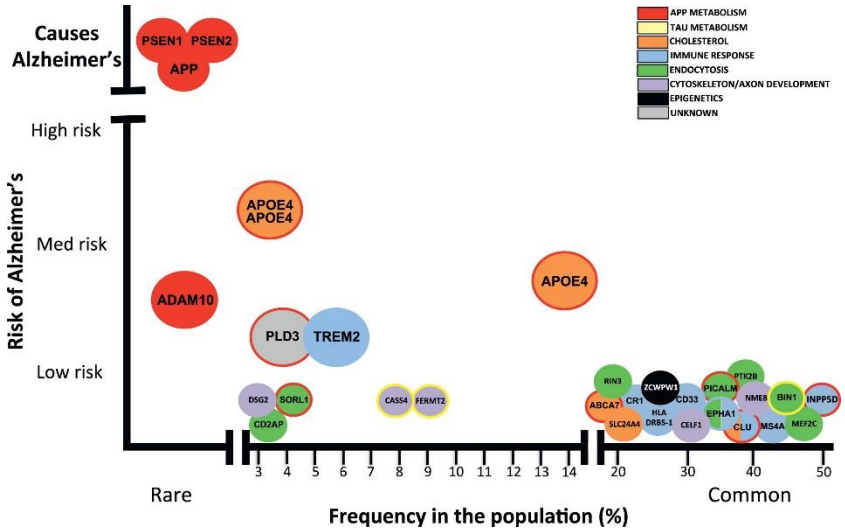

Figure 2. Rare and common variants implicated in AD neuropathology: prevalence in the population and disease risk. Image taken from the article authored by Karch and Goate, 2015 [68].

All things considered, rare and common variants implicated in pathological processing of Aβ, tau pathology, phagocytosis, lipid metabolism, microglial activation, neuroinflammation, mitochondrial dysfunction, oxidative stress, dendritic structure, synaptic dysfunction, axonal growth and transport, BBB disruption and vascular damage, amyloid angiopathy, along with other variants still to be described, can influence neuropathological events in AD [80].

1.2.5.2.2. Risk factors

While genetics plays a significant influence in AD, its onset is also influenced by several environmental factors. These factors encompass not only sociodemographic and behavioural aspects but also psychological variables and other medical risk factors (figure 3) [89], some of which exhibit modifiable features.

In some cases, when examining brain tissues from individuals diagnosed with AD, the histopathological observations do not always correlate the severity of the symptoms. The concept of brain reserve was carved out to describe brain's resilience to endure pathological insults, either because of stronger synaptic density or higher number of healthy neurons. The reserve allows the brain to maintain normal cognitive functions and compensate the damage. Additionally, the brain can also use its cognitive reserve, which involves engaging alternative neuronal networks or cognitive strategies to maintain normal cognition [32,89].

1.2.5.2.2.1. Education and social interactions

Education levels have been directly linked to brain and cognitive reserve, since education activities might protect against cognitive decline [90]. In fact, there is a dose-dependent correlation between higher levels of education and a reduced risk of dementia [91]. Cognitive activities like doing puzzles, reading books or playing games have been also suggested to be protective against cognitive decline and AD. In fact, cognitive activities could even prevent deleterious effects of APOEε4 and Aβ deposition [92].

Social engagement and social activities seem to reduce cognitive decline and risk of AD, while the sense of loneliness may increase late-life risk of dementia [93]. Psychological factors, including personality traits and individual emotional experiences or moods, have significant impact in AD risk. The habit of being organized, responsible, reliable, and goal-oriented reduces the risk of MCI and AD [94]. In addition, levels of depressive symptoms have been directly linked to accelerate cognitive decline [95].

1.2.5.2.2.2. Physical activities

Physical activity, including walking, house work, or aerobic and strength training, has beneficial effects on ageing and cognitive function. In fact, in AD patients, exercise can improve markers of functional independence and physical function, while also attenuating cognitive decline [96]. Although the exact mechanisms behind the beneficial effects of exercise on AD pathology remain partially understood, it is thought to potentially act through building of brain reserve [89,97]. Studies with the 3xTg-AD, indicate that exercise has a beneficial impact in BPSD-like disturbances and cognition, as well as in oxidative stress, which positively impacts synaptic function and plasticity [98].

1.2.5.2.2.3. Diet

It is well known what we eat affect our health and that a good nutrition can promote overall wellness. Maintaining a diet rich in nutrients from an early age onward is beneficial for healthy ageing, as metabolism slows down during aging and can worsen AD [5,99,100]. Adhering to a diet rich in berries, leafy greens, vegetables, whole grains, beans, legumes, nuts and seeds, and extra virgin oil presents protective effects for the brain and reduces the risk of developing AD [101].

The Mediterranean diet is influenced by the culinary style surrounding the Mediterranean Sea. It is thought to improve cognitive performance and to decrease the risk of suffering cognitive impairment, dementia or AD [102]. Polyunsaturated fatty acids (PUFAs), particularly omega-3 (ω-3) found in fish oils, reduce both systemic and brain inflammation. Additionally, PUFAs are believed to increase Aβ clearance [103]. Interestingly, diet can even affect intrauterine and postnatal environment. Maternal obesity and high-fat diet consumption can predispose its offspring to a more inflammatory and immune activated environment, and this can be reflected in epigenetic changes [104].

1.2.5.2.2.4. Exposure to contaminants

Exposure to toxic metals, insecticides or pesticides, industrial and commercial pollutants, antimicrobials, and air pollutants, have been directly linked to AD and its aggravation [105]. In fact, exposure to environmental pollutants such as lead, manganese, and mercury (Hg) during prenatal development has been linked to alterations in cognitive function, emotional regulation and other behavioural aspects later in life [106].

Air pollution has been directly associated with Aβ deposition, production of ROS and neuronal damage and death [107]. Several metals, such as aluminium, copper, zinc or iron, can be found in contaminated water or food, and they have been related to increased

Aβ deposition. Other metals like lead can have an impact in physiological development, leading to a higher risk of developing dementia [105]. Exposure to Hg ions enhances the formation of Aβ aggregates and phosphorylated tau, while also compromising membrane structural integrity of neurites and neuronal growth cones [108,109].

1.2.5.2.2.5. Sleep and circadian rhythm

Sleep and circadian rhythm disruption have been directly linked to neuroinflammation, lower Aβ clearance, increased oxidative stress, BBB deterioration, and direct connection to gut microbiota alterations [110]. Changes in sleep patterns can trigger inflammatory processes, such as NF-κB activation, leading to cellular ageing and further exacerbating inflammatory pathways. Additionally, short sleep duration have been related to higher presence of CRP and IL-6 [111]. It has to be taken into account that sleep can influence other risk factors such as physical activity or social interactions, aggravating AD risk.

1.2.5.2.2.6. Other diseases

Adults living with Down syndrome will develop the neuropathology hallmarks of AD and have 88-100 % risk of developing dementia after the age of sixty-five. This is attributed to the presence of an extra copy of chromosome 21, which contains the gene for APP. Consequently, individuals with Down syndrome may exhibit higher levels of amyloid angiopathy compared to those with sporadic AD. Over time, there is a significant accumulation of Aβ plaques and NFT, similar to patients with autosomal dominant forms of the disease. Interestingly, substantial oxidative damage in people with Down syndrome exacerbates neuroinflammation, which, accompanied by immune dysregulation, results in a chronic inflammatory state [112].

Changes in blood sugar levels and impairments in insulin signalling and resistance are associated with an elevated risk of developing dementia and AD. Diabetes can impact memory processing, brain atrophy and synaptic communications. The presence of Aβ, phosphorylated tau, oxidative stress, and neuroinflammation have been linked to insulin resistance, features that contribute to neurodegeneration [113].

There is a strong connection between stroke and sporadic AD. 60-90 % of AD brains show cerebrovascular pathology after autopsy. Hemodynamic changes, ionic gradients disruption and metabolic shifts after an ischemic brain injury will eventually cause neurons to degenerate, also due to a reduction in cerebral blood flow. Generally, vascular risk factors such as hypertension, atherosclerosis, or atrial fibrillation, increase AD risk. Besides, ischemic events can affect APP processing and increase Aβ plaques and NFT presence [114]. Furthermore, higher presence of Aβ can contribute to the progression of the ischemic brain injury, and over time to the development of AD-like dementia [115]. Additionally, patients already diagnosed with AD who experience stroke or cerebrovascular events indeed exhibit a more rapid decline in their pathology. Remarkably, deprivation of nutrients and oxygen can induce the activation of microglia, triggering the inflammatory response. Additionally, this deprivation can also damage the endothelial cells that form the BBB, so when an ischemic event arises, there is usually a BBB breakdown, not that commonly seen in AD [114,115].

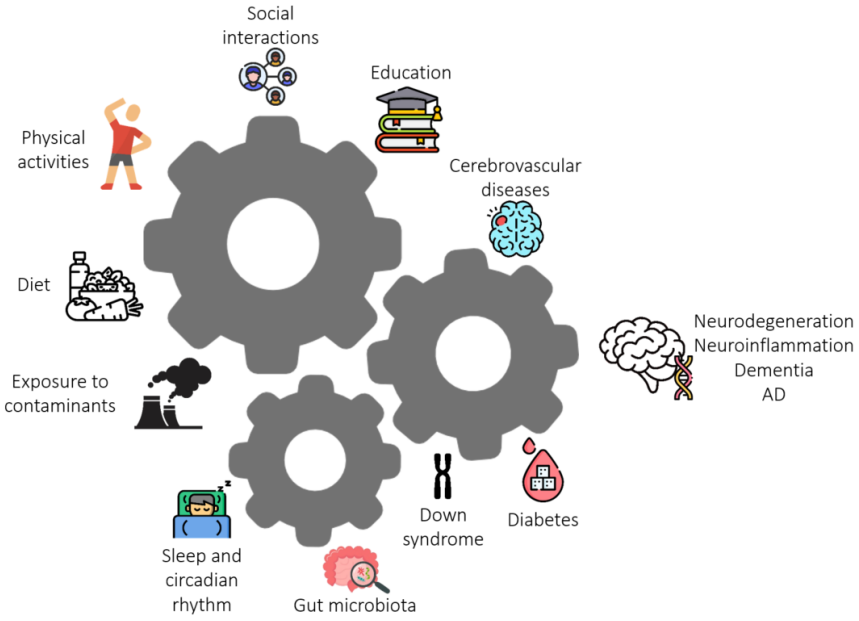

Figure 3. Selected external and internal factors, which in combination with genetic factors, modulate the risk and/or development of neurodegeneration, neuroinflammation, dementia, and AD.

In a nutshell, several external and internal factors can significantly impact human health outcomes, particularly concerning neuroinflammation, which is a key contributor to cognitive impairment, and AD. Notably, these factors may affect individuals throughout their lives, with effects often emerging in middle age and beyond. However, the mechanisms of action of this factors are still quite unknown, as well as its role in AD pathology.

1.3. Neuroinflammation and microglia

Neuroinflammation entails the inflammatory response in the peripheral or the central nervous system (CNS) after external or internal pathological insults. In the brain, inflammation is a defensive and protective mechanism. However, depending on the nature, intensity and the duration of this inflammation, it can transition into a chronic state, ultimately leading to detrimental effects on the brain [116].

The activation of glial cells, particularly astrocytes and microglia, followed by their increased release of cytokines, chemokines, and other inflammatory factors, mark the onset of neuroinflammation. Notably, microglia are considered the main leaders of inflammation in the brain, since they are the main producers of cytokines and they express numerous genes related to inflammation [117,118].

Microglia originate from mesodermal progenitors [119]. They are recognized as resident macrophages of the brain, constituting its primary line of defence [116]. Microglia exhibit a diverse range of phenotypic states. Traditionally, they were categorized as M0 or resting/homeostatic state, M1 or pro-inflammatory state, and M2 or anti-inflammatory state [119]. Nonetheless, in the recent years it has been suggested that microglia phenotypes should be divided into a broader spectrum. In 2023, Sun et al proposed 12 transcriptional states of microglia and its relevance to AD. These 12 clusters highlight

the significant involvement of microglia in inflammation and lipid metabolism, which contributes to the pathogenesis and the progression of AD [117].

When microglia respond to a stimulus, there is a shift in their gene expression that triggers the production of cytokines and other mediators. Moreover, microglia change their morphology, characterized by an amoeboid morphology, an increased soma size, and reduced ramifications [120]. Indeed, the decrease in the expression of homeostatic genes indicates a shift towards a more inflammatory state, which has been directly linked to neuronal loss [121]. Furthermore, in microglia from mouse models of AD, an upregulation of several genes related to chemokines and interferon-induced genes has been identified and directly associated to neuroinflammatory responses [122].

Interestingly, evidence suggests that microgliosis takes place near dense-core plaques of Aβ and NFT, and its intensity increases linearly with the duration of AD. While Aβ burden reaches a plateau in early stages of the disease, NFT accumulation continues to rise, paralleling with the increase of microgliosis, thus establishing a direct connection between these two factors [123]. The pathological characteristics of AD have been directly associated to an aggravation of the pathology, when combined with microglia response. As mentioned above, ApoE is primarily expressed by microglia along with astrocytes in the CNS. In presence of the APOE4 genotype, there is a predisposition of enhanced inflammatory response [76]. Furthermore, studies conducted with the microglial cell line BV2 have indicated that the presence of Aβ1-42 induces the activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome pathway, which exacerbates a neuroinflammatory state [124].

Microglia express several pattern recognition receptors (PRRs) that are responsible for the identification of exogenous or endogenous insults. These PRRs can activate numerous responses in microglia. For instance, lipopolysaccharide (LPS), a well-studied pro-inflammatory agent, activates microglia through the PRR TLR4 [125,126]. This triggers the activation of several transcription factors such as NF-kB, AP1, or STAT5, as well as the upregulation of cell membrane biomarkers associated with the immune response [127]. Additionally, it activates the production of several pro-inflammatory mediators, including tumour necrosis factors (TNF), interleukins (IL) such as IL-1, IL-6, IL-12, IL-17, IL-18, chemokines, and other mediators such as ROS, inducible nitric oxide synthase (iNOS) leading to the production of NO, and the activation of cyclooxygenase-2 (COX-2) (figure 4) [128–130].

All these mentioned processes are crucial for inducing an immune response and combating various insults. In the end, the main purpose is to accomplish immunological resolution and tissue repair and regeneration [131,132]. However, a continuous inflammatory stimulation can lead to neurodegenerative processes [116,133,134].

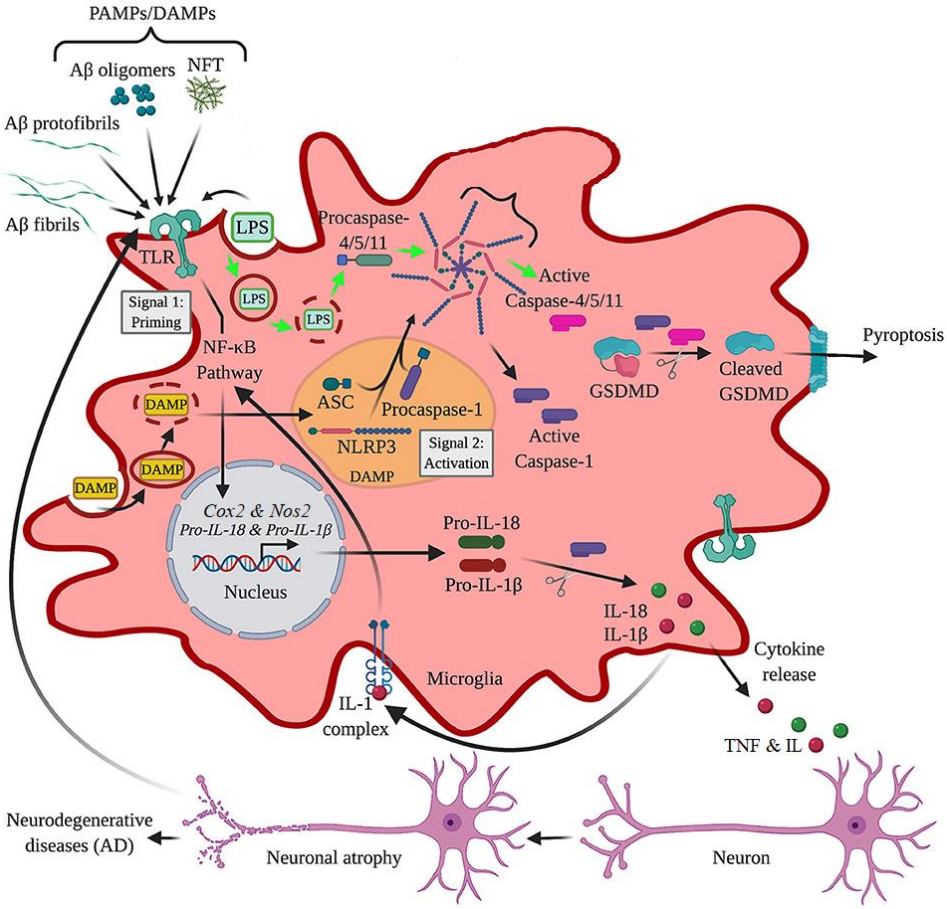

Figure 4. Microglia play a crucial role in neuroinflammation following the recognition of exogenous or endogenous insults such as LPS or Aβ by PRR. Activation of TLRs leads to the activation of the NF-κB pathway, initiating a signalling cascade that results in the expression of several proteins, including cytokines, COX-2, iNOS and NLRP3. Image taken and modified from the article authored by Hanslik and Ulland, 2020 [135].

1.3.1. The monomeric C-reactive protein (mCRP) as an inflammatory model of AD

CRP is a pentraxin composed of five 23 Kda subunits that is primarily synthesized by the liver and plays pivotal role as a mediator of the acute phase of infection (figure 5) [136–138]. Circulating CRP has been traditionally used as an indicator of inflammatory levels, particularly within cardiovascular and autoimmune disciplines [139]. Functioning as a pattern recognition molecule, CRP is considered part of the immune system, binding to damaged and inflamed cells to amplify the inflammatory response. In instances of inflammation, CRP levels can increase up to 10,000 fold within 24-72 h [137].

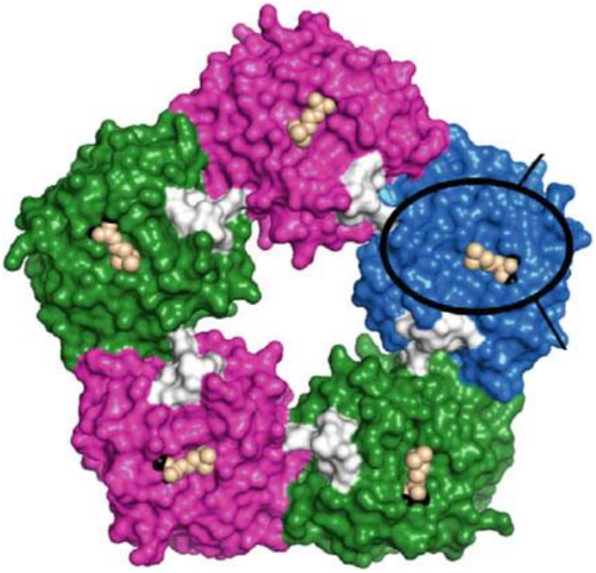

Figure 5. Structure of CRP. Representation of the structure of the pCRP pentamer with each individual monomer highlighted in a different colour (fuchsia, green, blue) and phosphocholine (circled) located at the ligand-binding site. Image taken from the book chapter authored by McFadyen et al. 2020 [137].

When CRP binds to phosphocholines on cellular membranes, it undergoes a conformational change to the pCRP* isoform. This isoform maintains its pentameric symmetry and exposes neoepitopes that can interact and activate complement C1q. The binding to the globular head of C1q can induce further dissociation of pCRP* subunits into the monomeric form of CRP (mCRP). While the pentameric isoform of CRP lacks pro-inflammatory effects, pCRP* and mCRP can further induce immune and pro-inflammatory responses [137,140,141].

Recent research has shown that the mCRP isoform is the primary mediator of the pro-inflammatory responses within the CRP system [137]. This form, for instance, is expressed in damaged tissue post-stroke, where it plays a crucial role in promoting angiogenesis and is directly associated with neuroinflammation and neurodegeneration [138].

In addition, while CRP has a half-life of 19 h in the bloodstream and does not go directly to the damaged tissues, mCRP, which has a lower solubility, can accumulate if not cleared effectively, causing tissue damage [141–143]. Circulating CRP dissociates irreversibly upon contact with tissues and cells, particularly in a context of hypoxia and necrosis. mCRP, capable of binding to vascular and inflammatory cells, triggers and perpetuates inflammation, angiogenesis and thrombus formation, stimulating cytokines production and activating intracellular signalling pathways (figure 6) [144–146]. The Molecular mechanisms by which mCRP exerts its effects are not fully understood, although in 2009, *Ji et al.* identified an interaction of this monomer with lipid rafts microdomains, involving the cholesterol binding site through residues 35-47 [147].

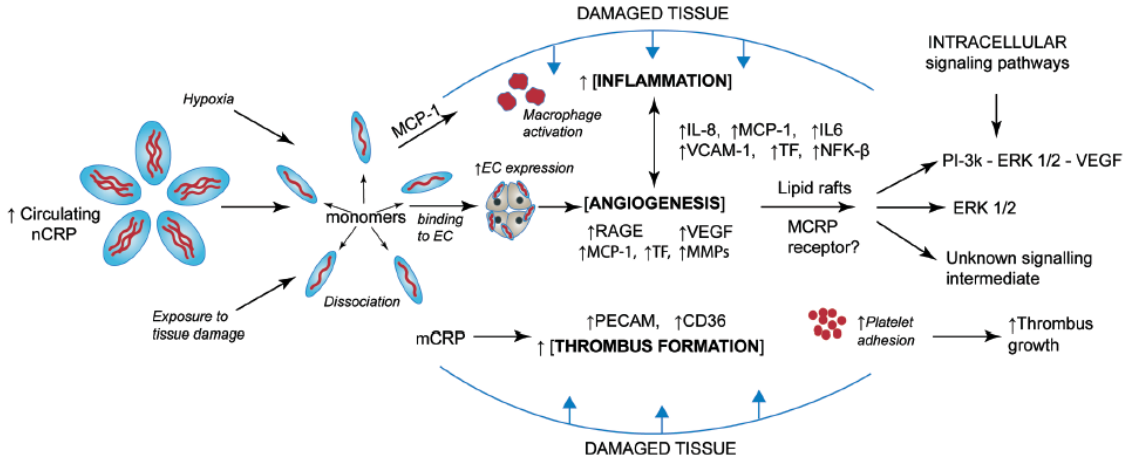

Figure 6. The pentraxin CRP is capable of quick dissociation upon contact with tissues and cells, especially under conditions of hypoxia and necrosis. This results in the formation of monomers (mCRP), which have the ability to attach to vascular and inflammatory cells. This attachment triggers inflammation, angiogenesis and thrombus formation, by stimulating cytokine production and initiating intracellular signal transduction pathways via unknown receptor-mediated mechanisms. Image taken from the article authored by Slevin and Krupinski, 2009 [146].

Regarding implications within the brain, although there are few studies, mCRP has been strongly linked to the pathogenesis of stroke and AD pathology. Elevated levels of mCRP have been observed in regions with previous haemorrhages or ischemic infarctions, associated with activated glial cells and macrophages, and abnormal neurons expressing p-tau and beta-amyloid [136,138]. These mentioned abnormal alterations can be associated with dementia onset. Hippocampal injection of mCRP in three-month old C57BL/6J mice induced impairment in recognition and spatial memory, as well as anxiety, accompanied by a reduction in in synaptic functions [148].

Furthermore, other crucial factors implicated in AD pathology have also been associated to mCRP. Aβ plaques were found to induce CRP dissociation, thus localizing inflammation around these plaques and potentially promoting AD progression [149]. mCRP induced pathological characteristics of AD, including neuroinflammation, cerebrovascular inflammation, extravasation of T lymphocytes into the brain, and tau phosphorylation, exhibiting an APOE-dependent pattern [150–152].

The mechanisms behind the increased levels of mCRP and its diffusion remain inconclusive. Studies suggest that mCRP becomes associated with circulating microparticules, macrovesicles, and cell-derived exosomes, facilitating its transport across the BBB, especially in cases of compromised microvasculature and/or BBB integrity [153–156]. Once within the brain, it is hypothesized to diffuse through extracellular spaces and by crossing the membranes of capillaries and arteries [142]. Additionally, as previously mentioned, to move in and out of the cells, mCRP seems to cross the plasma membrane involving cholesterol. Intriguingly, neurons possess the ability to synthetize mCRP, potentially contributing to structural support, intracellular signalling, and cell-to-cell communication. In the context of AD, this production is believed to be upregulated [157].

1.4. PUFAs and EETs in neurodevelopment and neuroprotection

Lipids are crucial biochemical components in the brain that play a wide range of functions. Alterations in the composition and metabolism of lipids have been related to several neuropsychiatric and neurodegenerative conditions [158,159].

PUFAs are lipids considered as essential nutrients, since mammals are not able to synthetize them and have to acquire them throughout dietary sources [160,161]. Moreover, PUFAs have crucial roles in brain development, neuroplasticity and neuroinflammation [158,162,163].

PUFAs are divided into two families, omega-6 (ω-6) and ω-3, based on the position of their double bond in the carbon chain [164]. Alpha-linoleic acid (ALA, ω-3) and linoleic acid (LA, ω-6) are the main precursors for the synthesis of several important long-chain PUFAs. ALA gives rise to two ω-3 fatty acids: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), while LA metabolizes into one ω-6: arachidonic acid (AA) [158].

The brain stores a great amount of long-chain PUFAs that are essential for physiological functions such as cerebral plasticity, cell survival and neuroinflammation [158,162,165]. The accumulation of these PUFAs begins early during the prenatal period, commonly termed as “accreation”. Depending on the gestational state, the foetal brain receives varying amounts of PUFAx from the mother through the placenta via free diffusion or specific transporters. Nonetheless, the role of EPA, DHA, and AA accreation during brain development and their impact on glial cells remains unknown [166]. In the adult brain, these PUFAs are sourced from plasma based on brain consumption and demand [167].

Once PUFAs enter the brain, long-chain-fatty-acid-CoA synthase is responsible for their activation. Subsequently, some of them, particularly DHA and AA, are esterified to phospholipid membranes, while other PUFAs like ALA and EPA undergo β-oxidation [168]. From the membranes, fatty acids can be selectively de-esterified and released by phospholipases (PL). In response to physiological stimuli or pathological injury, and through interaction with various receptors in the brain, PL-A2 de-esterifies AA and DHA from membrane phospholipids [158,169]. It is at this point that these PUFAs are further metabolized into the bioactive derivatives known as eicosanoids or docosanoids, which are considered mediators and form part of the broader group known as oxylipins (figure 7) [166]. The main enzymes involved in these reactions are cyclooxygenases (COX), lipooxygenases (LOX), and the cytochrome P450 system (CYP450) [170,171].

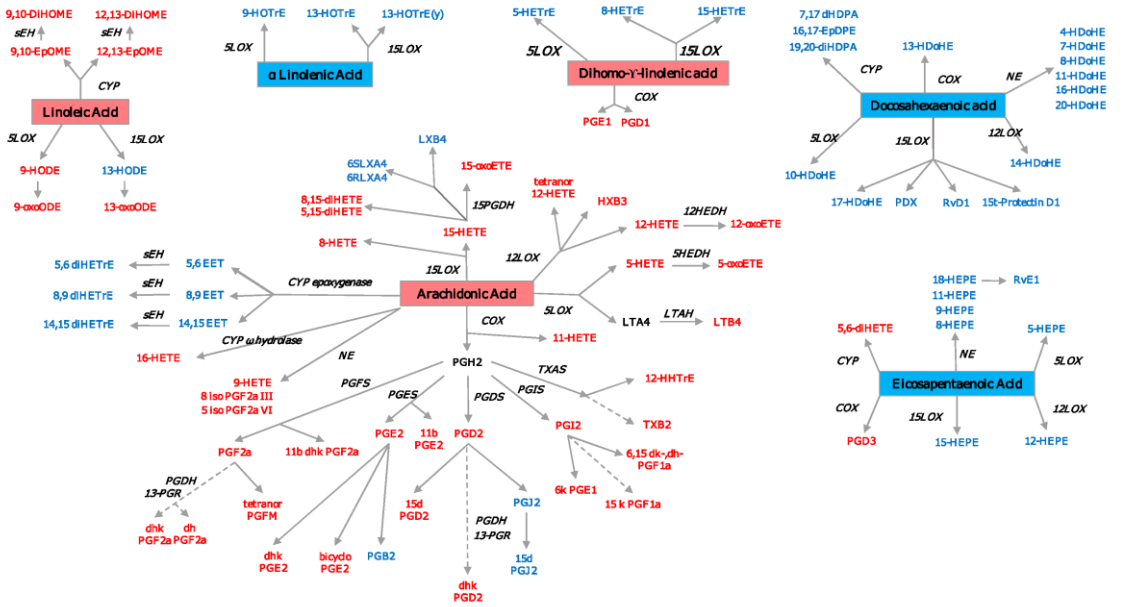

Figure 7. Oxylipins originating from PUFAs. Pro-inflammatory oxylipins are highlighted in red, whereas anti-inflammatory ones are highlighted in blue. Ω-3 precursors are squared in red, while ω-6 precursors in blue. Image taken from the article authored by Coras et al, 2020 [172].

In fact, these derived oxylipins can modulate neuroinflammation. In front of inflammatory circumstances, PUFAs are metabolized into pro- and anti-inflammatory oxylipins, hence influencing both the initiation and resolution of inflammation [159,165,173]. Here, the enzymes involved in this PUFAs metabolism play an essential role. For instance, after the administration of LPS to C57Bl6/J mice, there is an increase in the pro-inflammatory mediators derived from ω-6 PUFA, such as TxB2 and 8-HETE [162].

Despite the limited number of studies on DHA, EPA, and AA mediators, it is believed that these mediators are primarily responsible for the effects attributed to their precursors. For instance, exposure to EPA and/or its mediators has been associated with a reduction in IL-1β effects in rats, thereby improving memory deficits and anxiolytic behaviour [174]. Similarly, DHA and/or its mediators have demonstrated the ability to diminish the inflammatory profile and astrogliosis, as well as restore spatial memory deficits in aged mice [175].

Some AA-derived mediators have been extensively studied. When AA is metabolized by CYP450, four epoxyeicosatrienoic acids (EETs) regiosomers are produced. These EETs are rapidly metabolized by the soluble epoxide hydrolase (sEH) into less active metabolites called dihydroxyeicosatrienoic acids (DiHETs), having EETs a half-life of seconds or minutes [176,177]. Specifically, 11,12-EET has been found to decrease the protein expression levels of PL-A2, COX-2, and PGE2 in rat brains after cerebral ischemia-reperfusion. As a result, there is an attenuation of neuroinflammation, which reduces cerebral infarct volume and improves cerebral outcomes [178].

1.5. Pharmacological treatments against AD

There are presently no approved drugs capable of reversing or curing AD. Current research is focused on various mechanisms of the disease, targeting cholinergic damage, Aβ, NFT, oxidative damage, and neuroinflammation (Bhole et al., 2024; Ma et al., 2019). The main objective is not only to improve cognition and memory but also to prevent the progression and the symptoms of the disease.

Considering the complexity of the pathogenesis and the aetiology of AD, the establishment of combined therapies rather than monotherapies would be of great advantage. This way, patients would have several advantages in managing the progression of the disease while addressing the behavioural and social aspects of their lives. Additionally, early detection is crucial for well-timed interventions during the initial stages of AD. Further research plays a pivotal role in advancing our understanding of AD.

1.5.1. Current treatments

Currently, there are only two categories of drugs that have the FDA approval: acetylcholinesterase inhibitors (donepezil, galantamine, and rivastigmine) and N-methyl-D-aspartate (NMDA) receptor antagonist blockers (memantine). However, these drugs manage AD symptoms by replacing altered neurotransmitters, but are not able to stop the progression of the disease [179–181].

1.5.2. Aβ and tau as therapeutic targets

Several anti-Aβ drugs have reached phase III clinical trials. These drugs target β- and γ-secretase. In patients experiencing MCI or AD, these drugs effectively decrease Aβ accumulation in the brain. However, they have not succeeded in slowing cognitive decline in either group, or they presented several secondary effects [182–185]. Alternatively, immunotherapeutic drugs have been under investigation to promote Aβ clearance. Donanemab is currently in phase III trials as a promising treatment for early AD, as it clears Aβ deposition, reduces p-tau progression, and seems to delay cognitive impairment and functional deterioration [186,187].

The lack of success in Aβ trials suggests that simply removing Aβ from the brain might not be enough to prevent cognitive decline. This highlights the importance of targeting tau hyperphosphorylation. For that, there are several kinases and phosphatases under investigation that are considered potential pharmaceutical targets [181,188,189]. Additionally, there are some inhibitors that aim to reduce tau accumulation. For instance, hydromethanesulfonate is used for the treatment of mild to moderate AD and has been proposed as a complementary approach to existing therapies [190]. However, further research is still ongoing.

1.5.3. Anti-oxidant and anti-inflammatory treatments

1.5.3.1. Soluble epoxide hydrolase inhibitors (sEHI)

As previously mentioned, EETs possess several beneficial properties. The Inhibition of the sEH enzyme (figure 8) has been proposed as a therapeutic target for the treatment of AD [191,192]. In fact, studies in mouse models confirm that the genetic deletion of the sEH gene can slow down the progression of AD, alleviate its pathology, and reduce neuronal death [193], as well as apoptosis and BBB permeability after a traumatic brain injury [194]. Moreover, this inhibition seems to protect against Aβ-induced AD in mice, suggesting that its inhibition can stabilize EETs levels and confer protective effects [195,196].

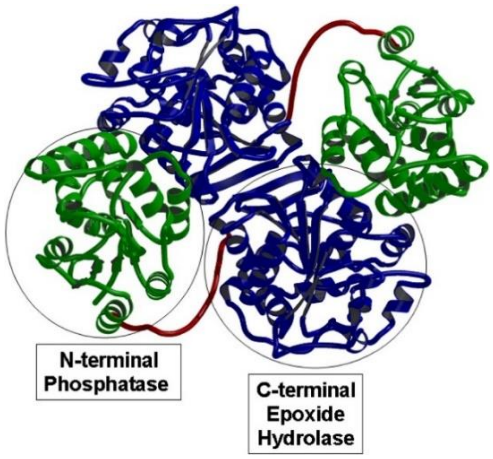

Figure 8. Crystal structure of the sEH enzyme. With a molecular weight of 62 kDa, consists of two globular regions connected by a brief proline-rich linker. sEHI compounds bind to the N-terminal domain, inhibiting sEH activity. Image taken from the article authored by Harris and Hammock, 2013 [197].

Notably, the inhibition of sEH in mice increases EETs half-life and stabilize levels of other oxylipins *in vivo*, enhancing their anti-inflammatory effects [198]. Each new generation sEH inhibitors (sEHI) improves its characteristics, and when well-designed, sEHI can efficiently cross the BBB and reach the brain in sufficient concentrations [199]. In mouse models, sEHI have demonstrated the ability to reduce cognitive impairment, Aβ deposition, tau hyperphosphorylation, BBB disruption, oxidative processes and neuroinflammation; all hallmarks of AD pathology [191,192,200,201]. Moreover, sEHI appear to directly modulate neuroinflammatory effects by reducing glial activation and the unfolded protein response [196]. However, further studies are needed to understand mechanisms involving the effects of sEHI on neuroinflammation.

1.5.3.2. Natural drugs: resveratrol

Natural drugs hold significant promise in AD treatment, offering multiple target properties with minimal secondary effects. However, many of these compounds are still under study to ascertain their efficacy, establish relevant study models, and determine optimal dosage [202]. Overcoming the BBB remains as a key challenge in effectively targeting pharmaceutical drugs to the brain [203]. The metabolites from bioavailable polyphenols can be transported across the BBB endothelium [204], and some of them have neuroprotective effects due to their anti-oxidant and anti-inflammatory properties [205].

Resveratrol (RV) is a polyphenol found abundantly in red wine, berries, peanuts, and various plants. It is recognized as a significant therapeutic agent because of its potent anti-oxidant and anti-inflammatory properties. RV has beneficial effects in oxidative stress, inflammation, apoptosis, mitochondrial function, proteostasis, gut microbiota diversity and lifespan, thus emerging as a pleiotropic agent [206]. In neuronal cells, RV demonstrates the ability to attenuate the inflammatory response, reduce lipid peroxidation, and increase heme oxygenase-1 levels to counteract further oxidation [207]. Moreover, RV has been observed to modulate cerebral blood flow, thereby influencing cerebral functions [208].

Long-term administration of RV in the age-related AD mouse model SAMP8 has been shown to reverse memory impairments and reduce the presence of Aβ deposits and p-tau [209], findings also corroborated in studies involving the 3xTg-AD mice [210]. Additionally, research with the mouse familial AD model AβPP/PS1 suggests that dietary RV may delay and alleviate AD onset, partly due to the modulation of the antioxidant machinery and cytokines levels [211]. Moreover, RV appears to exert protective effects against AD induced by a high fat diet regimen, in both wild-type and 5XFAD mouse models. This includes normalization of BPSD-like behaviour, protection against memory loss, reduction in Aβ and p-tau pathologies, and normalization of immuneproteasome activity [212].

Limited clinical trials conducted in AD patients suggest that RV may have a potential role in delaying cognitive impairment compared to the placebo group [213]. Nonetheless, while RV shows great promise as a treatment for AD, possibly via oxidative stress and inflammation modulation, its rapid metabolism in humans poses a challenge. Hence, multiple research initiatives and clinical trials are ongoing to explore its impact on neurological disorders.

Chapter 2. Hypothesis and objectives

There are currently limited therapies for neurodegenerative diseases. Considering the complexity of AD pathology and aetiology, it is essential to explore new therapeutic approaches. Neuroinflammation, being one of the primary neuropathological characteristics and triggers of AD, emerges as a promising target for treatment.

We hypothesize that by modulating EETs and other oxylipins, it may be possible to prevent microglia from transitioning into a more activated and pro-inflammatory state. This modulation, particularly when considered alongside genetic and/or environmental risk factors, could potentially reduce the risk of neurodegeneration and AD.

The hypothesis of this thesis is based on our understanding of oxylipins, with special focus to EETs regiosomers derived from AA, and their anti-inflammatory properties within the brain. Investigating the inhibition of sEH, the enzyme responsible for EETs rapid metabolism, emerges as a potential therapeutic strategy to mitigate cognitive decline *in vivo* and neuroinflammation both *in vivo* and *in vitro*. Furthermore, the impact of increased EETs levels and sEH inhibition on early-stage neuroinflammation could have long-lasting effects. Therefore, we propose that pharmacological agents acting as inhibitors of sEH (sEHI), when tested at an early age *in vivo*, could reduce the risk of developing AD in the adulthood by modulating inflammatory mechanisms. Additionally, using an *in vitro* system with mCRP, a proposed trigger of neuroinflammation, will be instrumental in studying molecular mechanisms involved.

To test our hypothesis, this thesis has been developed based on the following objectives:

1) Study the neurodevelopmental cognitive and inflammatory effects of maternal treatment with a sEHI by performing an evaluation in adult 5xFAD and WT offspring.
    - ❖ Assess if the maternal treatment with the sEHI TPPU can be effective in protecting against AD pathological characteristics of the 5XFAD offspring.
    - ❖ Confirm if maternal inflammatory status can define its offspring health outcome.
2) Investigate the correlations between levels of EETs or other PUFA-derived oxylipins in placenta and cord blood samples, and the neurodevelopmental outcomes of children, prenatally exposed to known levels of environmental Hg contamination.
    - ❖ Determine oxylipins levels in placenta and cord-blood samples according to environmental exposure to low or high Hg.
    - ❖ Study if the EETs and other oxylipins levels correlate with mental and psychomotor scores in the children at 14 months and 5 years of age, either under low or high prenatal Hg exposure.
3) Explore the mechanisms of mCRP in microglial cells and its protection by newly-synthetized sEHI, to characterize this proposed pro-inflammatory molecule and the sEH target in an AD *in vitro* setting.
    - ❖ Study if the inflammatory mechanisms of mCRP induced in microglia can be related to chronic neuroinflammation in AD.

- ❖ Validate whether sEHI can protect microglia against an inflammatory stimuli.
- ❖ Confirm the effectivity of newly-synthesized sEHI compared to the known TPPU.

4) Determine whether RV, which possesses anti-oxidant and anti-inflammatory properties, can mitigate mCRP detrimental effects in microglial cells.
   - ❖ Analyse if RV can cope with the harmful inflammatory response of microglia.

Chapter 3. Publications

Publication 1.

Neuroprotective epigenetic changes induced by maternal treatment with an inhibitor of soluble epoxide hydrolase prevents early Alzheimer's disease neurodegeneration

Clara Bartra, Alba Irisarri, Ainhoa Villoslada, Rubén Corpas, Samuel Aguirre, Elisa García-Lara, Cristina Suñol, Mercè Pallàs, Christian Griñán-Ferré and Coral Sanfeliu

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International Journal of *Molecular Sciences*

MDPI

Article

Neuroprotective Epigenetic Changes Induced by Maternal Treatment with an Inhibitor of Soluble Epoxide Hydrolase Prevents Early Alzheimer's Disease Neurodegeneration

Clara Bartra [1,2], Alba Irisarri [1,3], Ainhoa Villoslada [1], Rubén Corpas [1,2], Samuel Aguirre [1,3], Elisa García-Lara [1,2], Cristina Suñol [1,2], Mercè Pallàs [3,4], Christian Griñán-Ferré [3,4] and Coral Sanfeliu [1,2,*]

[1] Institut d'Investigacions Biomèdiques de Barcelona (IIBB), Consejo Superior de Científicas (CSIC), 08036 Barcelona, Spain; clara.bartra@iibb.csic.es (C.B.); alba.irisarri@ub.edu (A.I.); ainhoa.villoslada@estudiants.urv.cat (A.V.); rcorpas@oryzon.com (R.C.); saibmc@ibmb.csic.es (S.A.); elisa.lara@frontiersin.org (E.G.-L.); cristina.sunol@iibb.csic.es (C.S.)
[2] Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), 08036 Barcelona, Spain
[3] Department of Pharmacology and Therapeutic Chemistry, Faculty of Pharmacy and Food Sciences, 08028 Barcelona, Spain; pallas@ub.edu (M.P.); christian.grinan@ub.edu (C.G.-F.)
[4] Institut de Neurociències, Universitat de Barcelona, 08028 Barcelona, Spain
* Correspondence: coral.sanfeliu@iibb.csic.es; Tel.: +34-93-363-8338

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Abstract: Modulation of Alzheimer's disease (AD) risk begins early in life. During embryo development and postnatal maturation, the brain receives maternal physiological influences and establishes epigenetic patterns that build its level of resilience to late-life diseases. The soluble epoxide hydrolase inhibitor N-[1-(1-oxopropyl)-4-piperidinyl]-N'-[4-(trifluoromethoxy)phenyl] urea (TPPU), reported as ant-inflammatory and neuroprotective against AD pathology in the adult 5XFAD mouse model of AD, was administered to wild-type (WT) female mice mated to heterozygous 5XFAD males during gestation and lactation. Two-month-old 5XFAD male and female offspring of vehicle-treated dams showed memory loss as expected. Remarkably, maternal treatment with TPPU fully prevented memory loss in 5XFAD. TPPU-induced brain epigenetic changes in both WT and 5XFAD mice, modulating global DNA methylation (5-mC) and hydroxymethylation (5-hmC) and reducing the gene expression of some histone deacetylase enzymes *(Hdac1* and *Hdac2*), might be on the basis of the long-term neuroprotection against cognitive impairment and neurodegeneration. In the neuropathological analysis, both WT and 5XFAD offspring of TPPU-treated dams showed

lower levels of AD biomarkers of tau hyperphosphorylation and microglia activation (*Trem2*) than the offspring of vehicle-treated dams. Regarding sex differences, males and females were similarly protected by maternal TPPU, but females showed higher levels of AD risk markers of gliosis and neurodegeneration. Taken together, our results reveal that maternal treatment with TPPU impacts in preventing or delaying memory loss and AD pathology by inducing long-term modifications in the epigenetic machinery and its marks.

Keywords: Alzheimer's disease; prenatal risk; soluble epoxide hydrolase; maternal TPPU; epigenetics; long-term neuroprotection; memory; neuroinflammation; 5XFAD mice

1. Introduction

Therapies for Alzheimer's disease (AD) have been elusive despite some recent promising clinical trials with monoclonal antibodies against amyloid beta. However, all disease-modifying therapies assayed so far showed minor or no neuroprotective effects [1]. Many new drugs are in the pipeline, but prevention should be urgently addressed [2]. Early environmental influences are increasingly considered as modulators of late-life neurodegeneration and AD risk [3]. During gestation and breastfeeding, several maternal factors such as diet, consumption of drugs, and stress can affect the baby's brain development. An impact on plasticity and epigenetically-mediated programming of diverse brain cells has been proposed linking early influences and the late risk of neurodegenerative disorders [4,5]. Epigenetic mechanisms regulate gene transcription through DNA methylation and other DNA modifications, a variety of post-translational modifications of histones, chromatin remodelers, and several classes of non-coding RNAs. The imprinting of the genome by epigenetic marks is a dynamic process that can be modified by environmental factors such as drugs, nutrition, and mental stimulation [6]. Dysregulation of the brain epigenome has been strongly associated with memory impairment and neurodegeneration [7]. Accordingly, epigenetic mechanisms play a critical role in the genome–environment interaction in AD [8]. Likewise, maternal exposure to different environmental factors can affect the offspring cognitive outcome for the next generation [9]. For instance, experimental studies with a maternal diet supplemented with choline or with resveratrol have shown the relevance of the triggered epigenetic mechanisms in the long-term neuroprotection of the offspring of mouse models of cognitive impairment [10,11]. Specifically, choline is a major dietary donor of methyl groups essential for DNA and histone methylation that play an important role in fetal brain development [12]. Epigenetic mechanisms of maternal choline induce long-term changes in gene expression levels affecting neurotransmission, neurotrophism, and autophagy pathways in the Ts65Dn mouse model of Down's syndrome [10]. Moreover, resveratrol is a natural polyphenol with potential therapeutic use in neurodegenerative diseases that modulates DNA methyl transferase and histone deacetylase enzymes among other epigenetic mechanisms [13]. Similarly, maternal

treatment with resveratrol induces epigenetic regulation of redox master genes, autophagy, and mitochondrial biogenesis leading to cognitive improvement in the offspring of the senescent mice SAMP8 [11]. Thus, research on treatments affecting neurodevelopmental epigenetics may lead to new therapeutic strategies against AD. This may be particularly relevant when known risk factors are present, such as in families bearing risk alleles or mild late-onset AD mutations [14].

In a search for epigenetic-mediated therapies, we hypothesized that an anti-inflammatory drug that is neuroprotective in adult mouse models of AD would induce beneficial epigenetic imprinting of the innate immune system during embryonic and perinatal brain development. As a novel anti-inflammatory drug, we and others have shown that 1-(1-propanoylpiperidin-4-yl)-3-[4-(trifluoromethoxy)phenyl]urea (TPPU), an inhibitor of the soluble epoxide hydrolase (sEH) enzyme that converts anti-inflammatory oxylipins epoxyeicosatrienoic acids (EETs) to the corresponding less active dihydroxyeicosatrienoic acids (DHETs), is neuroprotective in experimental models of cognitive loss and dementia [15–20]. Furthermore, EETs have been associated with epigenetic changes in differentiating adipocyte cells leading to decreased inflamed adipocyte phenotypes [21]. Additionally, in terms of maternal diet, EETs are produced from the n-6 polyunsaturated fatty acid (PUFA) arachidonic acid and have important roles in the regulation of inflammation and immune response, vascular enhancement, and specific neuroprotective effects [22–25]. In general, PUFAs are essential fats for brain development and function. PUFAs are found in plant and animal foods, such as salmon, vegetable oils, and some nuts and seeds. PUFAs are metabolized to several families of oxylipins that are the main mediators of the beneficial effects [26]. In adults, alterations in fatty acid metabolism have been reported in AD patients and mouse models of AD [27]. Furthermore, lipidomic alterations have been associated with epigenetic changes and AD development [28].

In this study, pharmacological inhibition of sEH was induced in female wild-type (WT) mice mated with male heterozygous 5XFAD mice by TPPU treatment. Six WT females were dosed orally with 5 mg/kg/day of TPPU added to their drinking water and six WT females were dosed with the cyclodextrin vehicle. Treatment started at mating and was maintained during pregnancy and lactation. The litter pups that were raised together were either WT or transgenic AD mice of the 5XFAD strain. Male and female mice were included in the study. Our aim was to investigate the long-term protective effects of maternal TPPU dosing in young adult 5XFAD mice compared to WT mice, emphasizing epigenetic changes that may impact the early course of AD. To this end, we fully analyzed the offspring at two months of age, when 5XFAD mice show the first pathological features of AD. As a reference, we included a group of untreated 7-month-old male 5XFAD mice with advanced AD pathology for analysis of selected markers.

2. Results

2.1. Maternal TPPU Treatment Prevented Cognitive and Behavioral Changes of 5XFAD Offspring

2.1.1. Protection against Memory Loss

Two-month-old male and female 5XFAD mice from control litters whose dams were treated with vehicle (Ct) showed memory impairment in the object recognition test (ORT) and the object location test (OLT), as shown in Figure 1A,B. Interestingly, 5XFAD mice belonging to litters with maternal TPPU treatment (TPPU) showed absence of impairment, although the genotype factor did not reach significance in OLT. Furthermore, TPPU induced cognitive enhancement in WT mice, as shown by the statistical significance of the treatment factor in the two-way ANOVA. Thus, maternal TPPU protected against learning and memory impairment in two different paradigms: the recognition of a new object versus a familiar one and the recognition of a change in object position.

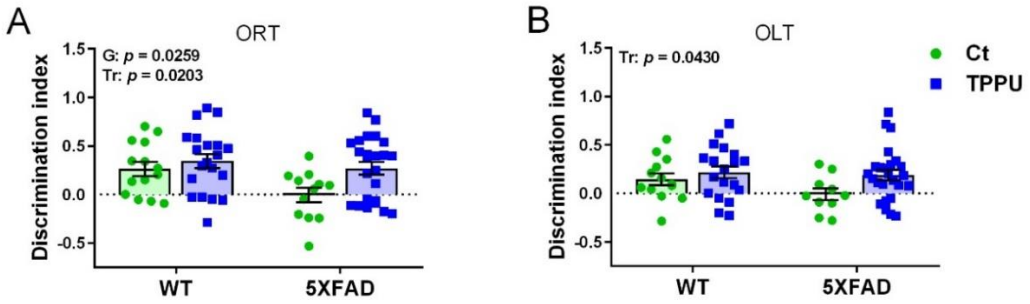

Figure 1. Cognitive testing in 2-month-old WT and 5XFAD mice from litters with maternal TPPU treatment or control (Ct). Breeding WT female mice were mated to 5XFAD male mice and dosed with 5 mg/kg/day of TPPU or vehicle during pregnancy and lactation. (**A**) Object recognition test (ORT); (**B**) object location test. Experimental maternal treatment: Ct (green circles), TPPU (blue squares); mouse genotype: WT (wild-type mice), 5XFAD (AD 5XFAD transgenic strain). Values are mean ± SEM of male and female mice ((**A**) *n* = 12−20; (**B**) *n* = 10−25). Statistics: two-way ANOVA. There was a significant effect of the treatment factor (Tr) in (**A**,**B**) and of genotype factor (G) in (**B**). *p* values of the significant factors are indicated in the corresponding graphs.

2.1.2. Maternal Treatment with TPPU Rescued Exploratory Activity in the 5XFAD Offspring

Male and female 5XFAD mice from control litters showed a tendency for higher exploratory activity than WT mice in the open field test (OFT). Horizontal activity is shown in Figure 2A,B. There was a borderline effect of the genotype factor in the total distance ambulated (p = 0.0603) and in the mean speed without rest intervals (p = 0.0530). However, 5XFAD offspring from TPPU-treated dams showed similar activity to WT mice, although the treatment factor did not reach significance. Therefore, these results suggested the presence of mild hyperactivity in young 5XFAD mice that was reduced by maternal TPPU treatment (Figure 2A,B). Vertical activity was significantly lowered by TPPU treatment in both WT and 5XFAD animals, as shown in Figure 2C. As a whole, a decrease in horizontal and vertical activities may indicate TPPU-induced changes in the exploratory pattern leading to lower excitability in a stressful environment. Finally, no changes were detected in grooming activity which discards the presence of stereotypic-like disorders, as shown in Figure 3D.

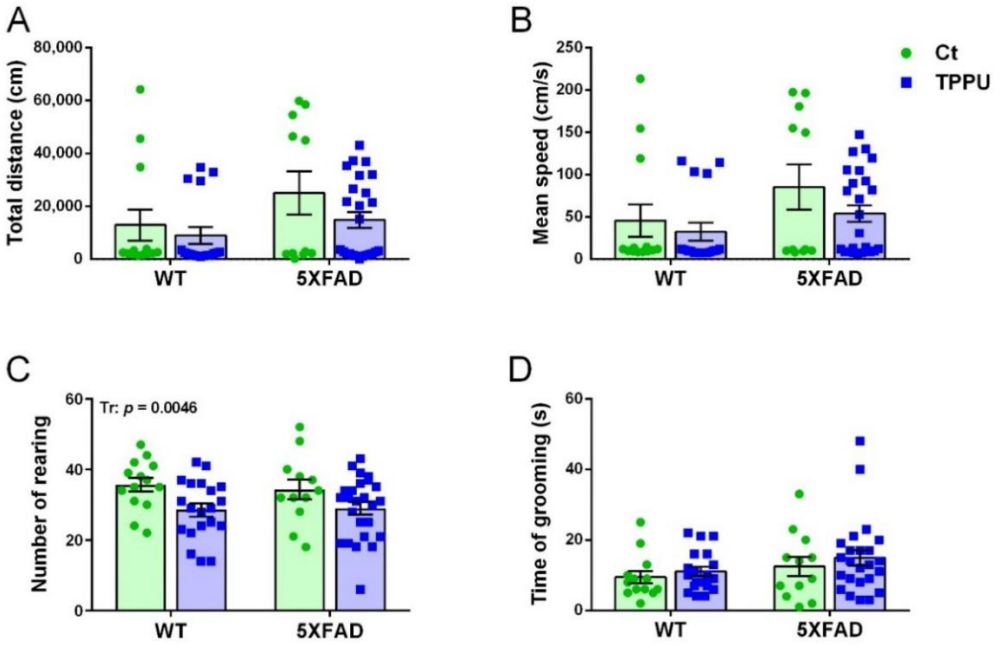

Figure 2. Exploratory behavior in the open field test of 2-month-old WT and 5XFAD mice from litters with maternal TPPU treatment or control (Ct). (**A**) Horizontal activity; (**B**) mean speed without rest intervals; (**C**) vertical activity; (**D**) grooming activity. Values are mean ± SEM of male and female mice ((**A**–**C**) *n* = 12–25; (**D**) *n* = 12–24). Statistics: ANOVA, significant effect of treatment (Tr) in (**C**), *p* value of the significant factor is indicated in the corresponding graph; borderline significant effect of genotype (G) in (**A**,**B**) (see text).

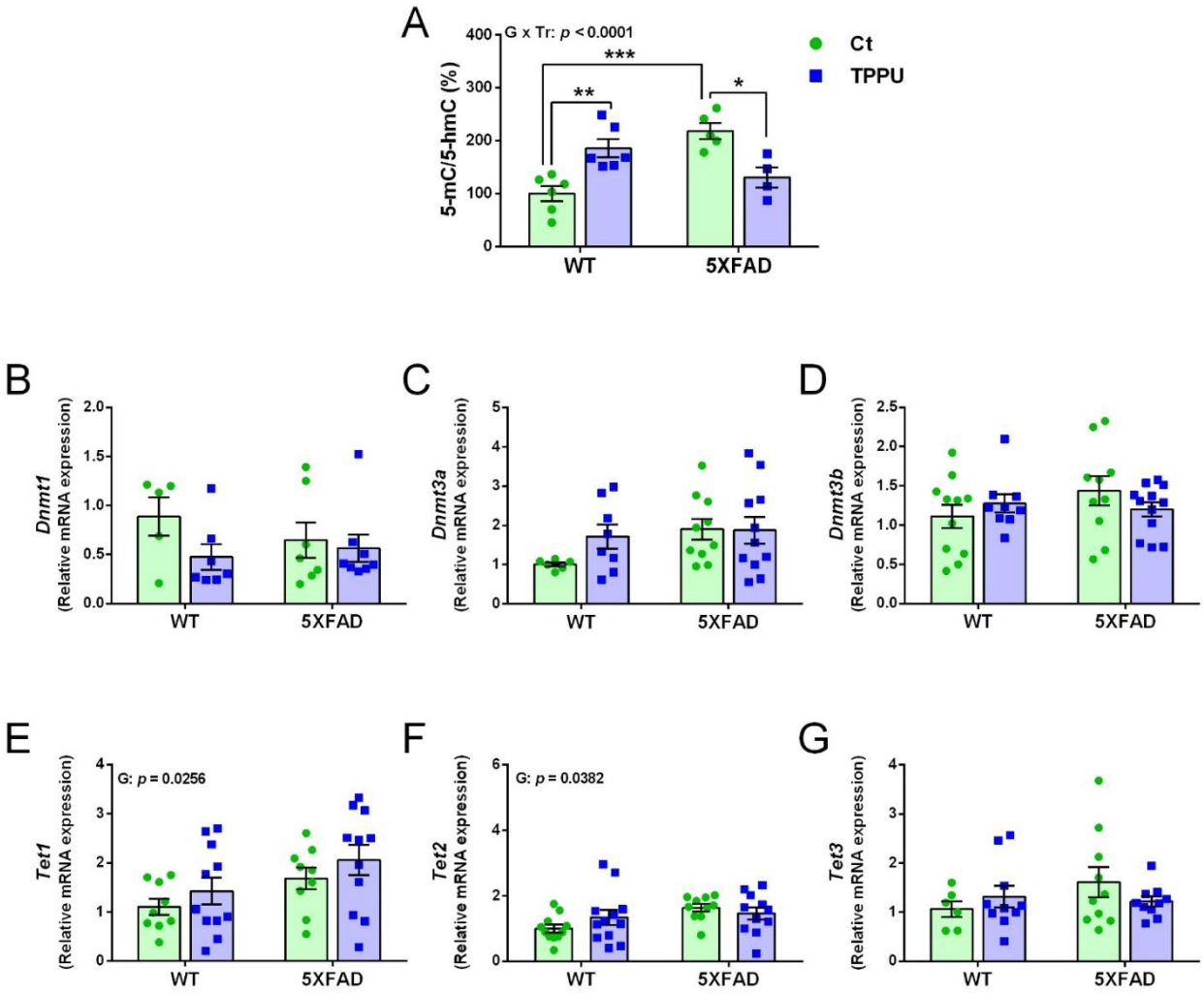

Figure 3. DNA methylation and demethylation changes in 2-month-old WT and 5XFAD mice from litters with maternal TPPU treatment or control (Ct). (**A**) Methylation profile obtained by the 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) ratio (%); (**B**–**D**) expression levels of the DNA methyltransferase genes *Dnmt1*(**B**), *Dnmt3a* (**C**), and *Dnmt3b* (**D**); (**E**–**G**) expression levels of the methylcytosine dioxygenase genes *Tet1* (**E**), *Tet2* (**E**), and *Tet3* (**G**). (**A**–**G**) Cerebral cortex samples. Values are mean ± SEM of male and female mice ((**A**) *n* = 4–6; (**B**) *n* = 5–8; (**C**) *n* = 6–11; (**D**) *n* = 9–12; (**E**) *n* = 9–11; (**F**) *n* = 10–12; (**G**) *n* = 7–10). Statistics: ANOVA, interaction genotype per treatment effect (G x Tr) in (**A**), effect of genotype (G) in (**E**,**F**), *p* values of the significant factor and factor interaction are indicated in the corresponding graphs; Bonferroni post-hoc test after a significant factor interaction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between groups as indicated.

2.2. Maternal TPPU Treatment Induced Neuroprotective Epigenetic Changes in 5XFAD and WT Offspring

2.2.1. Changes in the Global DNA Methylation Landscape

Epigenetic methylation of DNA silences gene expression by direct methylation of the cytosine carbon ring in the dinucleotide CpG repeats [29]. Impaired global DNA methylation has been associated with AD neuropathology and cognitive loss [30].

The global DNA methylation profile was calculated as the ratio between the 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) levels and the results are displayed in Figure 3A. There was an interaction between the ANOVA factors genotype

and treatment, indicating that maternal TPPU differently affected WT and 5XFAD mice. That is, it increased DNA methylation in WT mice but normalized the excessive methylation shown by 5XFAD mice. The gene expression of the specific DNA methyl transferase (DNMT) enzymes *Dnmt1*, *Dnmt3a*, and *Dnmt3b* is shown in Figure 3B–D, respectively, with no significant changes in any of them. However, ten-eleven translocation family (TET) genes *Tet1* and *Tet2* showed an effect of genotype indicating higher levels in 5XFAD mice. The statistical results and several trends shown in the displayed graphs indicate that there were global 5-mC differences between 5XFAD and WT offspring and the maternal treatment with TPPU would mitigate the 5XFAD epigenetic alterations.

2.2.2. Changes in the Histone Deacetylation Machinery

Several histone deacetylase enzymes (HDACs) have been implicated in memory and cognition [31]. Therefore, we analyzed the brain expression of the genes Hdac1 and Hdac2 by qPCR. The mRNA of these epigenetic enzymes was significantly decreased by maternal TPPU treatment in both 5XFAD and WT offspring, as shown in Figure 4A,B. However, no genotype differential effect was detected in the young male and female mice. Removal of acetyl groups from lysine residues on histones increases chromatin compaction into a transcriptionally repressed state. Therefore, TPPU-induced downregulation of Hdac1 and Hdac2 would activate gene transcription.

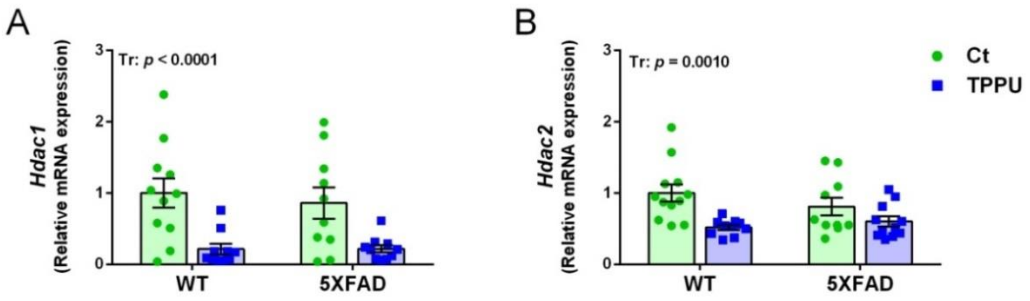

Figure 4. Histone deacetylation changes in the brain tissue of 2-month-old WT and 5XFAD mice from litters with maternal TPPU treatment or control (Ct). Expression levels of the histone deacetylase genes *Hdac1* (**A**) and *Hdac2* (**B**). (**A**,**B**) Hippocampus samples. Values are mean ± SEM of male and female mice ((**A**) *n* = 10–11; (**B**) *n* = 10–12. Statistics: ANOVA, effect of treatment (Tr) in (**A**,**B**), *p* values of the significant factor are indicated in the corresponding graphs.

2.3. Maternal TPPU Treatment Did not Modify Amyloid β42 but It Protected against Tau Hyperphosphorylation in 5XFAD Offspring

Female 5XFAD mice showed higher levels of amyloid β42 quantified by ELISA in brain homogenates than males as shown in Figure 5A. These differential levels at 2 months of age agree with those reported in the characterization of the strain 5XFDA, line Tg6799; [32]. The high dispersion of results at this early age might mask any treatment effect. However, TPPU treatment induced a general decrease in hyperphosphorylated tau (p-tau) as shown by the significant effect of treatment in the p-tau/total tau ratio (Figure 5B). Male and female 5XFAD mice showed similar levels of p-tau and the data were analyzed together. Levels of p-tau were low as expected at this age in a strain with

predominantly amyloid pathology. However, there was a trend to higher p-tau in 5XFAD mice that was suppressed by maternal TPPU treatment as shown in Figure 5B,C. Furthermore, the interaction between genotype and treatment reached a borderline significance in p-tau protein levels when normalized by the loading control (p = 0.0638, Figure 5C). Likewise, total tau levels were similar in the different experimental groups (Figure 5D). Thus, there was incipient p-tau pathology protected by maternal TPPU.

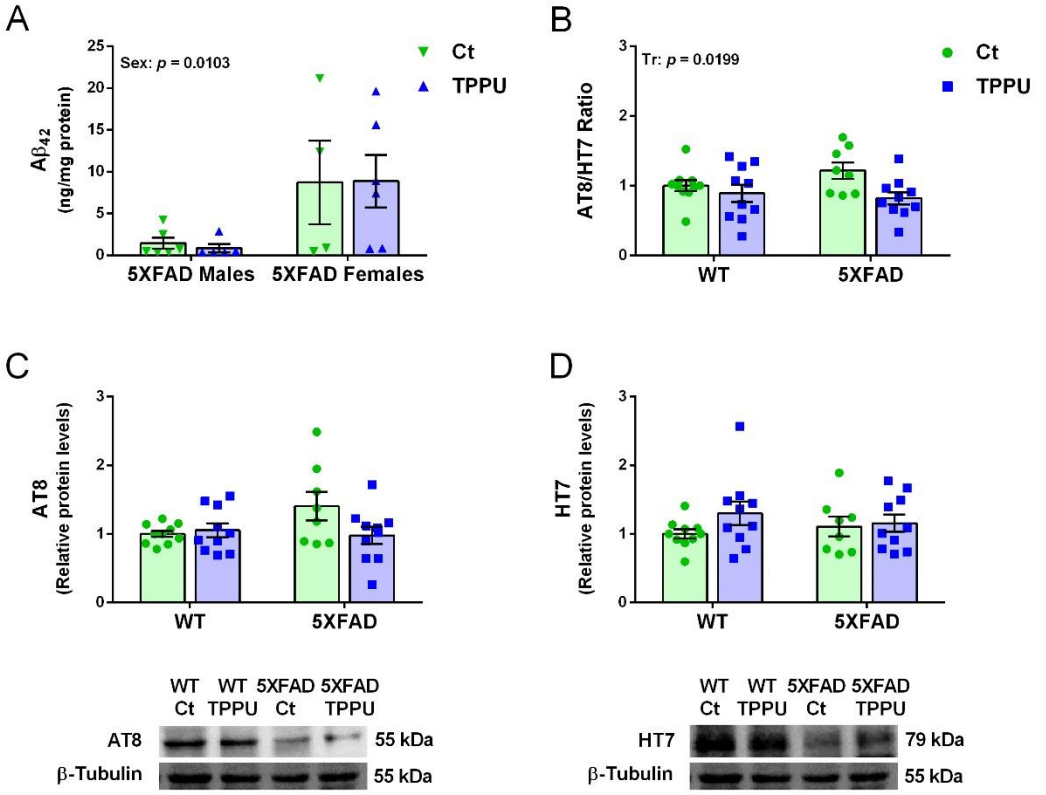

Figure 5. Amyloid β42 (Aβ42) and hyperphosphorylated tau (p-tau) in 2-month-old 5XFAD mice from litters with maternal TPPU treatment or control (Ct). (**A**) Differential Aβ42 concentration in male and female 5XFAD mice; (**B**) ratio of protein levels of p-tau immunodetected by AT8 antibody (values shown in (**C**)) and total tau immunodetected by HT7 antibody (values shown in (**D**)) in WT and 5XFAD offspring. Representative blots are shown below the corresponding histograms in (**C**,**D**). (**A**) Cerebral cortex samples; (**B**–**D**) hippocampus samples. Values are mean ± SEM ((**A**) *n* = 4–6; (**B**–**D**) *n* = 8–10). Statistics: ANOVA, effect of sex (Sex) in (**A**) and treatment (Tr) in (**B**), *p* values of significant factors are indicated in the corresponding graphs; borderline interaction effect (G × Tr) in (**C**) (see text).

2.4. Maternal TPPU Treatment Decreased Neuroinflammation in the Offspring Mice

A relevant microglial gene activated in AD is the triggering receptor expressed on myeloid cells 2 (*Trem2*). Expression of *Trem2* showed a trend to increase in 2-month-old 5XFAD mice as shown in Figure 6A. High dispersion of the data probably caused a lack of genotype significance. Interestingly, offspring of TPPU-treated dams showed a significant decrease of *Trem2* mRNA levels. Seven-month-old 5XFAD showed an 8.8-fold increase in *Trem 2* compared to age-matched WT levels (Supplementary Figure S1A). Regarding the gene epoxide hydrolase 2 (*Ephx2*) that codifies for the sEH enzyme, there is a decrease in the TPPU litters as shown in Figure 6B. This unexpected effect may contribute to decreased neuroinflammation in the offspring brain. Older 5XFAD mice did

not show changes in the *Ephx2* gene (Supplementary Figure S1B). Gene expression levels of the cytokine interleukin 6 (*Il6*) and the chemokine C-C motif ligand 3 (*Ccl3*) were similarly low in all of the young mice (not shown). However, analysis of these markers in the added reference group of 7-month-old 5XFAD mice showed respective fold increases of 2.3 and 36.6 compared to WT mice (Supplementary Figure S1C,D). Finally, the protein concentration of tumor necrosis factor α (TNFα) was similar in the different experimental groups. Therefore, despite a low brain inflammatory status at this early age, there was a protective effect of maternal TPPU treatment. Interestingly, the decrease in neuroinflammatory markers in the offspring was a specific TPPU effect, as it was not mediated by the lower inflammatory status of the dams (plasma levels of C reactive protein (CRP) and TNFα of female progenitors are shown in Supplementary Table S1).

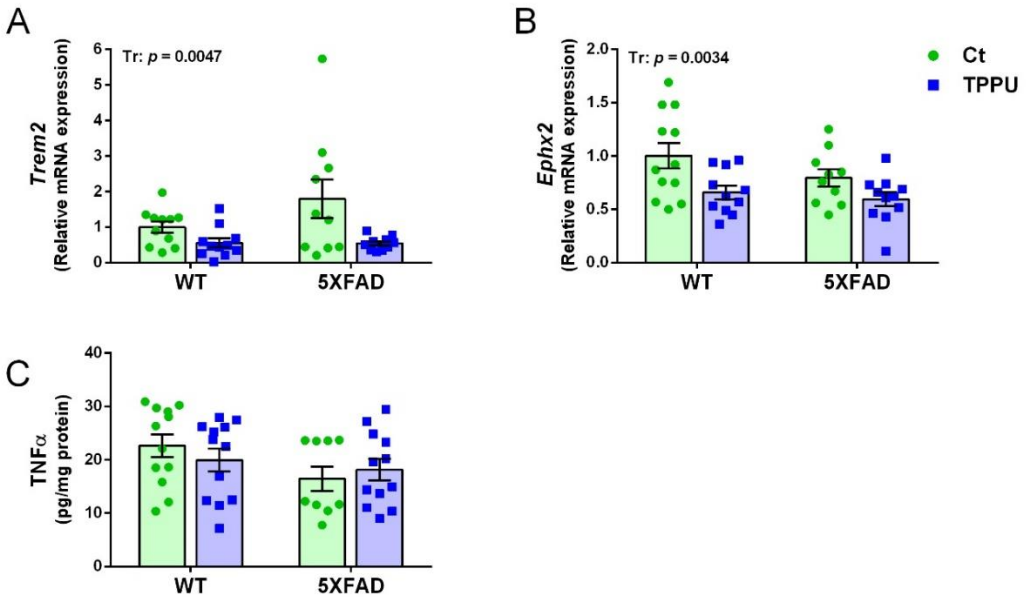

Figure 6. Neuroinflammatory markers in 2-month-old 5XFAD mice from litters with maternal TPPU treatment or control (Ct). Expression levels of the genes *Trem2* (**A**) and *Ephx2* (**B**); (**C**) tumor necrosis factor α (TNFα) concentration (pg/mL). (**A**,**B**) Hippocampus samples; (**C**) cerebral cortex samples. Values are mean ± SEM ((**A**) *n* = 10–11; (**B**) *n* = 10–12; (**C**) *n* = 9–12). Statistics: ANOVA, effect of treatment (Tr) in (**A**,**B**), *p* values of the significant factor are indicated in the corresponding graphs.

2.5. Female Mice Showed Higher Gliosis and Neurodegenerative Changes than Male Mice

Gene expression of the neurodegenerative markers chitinase-like 1 (*Chil1)* and sirtuin 2 (*Sirt2*) showed a significant effect of sex and the experimental groups of males and females were analyzed separately. No effect of genotype or treatment was detected for these genes as shown in Figure 7A,B. However, older male 5XFAD mice showed a 1.8-fold increase in *Chil1* mRNA and a 1.2-fold increase in *Sirt2* mRNA (Supplementary Figure S1E,F). Protein levels of the reactive astrocyte marker glial fibrillary acidic protein (GFAP) also showed a differential effect of sex and no further statistical differences as indicated in Figure 7C. Finally, protein levels of the neuroplasticity transcription factor early growth response 1 protein (EGR1) were also differential in male and female mice as

shown in Figure 7D. Generally, females showed higher levels of these markers than males, suggesting a greater susceptibility to neurodegenerative processes. Interestingly, female responses were not linked to specific treatment or genotype as shown by the absence of statistical differences in the post-hoc analysis of the four markers (Figure 7A–D).

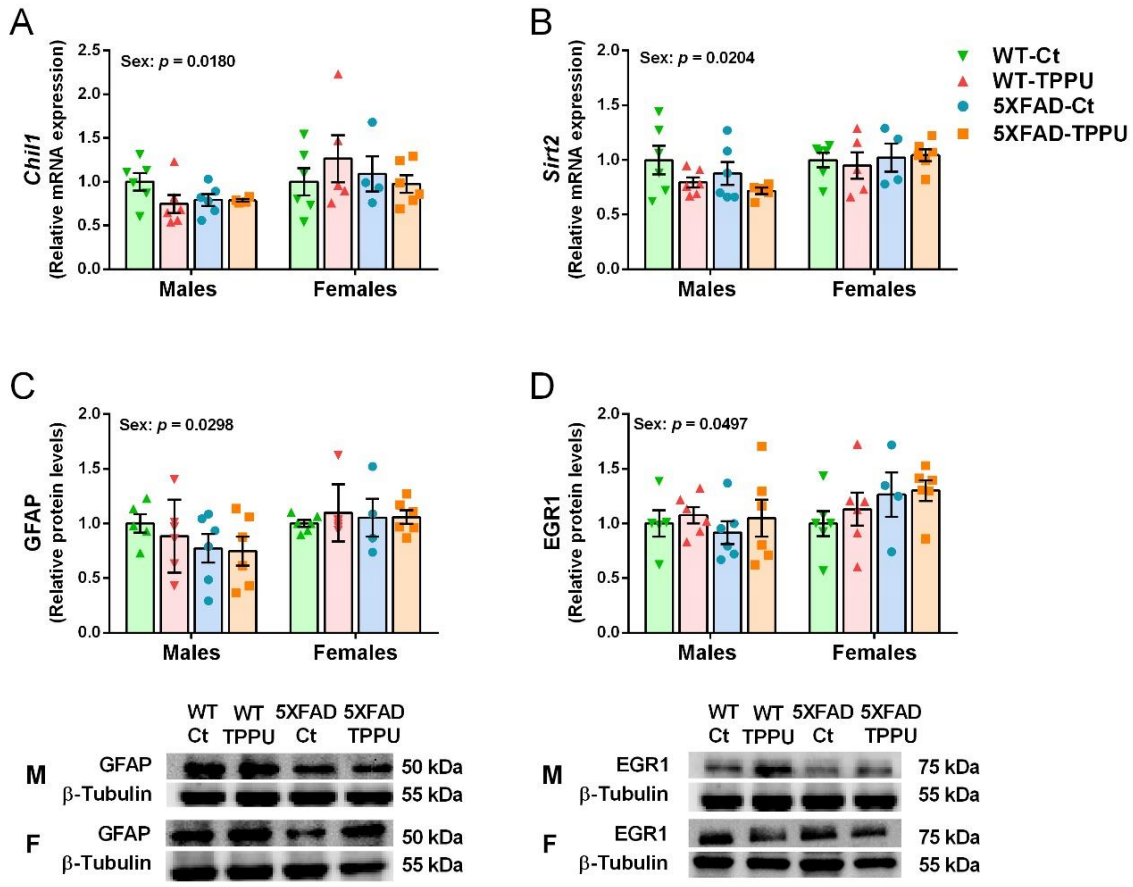

Figure 7. Differential neurodegenerative markers in male and female 5XFAD mice. Expression levels of the genes *Chil1* (**A**) and *Sirt2* (**B**). Relative protein levels of glial fibrillary acidic protein (GFAP) (**C**) and early growth response 1 protein (EGR1) (**D**). Representative blots for males (M) and females (F) are shown below the corresponding histograms in (**C**,**D**). (**A**,**B**) Hippocampus samples; (**C**,**D**) cerebral cortex samples. Values are mean ± SEM ((**A**–**D**) n = 4–6). Statistics: ANOVA, effect of sex in (**A**–**D**), p values of the significant factor are indicated in the corresponding graphs. The Bonferroni post-hoc test was not significant in the comparison between specific experimental groups of males and females.

3. Discussion

The present study examined the effects of a pharmacological maternal intervention with TPPU during pregnancy and lactation, showing that it could prevent or delay the course of AD in offspring that inherited familial AD mutations. Behavioral performance and underlying changes in epigenetic mechanisms and AD hallmarks were analyzed. Total preservation of learning and memory induced by maternal TPPU in the 2-month-old offspring with the 5XFAD genotype is remarkable. Cognitive loss in these mice is induced by early amyloidosis from 1.5–2 months of age linked to five familial AD mutations, as reported in the strain characterization [32]. These authors did not detect impairments in the Y-maze at that early age. However, our current results of early deficits of

recognition memory in the ORT and spatial memory in the OLT agree with previous findings of 5XFAD deficits in the Morris water maze test at 2 months of age [33]. Furthermore, the decrease of a hyperactivity trend by TPPU indicates maintenance of brain circuitry. Hyperactivity later turns to decreased motor activity in AD mice as seen in the reported comparison between 2-month-old and 8-month-old 5XFAD [33]. This mild neuropathology sign may be related to synaptic excitability reported in other mouse models [34] and the neuropsychiatric symptom of agitation, common in AD patients [35]. Here the treatment with TPPU, as an inhibitor of the sEH enzyme, would increase the maternal endogenous levels of EET arachidonic acid metabolites to exert its beneficial effects. In addition, TPPU is expected to reach fetuses and lactating pups as it crosses the blood-brain barrier [36]. As mentioned above, there is no doubt about the importance of maternal influences on offspring neurodevelopment [37]. For instance, dietary supplementation of rat dams with the essential PUFAs linoleic and linolenic fatty acid during gestation and lactation was reported to anticipate reflex maturation and improve memory in the offspring [38]. However, reports of beneficial effects of pharmacological maternal treatments on offspring at risk for brain disease are scarce. In a similar experimental setting, TPPU has protected juvenile mice from neuropsychiatric-like traits induced by maternal infection during pregnancy [39].

In the epigenetic analysis, our finding of higher global DNA methylation as the 5-mC/5-hmC ratio in the 2-month-old 5XFAD mice compared to the WT mice suggests an abnormal increase in DNA methylation leading to a decrease in gene transcription. In addition, relative gene expression of both DNMTs and TETs enzymes compared to WT mice generally showed a tendency to increase as compared to WT. These results are in agreement with previous findings in 5XFAD at this young age [33]. Furthermore, accumulation of both 5-mC and 5-hmC with increasing age has been reported in an AD mouse model [40]. Interestingly, maternal TPPU reduced the 5XFAD 5-mC/5-hmC ratio to WT levels and on the contrary increased that of WT. In the end, offspring of both genotypes of TPPU-treated dams reached similar levels. DNA methylation is a major regulator of gene transcription [41] and an aberrant DNA methylation pattern is known in AD brain [42]. It is known that an improved regulation of this epigenetic mechanism during development benefits synaptic plasticity and cognitive function in the offspring [41]. This may be the case here, as we found an effect of the treatment factor in memory tests, corroborating the general cognitive enhancement in the offspring of TPPU-treated dams.

On the other hand, we did not detect changes in the expression of the genes Hdac1 and Hdac2 in 5XFAD young mice compared to WT mice. However, some authors have reported HDAC2 elevation in mouse models and AD brain and demonstrated its involvement in the blockade of cognitive functions [33,43]. Other authors reported a decrease in HDAC1 and 2 in AD brain [44] that may be associated with advanced pathology. Interestingly, general inhibition of Hdac1 and Hdac2 by maternal TPPU may induce preventive and protective mechanisms against neurodegeneration in WT and 5XFAD mice, respectively. Histone deacetylation is a crucial epigenetic mechanism that regulates gene expression and can modulate neurodevelopment and brain function

[45]. Particularly, dysregulation of the transcriptional repressors HDAC1 and 2 are important drivers of neurodegeneration and AD [42,46]. HDAC1 and 2 are negative regulators of synaptic plasticity and memory formation and circumventing its epigenetic blockade would be beneficial against neurodegeneration [47,48]. Inhibition of both HDAC1 and 2 has shown synaptic improvement in experimental systems that model the early stages of AD [49]. In addition, HDAC inhibition has been shown to be protective against Aβ-induced hyperphosphorylation of tau through inhibition of tau kinase enzymes [50].

The epigenetic changes induced by maternal TPPU in 5XFAD offspring suggest involvement of epigenetic signaling in the modulation of AD traits during brain development. Furthermore, epigenetic changes in WT offspring suggest improved neurodevelopment as an acquired resilience against neurodegeneration. The implications of epigenetic changes in the initiation and development of AD are not well understood, but they may fill the gap between genetic and environmental risk [42].

Remarkably, we found a decrease in p-tau by maternal TPPU treatment. However, TPPU did not decrease genetically-driven Aβ42 in 5XFAD offspring. Tau hyperphosphorylation is induced by proinflammatory mediators released by reactive microglia under amyloid pathology [51]. Therefore, we hypothesize that both HDAC inhibition of tau kinase activity and the absence of microglial pro-inflammatory mediators, as discussed below, decreased p-tau levels downstream of Aβ generation in the 2-month-old mice. Cognitive benefices paralleling p-tau decrease in the offspring are in agreement with the fact that PET detection of tau tangles is the pathological sign most consistent with cognitive impairment and neurodegeneration in AD patients [52].

Neuroinflammation was detected at the age of 2 months as a trend towards an increase in the microglial activation marker gene *Trem2*. However, neuroinflammation was a prominent sign in the 7-month-old 5XFAD mice, in agreement with its relevance in AD triggering and progression [53,54]. Therefore, polarization of microglia to a reactive phenotype is an early event after Aβ42 accumulation in these mice. TREM2 has also been found increased in the cerebral cortex of autosomal dominant AD patients [55]. Microglia play an important role in the innate immune response of the brain and their phenotypic changes may protect against neurodegeneration or worsen it when chronically activated [55,56]. Interestingly, maternal TPPU maintained low levels of Trem2 in both 5XFAD and WT mice. Microglia plasticity is modulated by epigenetic modifications including DNA methylation and histone acetylation reported here [57,58]. Therefore, we may speculate that microglia were imprinted early by epigenetic changes induced by TPPU treatment to a neuroprotective phenotype, although further investigations would be required to confirm the involvement of either HDACs inhibition or specific DNA gene methylation changes.

Strikingly, TPPU treatment during brain development induced long-term downregulation of the sEH gene *Ephx2*. This would maintain an anti-inflammatory milieu in the brain of the offspring. Several epigenetic mechanisms may modulate *Ephx2* expression, including DNA methylation [59]. On the other hand, no change in the

peripheral inflammatory marker CRP [60] was found in the dams at the end of their treatment with TPPU.

Finally, the increase in female mice in four biomarkers of neurodegeneration compared to males indicates early sex differences in the mouse phenotype. Expression of the genes *Chil1* and *Sirt2* was increased in the 7-month-old 5XFAD mice as compared to the WT mice, but we found a general increase in young female offspring as compared to the males. Sirtuin 2 is a histone deacetylase belonging to the sirtuin family. In contrast to other sirtuins, sirtuin 2 promotes neurodegeneration in experimental models [61] and some SIRT2 variants increase AD risk [62]. Chitinase-like 1 is a marker of reactive gliosis and the innate immune response which shows human counterpart (chitinase 3-like 1 or YKL-40) increases in the CSF of AD patients, although changes in postmortem AD brain are controversial [63,64]. Young female offspring also showed a general increase in protein levels of GFAP and EGR1 as compared to the male offspring. The reactive astrocyte marker GFAP is increased in AD brain and in AD mouse models [32,63]. GFAP and chitinase-like 1 are differentially related to AD but both may mediate brain atrophy and cognitive impairment [65]. In addition, indicating some neurodegeneration risk, the female increase in EGR1 may be analogous to the reported increase in EGR1 transcript preceding pathology in the early stages of AD brain [66]. Next, there is a decreased expression of this neuroplasticity gene with advanced pathology as we previously reported in 7-month-old 5XFAD mice [17]. A mild increase in these AD-related pathological markers in 2-month-old female mice compared to male mice is consistent with a higher risk of developing AD in the female sex. Indeed, women are more likely to suffer AD than men [67,68].

4. Materials and Methods

4.1. Animal Breeding and Experimental Design

Heterozygous 5XFAD male mice were bought from Jackson Laboratories (Bar Harbor, ME, USA) and B6SJLF1/J hybrid female mice were bought from JANVIER LABS (Le Genest-Saint-Isle, France), Male 5XFAD and female B6SJLF1/J were crossed to breed WT and 5XFAD offspring, following the breeding directories from Jackson Laboratories. Each male was mated overnight with two females. Then, each female was transferred to a new cage to start the treatment.

TPPU (Sigma-Aldrich, Darmstadt, Germany) was administered orally within the drinking water during pregnancy and lactation, mixed with cyclodextrin 3% (Sigma-Aldrich) to improve its solubility. Control females had the same dose of cyclodextrin in the drinking water. TPPU dose was decided according to previous studies from our group 1; hence, treated females were ensured with a constant daily dose of 5 mg/kg of TPPU. For this purpose, the concentration of the compound in the drinking water was adjusted twice per week according to the body weight and water consumption. Six TPPU-treated and six control dams and their respective litters remained undisturbed until pup weaning at 21 days of age. All pups were sexed and identified by ear punching. Tissue samples obtained from ear punches were used for genotype identification by PCR. Mice were

housed according to sex and litter. No further treatment was administered to the offspring.

Male and female offspring at 2 months of age were used in this study. The total of 71 animals obtained from the control or TPPU-treated dams were distributed into the corresponding experimental groups as follows: WT Control (WT-Ct), *n* = 7 males and *n* = 7 females; WT TPPU (WT-TPPU), *n* = 10 males and *n* = 10 females; 5XFAD Control (5XFAD-Ct), *n* = 7 males and *n* = 5 females; 5XFAD TPPU (5XFAD-TPPU), *n* = 13 males and *n* = 12 females. All of the mice were submitted to behavioral testing and then euthanized to obtain brain tissue for molecular analysis. A scheme of the experimental design is shown in Figure 8.

A group of untreated male 5XFAD mice (*n* = 8) at the age of 7 months with advanced AD pathology and an age-matched group of WT mice (*n* = 8) were added to the study as a reference for selected molecular analysis of brain tissue.

Animal procedures were carried out in the Animal Unit of the University of Barcelona (UB), Spain. The mice were housed in Makrolon® cages (Tecniplast, Buguggiatta, Italy), with free access to food and water and a room temperature maintained at 22 ± 2 °C and a 12 h light/12 h dark cycle. Approval for the study and experimental protocols was confirmed by the local animal experimentation ethics committee (CEEA-UB) under the guidelines of the Animal Experimentation Commission of the Autonomous Government of Catalonia (approval reference: 22/20 from date 5 November 2020). All procedures were performed in accordance with the European Commission Council Directive 86/609/EEC on this subject.

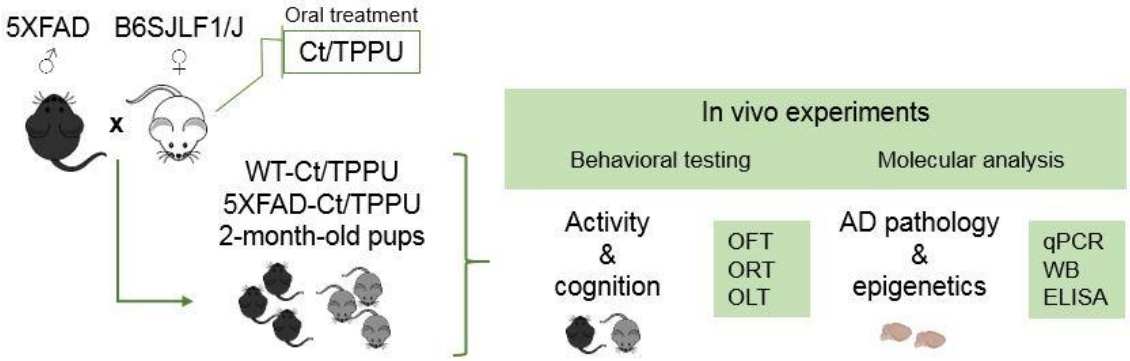

Figure 8. Experimental design scheme. Two-month-old WT and 5XFAD offspring of dams treated with vehicle (Ct) or TPPU were submitted to behavioral testing and molecular analysis. Abbreviations: ELISA: enzyme-linked immuno sorbent assay, OFT: open field test, OLT: object location test, ORT: object recognition test, qPCR: real-time quantitative polymerase chain reaction, WB: Western blotting.

4.2. Sample Collection

Immediately after the pups were weaned, the dams were anesthetized with isoflurane to obtain blood samples by cardiac puncture and euthanized. The blood was collected in heparinized tubes and centrifuged for 10 min at 2000× *g* using a refrigerated centrifuge. The plasma was collected and stored at −80 °C until testing.

Brain tissue samples were obtained from 2-month-old mice at necropsy. The brain was dissected out on a cold plate, and the cerebral cortex (CC) and hippocampus (HC) tissue were snap-frozen in liquid nitrogen and stored at −80 °C until molecular testing. Relative expression of specific genes and semiquantitative protein levels were analyzed in both CC and HC samples with generally similar results. For better clarity, the results of one of the regions were selected as indicated in the figure legends. HC tissue from 7-month-old mice was used as a reference for the expression of selected genes. ELISA determinations were performed in CC.

4.3. Behavioral Procedures

Open field (OF). Locomotor activity and general behavior were analyzed in a 50 × 50 × 20 cm white maze. Each mouse was gently placed in the center of the arena and allowed to move freely for 5 min. A computerized video tracking system (SMART v3.0, Panlab S.A., Barcelona, Spain) was used to measure the traveled distance and the ambulatory pattern. Rearing and grooming activities were also analyzed.

Object recognition test (ORT). The assessment of recognition memory is based on the mouse's tendency to explore novel objects longer than familiar ones. Each mouse went through successive 10-min sessions in a 30 × 40 × 30.5 cm black maze. In the habituation, the mouse was allowed to explore the arena without any objects for two consecutive days. The next day, in the acquisition trial, the mouse was allowed to explore two identical plastic objects (A + A) that were located equidistant from the corners. Two h later, the mouse was tested in the retention trial where one of the objects was replaced by another object with a different shape and color (A + B). All of the sessions were videotaped and blindly analyzed to measure the exploration time (t) of each object. Object exploration was defined as the orientation of the nose to the object at a distance smaller than 2 cm. A discrimination index was calculated as [novel object (t)—familiar object (t)]/[total (t) novel + familiar].

Object location test (OLT). The assessment of spatial memory is based on the mouse's tendency to explore a newly located object for a longer time than an unmoved object. The test uses the same apparatus and general procedure as ORT with some modifications. The habituation and test sessions lasted 5 min. In the acquisition trial, the mouse explored two identical objects (A1 + A2) located equidistant from each other and near one of the long walls. In the 2 h retention trial, the mouse was allowed to explore the same objects but one of them was moved to a position near the opposite wall (A1 + A3). In OLT, the DI was calculated as [novel location (t)—familiar location (t)]/[total (t) novel + familiar].

4.4. Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Extraction of total RNA from the brain tissue samples was carried out using a mirVanaTM mRNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. RNA content was determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Reverse transcription from RNA to first-strand complementary DNA (cDNA) was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific, Waltham, MA, USA). Three hundred ng of RNA per sample were loaded in a thermal cycler (FlexCycler, Analytikjena, Jena, Germany). cDNA samples were stored at −20 °C until use.

Gene expression was analyzed by qPCR using TaqMan® Fluorescein amidite (FAM)-labeled specific probes (Thermo Fisher Scientific, Waltham, MA, USA) and a Quantimix Easy Probe kit (Biotools, Madrid, Spain). The reaction mix containing 6.75 ng of cDNA was loaded in a CFX96TM Real-Time System (Bio-Rad, Hercules, CA, USA). Alternatively, SYBR Green qPCR was performed in a Step One Plus Detection System (Applied Biosystems). Each reaction mixture contained 6.75 µL of cDNA (which concentration was 2 µg), 0.75 µL of each primer (the concentration of which was 100 nM), and 6.75 µL of SYBR Green PCR Master Mix (2X) (Applied Biosystems). The Taqman assay probes and primers used are listed in Supplementary Tables S2 and S3, respectively. The samples were analyzed in duplicate.

The results were normalized to actin beta (*Actb*) gene expression using the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method.

4.5. Western Blotting (WB)

Protein extracts were obtained from the brain tissue samples. The tissues were homogenized in a cold RIPA lysis buffer supplemented with protease and phosphatase inhibitors, ultrasonicated, and centrifuged. The protein concentration of the supernatants was determined using the Bradford protein assay (Bio-Rad). Forty µg of protein per sample were denatured by boiling, loaded into polyacrylamide gels, and separated by SDS-PAGE at 100 V. Electrophoresed proteins were transferred to PVDF membranes by electroblotting at 200 mA for 90 min. Then, the membranes were incubated with a blocking buffer for 1 h followed by 4 °C overnight incubation with primary antibodies. Secondary antibodies were peroxidase-conjugated. The antibodies used are listed in Supplementary Table S4. The proteins were visualized by enhanced chemiluminescence detection in a Chemidoc™ Imaging System (Bio-Rad). The densitometric analysis was performed using Image Lab software (v3.0.1, Bio-Rad). The protein levels were normalized using actin, β-tubulin, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

4.6. Enzyme-Linked Immunosorbent Assay (ELISA)

The plasma levels of CRP were measured with the Mouse CRP ELISA Kit (Invitrogen, Thermo Fisher Scientific).

TNFα was measured with the Mouse TNF alpha uncoated ELISA kit (Invitrogen, Thermo Fisher Scientific) in plasma and brain tissue samples.

Global DNA methylation was determined with the MethylFlash Methylated DNA 5-mC Quantification kit (Colorimetric) (EpiGentek, Farmingdale, New York, NY, USA). Global DNA hydroxymethylation was determined with the MethylFlash Global

Hydroxymethylation (5-hmC) ELISA Easy Kit (Colorimetric) (EpiGentek). DNA methylation and hydroxymethylation were measured in brain samples.

The Human $A\beta_{42}$ ELISA Kit (Invitrogen, Thermo Fisher Scientific) was used to quantify $A\beta_{42}$ in 5XFAD mouse brains.

Sandwich ELISA assays were performed according to the specific manufacturer's instructions. Absorbances were measured in a spectrophotometer Multiskan SkyHigh (Thermo Fisher Scientific) and the corresponding concentrations were calculated from standard curves. The samples were tested in duplicate.

4.7. Statistical Analysis

The results are expressed as mean ± SEM. The normality of the data was tested with the Shapiro–Wilk test. The data were analyzed by ANOVA with the factors: sex, genotype, and treatment. In the absence of a sex effect, male and female data were jointly analyzed by two-way ANOVA (main factors: genotype and treatment, and its interaction). The presence of a statistical significance of the ANOVA factors or its interaction is indicated in the figures. In this two x two ANOVA design, a comparison between the group means was performed after a significant interaction of main factors using Bonferroni's post-hoc test. In the presence of a sex effect, male and female experimental groups were analyzed separately. We first checked for genotype or treatment effects for each sex. In their absence, we analyzed the male and female data by two-way ANOVA (main factors: sex and experimental group, and its interaction). The Bonferroni post-hoc test was used to discern whether sex differences could be attributed to any of the four experimental groups. The Student's t-test was used to analyze the significance between the two experimental groups, where indicated (supplementary groups of 7-month-old animals). Statistical outliers were identified by Grubbs' test ($\alpha = 0.5$). The results were considered significant when the p-value < 0.05. The data were analyzed using the GraphPad Prism 6.01 package (GraphPad Software, Inc, La Jolla, CA) and IBM SPSS Statistics v23 (IBM Corp., Armonk, New York, NY, USA).

5. Conclusions

Two-month-old 5XFAD mice showed memory loss and mild AD-like neuropathology comparable to the early stages of AD. A maternal pharmacological intervention with TPPU, which increases endogenous EETs, protected the mice against cognitive impairment and reduced p-tau and microglial reactivity. This protection was achieved despite sustained $A\beta_{42}$ generation driven by familial AD mutations in the 5XFAD mouse model. Changes in 5-mC/5-hmC and histone deacetylase machinery in both WT and 5XFAD offspring demonstrated that the epigenetic mechanism substantially contributed to brain resilience and neuroprotection, thus reinforcing the importance of beneficial maternal interventions. Furthermore, female offspring mice reproduced the higher AD risk described in women, as shown by higher amyloid levels in 5XFAD mice and increased AD risk markers in all of the females. Hence, the results obtained may contribute to understanding the connection between early events and late neurodegeneration and designing effective therapies to increase brain resilience and prevent AD. Finally, the

interest of a time-course study in the offspring should be considered to confirm the maternal TPPU neuroprotection found in early AD up to advanced pathological stages.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1.

Author Contributions: Conceptualization, C.S. (Coral Sanfeliu); methodology, R.C. and C.B.; validation, C.S. (Cristina Suñol) and C.G.-F.; formal analysis, C.B., A.I., A.V., S.A., and E.G.-L.; investigation, R.C., M.P., C.G.-F., and C.S. (Coral Sanfeliu); data curation, C.B., C.S. (Cristina Suñol), and C.S. (Coral Sanfeliu); writing—original draft preparation, C.S. (Coral Sanfeliu); writing—review and editing, M.P., C.G.-F., and C.S. (Coral Sanfeliu); visualization, C.B.; supervision, C.G.-F. and C.S. (Coral Sanfeliu); project administration, C.S. (Coral Sanfeliu); funding acquisition, M.P. and C.S. (Coral Sanfeliu). All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of Animal Experimentation (CEEA) of the University of Barcelona (authorization number 22/20 on 5 November 2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are included in the article and Supplementary Materials.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Materials

Neuroprotective epigenetic changes induced by maternal treatment with an inhibitor of soluble epoxide hydrolase prevents early Alzheimer's disease neurodegeneration

Clara Bartra, Alba Irisarri, Ainhoa Villoslada, Rubén Corpas, Samuel Aguirre, Elisa García-Lara, Cristina Suñol, Mercè Pallàs, Christian Griñán-Ferré, Coral Sanfeliu.

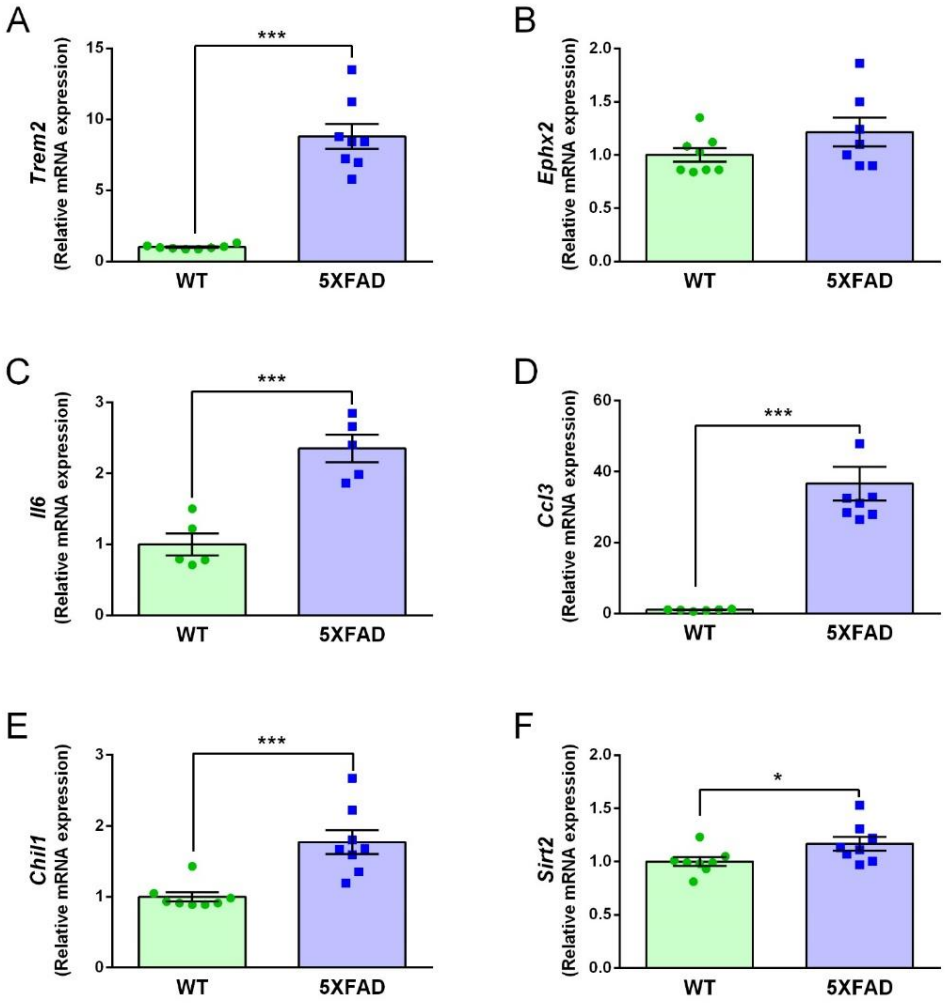

Figure S1. Gene expression of inflammatory and neurodegenerative biomarkers in the hippocampus of 7-month-old 5XFAD male mice. Increased relative mRNA values of 5XFAD mice compared to wild-type (WT) littermates of the genes: (**A**) Triggering receptor expressed on myeloid cells 2, *Trem2*; (**B**) Epoxide hydrolase 2, *Ephx2*; (**C**) Chitinase-like 1, *Chil1*; (**D**) Sirtuin 2, *Sirt2*; (**E**) Interleukin 6, *Il6*; (**F**) Chemokine C-C motif ligand 3, *Ccl3*. Values are mean ± SEM (n = 5 - 8). Statistics: Student's t test, *$p < 0.05$, ***$p < 0.001$.

Table S1. CRP and TNFα ELISA results from mouse dams plasma samples

		Ct	TPPU
CRP	µg/mL (mean ± SEM)	10.36 ± 0.81	12.77 ± 1.36
	n	6	6
TNFα	pg/mL (mean ± SEM)	< 2	< 2
	n	6	5

Abbreviations: CRP, C-reactive protein; TNFα, Tumor necrosis factor α.

Table S2. TaqMan Assays used in qPCR analysis

Gene Name	Gene Symbol	Assay ID
Actin, beta	*Actb*	Mm02619580_g1
Brain derived neurotrophic factor	*Bdnf*	Mm04230607_s1
Chemokine C-C motif ligand 3	*Ccl3*	Mm00441259_g1
Chitinase-like 1	*Chil1*	Mm00801477_m1
Epoxide hydrolase 2	*Ephx2*	Mm01313813_m1
Histone deacetylase 1	*Hdac1*	Mm02391771_g1
Histone deacetylase 2	*Hdac2*	Mm00515108_m1
Interleukin 6	*Il6*	Mm00446191_m1
Sirtuin 2	*Sirt2*	Mm01149204_m1
Triggering receptor expressed on myeloid cells 2	*Trem2*	Mm00451744_m1

Table S3. Primers used in SYBR Green qPCR analysis

Gene name	Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')
Actin, beta	*Actb*	CAACGAGCGGTTCCGAT	GCCACAGGTTCCATACCCA
DNA methyltransferase (cytosine-5) 1	*Dnmt1*	GGGCTGTGCTTCCTGTCG	GGTGTCCCCAAGCTTGTTCT
DNA methyltransferase 3A	*Dnmt3a*	GGGCCACACGGCAGAG	CACGGTTCTCCTCCTGTTCC
DNA methyltransferase 3B	*Dnmt3b*	TGCCAGACCTTGGAAACCTC	GCTGGCACCCTCTTCTTCAT
Tet methylcytosine dioxygenase 1	*Tet1*	CCTGCCTCTTCTACGGGAAC	GATTTGGAAGGCTTTGCGGG
Tet methylcytosine dioxygenase 2	*Tet2*	CCATCATGTTGTGGGACGGA	ATTCTGAGAACAGCGACGGT
Tet methylcytosine dioxygenase 3	*Tet3*	GGGCAGGCAGCGTAGC	CAGGATCTGGGGCAAGACAG

Table S4. Antibodies used for Western blot analysis

Antibody	Host	Source (catalog)	WB dilution
Actin	Rabbit	Sigma-Aldrich (#A5060)	1:10,000
Early growth response protein 1 (EGR-1)	Rabbit	Cell Signaling (#4153)	1:1000
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Mouse	Assay designs (#CSA-335)	1:5000
Glial fibrillary acidic protein (GFAP)	Mouse	Sigma-Aldrich (#G3893)	1:500
p-Tau clone AT8	Mouse	Thermo Fisher Scientific (#MN1020)	1:1000
ß-Tubulin	Mouse	Abcam (#ab21754)	1:10,000
Total tau clone HT7	Mouse	Thermo Fisher Scientific (#MN1000)	1:1000
Sheep-anti-mouse HRP conjugated		Amersham (GE) (#NA931)	1:2000
Donkey-anti-rabbit HRP conjugated		Amersham (GE) (#NA934)	1:2000

Publication 2.

High levels of anti-inflammatory epoxyeicosatrienoic acids may improve neurodevelopment in children prenatally exposed to mercury

Clara Bartra, Sabrina Llop, Julia Kuligowski, Abel Albiach-Delgado, Cristina Suñol, Eduard Rodríguez-Farré, Ferran Ballester, Raquel Soler-Blasco, Beatrice Jora, Júlia Jarné-Ferrer, Christian Griñán-Ferré, Santiago Vázquez, Mercè Pallàs and Coral Sanfeliu

Pending for submission.

High levels of anti-inflammatory epoxyeicosatrienoic acids may improve neurodevelopment in children prenatally exposed to mercury

Clara Bartra[1,2], Sabrina Llop [3,4], Julia Kuligowski[5], Abel Albiach-Delgado[5], Cristina Suñol[1], Eduard Rodríguez-Farré[1], Ferran Ballester[3,4,6], Raquel Soler-Blasco[3,4,6], Beatrice Jora[7], Júlia Jarné-Ferrer[7], Christian Griñán-Ferré[7,8], Santiago Vázquez[7], Mercè Pallàs[7,8], Coral Sanfeliu[1]

[1] Institut d'Investigacions Biomèdiques de Barcelona (IIBB), CSIC, Barcelona, Spain

[2] PhD Program in Biotechnology, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, 08034 Barcelona, Spain

[3] Epidemiology and Environmental Health Joint Research Unit, FISABIO-UJI-UV, Valencia, Spain

[4] CIBERESP, Instituto de Salud Carlos III, Madrid, Spain

[5] Neonatal Research Group, Health Research Institute La Fe, Valencia, Spain

[6] Dept. of Nursing, Universitat de València, Spain

[7] Dept. Pharmacology and Therapeutic Chemistry, Faculty of Pharmacy and Food Sciences, UBNeuro and IBUB, Universitat de Barcelona, Spain

[8] CIBERNED, Instituto de Salud Carlos III, Madrid, Spain

Keywords: Neurodevelopment, human perinatal samples, oxylipins, mercury, 5XFAD mice, soluble expoxyde hydrolase

Abstract: A balanced concentration of polyunsaturated fatty acids (PUFA) during the prenatal stage is essential for children's neurodevelopment. Also, animal studies have shown that high levels of anti-inflammatory PUFA metabolites in the embryonic brain protect against genetic or environmental insults that could otherwise cause cognitive impairment. Here we used liquid chromatography - mass spectrometry to analyze oxylipins, the oxygenated metabolites of PUFA, in the placenta tissue (N=12) and cord blood plasma (N=39) of children from the INMA cohort (Valencia, Spain). Both tissues were from independent donors with whole cord blood characterized for total mercury levels as a reference for developmental neurotoxic methylmercury (MeHg) exposure during pregnancy. Children's samples were subsequently categorized into low and high mercury exposure groups. Deleterious effects of high mercury were confirmed in the placenta, evidenced by decreased proteasome activity and inhibitory epigenetic changes. Children's neurodevelopment was assessed using the Bayley and McCarthy

scales at 14 months and 5 years of age, respectively. Spearman correlation coefficients were calculated between the scores and oxylipins levels. We also analyzed cortical brain tissue from wild type and transgenic Alzheimer's disease 5XFAD mice that were dosed for 2 months with either a vehicle or the soluble epoxide hydrolase enzyme (sEH) inhibitor UB-BJ-02 (5 mg/Kg, N=5/group). Inhibition of sEH increases the levels of the epoxyeicosatrienoic acid isomers (EETs) by inhibiting their hydrolysis into dihydro-EETs (DiHETs). EETs are potent anti-inflammatory oxylipins derived from arachidonic acid (AA). We found that DiHETs were increased in the placenta of high mercury exposure group. Furthermore, both EETs and DiHETs in placenta tissue showed general positive correlations with Bayley's mental scale scores in 14-month-old children exposed prenatally to low mercury levels. Interestingly, cord blood DiEHTs positively correlated with McCarthy's working memory and numerical score at 5 years in the high mercury exposure group. Oxylipins derived from docosahexaenoid acid (DHA) and eicosapentanoic acid (EPA) showed lesser positive correlations with neurodevelopment. Therefore, EETs and other anti-inflammatory oxylipins showed a differential signaling according to age and mercury exposure levels. Additionally, other findings highlights the prenatal signaling importance of prostaglandins and thromboxanes in both mercury exposure groups. Finally, mouse treatment with the drug UB-BJ-02 showed increased EETs levels and decreased pro-inflammatory markers in brain tissue as expected. Both human and experimental results showed that EETs are prominent anti-inflammatory metabolites and support maternal EETs supplementation or sEH inhibition as an anti-inflammatory treatment to increase brain resilience against any environmental risk of neurodevelopmental damage.

1. Introduction

Polyunsaturated fatty acids (PUFAs) are essential fatty acids obtained from food sources. In the body, they mainly localize in cell membranes and exert multiple regulatory functions. Specifically, the long chain PUFA arachidonic acid (AA, C20:4, ω-6), eicosapentanoic acid (EPA, C20:5 ω-3), and docosahexaenoic acid (DHA, C22:4 ω-3) are involved in building the structure and functioning of the central nervous system (1). A balanced concentration of ω-6 and ω-3 PUFAs during the prenatal stage is crucial for the development of the children's brain. Deficiency of ω-3 fatty acids or excess of ω-6 / ω-3 ratio is linked to neurodevelopmental disorders (2). Deficiency of ω-6 PUFAs is rare, and moreover, their consumption has progressively increased to unhealthy levels, as indicated for excessive intake of linoleic acid (LA, C18:2 omega-6) in the Western diet (3).

Oxylipins, which are oxidized metabolites of PUFAs, are formed locally on demand via cytochrome P450 (CYP), cyclooxygenase (COX) and lipoxygenase (LOX) family enzymes, and they are the main effectors of PUFAs activity. Free oxylipins interact with cell receptors or intracellular effectors to mediate their biological effects (4). The COX and LOX pathways produce oxylipins that act primarily on inflammatory and vascular mechanisms, with either adverse or beneficial effects depending on their precursor fatty acid: AA or EPA, respectively (1). Additionally, LOX metabolism of DHA also produces

oxylipins with generally anti-inflammatory and beneficial vascular effects (4). The CYP family enzymes can have epoxygenase or ω-hydroxylase activity. CYP ω-hydroxylation results in a series of EPA metabolites with resolution of inflammation properties. Conversely, CYP epoxygenase products from LA have proinflammatory and generally adverse effects. However, the expoxy metabolites from EPA, DHA and AA are considered beneficial oxylipins. Specifically, AA-derived epoxyeicosatrienoic acids (EETs) are potent anti-inflammatory and neuroprotective agents (5,6). Epoxyde oxylipins are rapidly metabolized by the soluble epoxyde hydrolase enzyme (sEH) into their less active diol metabolites, dihydroeicosatrienoic acids (DiHETs). Experimental treatments that increase EETs concentration by inhibiting sEH activity have demonstrated significant therapeutic potential against a range of inflammatory, vascular and neurodegenerative diseases (7–10). Interestingly, EETs may also protect the embryonic brain against genetic or environmental insults that would otherwise cause cognitive impairment. Female mice treated during pregnancy and lactation with a sEH inhibitor (sEHI) prevented or delayed memory loss in Alzheimer's disease transgenic (TgAD) mouse offspring at 2 months of age, compared to the offspring of females treated with vehicle (11). Furthermore, a similar treatment of pregnant mice with sEHI protected offspring against neurodevelopmental disorders and cognitive deficits caused by maternal immune activation (12).

Environmental exposure to mercury and specifically methylmercury (MeHg), the most prevalent organic form of mercury, is a harmful risk for embryonic neurodevelopment. MeHg is highly neurotoxic (13) and has the capacity to cross the placenta (14) and the blood-brain barrier (15). Several poisoning episodes caused severe neurodevelopmental impairment in children born in the affected areas (16,17). However, current safety concern focusses on the chronic low to moderate maternal intake of MeHg through diet during pregnancy. The reference dose (RfD) for safe dietary intake of MeHg is estimated in 0.1 µg/kg body weight/day by the U.S. Environmental Protection Agency (18). The corresponding safe level in blood is 5.8 µg/L. Heavy consumers of seafood may easily reach this level. Oily fish at the higher end of the trophic chain contain elevated levels of MeHg (19). However, it is suggested that the RfD for pregnant or lactating women be halved. Total mercury (T-Hg) levels in blood are widely used as a surrogate for MeHg levels. MeHg in blood accounts for around 72% of the T-Hg in the general population (20), but it may reach up to 96% in a population of heavy fish consumers (21). A correlation between prenatal mercury exposure and decreased fetal brain growth has been proved in epidemiological studies (22,23). However, the impact on the neurodevelopment of children is controversial, with reports of minor impairment or delay (24) and late cognitive deficits (25). Furthermore, two recent systematic reviews reached differing conclusions. One study suggested that dietary mercury exposure during pregnancy was an unlikely risk factor for low neurodevelopmental functioning in children up to 5 years of age (26), while the other found limited evidence of neurodevelopmental impairment in cohorts of children up to 12 years old (27). Genetic factors related to higher antioxidant and neuroprotective capacity may contribute to discrepancies between epidemiological studies in different fish-consuming populations

(28). Another confounding factor may be the content of PUFAs and thiol antioxidants, such as selenium in the fish that would protect against mercury neurotoxicity. Interestingly, it has been reported an interaction between mercury exposure and fish nutrients in child neuropsychological development (29). Therefore, it might be enlightening to analyse the fetal PUFA metabolite profile in comparison with postnatal neurodevelopment.

We aimed to uncover the influence of oxylipins reaching the foetuses concurrently exposed to high or low levels of mercury on the postnatal neurocognitive and psychomotor development of children. For this purpose, we analyzed the oxylipins present in placental tissue and cord-blood plasma of children in a population birth cohort in Valencia, a Spanish region with relatively elevated fish intake. This cohort is part of the multicentre INMA Spanish Study on the effects of prenatal environmental exposures in child health (30,31). Next, we statistically correlated their oxylipins levels with the scores obtained with the Bayley scales of infant and toddler development at 14 months of age and McCarthy scales of children's abilities at 5 years of age.

We also aimed to discover changes in the oxylipin profile of the mouse brain after treatment with a sEHI neuroprotective agent. For that purpose we specifically used the sEHI UB-BJ-02 (32).Therefore, we analyzed cerebral cortical oxylipins of wild type (WT) mice and TgAD mice of the 5XFAD strain after 4 weeks of sEHI pharmacological treatment.

2. Materials and Methods

2.1. Human samples of placenta and cord blood

The use of human samples and all the procedures of the study were approved by the 'Comité Coordinador de Ética de la Investigación Biomédica de Andalucia' (approval codes: S1900596 and 2218-N-19), the Ethics Committee of the CSIC (approval code: 072/2019) and the Ethics Committees of La Fe Hospital in Valencia. The study followed the directives from the Declaration of Helsinki.

Tissue samples of placenta (pairs of fetal and maternal parts) and cord blood plasma of children from the INMA cohort in the Valencia region (Spain) were obtained from the the Biobanco del Sistema Sanitario Público de Andalucía. The two tissues were from independent donors. Whole cord blood from all donors had previously been analyzed for total mercury (T-Hg) as a reliable biomarker of MeHg exposure during pregnancy (33). A high proportion of children had cord blood T-Hg levels exceeding the current recommended RfD for MeHg intake (29,31). Two groups of samples with low T-Hg level and high T-Hg level, respectively, were selected for analysis in order to stress any difference between low and high prenatal exposure to mercury. Samples are listed in Supplementary Table 1. Mean values of T-Hg per group of low or high exposure to environmental mercury are shown in Table 1.

Table 1 – Whole cord blood mercury levels in the study groups

	Low T-Hg	High T-Hg
Pairs of fetal and maternal placenta	4.38 ± 0.65 µg/L (6)	23.83 ± 1.49 µg/L (6)
Cord blood plasma	2.43 ± 0.42 µg/L (20)	43.68 ± 2.86 µg/L (19)

Note: Data are expressed as Mean ± SEM (N); T-Hg, total mercury

2.2. Neuropsychological assessment of children

Child neurodevelopment has previously been evaluated though neuropsychological tests conducted in the INMA Project (34). Specifically, cognitive and psychomotor development was fully assessed around 14 months of age using the Bayley Scales of Infant and Toddler Development (BSID) and around 5 years of age using the McCarthy Scales of Children's Abilities (MSCA). The latter was a standardized version adapted to the Spanish population. Procedures were performed as detailed elsewhere (29,35). In brief, BSID consists of the Mental scale (163 items) and the Psychomotor scale (81 items). MSCA comprises 18 subtests that yield test scores for cognitive and motor domains, including: verbal information processing, perceptual performance, numerical abilities, short-term memory, and gross and fine motor abilities. Scores of executive function, working memory and general cognition were also calculated. All neuropsychological data were adjusted for children's age.

2.3. Mouse brain samples

Cortical brain tissue samples were obtained from male and female WT and 5XFAD mice treated with the sEHI UB-BJ-02 at the dose of 5 mg/Kg or with vehicle (N = 5 per group). The agent was administrated by daily oral gavage for 4 weeks. The used vehicle was 2% DMSO/25% of 2-hydroxypropyl-β-cyclodextrin. Mice were 6 month old at termination. The treatment was reported to decrease neuroinflammation markers and memory loss in 5XFAD mice (32).

2.4. Molecular markers of placenta tissue

Presence of deleterious effects of mercury in the high T-Hg group was analyzed in the placental tissues. Results for each placenta donor were the average of the corresponding pair of fetal and maternal placental tissue results.

Enzymatic activity of the cell proteasome system was analyzed with the Proteasome-Glo™ Assay Systems (Promega, USA). Specific luminogenic substrates were added to the supernatants of placenta lysates to determine the corresponding chymotrypsin-like activity, trypsin-like activity, and caspase-like activity. Assays were performed as detailed elsewhere (36).

Epigenetic changes of histone acetylation and methylation were analyzed with ELISA colorimetric methods. Tissue homogenates were assayed for global acetylation of

Histone H3 and H4 using EpiQuik™ Global Histone H3 Acetylation Assay Kit and EpiQuik™ Global Histone H4 Acetylation Assay Kit (EpigenTek, Farmingdale, NY, USA), respectively. Changes of histone methylation were assayed by tissue histone extraction followed by measures of two key specific sites using EpiQuik™ Total Histone Extraction Kit, EpiQuik™ Global Tri-Methyl Histone H3-K27 Quantification Kit and EpiQuik Global Tri-Methyl Histone H4K20 Quantification Kit (EpigenTek), respectively. Procedures were performed following manufacturer's instructions, and final optical density was measured in duplicate wells using a plate spectrometer at 450 nm (Multiskan Sky, Thermo Fisher Scientific).

2.5. Oxylipins analysis

The oxylipin profile of all tissue samples was determined by ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). This methodology allows the accurate determination of a wide panel of oxylipins (37). The method had previously been established and validated for the tissues to be analyzed in the study. Standards and reagents employed and the detailed procedure are described in Supplementary Materials. In brief, chemicals were of the highest purity available. Placenta and cortex samples were submitted to an extraction with 0.1% ammonium acetate. Then, 200 µL of supernatant of tissues or plasma samples were spiked with the standard agents. Extraction and preconcentration of oxylipins were performed after the addition of 200 µL of methanol (0.1 % formic acid) to the samples. Final extracts were evaporated and redicolved in 50 µL of MeOH:CH3CN (50:50) % (v/v) and analysed by UPLC-MS/MS. For data analysis, raw chromatograms were processed with the MassLynx v4.2 software from Waters (Milford, MA, USA) including the peak integration and calibrations. The method allowed the simultaneous determination of 32 PUFA oxygenated metabolites. However, some values were below the limits of detection, which were 0.665 nmol/mg in tissue extracts and 0.04 nM in plasma samples, and could not be determined. Results for each pair of fetal and maternal placental tissues were averaged after separate analysis.

2.6. Statistics

The association between oxylipin levels and neurodevelopmental scores was studied through correlation analysis, and is shown as the corresponding Spearman ρ coefficients and their significances. Correlations were considered moderate for ρ values between 0.40 and 0.59, strong for ρ between 0.60 and 0.79, and very strong for ρ values between 0.80 and 1.00. All other data are shown as mean ± SEM for each group, and their distribution was assessed using the Shapiro–Wilk test. Accordingly, significant differences between two groups were analyzed using the Mann-Whitney test or two-tailed Student's t test, as appropriated. ANOVA followed by Tuckey's post hoc test was used for experimental designs with multiple groups.

3. Results

3.1. Prenatal mercury exposure induced molecular changes in the placenta

The analysis of the placental tissues showed mild deleterious effects of mercury on both proteasome and epigenetics biomarkers (Figure 1). Proteasome enzymatic activities were mildly impaired by high environmental mercury exposure, as inhibition was detected in the caspase-like activity (Figure 1A) but not in the trypsin-like activity or chymotrypsin-like activity (Supplementary Figure 1B, C, respectively). Regarding epigenetic histone changes, samples from donors with higher mercury exposure showed a significant increase in H3 trymethylation at K27, which may led to transcription impairment (Figure 1B). However, no significant changes were detected in the other histone epigenetics markers analyzed (Supplementary Figure 1D-F).

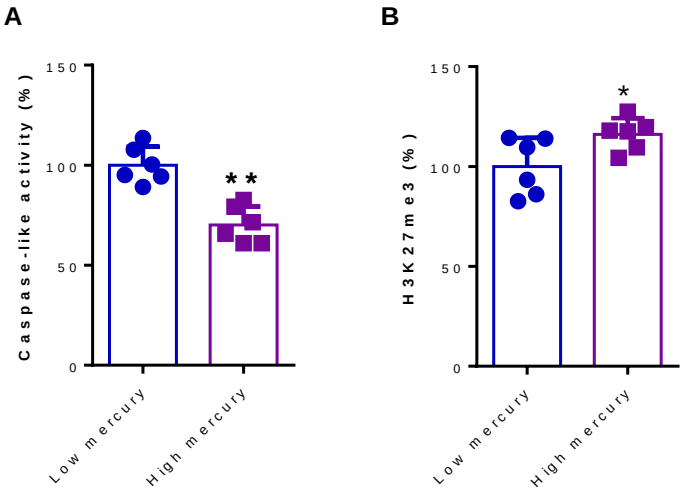

Figure 1. High prenatal exposure to mercury induced deleterious changes in proteasome function and epigenetics in the placental tissue. (A) Caspase-like activity of the proteasome. (B) Relative increase of H3K27me3. Statistics: Student's *t* test, * p <0.05, ** p< 0.01.

3.2. Prenatal mercury exposure induced changes in the oxylipin profile of placenta and cord blood plasma

3.2.1. Oxylipin profile in pairs of fetal and maternal placenta tissue

The LC-MS analysis of the placental tissues showed detectable levels of 17 oxylipins. (Supplementary Table 2A). Paired comparisons between low and high environmental exposure to mercury did not yield statistical differences in the oxylipin levels. However, there was a trend towards a mercury-induced increase in dihydroxyeicosatrienoic acids (DiHETs) that was statistically significant according to ANOVA (Figure 2A). These metabolites are yielded by the hydrolysis of the corresponding epoxyeicosatrienoic acid isomer by soluble epoxy hydrolase (sEH).

3.2.2. Oxylipin profile in cord-blood plasma

The analysis of cord blood plasma samples showed detectable levels of 9 oxylipins (Supplementary Table 2B). From these, the epoxygenase metabolite from

docosahexaenoic acid (DHA), (±)19,20-dihydroxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid (19,20-DiHDPA) showed a mercury-induced increase (Figure 2B).

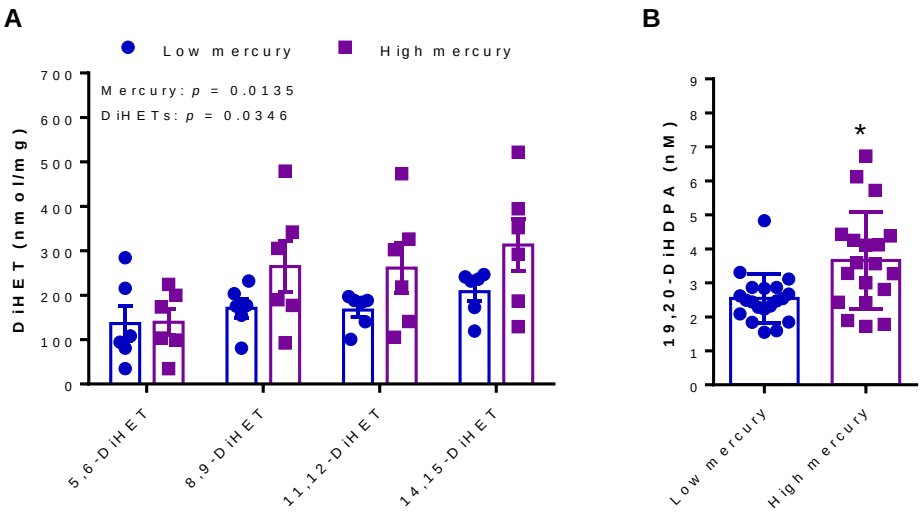

Figure 2. High prenatal exposure to mercury induced an increase in some stable PUFA metabolites. (A) Increase in the levels of DiHETs determined in placental tissue; statistics: two-way ANOVA, significance of factors is shown in the graph. (B) Increase in the level of 19,20-DiHDPA determined in plasma from cord blood; statistics: Student's *t* test, * p< 0.01.

3.3. Correlations of oxylipin profile of placenta and cord blood plasma with neurodevelopmental scores were modified by prenatal mercury exposure

3.3.1. Exposure to mercury and neurodevelopmental scores

Direct comparison of score values of Bayley and McCarthy scales between children prenatally exposed to low or high mercury levels did not shown statistical differences (Supplementary Table 3A and B, for cohorts of children of placenta and cord-blood samples respectively). However, there were remarkable correlations between some oxylipins levels in the tissue samples and children neurodevelopment, as summarized below.

3.3.2. Correlations between Bayley scales and placenta oxylipins

At 14 months of age, significant correlations were observed between the neurodevelopmental score and some key oxylipins analyzed in the pairs of fetal and maternal placental tissues (Table 2). All values are shown in Supplementary Table 4A. Spearman's ρ showed a strong or very strong positive correlation in the low mercury group between Bayles' Mental scale and all four EETs isomers, as well as one DiHET isomers, along with other oxylipins with anti-inflammatory functions derived from DHA or EPA. However, Mental scale also showed strong or very strong positive or negative correlation with prostaglandins and linoleic acid derived oxylipins. There was also a very strong negative correlation between the Mental scale and a marker of the proinflammatory eicosanoid family. No correlations were observed for the Psychomotor scale. In contrast, children with high prenatal exposure to mercury showed some strong or very strong negative correlations between Bayles' Psychomotor scale and oxylipins

derived from the AA prostaglandin pathway, linoleic acid, or EPA, but no correlation was observed for the Mental scale.

Table 2 – Selected correlations between Bayley scales of infant and toddler development at 14 months of age and oxylipins in placental tissue

	Low T-Hg				**High T-Hg**			
	Mental scale		Psychomotor scale		Mental scale		Psychomotor scale	
Metabolites (nmol/mg)	Spearman ρ	p	Spearman ρ	p	Spearman ρ	p	Spearman ρ	p
5,6-EET	**.943****	.005	.143	.787	.145	.784	.029	.957
8,9-EET	**.771**	.072	.143	.787	.058	.913	-.257	.623
11,12-EET	**.886***	.019	-.029	.957	.000	1.000	.086	.872
14,15-EET	**.771**	.072	.143	.787	-.087	.870	-.200	.704
5,6-DiHET	**.829***	.042	.257	.623	.551	.257	-.600	.208
PGF2a	**.771**	.072	.371	.468	.406	.425	**-.886***	.019
PGE2	**-.886***	.019	.029	.957	.058	.913	-.543	.266
9,10-DiHOME	**.943****	.005	.143	.787	.203	.700	-.200	.704
14-HDoHE	**.886***	.019	.143	.787	.290	.577	**-.829***	.042
17-HDoHE	**.771**	.072	.086	.872	.232	.658	**-.771**	.072
18-HEPE	**.943****	.005	-.086	.872	.290	.577	**-.829***	.042

Statistics: * $p < 0.05$, ** $p < 0.01$

3.3.3. Correlations between McCarthy scales and placenta oxylipins

Levels of oxylipins in placenta tissues showed some trend but did not reach significant correlations with scores of McCarthy scales at 5 years of age, probably because of the low number of data. Values are shown in Supplementary table 4B.

3.3.4. Correlations between Bayley scales and cord-blood plasma oxylipins

Levels of oxylipins in cord-blood plasma showed some moderate correlations with scores of Bayley scales at 14 months of age. The Mental scale positively correlated with TXB2 ($\rho = 0.492$, $p = 0.038$) and 11,12-DiHET ($\rho = 0.515$, $p = 0.029$) levels in the high mercury exposure group. The Psychomotor scale positively correlated with TXB2 ($\rho = 0.453$, $p = 0.078$) and 5,6-DiHET ($\rho = 0.506$, $p = 0.046$) levels in the low mercury exposure group. All values are shown in Supplementary table 4C.

3.3.5. Correlations between McCarthy scales and cord-blood plasma oxylipins

At 5 years of age, some moderately strong correlations were obtained between several McCarthy scales and oxylipins in cord-blood plasma (Table 3). All values are shown in Supplementary Table 4D. Interestingly, at this age, the correlations were positive and more abundant with higher prenatal mercury exposure compared to lower exposure

Numerical scores and working memory positively correlated with two DiHET isomers in the high mercury group. However, in this group, both latter scores and general cognitive scores, as well as perceptual-performance score, also correlated with the AA-derived pro-inflammatory thromboxane. Furthermore, there was a correlation between a DHA derived oxylipin and both memory score and working memory in the low mercury group.

Table 3 – Selected correlations between McCarthy scales of children's abilities at 5 years of age and oxylipins in cord-blood plasma

	Low T-Hg									
	General cognitive score		Perceptual-performance score		Numerical score		Memory score		Working Memory	
Metabolites (nM)	Spearman ρ	p	Spearman ρ	p	Spearman ρ	p	Spearman ρ	p	Spearman ρ	p
11,12-DiHET	.024	.955	**-.762***	.028	-.024	.955	-.048	.911	-.048	.911
14,15-DiHET	-.476	.233	-.452	.260	-.619	.102	-.143	.736	-.286	.493
TXB2	.167	.693	.262	.531	.095	.823	-.381	.352	-.214	.610
14-HDoHE	-.143	.736	.024	.955	-.524	.183	**-.690**	.058	**-.714***	.047
	High T-Hg									
	General cognitive score		Perceptual-performance score		Numerical score		Memory score		Working Memory	
Metabolites (nM)	Spearman ρ	p	Spearman ρ	p	Spearman ρ	p	Spearman ρ	p	Spearman ρ	p
11,12-DiHET	.360	.155	.118	.653	**.446**	.073	.203	.434	**.515***	.035
14,15-DiHET	.191	.462	-.044	.866	**.453**	.068	-.042	.874	**.608****	.010
TXB2	**.434**	.082	**.426**	.088	**.534***	.027	**.525***	.031	**.444**	.074
14-HDoHE	-.346	.174	-.184	.480	.037	.889	.172	.510	-.069	.794

Statistics: * $p < 0.05$, ** $p < 0.01$

3.4. sEHI induced changes in the oxylipin profile of mouse brain

WT mice treated with the sEHI UB-BJ-02 showed an increase in the 5,6-EET isomer in the cerebral cortical tissue, but no change was detected in the 5XFAD TgAD mice. (Figure 3A). However, a significant increase induced by UB-BJ-02 in the beneficial lipokine 12,13-DiHOME was a general effect observed in both WT and 5XFAD mice (Figure 3B). Furthermore, there was also a significant effect of sEHI in decreasing pro-inflammatory oxylipins from the prostaglandin proinflammatory pathway (Figure C-E). All oxylipin values are shown in Supplementary Table 5.

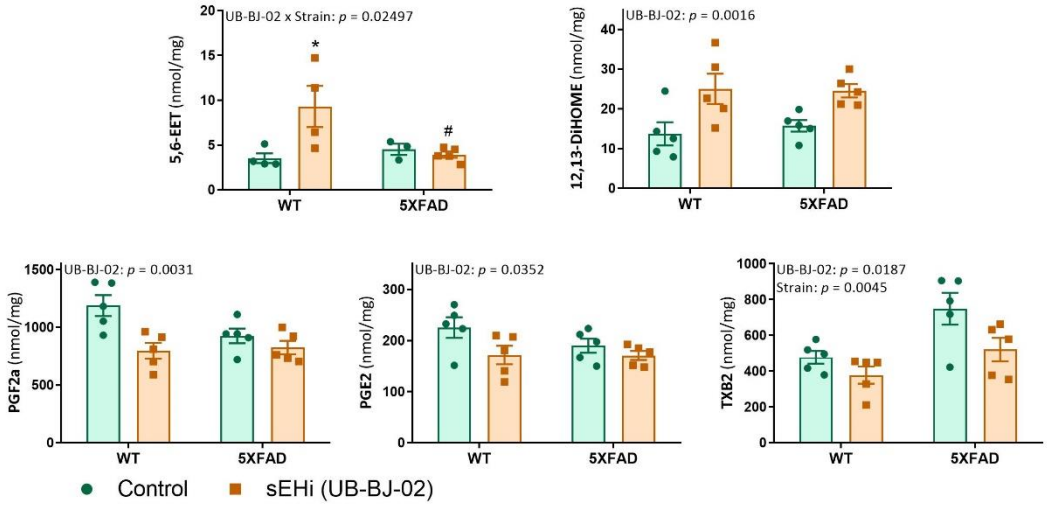

Figure 3. Mice dosed with sEHI for 4 weeks showed anti-inflammatory changes in the cerebral cortical oxylipin profile. (A) Borderline significant effect on the 5,6-EET levels, increasing in WT mice. (B) General effect increasing 12,13-DiHOME in WT and 5XFAD mice. (C) General effect decreasing PGF2a. (D) General effect decreasing PGE2. (E) Higher levels of TBX2 in 5XFAD mice than WT mice and general decrease induced by sEHI treatment. Statistics: two-way ANOVA, significance of factors or their interaction is shown in the graph; Tukey's post-hoc test, *p < 0.05 compared to control treatment, and #p < 0.05 compared to the corresponding treatment of WT mice.

3.5. Absence of sex related changes

Samples used for placenta analysis and for cord blood plasma analysis included a similar number of male and female children. Sex is indicated in the corresponding supplementary files.

Mouse brain tissue included a similar number of male and female mice. No effect of sex was detected and data from males and females were pooled together for final analysis.

4. Discussion

The analysis of the oxylipin profile in placenta and cord blood plasma, and their correlation with children's neurodevelopment scores, showed numerous changes associated with prenatal mercury exposure in both EETs and DiHETs. Metabolites from DHA and EPA showed fewer changes. The results suggest that the oxylipins EETs and their hydrolyzed derivatives, DiHETs, play a significant role in prenatal neurodevelopment and protection against prenatal brain damage. The oxylipins that

showed changes in the tissues analyzed, either in human placenta, cord blood plasma, or the cerebral cortex of mice, are displayed in Figure 4.

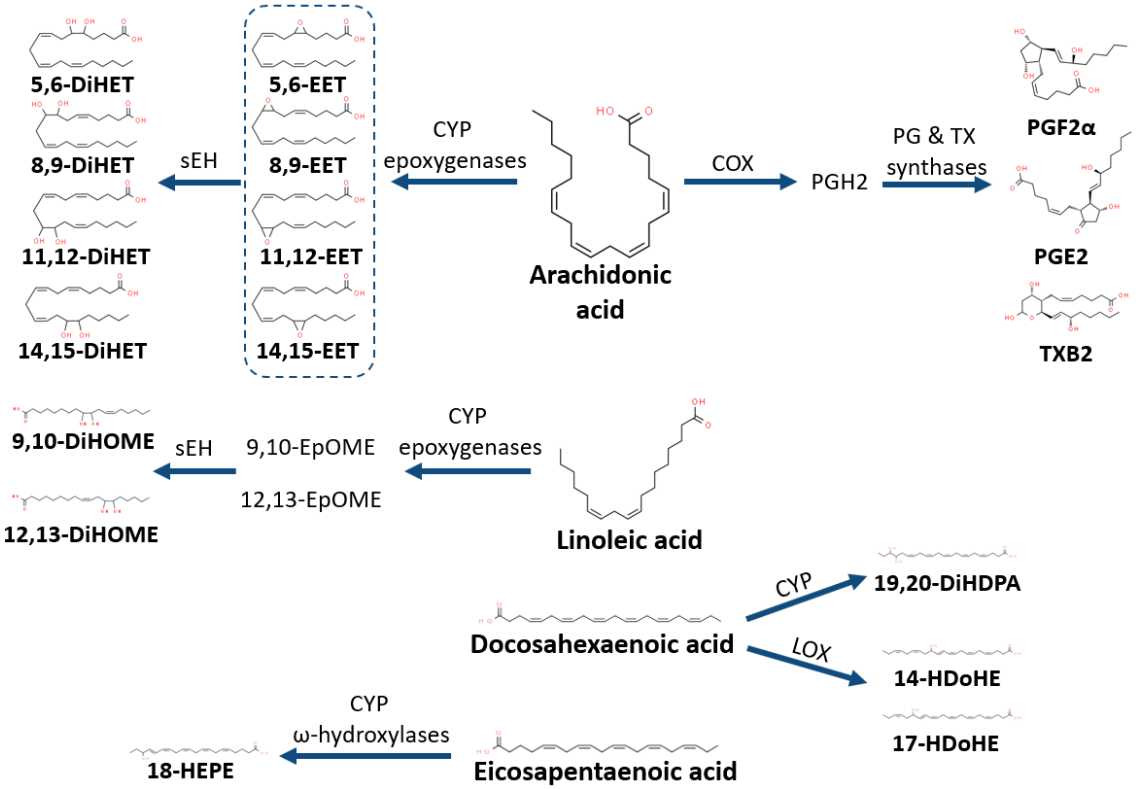

Figure 4. Oxylipins mentioned in the study and the corresponding metabolic scheme from their respective parent PUFAs.

Children with high level of prenatal exposure to mercury were submitted to a 3 – 8 fold increase above the RfD for safe maternal dietary intake of MeHg. Therefore, they suffered a prenatal toxic stress, as shown by mild deleterious changes in the placenta. The inhibition of proteasome caspase-like activity causes a reduction in the degradation of abnormal intracellular proteins and subsequent disruption of protein turnover. However, only mild effects were expected, as the trypsin-like and chymotrypsin-like activities were not affected. Mercury is a known proteasome inhibitor in a dose-dependent manner (38). Regarding the analysis of epigenetic changes in histone acetylation and methylation, a mild change was detected, specifically an increase in trimethylated histone H3K27. H3K27me3 is a repressive mark that silences genes during early development (39). Epigenetics changes caused by mercury or methylmercury are mainly known as methylations in the human placenta (40). However, experimental studies have revealed histone modifications induced by MeHg (41). Therefore, the prenatal high mercury exposure imprinted toxic biomarkers in the placenta and has probably affected neurodevelopment of the children. However, no significant changes have been detected in the analyzed neurodevelopmental scores between children prenatally exposed to low and high mercury levels. Nevertheless, studies with the full INMA cohort, including a larger number of children, have detected minor cerebral impairments or delays, as mentioned above (23,24).

The increase found in the levels of some anti-inflammatory and protective oxylipins in the placenta or cord blood may be related to an increase of PUFA-derived signaling to protect against MeHg damage. These changes were probably reproduced in the fetal CNS and other tissues. The increase of DiHETs in the placenta can be attributed to a higher generation of the neuroprotective EETs which suffer a rapid metabolization to DiHETs, as mentioned above. In the mice study, there was an increase of an EET isomer in the brain after treatment with a sEHI. However, higher EET levels were found in WT but not in TgAD mice, despite the known neuroprotective effects of sEHI treatment in AD mouse models (9,23,24,42). We may speculate that there is full use and degradation of EETs in the TgAD mouse brain. In the cord blood plasma, an increase of 19,20-DiHDPA, a final stable metabolite of DHA, was found in the high mercury exposure group, indicating a mobilization of DHA that also protects against toxic damage. Accordingly, the increase of 19,20-DiHDPA and other DHA metabolites in mouse pup brain has been related to the neuroprotection afforded by maternal DHA supplementation against MeHg (43).

The correlations between all four EETs and one DiHETs isomers, and the Bayley' Mental scale in the low mercury exposure group, confirm the significance of these AA metabolites in brain neurodevelopment. Unexpectedly, these correlations were not observed in the high mercury exposure group. The lack of apparent metabolite signaling in the latter group may be related to the full use of these protective molecules, as proposed for TgAD mice. Furthermore, positive correlations of the Mental scale scores with DHA and EPA anti-inflammatory and protective metabolites (14-HDoHE, 17-HDoHE and 18-HEPE) in the low mercury group turned into negative correlations with the Bayley's Psychomotor scale in the high exposure group. Interestingly, MeHg is primarily neurotoxic for the granule cell neurons of the cerebellum, which has a significant impact on motor areas during brain development in addition to cognitive impairment (13,44,45). Therefore, fine motor activities of the children may be highly sensitive to the crosstalk signaling between high mercury exposure and PUFA activation.

At 5 years of age, two DiHET isomers consistently correlated with McCarthy scales for numerical score and working memory in the high mercury exposure group, whereas the low mercury exposure group showed scattered negative correlations with one DiHET metabolite and the 14-HDoHE DHE metabolite. The differential correlations according to the children's age suggest a neurodevelopment delay induced by prenatal MeHg, leading to the appearance of beneficial effects from EETs and DiHETs signaling at older ages. This delay suggests mild neurodevelopmental neurotoxicity of MeHg at prenatal exposures higher than the RfD.

Finally, the oxylipin analysis highlighted some correlations between neurodevelopmental scores and pro-inflammatory metabolites. Prostaglandins PGF2α and PGF2 interacted with children's neurodevelopment at 14 months, without a consistent pattern associated with mercury exposure levels. However, the thromboxane TXB2 showed a consistent positive correlation with scores at 5 years of age. All three oxylipins are derived from AA through activation of the proinflammatory COX pathway,

and sustained activation of this pathway may lead to neuroinflammation. These three oxylipins showed reduced levels in both WT and TgAD mice after anti-inflammatory treatment with sEHI. Increased levels of prostaglandins and thromboxanes have been reported in rat pups exposed prenatally to lead (46), which is another known developmental neurotoxic agent. However, these molecules also play a signaling role in pregnancy progression and resolution, which should be considered (47). The toxic mediator 9,10-DiHOME, derived from linoleic acid, showed a single positive correlation with mental scale scores at 14 months of age, of unknown implication in the low mercury exposure group. In contrast, the isomer 12,13-DiHOME showed an increase in the brains of both WT and TgAD mice after treatment with sEHI, in agreement with its suggested beneficial effects (48).

LIMITATIONS: The first limitation is the relatively small sample size, which limited the statistical power of the study. Additionally, the use of different donors for placenta and cord blood samples did not allow to draw further conclusions about prenatal mercury exposure. As known, the placenta acts as a reservoir of beneficial nutrients and toxic chemicals, reflecting chronic exposure during pregnancy. However, cord blood indicates current levels of toxins and metabolites.

5. Conclusions

Prenatal exposure to mercury induced changes in PUFA oxidized metabolites in placental tissue and cord blood plasma that correlated with postnatal neurodevelopment in children. EETs and their subsequent metabolites, DiEHTs, were the main oxylipins detected as differentially modulated by prenatal mercury exposure. The oxylipin profile in the cerebral cortical tissue of adult mice treated with a sEHI showed increased levels of neuroprotective oxylipins and decreased levels of pro-inflammatory oxylipins. Both human and experimental mouse results showed that EETs are prominent anti-inflammatory metabolites, supporting EETs supplementation through diet or sEH inhibition as an anti-inflammatory treatment to enhance brain resilience against environmental risk of neurodevelopmental damage.

6. Funding

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Publication 3.

Microglial pro-inflammatory mechanisms induced by monomeric C-reactive protein are counteracted by soluble epoxide hydrolase inhibitors

Clara Bartra, Kristijan Vuraić, Yi Yuan, Sandra Codony, Haydeé Valdés-Quiroz, Carme Casal, Mark Slevin, Christian Griñán-Ferré, Mercè Pallàs, Christophe Morisseau, Bruce Hammock, Santiago Vázquez, Cristina Suñol and Coral Sanfeliu

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Microglial pro-inflammatory mechanisms induced by monomeric C-reactive protein are counteracted by soluble epoxide hydrolase inhibitors

Clara Bartra[1,2,*], Kristijan Vuraić[1], Yi Yuan[1], Sandra Codony[3,4], Haydeé Valdés-Quiroz[1], Carme Casal[5], Mark Slevin[6.7], Christian Griñán-Ferré[8.9], Mercè Pallàs[8,9], Christophe Morisseau[10], Bruce D. Hammock[10], Santiago Vázquez[3,4], Cristina Suñol[1], Coral Sanfeliu[1,*]

[1] Department of Neuroscience and Experimental Therapeutics, Institute of Biomedical Research of Barcelona (IIBB), CSIC, 08036 Barcelona, Spain.

[2] PhD Program in Biotechnology, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, 08034 Barcelona, Spain.

[3] Laboratory of Medicinal Chemistry (CSIC Associated Unit), Faculty of Pharmacy and Food Sciences, Universitat de Barcelona, Barcelona, Spain.

[4] Institute of Biomedicine of the University of Barcelona (IBUB), Universitat de Barcelona, Barcelona, Spain.

[5] Microscopy Service, Institute of Biomedical Research of Barcelona (IIBB), CSIC, Barcelona, Spain.

[6] School of Life Sciences, John Dalton Building, Manchester Metropolitan University, Manchester M15 6BH, UK.

[7] CCAMF, George Emil Palade Universitatea de Medicina, Farmacie, Stiinte se Technologie, "George Emil Palade" din Targu-Mures, 540142 Tirgu Mures, Romania.

[8] Department of Pharmacology and Therapeutic Chemistry, Institut de Neurociències-Universitat de Barcelona, Barcelona, Spain.

[9] Centro de Investigación en Red, Enfermedades Neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, Madrid, Spain.

[10] Department of Entomology and Nematology and Comprehensive Cancer Center, University of California, Davis, California 95616, United States.

*** Correspondence:**

Coral Sanfeliu, coral.sanfeliu@iibb.csic.es

Clara Bartra, clara.bartra@iibb.csic.es

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1. Abstract

Background: Monomeric C-reactive protein (mCRP) was shown to induce Alzheimer's disease (AD) pathology and cognitive loss. However, its neuroinflammatory mechanisms have not been proven. Microglia are the first line innate immune cells inducing neuroinflammation in response to an injury. However, a sustained pro-inflammatory response may cause or even potentiate neurodegeneration. Innovative therapies are desperately needed, and inhibitors of the enzyme soluble epoxide hydrolase (sEH) might be a new generation of anti-neuroinflammatory drugs.

Methods: Mouse primary microglia and BV2 cell line cultures were incubated with 25 µg/mL or 50 µg/mL of mCRP. sEH inhibitors, both newly synthesized UB-SCG-55 and UB-SCG-65, and the reference agent TPPU, were tested for anti-inflammatory action at 50 µM. Phenotypic changes were analyzed through cell imaging techniques, as well as molecular analysis of inflammatory mediators and gene activation pathways.

Results: Results show that mCRP triggers a pro-inflammatory response in primary microglia through the three main inflammatory pathways: iNOS, NLRP3, and COX-2, followed by increased release of TNF-α and IL-6. Polarization of microglia toward a pro-inflammatory M1-like phenotype was confirmed by image analysis. Also, mCRP can bind to and cross the BV2 cell membrane, providing further insights into its mechanisms of action. sEH inhibitors were tested in BV2 cells. While all three compounds were efficacious, UB-SCG-55 emerged as the most potent anti-inflammatory against mCRP injury. UB-SCG-55 also inhibited morphological changes indicative of an M1-like phenotype in primary microglia treated with mCRP, thus confirming its anti-inflammatory efficacy.

Conclusion: The direct activation of microglia by mCRP provides evidence of its role in triggering and exacerbating neurodegenerative diseases with a neuroinflammatory component, such as AD. Furthermore, the protection given by inhibitors of sEH confirms its potential as innovative drugs against deleterious effects of neuroinflammation.

2. Introduction

Neuroinflammation has attracted increasing attention for its role in several neurodegenerative conditions such as Alzheimer's disease (AD) (1, 2). In fact, neuroinflammation is a natural defensive, protective and restorative mechanism, orchestrated by microglia and astrocytes in front of endogenous (e.g. ischemia) and exogenous (e.g. microbial infection) insults (2–6). Nevertheless, if this inflammatory response persists and becomes chronic it can lead to synaptic dysfunction, decrease in adult neurogenesis and ultimately neuronal death (3, 5). Neuroinflammation is not the primary cause of AD but a consequence of a related pathology and a risk factor associated with the disease (1, 7, 8). However, this inflammation can worsen the outcome of the main two AD pathologies: the presence of amyloid-β (Aβ) plaques and neurofibrillary tangles (7).

C-reactive protein (CRP) is an acute phase reactant pentraxin produced mainly by the liver, induced by IL-6 and enhanced by IL-1β (9, 10). CRP binds to phosphocholines on cellular membranes, inducing a conformational change that forms an isoform known as pCRP*. This pCRP* isoform interacts with the complement C1q, triggering the dissociation of pCRP* into its monomeric form (mCRP), which possesses pro-inflammatory and pro-thrombotic properties (11).

In human stroke tissue, mCRP has been found in damaged areas of the brain, and its presence has been related to angiogenic neo-vessels and ischemic neurons (10). Additionally, this protein has been directly linked to a higher likelihood of dementia development after ischemic or vascular brain damage. In fact, Slevin et al. demonstrated that the presence of mCRP not only perpetuated inflammation in the brain throughout abnormal angiogenesis, but also increased Aβ plaques and p-tau in the 3xTg-AD mouse model of AD (12). Other studies with wild type C57BL/6J mice suggest that a 6-month exposure to mCRP via injection in the hippocampus produced long-lasting cognitive impairment and late anxiety (13), which are common characteristics of older AD mouse models. Recent findings have confirmed that mCRP directly triggers AD pathogenesis in mouse cortical neurons and brain vascular endothelial cells with the APOEε4 genotype (14, 15), pointing to an unswerving connection between mCRP and AD risk.

In the brain of AD patients, mCRP has been detected in regions exhibiting cerebrovascular damage, accompanied by the presence of probable macrophages and microglia (16). Moreover, mCRP has been proposed to increase the risk of AD following stroke (12). As a matter of fact, mCRP has already been proven to induce pro-inflammatory responses in monocytes, either in the U937 cell line or primary peripheral blood monocytes (17, 18), and in BV2 microglial cells (13, 19). Nonetheless, the effects of mCRP on microglia, the primary innate immune cells of the brain, have not been clarified so far.

Epoxyeicosatrienoic acids (EETs), produced from the arachidonic acid via a CYP P450 monooxygenase reaction (20, 21), are known for presenting several beneficial properties. EETs and other epoxides of long chain fatty acids (EpFA) are well known for resolving inflammation, pain and cell death. More specifically, in the brain, EpFA can reduce inflammation, ER stress and oxidative stress (22). All three are pathological processes involved in AD. However, these acids are rapidly metabolized by the soluble epoxide hydrolase enzyme (sEH), which converts EETs to the less bioactive dihydroxyeicosatrienoic acids (23). Moreover, in human AD postmortem brains and AD mouse models, sEH levels are elevated (24, 25), which can be related to subsequent lower levels of EETs.

sEH inhibitors (sEHI) have already been proposed as possible therapies to enhance benefits of EETs and other EpFA. In sEH KO mice with traumatic brain injury, sEHI 12-(3-adamantan-1-ylureido)-dodecanoic acid (AUDA) administration reduced microglia activation, thereby attenuating inflammation and reducing neuronal death (21). In WKY rats with an ischemic brain injury, AUDA polarized microglia to an M2-like phenotype,

decreasing the expression of pro-inflammatory cytokines and increasing the expression of antioxidative enzymes (23). In the AD mice model 5XFAD mice, pre-administration of 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl)-urea (TPPU), a well characterized sEHI, protected against LPS-induced neuroinflammation (24). Furthermore, maternal treatment with TPPU had a beneficial impact against memory loss and AD pathology in its 5XFAD offspring (26). A study using the senescence-accelerated mouse prone 8 (SAMP8) and 5XFAD mice models, treated with three structurally different sEHI: AS-2586114, TPPU, and UB-EV-52, suggested that inhibiting sEH reduces neuroinflammation, ER stress, and oxidative stress, as well as Aβ plaques and p-tau pathologies (25).

Herein, we propose mCRP-activated microglia as an *in vitro* model to characterize pro-inflammatory mechanisms and potential protective agents against neuroinflammation in AD and other brain pathologies. Firstly, we wanted to elucidate the pro-inflammatory mechanisms activated by mCRP in primary microglia cultures. As mentioned above, preliminary investigations were reported in monocytes and BV2 cell lines. However, microglia are the main player of the brain innate immune system (27) and should be analyzed in the scenario of mCRP related injury. Next, we propose BV2 cells, a widely used immortalized microglia, to study the newly synthesized sEHI UB-SCG-55 and UB-SCG-65 alongside with TPPU for their anti-inflammatory potency against mCRP. Finally, confirmation of the anti-inflammatory effects of the most promising sEHI was required in primary microglia.

3. Materials and methods

3.1. Cell culture

3.1.1. Primary microglia

Primary microglia cultures were obtained following established procedures (28). Mixed glial cultures were prepared from cerebral cortices of C57BL/6 mice at 2–4 days of age. In brief, through a combination of enzymatic and mechanical processing, cortices were minced and disaggregated into a single cell solution. Afterwards, cells were resuspended in DMEM:F12 with HEPES and L-glutamine, supplemented with gentamycin 50 µg/mL and 10% FBS (all culture reagents were from Gibco, Thermo Fisher Scientific). Cells were seeded in 12-well plates (Nunc, ThermoFisher Scientific, Waltham, MA, USA) at 8 × 104 cells/cm2 or in chamber slides (µ-Slide 8 Well high Glass Bottom, Ibidi, Gräfelfing, Germany, or Permanox Lab-Tek Chamber Slides, Nunc, Thermo Fisher Scientific) at 6 × 104 cells/cm2. Surfaces were previously coated with poly-D-lysine. Medium was renewed every 5–7 days. At 21 days *in vitro*, conditioned medium was preserved and cultures were submitted to a mild trypsinization with a solution of 0.25% trypsin and 1 mM EDTA diluted with DMEM-F12 1:4. After 20-40 min, the upper layer of mixed glial cells detaches and it is discarded, while a layer of microglia cells remains attached to the bottom. Wells were replenished with conditioned medium of the mixed glial cultures. This procedure yielded a high purity of microglia cells (> 98%) (29). Cells were used for experiments the following day.

3.1.2. BV2 cells

BV2 cell line is derived from primary microglia of C57BL/6 mice (30). BV2 cells were purchased from the Biological Resource Center ICLC Cell bank (Genova, Italy; code ATL03001). Cells were cultured in T25 flasks (NuncTM, Thermo Fisher Scientific) in RPMI-1640 medium w/ L-glutamine (Biowest, Riverside, Newry and Mourne, UK) supplemented with 10% of heat inactivated fetal bovine serum (FBS) and gentamycin 50 µg/ml (GibcoTM, Thermo Fisher Scientific). Cultures were passaged when they reached 70-80% confluency.

For experimental procedures, cells were seeded at 5 x 104 cells/cm2 in 12-, 24-, or 96-well plaques (NuncTM, Thermo Fisher Scientific) or in 8-well chamber slides Lab-Tek Chamber Slides (Nunc, Thermo Fisher Scientific) at 1 x 104 cells/cm2. After 24 h, cells were treated in medium without FBS and the corresponding inflammatory or/and anti-inflammatory agent for further 24 h. Twenty-four hours later, analyses were carried out. Experiments were conducted and repeated using cells from three independent passages.

3.2. Experimental Agents

3.2.1. Monomeric C-reactive protein

A 2.5 mg/mL stock solution of mCRP diluted in PBS was obtained using the Potempa method (31), which involves urea/EDTA chelation of pentameric native CRP and dialysis procedures to obtain a solution of pure monomers. For treatments, mCRP was diluted with the corresponding medium at a concentration of 25 µg/mL and 50 µg/mL.

3.2.2. Soluble epoxide hydrolase inhibitors

sEHI compounds UB-SCG-55 and UB-SCG-65 (figure 1) were synthesized as described in Codony et al. (32, 33). TPPU (MedChemExpress, Monmouth Junction, NJ, USA) was used as a reference sEHI compound. All sEHI agents were dissolved in DMSO (Invitrogen, Thermo Fisher Scientific) to prepare 100 mM stock solutions, and therefore DMSO was also added to the control wells. For experimental procedures, agents were further diluted with culture medium. The final concentration of DMSO for all the wells was 0.1 %. Cells were treated with sEHI at 10 µM up to 100 µM.

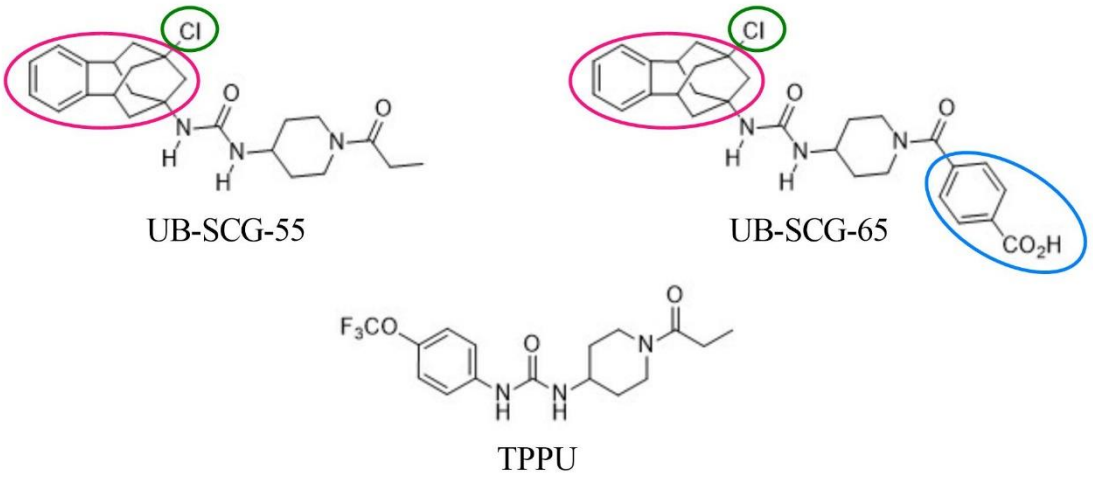

FIGURE 1. sEHI structures. As the catalytic center of sEH is large and highly hydrophobic, sEHI need to be highly hydrophobic. In UB-SCG-55 and UB-SCG-65 the aromatic ring of TPPU was fused with an adamantane, leading to a very hydrophobic and bulky benzohomoadamantane unit (in pink) which led to increased potency (32, 33, 57). The chlorine atom (in green), enhances microsomal stability (33, 57). UB-SCG-65 features a carboxylic acid group (in blue) which fostered additional interactions with the catalytic pocket of sEH.

3.3. Cytotoxicity Assay

In order to characterize the safety of the sEHI compounds, TPPU, UB-SCG-55 and UB-SCG-65 were tested in the BV2 cell line. The compounds were added to 96-well plates at various concentrations, up to 100 µM, with 0.1% DMSO as the vehicle. After 24 h, to determine cell death, propidium iodide (PI) was added to each well at a final concentration of 7.5 µg/ml and incubated for 1 h. Fluorescence was measured in a fluorimeter (SAFAS, Monte-Carlo, Monaco) at 530 nm excitation and 645 nm emission. Subsequently, cell viability and proliferation were evaluated by adding 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 50 µg/ml. Cells were incubated for 2 h, lysed, and maintained at 37 ºC overnight. Optical density given by MTT reduction was measured at 570 nm in a plate spectrometer (Multiskan Sky, Thermo Fisher Scientific). The reference wavelength was 630 nm.

Percentage of cell death from each well (Ft) was calculated based on the fluorescence values coming from wells with Triton X100 (Fmax; 100 % cell death), and on the values of control wells (Fmin; 0 % cell death) [% = ((Ft-Fmin)/(Fmax-Fmin)) x 100]. Percentage of cell viability and proliferation from each well (OD) was measured according to the optical density values of cells without MTT (ODo; 0 % MTT reduction) and the values of the control cells (ODc; 100 % MTT reduction). The 50% cytotoxic concentration (CC50) was determined for both assays using GraphPad Prism 6.01 package (GraphPad Software, La Jolla, CA, USA).

3.4. Western Blotting

Medium was removed from 12-well plates and cells were washed with cold PBS and homogenized with RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Extracts were collected for protein analysis. Protein concentration of cell

lysates was determined with Bradford protein assay (Bio-Rad, Hercules, CA, USA) and absorbance was measured at 595 nm.

Twenty µg of protein for primary microglia samples and 15 µg for BV2 cells samples were mixed with loading buffer, 5x DTT and distilled water. Samples were boiled at 95 °C for 5 min. Polyacrylamide gels were prepared ranging from a concentration of 8 to 15% of 30% Acrylamide/Bis Solution 29:1 (Bio-Rad, Hercules, CA, USA). Prepared samples and ladder (Precision Plus Protein Dual Color Standards; Bio-Rad) were loaded and ran at 100 V. Electrophoresed proteins were transferred onto methanol-activated PVDF 0.45 µm membranes (Millipore, Merck, Darmstadt, Germany) at 200 mA for 90 min. Membranes were blocked in 5 % milk and washed in 1X Tris-Buffered Saline 0.5 % Tween® 20 Detergent (TBS-T). Membranes were later incubated overnight with the corresponding primary antibody diluted in WestVisionTM Block and Diluent (Vector Laboratories, Newark, CA, USA). The primary antibodies used were: anti-inducible nitric oxide synthase (iNOS) (1:1000; #610421, BD Bioscience, BD Transduction Laboratories, Franklin Lakes, NJ, USA), anti-nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP3) (1:1000; #NBP2-12446SS, Novus Biologicals, Bio-Techne, Centennial, CO, USA), anti-cyclooxygenase 2 (COX-2) (1:1000; # aa 584-598, Cayman Chemical, Ann Arbor, MI, USA) and anti-ß-tubulin (1:10,000; #T4026; Sigma-Aldrich, Merck). The day after, membranes were incubated with the appropriate secondary antibody diluted in TBS-T: sheep-anti-mouse HRP conjugated (1:2000; #NA931, Amersham, General Electric, Boston, MA, USA) or donkey-anti-rabbit HRP conjugated (1:2000; #NA934, Amersham).

For membrane reveal, membranes were incubated with two solutions containing luminol salt sodium and p-coumaric acid (Sigma-Aldrich, Merck), in a 1:1 ratio. Proteins were visualized using ChemidocTM Imaging System (Bio-Rad). Image LabTM 6.0.0 (Bio-Rad) was employed for band analysis and quantification. Protein levels were normalized with house-keeping gene β-tubulin.

3.5. Enzyme Linked Immunosorbent Assay

Conditioned supernatants were collected from either 12- or 24-well plates and frozen at -70 ºC until analysis. Tumour Necrosis Factor alpha (TNF-α) release was measured using Mouse TNF-alpha Uncoated ELISA Kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol, with samples diluted in a 1:10 ratio for both primary microglia and for BV2 supernatants. The levels of Interleukin 6 (IL-6) were measured with the Mouse Interleukin-6 (IL-6) Uncoated ELISA Kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol, with primary microglia supernatants diluted in a 1:100 ratio.

3.6. Immunofluorescence and cell imaging

Interaction of mCRP with cell membrane was analyzed by confocal immunofluorescence in BV2 cells. Cells in chamber slides were fixed in 4 % paraformaldehyde for 30 min and permeabilizated in 0.2 % Triton X-100 for 8 min. Following that, blocking of unspecific unions was carried out with 10 % goat serum in 0.2% Triton X-100 in TBS for 1 h.

Thereafter, cells were incubated overnight with the mouse monoclonal antibody against human mCRP clone 3H12 (1:100) from Dr L.A. Potempa (10, 31) and 1 % goat serum, diluted in TBS. Primary antibody solution was washed and cells were incubated for 1 h with an Alexa Fluor 488-conjugated secondary antibody (1:1000; #A-11001, Molecular Probes, Thermo Fischer Scientific) and 1 % goat serum, diluted in TBS. After that, for cell membrane staining, cells were incubated for 2 h with diluted 1:40 Rhodamine phalloidin-TRITC (Hello Bio, Avonmouth, Bristol, UK). Slides were washed and mounted with Fluoroshield™ with DAPI (Sigma-Aldrich, Merck). Imaging was performed with an Andor Dragonfly 200 Spinning Disk confocal (Oxford Instruments, Abingdon, Oxfordshire, UK). Diode lasers 405, 488 and 561 nm were used. Images were taken every 0.2 µm at 60x. The images selected for the analysis had the cellular nuclei in the central view. From the chosen images, the position of mCRP was examined using orthogonal views (ZY and ZX positions). Images were analyzed using ImageJ (34).

Morphological phenotype changes in response to treatments were analyzed in primary microglia cultures stained with lectin from Bandeiraea simplicifolia. In brief, cells in chamber slides were fixed and permeabilized as described for BV2 cells. BSA was used to block nonspecific binding. Isolectin B4, FITC conjugated (1:400; #L2895, Sigma-Aldrich, Merck) was incubated overnight, nuclei were stained with bisbezimide and the slides were mounted. Furthermore, primary microglia cultures stained with Vybrant™ CFDA SE Cell Tracer Kit were used for morphological measures. Cells in glass chamber slides were washed with FBS-free medium and stained with CFDA SE (carboxyfluorescein diacetate succinimidyl ester) at 5 µM concentration for 20 min. Afterwards, cells were washed twice with complete medium and placed in the incubator for 5 min. Any unreacted CFDA SE was discarded with a last wash. Cultures were treated with the agents and immediately transferred to a microscope chamber maintained at 37 ºC and 5% CO2. Images were taken every 10 min for 15 h at four predetermined sites per well at 10x with an Andor Dragonfly 200 Spinning Disk confocal, using a 488 nm diode laser. The last time point was selected for image analysis with Trackmate 7 algorithms (35).

3.7. Determination of nitric oxide generation

Nitric oxide generation was measured by the colorimetric Griess reaction (36). This method detects the production of nitrite (NO2-), coming from the combination of molecular oxygen and nitric oxide. The Griess reagent is prepared by mixing a 1:1 solution of Griess A (100 ml of Milli-Q water, 1 % sulfanilamide, 5 % orthophosphoric acid) and Griess B solution (100 ml of Milli-Q water, N-(1-naphthyl) ethylenediamine dihydrochloride 0.1 %).

Cell plates were centrifuged at 153 rcf for 5 min at 4 ºC. Then, 50 µl of conditioned medium from each of the 96 wells were incubated in a new plate with 50 µl of the Griess reagent for 10 min at room temperature. Optical density was measured at 540 nm. Nitrite concentration was determined with a sodium nitrite standard curve, normalized by cell protein content in each well, and subsequently expressed as a percentage of the average maximal values given by the pro-inflammatory agent mCRP.

3.8. Real time quantitative Polymerase Chain Reaction

Extraction of total RNA from BV2 cells from 24-well plates, was carried out using TRIsureTM Reagent (Meridian Bioscience, Bioline, London, UK), following the manufacturer's protocol. RNA quantity and quality of the extractions was determined in the spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260 nm. The quantitative conversion of total RNA to single-stranded cDNA was performed using a high-capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific). Three hundred ng of RNA per sample were loaded in a thermal cycler (FlexCycler, Analytikjena, Jena, Germany), following the protocol: 25 ºC 10 min; 37 ºC 120 min; 85 ºC 5 min; and 4 ºC ∞. cDNA samples were diluted 1:4 with nuclease-free water and stored at -20 ºC.

Gene expression assays were performed using a mix of TaqMan® Fluorescein amidite (FAM)-labeled specific probes (Thermo Fisher Scientific), Quantimix Easy Probe kits (Biotools, Jupiter, FL, USA) and 6.75 ng of cDNA per well, in a CFX96TM or a CFX384TM real-time system (Bio-Rad) using the following protocol: 95 ºC 5 min; 40 cycles at 95 ºC 10 min; 60 ºC 1 min. The used Taqman assay probes were: *Actb* (Actin beta, #Mm02619580_g1), *Ccl2* (chemokine (C-C motif) ligand 2, Mm00441242_m1), *Cox2* (Cyclooxygenase 2, Mm00478374_m1), *Il6* (Interleukin 6, #Mm00446191_m1), *Nos2* (Inducible nitric oxide synthase, Mm00440502_m1). From each sample, gene expression was analyzed in duplicates. Data were normalized to actin beta gene expression using the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method.

3.9. Statistical Analysis

Results are expressed as mean ± SEM. Shapiro–Wilk test was used to assess data distribution. Data were analyzed by one-way ANOVA or two-way ANOVA with the factors: treatment and mCRP. When significant, Tukey's post-hoc test was carried out allowing multiple comparisons between groups.

Statistical significance of the ANOVA factors is indicated in the figures, results were considered significant when $p < 0.05$. Statistical outliers were identified by Grubbs' test ($\alpha = 0.05$). Sample N per group is written in the figure legends. GraphPad Prism 6.01 package (GraphPad Software) and IBM SPSS Statistics v23 (IBM Corp., Armonk, New York, NY, USA) were used for the statistical analysis of data.

4. Results

4.1. mCRP activates inflammatory pathways and stimulates cytokine release in primary cultures of microglia.

Primary microglia were exposed to mCRP at two different concentrations: 25 µg/mL and 50 µg/mL. Both concentrations of mCRP increased protein levels of iNOS (Figure 2A), whose activation is known to be mediated by pro-inflammatory agents, and leads to an increase of NO production (37). mCRP also increased protein levels of NLRP3 inflammasome (Figure 2B), which is a protein complex that modulates the innate immune system and the inflammatory response (38). A similar increase was observed in the cyclooxygenases isoform COX-2 (Figure 2C), which is constitutively expressed in the

brain, and its products act as inflammatory neuromodulators (39). Both concentrations of mCRP presented similar effects. Additionally, when stimulating primary microglia with 25 µg/ml and 50 µg/mL mCRP, an increase in TNF-α (Figure 2D) and IL-6 (figure 2E) release was observed. Both of these cytokines have important roles in neuroinflammatory processes (40, 41). As before, both concentrations of mCRP had similar effects in the microglia.

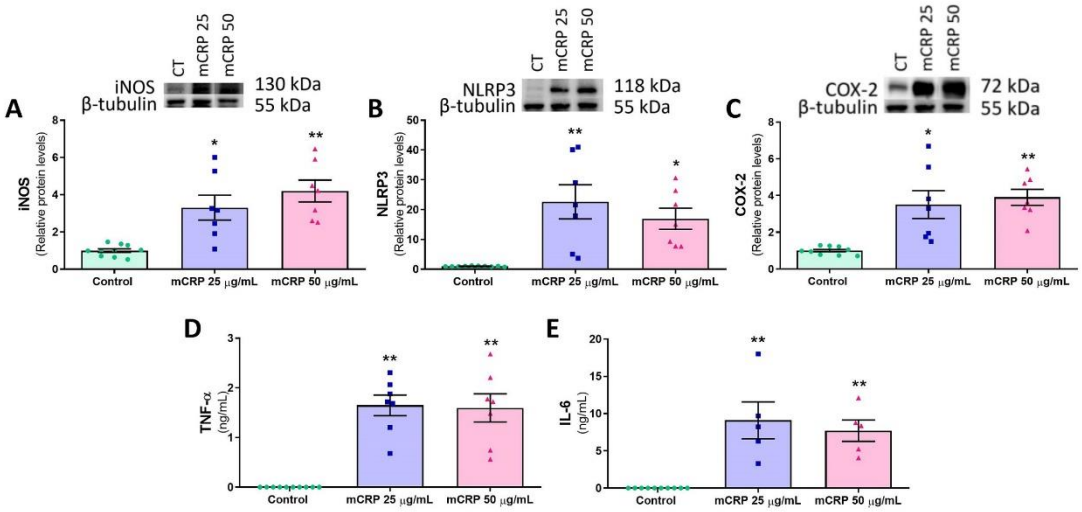

FIGURE 2. Activation of three of the main inflammatory pathways and cytokine release triggered by mCRP in primary microglia. (A-C) Relative protein levels of iNOS enzyme (*n* = 7-10, A), NLRP3 inflammasome (*n* = 7-10, B), and COX2 (*n* = 7-10, C) and its respective representative blots showed above the corresponding histogram. (D) TNFα (ng/mL) (*n* = 7-10) and (E) IL6 (ng/mL) (*n* = 5-10) release. Statistics: One-way ANOVA, Tukey post-hoc test significances: * $p < 0.01$, ** $p < 0.001$ vs. control.

4.2. mCRP modulates BV2 morphology and interacts with cell membrane.

BV2 cells were incubated with mCRP for 24 h. When comparing control cells (Figure 3A) and cells incubated with mCRP (Figure 3B), there was a moderate morphological difference. While control cells showed a rounded configuration, activated cells showed an enlarged and less spherical cell body, as described in our previous work (19). Surprisingly, when checking mCRP presence with our target antibody 3H12 (in green) (Figure 3C-E), BV2 orthogonal views, which allow a perpendicular and right perspective to the primary imaging plane, revealed that mCRP could be positioned attached (Figure 3D), into the cell membrane (Figure 3C), or completely internalized into the cell (Figure 3E).

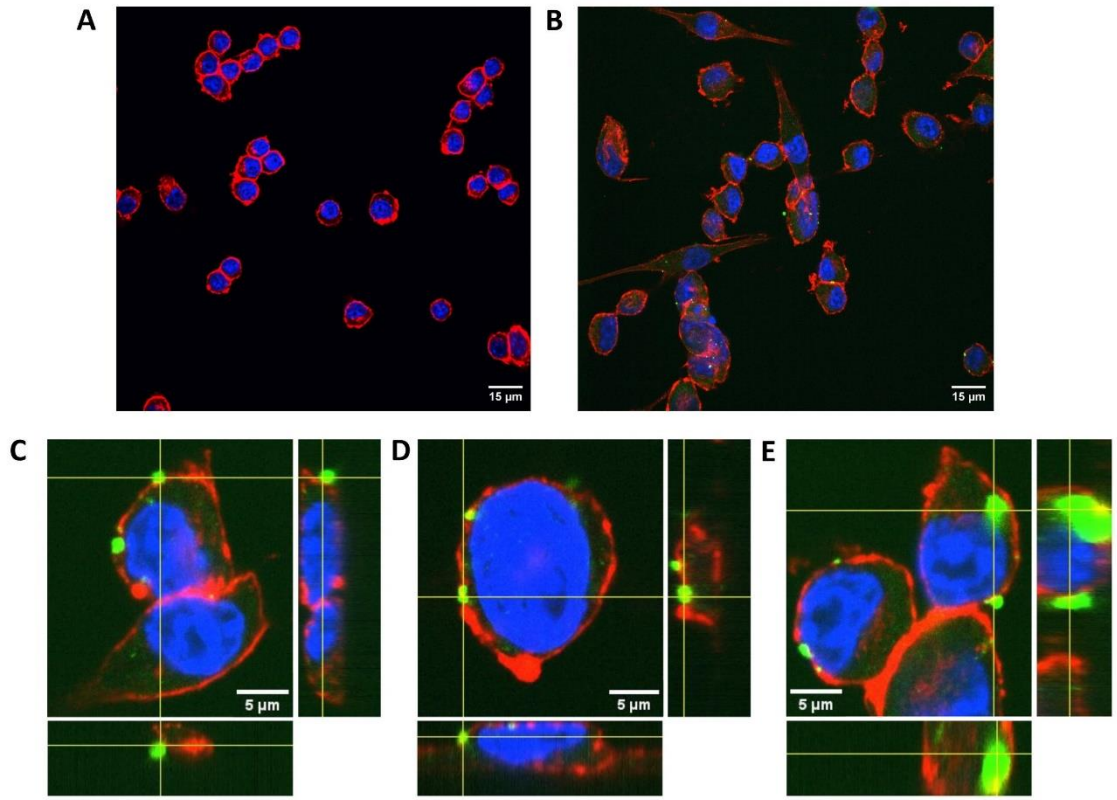

FIGURE 3. Immunofluorescence labelling of BV2 cells incubated 24 h with 50 µg/mL of mCRP. (A, B) A change of morphology is observed between control cells (A) and cells incubated with mCRP (B). (C-E) A main image with a detailed view of individual BV2 cells and their orthogonal views (ZY, right main image; ZX, under main image). mCRP can be attached to the cell membrane (C), into the membrane (D) or internalized (E). Here are shown representative confocal images from two independent experiments in duplicate wells. The green color indicates the presence of mCRP labelled with mAb 3H12, while the red color shows the membrane stained with rhodamine phalloidin-TRITC, and the blue color represents the nuclei labelled with DAPI.

4.3. sEHI counteracts mCRP-induced pro-inflammatory mechanisms in BV2 cells.

4.3.1. sEHI exhibit no cytotoxic effects on BV2 cells.

Safety of sEHI compounds was tested in BV2 cells and no cytotoxic effects were observed in either UB-SCG compounds or TPPU. After 24 h of incubation with different sEHI concentrations up to 100 µM, cytotoxicity was assessed using both PI staining and the MTT assay. Both methods yielded CC50 > 100 µM for all three tested compounds, so no cell death or effects on viability were observed. There was a wide margin of concentrations between safety and inhibitory potency since the IC50 in murine sEH is 1.0 nM for UB-SCG-55 (32), 0.6 nM for UB-SCG-65 (33), and 2.8 nM for TPPU (42).

4.3.2. Treatment with sEHI protects against iNOS pathway activation induced by mCRP.

BV2 cells activated by mCRP showed not only higher transcription of *Nos2* gene (Figure 4A-C) but also a higher translation of the iNOS enzyme (Figure 4D-F). UB-SCG-55 decreased both *Nos2* transcription and iNOS activation (Figure 4A, D), while UB-SCG-65

showed no protective effects (Figure 4B, E). Yet, TPPU tended to lower *Nos2* expression (p = 0.1338) and iNOS presence (p = 0.1592) (Figure 4C, F). Regarding Nitric oxide release, mCRP increased its release, and all sEHI blocked this effect (Figure 4G-I), but UB-SCG-55 protection stood out among the others (Figure 4G).

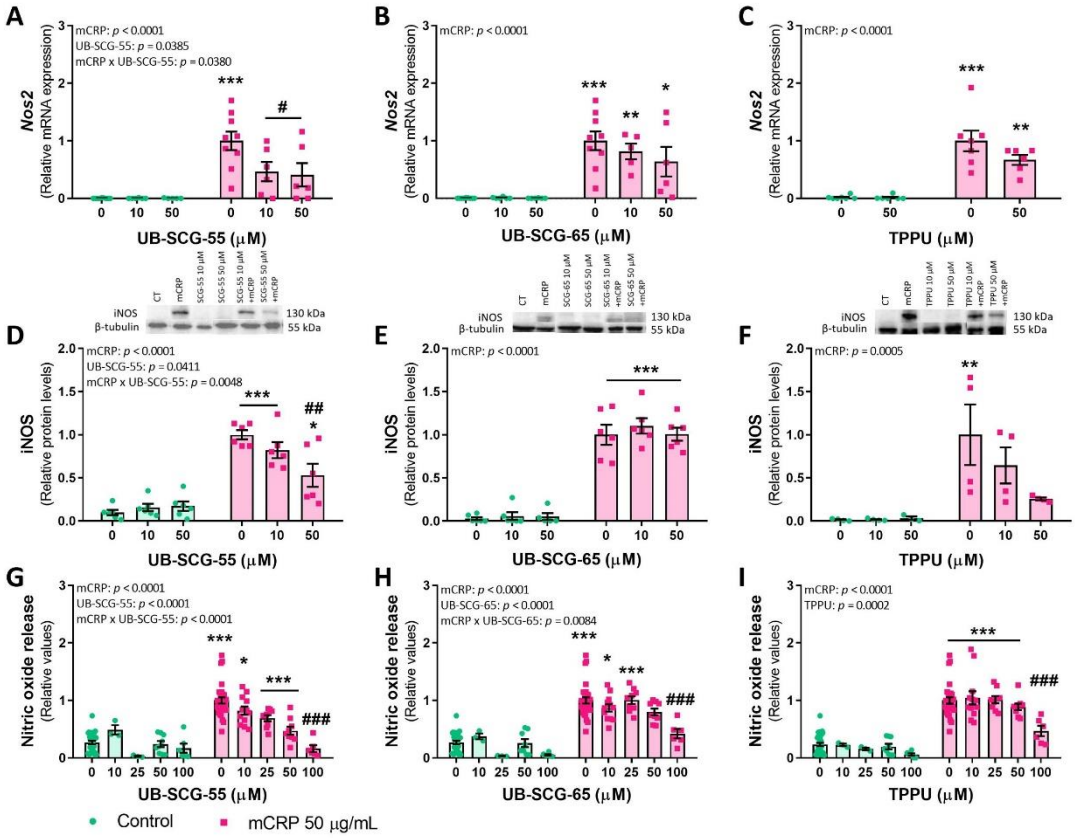

FIGURE 4. Activation of microglial iNOS pathway by mCRP and sEHI protection in BV2 cells. (A-C) expression levels of *Nos2* (*n* = 6-9), (D-F) protein levels of iNOS enzyme (*n* = 4-6) and its respective representative blots showed above the corresponding histogram, (G-I) nitric oxide release (*n* = 6-30); of BV2 cells stimulated with mCRP and treated with UB-SCG-55 (A, D, G), UB-SCG-65 (B, E, H) and TPPU (C, F, I). Statistics: Two-way ANOVA, *p* values of significant factors and its interaction are indicated in the corresponding graph; Tukey post-hoc test significances: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. corresponding concentration of sEHI without mCRP; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. sEHI 0 μM concentration of the mCRP group.

4.3.3. Treatment with UB-SCG-55 protects against NLRP3 inflammasome pathway activation induced by mCRP and protects against Cox2 transcription enhanced by mCRP.

In BV2 cells, mCRP activated the NLRP3 inflammasome complex (Figure 5A-C), and UB-SCG-55 counteracted this inflammatory effect (Figure 5A). Similarly, mCRP activated *Cox2* transcription in the BV2 cells (Figure 5D-F), and UB-SCG-55 decreased *Cox2* transcript (Figure 5D).

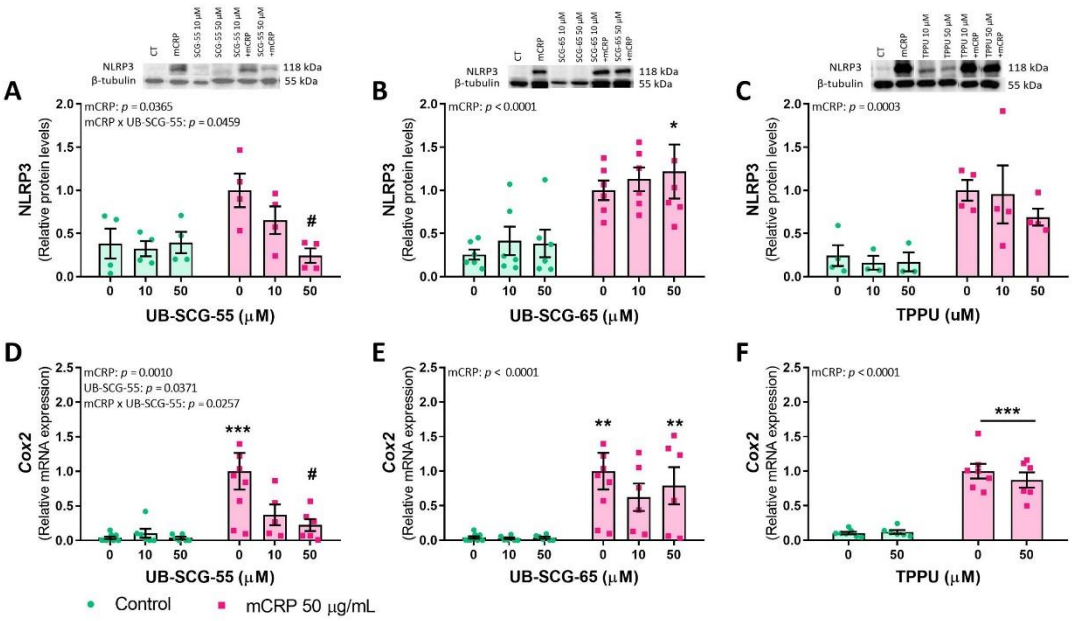

FIGURE 5. mCRP activates NLRP3 inflammasome and *Cox2* transcription in BV2 cells and sEHI protection. (A-C) protein levels of NLRP3 inflammasome protein complex (*n* = 3-6) with its respective representative blots showed above the corresponding histogram, and (D-F) relative mRNA expression of *Cox2* (*n* = 6-9) of BV2 cells stimulated with mCRP and treated with UB-SCG-55 (A, D), UB-SCG-65 (B, E) and TPPU (C, F). Statistics: Two-way ANOVA, *p* values of significant factors and its interaction are indicated in the corresponding graph; Tukey post-hoc test significances: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. corresponding concentration of sEHI without mCRP; # $p < 0.05$ vs. sEHI 0 µM concentration of the mCRP group.

4.3.4. Treatment with sEHI diminishes pro-inflammatory cytokines synthesis and release triggered by mCRP.

mCRP activated TNF-α production (Figure 6A-C) in BV2 cells. Interestingly, all three sEHI agents managed to counteract TNF-α increase (Figure 6A-C). BV2 cells also increased *Il6* transcription when being stimulated with mCRP (Figure 6D-F) and all sEHI agents were able to decrease this transcription (Figure 6D-F), especially UB-SCG-55 in a more significant manner (Figure 6D). With regard to CCL2, it is a chemotactic cytokine that can control microglial migration to neuroinflammatory areas (43). The transcription of the later cytokine was increased when microglia was activated with mCRP (Figure 6G-I) and UB-SCG-55 significantly decreased this effect (Figure 6G), while TPPU showed a tendency to behave in a similar fashion (p = 0.1706) (Figure 6I).

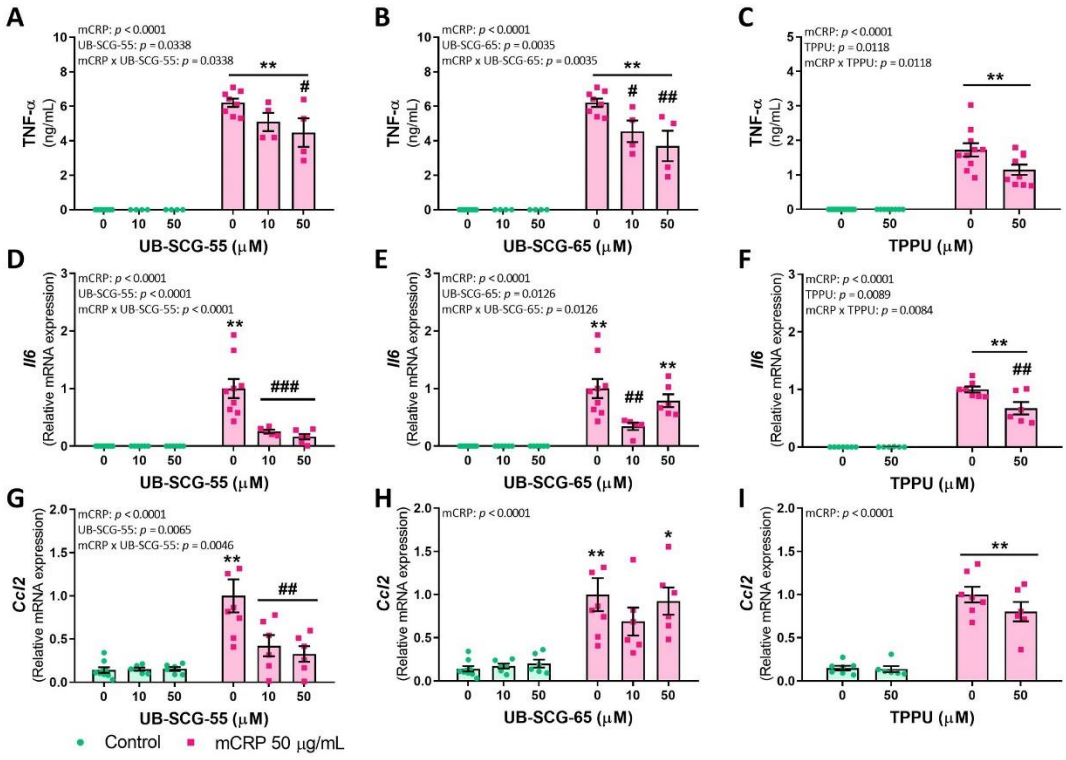

FIGURE 6. Cytokines release in BV2 cells stimulated with mCRP and sEHI protection. (A-C) TNFα release (ng/mL) (*n* = 4-17), (D-F) relative mRNA expression of *Il6* (*n* = 5-9), and (G-I) relative mRNA expression of *Ccl2* (n = 6-9); of BV2 cells stimulated with mCRP and treated with UB-SCG-55 (A, D, G), UB-SCG-65 (B, E, H) and TPPU (C, F, I). Statistics: Two-way ANOVA, *p* values of significant factors and its interaction are indicated in the corresponding graph; Tukey post-hoc test significances: * $p < 0.01$, ** $p < 0.001$ vs. corresponding concentration of sEHI without mCRP; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. sEHI 0 μM concentration of the mCRP group.

4.4. First-line sEHI UB-SCG-55 protects against phenotype activation of primary microglia by mCRP.

Pro-inflammatory pathways activated by mCRP in primary microglia cells were similar to those in BV2 cells, as shown above. This was not unexpected as the BV2 cell line has been proven to be a good microglia model to study neuroprotective and anti-inflammatory agents in an *in vitro* setting (44, 45). However, BV2 cells showed an amoeboid form in control conditions and small phenotype changes under pro-inflammatory treatment, in contrast to the morphology changes of microglia. Therefore, the anti-inflammatory effects of the best positioned sEHI agent UB-SCG-55 were confirmed through morphological analysis of the primary microglia phenotype.

Primary microglia cultures were incubated with mCRP at a concentration of 25 μg/mL for 24 h, with or without co-treatment with UB-SCG-55 at 50 μM. Posterior analysis using confocal microscopy revealed distinct morphological alterations when comparing cells exposed to mCRP with the control group (Figure 7A). Microglia treated with mCRP displayed a significant increase in size, as evidenced by increased cellular area (Figure 7B). They concurrently adopted a round, amoeboid shape, indicative of an M2-like phenotype, as evidenced by the increase in the measurements of the ellipse short axis

(Figure 7C). Noticeably, UB-SCG-55 significantly restored cell size and morphology (Figure 7A, B).

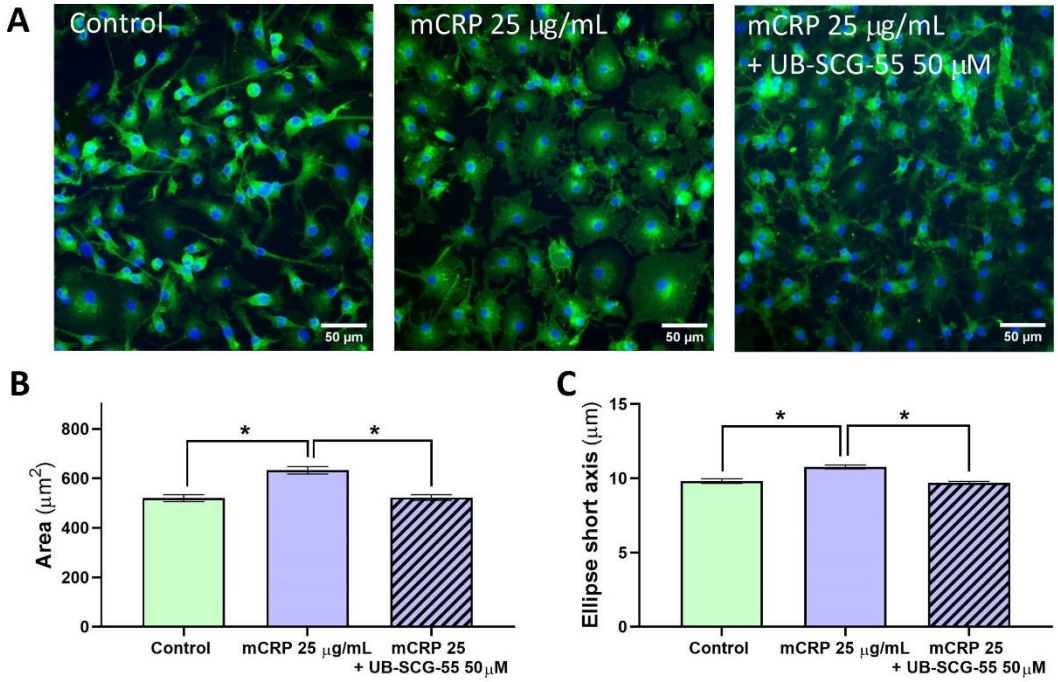

FIGURE 7. Morphological changes in primary cultures of microglia following mCRP incubation, and the restorative effects of UB-SCG-55. (A) Representative microphotographs showing microglia cultures (lectin, green fluorescence) submitted to treatments as indicated in the figure. (B, C) Cellular parameters measured are area (B) and ellipse short axis (C), both *n* = 661 - 1615. Statistics: One-way ANOVA, Tukey post-hoc test significance: * $p < 0.001$.

5. Discussion

A potent pro-inflammatory activity of mCRP has been unveiled here in primary microglia cultures following previous preliminary studies with the BV2 microglial cell line. Mechanisms shown to be activated by mCRP likely contribute to neuroinflammation in AD after vascular injury that allow CRP entry and disaggregation. Furthermore, the protective effects of sEHI agents against mCRP exposure, shown in both microglia and BV2 cells, are highly relevant for developing pharmacological strategies against neuroinflammatory diseases.

Exposure to mCRP triggers a pro-inflammatory response in primary microglia which has not been reported so far. Interestingly, the increased expression of key inflammatory proteins analyzed in these cultures was similar to that in BV2 cells even at a lower concentration of mCRP. The mCRP activated three of the main inflammatory pathways: iNOS, NLRP3, and COX-2. The iNOS enzyme plays a crucial role in the production of NO within microglia. This molecule signals through the receptor sCG, which activates the cGMP/PKG pathway that can modulate NF-κB activation (46). Shifting focus to the NLRP3 inflammasome pathway, it has been directly linked to AD pathology, as it arbitrates Aβ-induced tau pathology in Tau22 mice (47). The NLRP3 inflammasome

activates the NF-κB pathway and promotes IL-1β and IL-18 production in microglia, thereby initiating a pro-inflammatory signaling cascade (48, 49). On top of that, COX-2 is the major pro-inflammatory and inducible isoform of COX (50) and it is expressed in the brain (39). Ultimately, the activation of these three pathways can be related to activation and polarization of the microglia to a pro-inflammatory M1-like phenotype (51), which as previously noted, is directly related to neurodegenerative diseases.

Importantly, primary microglia increased TNF-α and IL-6 release after mCRP stimulation. TNF-α is a cytokine produced by the microglia to regulate several activities in the brain. The binding of this cytokine to its receptor TNFR1 can mediate apoptosis, leading to neurodegeneration, and chronic inflammation (40). Additionally, a study of systemic lupus erythematosus in BV2 showed that TNF-α activates the NF-κB inflammatory pathway and also induces the release of cytokines such as IL-6 (52). IL-6 binds to its receptor IL-6R and it can enhance microglia priming (53), which is typical of aged microglia or a neuroinflammatory environment. Indeed, in a previous study in BV2, mCRP also induced the release of TNF-α and the nuclear translocation of NF-κB in these cells (26).

One might assume that the dynamics of mCRP, when in contact with the cell membrane, is similar between primary microglia and BV2 immortalized microglia. The discovery that mCRP can bind to and cross the BV2 cell membrane may help to understand its mechanisms of action. So far, there have not been any previous reports of cell internalization. In endothelial cells, mCRP is able to enter the membrane lipid rafts and, from there, trigger cytokines, reactive oxygen species and membrane surface expression of ICAM-1, VCAM-1 and E-selectin (54). In addition, *in vitro* studies conducted with the HAEC cell line revealed that a cholesterol-binding sequence (a.a. 35-47) regulates the interaction between mCRP and various ligands (55). This particular sequence was identified as the key mediator facilitating the binding of mCRP to lipoproteins and the complement component C1q, among other constituents (11, 55). With the information just provided, we hypothesize that in microglia, as in endothelial cells, mCRP could enter membrane rafts as a way of interacting with its several ligands. Additionally, mCRP was observed in the cytoplasm, which could mean that either it is independently diffused, which is doubtful due to its low aqueous solubility (56). The other possibility is that it traveled throughout the cell via vesicles or motor proteins. Nevertheless, one may wonder, since mCRP appears to be forming a large aggregate inside the cell, if it could have mechanical negative effects.

Taking into account the similar mechanisms between microglia and BV2 cells, the latter cells were used to test the anti-inflammatory effectiveness of sEHI agents. Two newly-synthesised sEHI compounds with increased potency and microsomal stability (32, 57) were studied and compared with the well-known sEHI compound TPPU. After confirming no cytotoxic effects, they were tested against mCRP inflammatory effects. As expected, according to primary microglia results, mCRP stimulated the activation of the iNOS, NLRP3 inflammasome and COX-2 pathways in BV2. sEHI compounds differed in their level of effectiveness in counteracting the increase of the analysed markers. As a

whole, UB-SCG-55 showed the highest anti-inflammatory potency for the three inflammatory pathways.

Inhibition of these three pathways may undoubtedly help against the development of neuroinflammation associated with AD. Indeed, there is evidence linking these inflammatory pathways to AD. For instance, NO has already been linked to neuronal cell loss, neuronal injury and protein misfolding in this disease (58). Also, NLRP3 inhibition was reported to enhance the presence of a microglia with an M2-like phenotype and decrease NOS2 expression (59). In addition, levels of PGE2 increase in the cerebrospinal fluid of AD patients and this eicosanoid is synthetized in a two-step reaction involving the COX enzyme (60, 61). COX-2 inhibition could explain the reduction of NLRP3 inflammasome protein levels, since PGE2 can mediate its activation (62). Furthermore, Aβ pathology has been directly associated with this inflammatory pathway. In fact, when suppressing the signal of PGE2 receptor EP2 in the microglia of an AD mouse model, an enhanced clearance of this peptide (63), and a prevention of cognitive impairment and synaptic loss (60) can be observed.

Moreover, TNF-α and IL-6 levels were increased in the BV2 microglia after mCRP stimulation, and all three sEHI compounds were able to counteract these effects. The monocyte chemoattractant protein CCL2, which regulates microgliosis (64), was also studied in a mCRP-inflammatory context. mCRP appeared to stimulate this chemokine transcription, and UB-SCG-55 successfully diminish this increase. In this context, CCL2 has been previously described as not being able to modify other cytokines or NO release in rat microglia (64), although its expression is directly regulated via NF-κB (65).

Finally, the sEHI UB-SCG-55 also inhibited morphological changes indicative of M1-like phenotype in primary microglia incubated with mCRP. Thus, corroborating its anti-inflammatory effectiveness as assayed in BV2 cells.

How sEHI is able to counteract mCRP effects could be related to the repressed activation of NF-κB due to EETs increase (66), which is the main conductor of these three inflammatory pathways: iNOS, NLRP3 inflammasome, and COX-2/PGE2 pathways. With a sEHI treatment, the half-life and biological activity of EETs are positively increased. In Sprague-Dawley rats with central post-stroke pain, treatment with TPPU or TPPU along with a 14,15-EET supplement showed a diminished glial cell activation in the CNS and release of pro-inflammatory cytokines (67). Otherwise, EETs have been proposed as inhibitors of PGE2 production and action by reducing prostaglandin synthase, COX-2 and some prostaglandin receptors (68, 69). Nonetheless, it remains unclear whether EETs passively diffuse through the cell membrane or if this cellular entry involves a membrane transporter (69). In microglia, the role and mechanisms of lipids are poorly understood, but its metabolism is linked to cell migration, phagocytosis and inflammation, which affect neurons and other glial cells (70). EETs might alter the structure of cell membranes, interact directly with ligands or proteins, thereby activating several NFκB–driven inflammatory pathways, among other effects. However, further investigation into that matter, specifically in microglia cells, would entail a separate research study.

Interestingly, all three sEHI were more effective reducing release of cytokines than inhibiting the production and activity of some of the inflammatory-related enzymes. That could be explained either because EETs may act through molecules or receptors involving cytokines release, because the EETs are acting on enzyme systems such as those mentioned above which produce inflammatory eicosanoids, or because EETs act via other inflammatory pathways that reduce enhancement release of cytokines. Whether UB-SCG-55 was more effective than UB-SCG-65 in most of the variables studied might be related to its chemical structure. Since UB-SCG-65 has an added acid group, this acid may be found in either its protonated or deprotonated form in the culture media, potentially altering the molecule's permeability to enter the cell and affecting its efficacy. Hence, the structure of UB-SCG-55 may be a most suitable baseline for the design of more refined versions of sEHI.

6. Conclusion

In summary, the pro-inflammatory phenotype induced by mCRP was characterized in primary cultures of microglia, which showed a similar response in the BV2 cell line and assayed the anti-inflammatory efficacy of newly synthesized sEHI compounds. UB-SCG-55 stood out as first line sEHI. mCRP is emerging as a pro-inflammatory agent in neuroinflammatory diseases, while inhibition of sEH and subsequent increase of EETs is a valuable target for neuroprotection.

This mCRP-inflammatory scenario in microglia may be reflected in the AD pathology. While previous studies have identified several mechanisms of inflammation in AD, there is still much to uncover regarding the implications of CRP/mCRP in this pathology and the microglial response to these insults. Here, we have better characterized the inflammatory mechanisms activated by mCRP, including iNOS, NLRP3 inflammasome, and COX-2/PGE2 pathways, which may strongly contribute to microglial activation and polarization to a pro-inflammatory M1-like phenotype, and ultimately to neuroinflammation (Figure 8). Additionally, mCRP was shown to bind and cross BV2 cell membrane, which may help unravel its process of action. Understanding these mechanisms is crucial to develop new pharmacological strategies to mediate neuroinflammation. Based on these results, we firmly propose sEHI as a possible treatment for inflammation in AD and other neuroinflammatory diseases, since they have been shown to efficiently reduce the increase in inflammatory markers and morphological changes observed after exposure to mCRP in both microglia and BV2 cells.

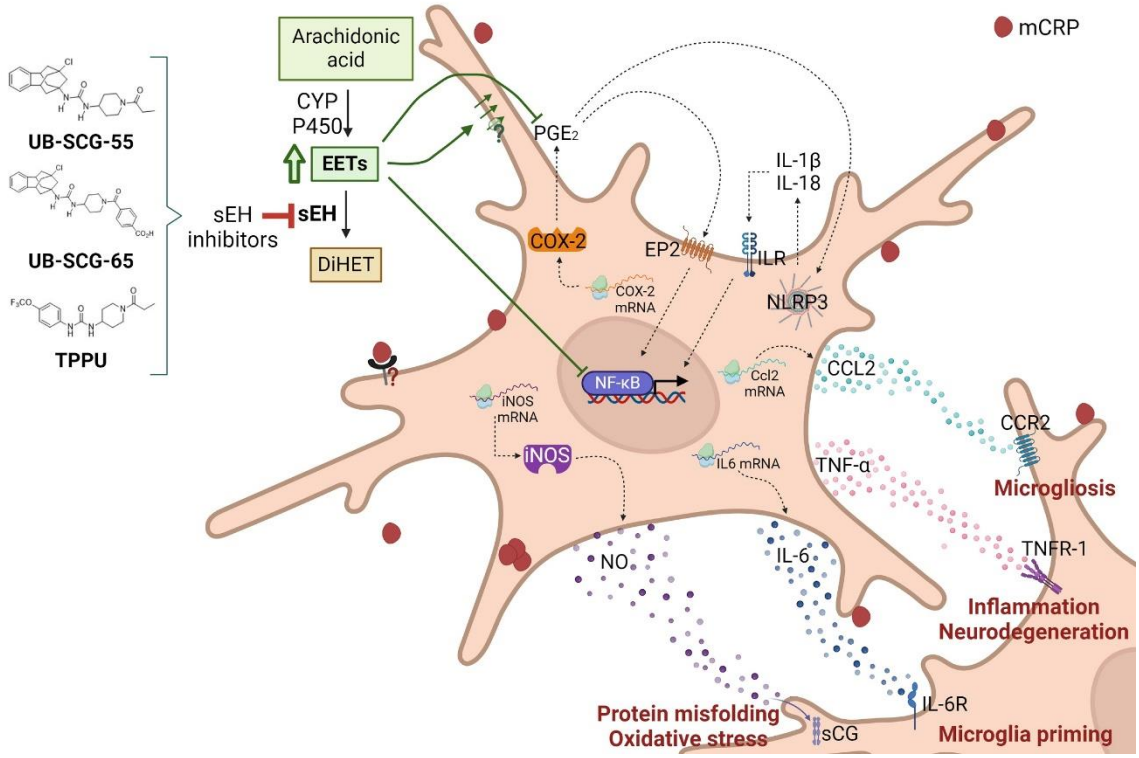

FIGURE 8. Proposed mechanisms of mCRP-activated inflammatory pathways in a microglial cell and sEH inhibition. Created with BioRender.com

7. Conflict of Interest

S.C., M.P., C.G.-F. and S.V. are inventors of the Universitat de Barcelona patent applications on sEH inhibitors WO2019/243414 and WO2022/200105. C.M. and B.D.H. are inventors of the University of California patents on sEH inhibitors licensed to EicOsis. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8. Author Contributions

Conceptualization, C.Sa.; methodology, M.P., S.V., and C.Su; formal analysis, C.B., K.V., Y.Y., H.V.-Q., and C.C.; investigation, C.B., C.G.-F., and C.Sa.; data curation, C.B., C.Su, and C.Sa.; resources, S.C., M.S., C.M., B.D.H. and S.V.; writing—original draft preparation, C.B. and C.Sa; writing—review and editing, C.B., C.Sa., K.V., S.V., C.M., B.D.H.,C.G.-F., and C.Su.; funding acquisition, C.S. All authors have read and agreed to the published version of the manuscript.

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11. Data Availability Statement

The Data supporting the conclusions of this article are contained in the corresponding figures. Full raw data will be made available by the authors, without undue reservation.

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Publication 4.

Resveratrol activates antioxidant protective mechanisms in cellular models of Alzheimer's disease inflammation

Clara Bartra, Yi Yuan, Kristijan Vuraić, Haydeé Valdés-Quiroz, Pau Garcia-Baucells, Mark Slevin, Ylenia Pastorello, Cristina Suñol and Coral Sanfeliu

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Resveratrol Activates Antioxidant Protective Mechanisms in Cellular Models of Alzheimer's Disease Inflammation

Clara Bartra [1,2,3], Yi Yuan [1], Kristijan Vuraić [1], Haydeé Valdés-Quiroz [1], Pau Garcia-Baucells [1], Mark Slevin [4,5], Ylenia Pastorello [6], Cristina Suñol [1,2] and Coral Sanfeliu [1,2,*]

[1] Institut d'Investigacions Biomèdiques de Barcelona (IIBB), CSIC, 08036 Barcelona, Spain;
clara.bartra@iibb.csic.es (C.B.); yuan_yi07@126.com (Y.Y.); kvuraic@stud.biol.pmf.hr (K.V.);
ayd.valdes@gmail.com (H.V.-Q.); paugarbau@gmail.com(P.G.-B.);
cristina.sunol@iibb.csic.es (C.S.)
[2] Institut d'Investigacions Biomèdiques August Pi i Sunyer (DIBAPS), 08036 Barcelona, Spain
[3] PhD Program in Biotechnology, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, 08034 Barcelona, Spain
[4] School of Life Sciences, John Dalton Building, Manchester Metropolitan University, Manchester M15 6BH, UK; m.a.slevin@mmu.ac.uk (M.S.)
[5] Centru Avansat de Cercetari Medicale si Farmaceutice (CCAMF), Universitatea de Medicina, Farmacie, Stiinte si Tehnologie "George Emil Palade" din Targu Mures, Targu Mures, 540142, Romania
[6] Department of Anatomy and Embryology, Universitatea de Medicina, Farmacie, Stiinte si Tehnologie "George Emil Palade" din Targu Mures, Targu Mures, 540142, Romania; ylenia.pastorello@umfst.ro (Y.P.)
* Correspondence: coral.sanfeliu@iibb.csic.es (C.S.); Tel.: +34-933638338

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Abstract: Resveratrol is a natural phenolic compound with known benefits against neurodegeneration. We analyzed *in vitro* the protective mechanisms of resveratrol against the proinflammatory monomeric C-reactive protein (mCRP). mCRP increases the risk of AD after stroke and we previously demonstrated that intracerebral mCRP induces AD-like dementia in mice. Here, we used BV2 microglia treated with mCRP for 24 h in the presence or absence of resveratrol. Cells and conditioned media were collected for analysis. Lipopolysaccharide (LPS) has also been implicated in AD progression and so LPS was used as a resveratrol-sensitive reference agent. mCRP at the concentration of 50 µg/mL activated the nitric oxide pathway and the NLRP3 inflammasome pathway. Furthermore, mCRP induced cyclooxygenase-2 and the release of proinflammatory cytokines. Resveratrol effectively inhibited these changes and increased the expression of the antioxidant enzyme genes *Cat* and *Sod2*. As central mechanisms of defense, resveratrol activated the hub genes *Sirt1* and *Nfe2l2* and inhibited the nuclear translocation of the signal transducer NF-κB. Proinflammatory changes induced by mCRP in

primary mixed glial cultures were also protected by resveratrol. This work provides a mechanistic insight into the protective benefits of resveratrol in preventing the risk of AD induced by proinflammatory agents.

Keywords: resveratrol; monomeric C-reactive protein; lipopolysaccharide; oxidative stress; inflammation; BV2; primary mixed glial cultures

1. Introduction

Resveratrol is a phenolic compound of the stilbene family. Its active *trans* form (*trans*-3,5,4'-trihydroxystilbene) has multiple health benefits shown in years of preclinical research. It is a powerful antioxidant that also exhibits anti-inflammatory, anti-aging and anti-neurodegenerative properties [1–4], among others. Animal studies showed that *trans*-resveratrol is able to cross the blood–brain barrier [5]. This supports the possibility that resveratrol could be protective for brain cells. Resveratrol is synthesized by more than 70 species of plants to stimulate cell defense in response to stress conditions. It is present in considerable amount in mulberries, lingonberries, cranberries, peanuts, the skin of red grapes, and in some medicinal herbs. In addition, resveratrol was initially associated with red wine as its main source. This misleading perception might lead to resveratrol being discredited after new studies showed no significant reductions in all-cause mortality with moderate drinking [6] and for concerns about possible alcohol addiction. However, moderate drinking in old men and women is consistently associated with a lower risk of dementia when compared with lifetime abstainers [7]. Whatever the active ingredient is in wine, resveratrol should be studied as a pure nutraceutical-type compound. In fact, therapeutic doses cannot be obtained from diet and require supplementation. Resveratrol doses in clinical trials with positive outcome were in the range of 150–250 mg/d for lowering blood pressure [8], for cerebrovascular and cognitive benefits in postmenopausal women [9,10], and cognitive benefits in overweight old men [11]. These authors hypothesized that the benefits found are at least partially based on the ability of resveratrol to modulate cerebral blood flow and vessel responsiveness during demand [9–11], and in association with improvement of the metabolic profile [8,11]. The proposed underlying molecular mechanisms include modulation of endothelial nitric oxide, decrease in oxidative stress, and activation of calorie restriction-like pathways (i.e., sirtuin 1 (SIRT1), AMP-activated protein kinase (AMPK) and nuclear factor-like 2). However, a number of clinical trials did not report benefits and thus, the results of meta-analysis are controversial [12]. We can speculate that either higher dosages or combination with other compounds to improve bioavailability could have worsened the outcome. Resveratrol is a hormetic agent and is not devoid of adverse effects at elevated concentrations [13,14]. Noticeably, a recent review of the limited number of clinical trials performed in Alzheimer's disease (AD) patients concluded a delay of cognitive impairment following resveratrol supplementation in comparison with placebo [15]. However, the bioavailability and

pharmacokinetics of resveratrol after oral administration are poor. Circulating resveratrol is mainly bound to albumin [16,17]. The hydrophobic structure of resveratrol may limit its absorption into target tissues. A main drawback is its rapid metabolism to less active glucuronide and sulfate conjugates [5,18,19]. The resulting low plasma and brain levels of *trans*-resveratrol after oral doses [5] have raised uncertainty about the administration regimen. Experimental strategies with innovative nanocarriers to improve the aqueous solubility and bioavailability of resveratrol have already shown benefices in the field of cancer therapeutics [20] and in brain delivery [21]. Research on innovative carriers and formulations of resveratrol to improve its bioavailability in humans is ongoing [22–24]. Improvements in pharmacokinetics will overcome the safety concerns in resveratrol dosing [25].

There is an interplay between oxidative stress and inflammation, as one may promote the other leading to a damaging spiral. With advancing age, an imbalance between the generation of reactive oxygen species (ROS) and its clearance leads to chronic inflammation. In turn, sustained inflammatory processes contribute to an increased risk of age-related diseases, including AD [26,27]. Resveratrol is effective in reducing oxidative stress in AD *in vitro* neuronal models [28–32]. These studies show that amyloid ß peptides induce oxidative stress in PC12 cells [28,29], human neural stem cells [30], HT22 cells [31] and primary neurons [32] that parallels cytotoxicity. The antioxidant protective mechanisms of resveratrol in these models included an increase in mitochondrial enzyme manganese superoxide dismutase (SOD2), inhibition of the redox-regulated transcription factor NF-kB, and activation of the master regulator of cell energy AMPK. Furthermore, resveratrol is effective against oxidative stress in immortalized lymphocytes from AD patients by increasing expression of genes encoding antioxidant enzymes and survival factors [33]. Resveratrol also shows antioxidant properties in *in vivo* rodent models of AD [31,32,34–36]. Transgenic mouse models of AD [31,32] and rat chemical AD models induced by colchicine [34,36] or angiotensin II [35] show oxidative stress as a deleterious mechanism associated with cognitive loss. Chronic intake of resveratrol inhibits oxidative damage by activating antioxidant systems in the brain, including SOD2 and reduced glutathione. Furthermore, as a phenolic agent it can also induce direct antioxidant effects.

Resveratrol has anti-inflammatory capacity in models of neurodegenerative disease, as shown by its downregulation of tumor necrosis factor α (TNFα) and other proinflammatory molecules [4,37–39]. Microglia are the key cells in the inflammatory processes associated with AD and in the interplay with oxidative stress and inflammation [40]. Previous studies showed that resveratrol inhibited amyloid ß-induced phenotype activation in microglial cell lines BV2 and N9 [41,42]. Several studies also showed that resveratrol protected against the proinflammatory effects of lipopolysaccharide (LPS) in microglial cells, using an *in vitro* model of neuroinflammatory diseases [43–50]. However, protective mechanisms of resveratrol that dampen microglial over-reactivity during AD progression are poorly characterized.

Here, we aimed to analyze the antioxidant protection mechanisms of resveratrol in BV2 microglia activated by monomeric C-reactive protein (mCRP) as a novel model of AD. The monomeric form of CRP is produced by activation and further disaggregation of the circulant pentameric CRP and has strong proinflammatory properties [51]. mCRP expression is strongly associated with the risk and progression of diseases dependent upon chronic inflammation [52–54]. In the brain it may increase AD risk after stroke [55,56]. *In vivo* and *in vitro* preclinical studies showed that mCRP induced the two main AD pathological markers, amyloid ß and hyperphosphorylated tau, and AD-like dementia in mice [56–58]. Resveratrol intake is known to decrease peripheral blood CRP levels in humans [59,60]. However, to our knowledge, the effects of resveratrol on mCRP within brain cells has not been studied to date. In addition, we used LPS as a reference agent involved in AD and other neurodegenerative diseases [61]. The proinflammatory effects of LPS on microglial cells are inhibited by resveratrol, as indicated above, which we analyzed further here. Resveratrol is effective against phenotype activation by LPS in both microglial cell lines and primary cultures. Therefore, we also aimed to confirm resveratrol inhibition of BV2 activation by mCRP in microglia grown in primary mixed glial cultures.

2. Materials and Methods

2.1. Cell Culture

BV2 microglial cells were used in this study (#ATL03001, ICLC, Banca Biologica e Cell Factory, Genova, Italy). The BV2 cell line was established from C57BL/6 mouse microglia, and it is a valued microglial model for the study of brain neuroinflammatory mechanisms [62]. BV2 cells were grown in RPMI 1640 medium (Biowest, Riverside, Newry and Mourne, UK) supplemented with L-glutamine 2 mM, gentamycin 50 µg/mL and 10% heat-inactivated fetal bovine serum (FBS) (all supplements were from Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Cells were seeded in T25 flasks (NuncTM, Thermo Fisher Scientific) and sub-cultured 1:10 when they reached 80–90% of confluence.

Primary glial cultures were used for selected experiments to confirm the benefits of resveratrol against glia activation by mCRP. For these purpose, mixed glial cultures were prepared from cerebral cortices of C57BL/6 mice at 2–4 days of age following established procedures [63]. Briefly, cortices were minced and disaggregated into a single cell solution by enzymatic and mechanical processing. Cells were resuspended in DMEM:F12 with HEPES and L-glutamine (#31330038, Gibco). This medium was supplemented with gentamycin 50 µg/mL and 10% FBS (all culture reagents were from Gibco, Thermo Fisher Scientific). Cells were seeded in 96- or 24-well plates (Nunc, ThermoFisher Scientific) at 8×10^4 cells/cm^2 or in 8-well chamber slides (Lab-Tek Chamber Slides; Nunc, ThermoFisher Scientific) at 6×10^4 cells/cm^2. The medium was replaced every 5–7 days. Cultures contained mainly astrocytes and microglia.

2.2. Drugs and Experimental Design

mCRP was generated from the native CRP protein (YO Proteins, Ronninge, Sweden). A pure solution of CRP monomers was obtained by urea/EDTA chelation and subsequent dialysis, as previously described [57].

The LPS strain was E. coli 026:B26 (L-2654, batch #120M4028, Sigma-Aldrich, St. Louis, MO, USA). All other reagents were also from Sigma-Aldrich where not otherwise indicated.

For experiments, BV2 cells were seeded in 96- or 12-well plates (Nunc, ThermoFisher Scientific) at 5×10^4 cells/cm^2 or in 8-well chamber slides (Lab-Tek Chamber Slides; Nunc, Thermo Fisher Scientific) at 1×10^4 cells/cm^2. After 24 h, the medium was replaced with fresh culture medium without FBS, containing vehicle (DMSO) or resveratrol (1 µM to 50 µM, dissolved in DMSO). The final concentration of DMSO in all wells was 0.1%. After 1 h of pretreatment, cells were stimulated with the proinflammatory agents mCRP (50 µg/mL) or LPS (0.1 µg/mL, 1 µg/mL) and incubated for 24 h. Each treatment was performed in 2–3 wells and the whole experiment was repeated 3–5 times in cultures of different cell passage.

Primary glial cultures in well plates and chamber slides were used at 18–21 days *in vitro*. Treatments with resveratrol (10 µM to 50 µM) and mCRP (50 µg/mL) were performed following the same procedure used in BV2 cells. However, primary cultures were not subjected to serum starvation to avoid astrocyte damage. Each treatment was performed in 3–4 wells and the whole experiment was repeated in two independent primary cultures.

2.3. Nitrites Assay

Nitric oxide generation was determined with the colorimetric Griess reaction that detects nitrite (NO_2^-), a stable reaction product of nitric oxide and molecular oxygen, following standard procedures [57]. Nitrite levels in the fresh conditioned media were calculated with a nitrite curve in each 96-well plate. The results were expressed as a percentage of mCRP 50 µg/mL or LPS 1 µg/mL values, as peak values.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

Conditioned media were collected and preserved at −70 °C until analysis. Levels of TNFα and interleukin 1ß (IL1ß) were measured using the Mouse TNF alpha uncoated ELISA kit and IL-1 beta Mouse Uncoated ELISA Kit, respectively (Invitrogen, Thermo Fisher Scientific). Samples were analyzed in duplicate. TNFα results were obtained in ng/mL and IL1β results in pg/mL.

2.5. Western Blotting

Protein extracts were obtained from the treated BV2 cells in 12-well plates at termination. Cultures were washed with cold PBS, homogenized in a cold RIPA buffer supplemented with protease and phosphatase inhibitors, and centrifuged. The supernatant concentration of proteins was determined by the Bradford protein assay

(Bio-Rad, Hercules, CA, USA). Western blotting analysis of specific proteins was performed following standard procedures. Briefly, 10 µg of denatured protein samples were separated by SDS-PAGE at 100 V. Electrophoresed proteins were blotted onto PVDF membranes at 200 mA for 90 min. The membranes were then incubated for 1 h with a blocking agent followed by overnight incubation with the primary antibody at 4 °C. The primary antibodies used were anti-inducible nitric oxide synthase (iNOS) (1:1000; #610421, BD Bioscience, BD Transduction Laboratories, Franklin Lakes, NJ, USA), anti-nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP3) (1:1000; #NBP2-12446SS, Novus Biologicals, Bio-Techne, Centennial, CO, USA), anti-actin (20-33) (1:10,000; #A5060, Sigma-Aldrich) and anti-ß-tubulin (1:10,000; #T4026; Sigma-Aldrich). Membranes were rinsed and incubated for 1 h with the secondary antibodies. The antibodies used were sheep-anti-mouse HRP conjugated (1:2000; #NA931, Amersham, General Electric, Boston, MA, USA) and donkey-anti-rabbit HRP conjugated (1:2000; #NA934, Amersham). The proteins were visualized by enhanced chemiluminescence detection in a Chemidoc™ Imaging System (Bio-Rad). The densitometric analysis was performed using Image Lab software (v3.0.1, Bio-Rad). The protein levels were normalized using ß-tubulin. Samples from all treatments were included on each membrane.

2.6. Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Extraction of RNA from BV2 cells was carried out using TRIsureTM reagent (Meridian Bioscience, Bioline, London, UK), following manufacturer's instructions. Samples were checked for RNA concentration and quality using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription from RNA to cDNA was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific, Waltham, MA, USA). Three hundred ng of RNA per sample were loaded in a thermal cycler (FlexCycler, Analytikjena, Jena, Germany). cDNA samples were stored at −20 °C until analysis.

Gene expression was analyzed by qPCR using TaqMan® Fluorescein amidite (FAM)-labeled specific probes (Thermo Fisher Scientific) and a Quantimix Easy Probe kit (Biotools, Madrid, Spain). The reaction mix containing 6.75 ng of cDNA was loaded in a CFX96TM Real-Time System (Bio-Rad). The Taqman assay probes were *Actb* (Actin beta, #Mm02619580_g1), *Cat* (Catalase, #Mm00437992_m1), *Clec7a* (C-Type lectin domain containing 7A, Mm01183349_m1), *Cox2* (Cyclooxygenase 2, Mm00478374_m1), Il6 (Interleukin 6, #Mm00446191_m1), *Nfe2l2* (NFE2 like BZIP transcription factor 2, Mm00477784_m1), *Nos2* (Inducible nitric oxide synthase, Mm00440502_m1), *Sirt1* (Sirtuin (silent mating type information regulation 2 homolog) 1, #Mm00490758_m1), and *Sod2* (Superoxide dismutase 2, #Mm01313000_m1). Samples were analyzed in duplicate. Results were normalized to actin beta gene expression using the comparative cycle threshold method (ΔΔCT).

2.7. Immunofluorescence assay

Cells in chamber slides were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100 for 8 min and incubated with a blocking solution for 1 h.

BV2 were then incubated overnight at 4 °C with anti-nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) antibody, p65 subunit, active subunit, clone 12H11 (1:200; #MAB3026, Chemicon, Merck, Darmstadt, Germany). Slides were washed and incubated with Alexa Fluor 488-conjugated secondary antibody (1:1000; #A-11001, Molecular Probes, Thermo Fischer Scientific). Slides were mounted with Fluoroshield with DAPI (Sigma-Aldrich, Merck). Imaging was performed with an Andor Dragonfly 200 Spinning Disk confocal (Oxford Instruments, Abingdon, Oxfordshire, UK). 405 nm and 488 nm diode lasers were used to visualize DAPI nuclear fluorescence and NF-κB p65 fluorescence, respectively. Images were taken every 0.5 µm at 40x. Cell images at the midline of the nucleus were used for each cell analysis. Nuclear NF-κB p65 fluorescence was analyzed using ImageJ [64].

Primary cultures were incubated for 1 h at room temperature with anti-glial fibrillary acidic protein (GFAP) antibody (1:500; #Z0334, Dako, Agilent, Santa Clara, CA, USA) to stain astrocytes. The secondary antibody was conjugated with Alexa Fluor 546 (1:1000; Thermo Fisher Scientific). Microglia were stained with lectin from *Bandeiraea simplicifolia* conjugated with fluorescein (1:400; #L2895, Sigma-Aldrich). Slides were mounted with Fluoroshield (Sigma-Aldrich, Merck). Microphotographs to visualize changes in cell morphology were taken on a Nikon E1000 microscope.

2.8. Statistical Analysis

Results are expressed as the mean ± SEM. Normal distribution was checked with the Shapiro–Wilk test. Data were analyzed by two-way ANOVA with the factors: resveratrol and either mCRP or LPS. Tukey's multiple comparisons test was used for the post hoc analysis. Statistical outliers were identified by Grubbs' test ($\alpha = 0.5$). The results were considered significant with a value of $p < 0.05$. The software used were GraphPad Prism v6.01 (GraphPad Software, La Jolla, CA, USA) and IBM SPSS Statistics v23 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Resveratrol Inhibited TNFα Production Induced by mCRP and LPS

TNFα is a master proinflammatory cytokine whose production by activated microglia in the brain is induced by a variety of agents and conditions.

mCRP at 50 µg/mL potently induced TNFα release by microglial BV2 cells which was inhibited by resveratrol to levels close to the control treatment (Figure 1a). LPS at 0.1 µg/mL induced less TNFα release than mCRP, while resveratrol showed a non-significant tendency to reduce the LPS effect at the tested concentration of 25 µM (Figure 1b).

Therefore, resveratrol protected against TNFα release from BV2 microglia after a proinflammatory injury, implying reduced activation of the inflammatory phenotype and subsequent downstream signaling.

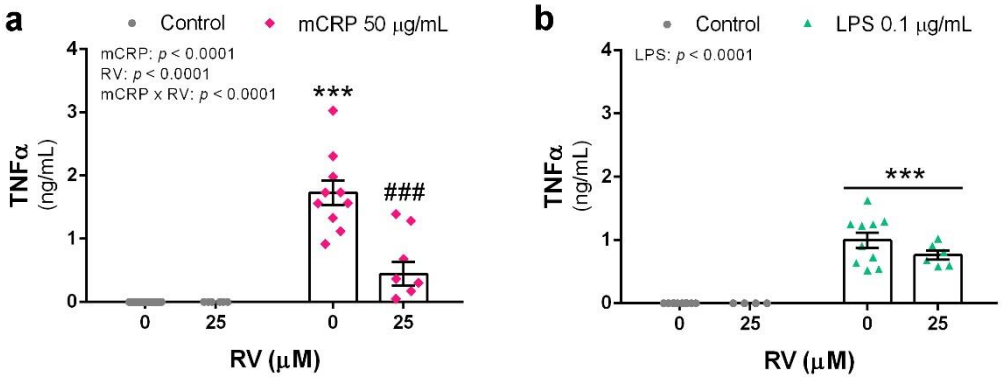

Figure 1. TNFα release by BV2 microglial cells activated with the proinflammatory agents mCRP (**a**) or LPS (**b**), in the absence or presence of resveratrol. Statistics: two-way ANOVA, significance of factors is indicated in graph; post hoc test, *** $p < 0.001$ compared to control, ### $p < 0.001$ compared to mCRP in the absence of resveratrol. RV, resveratrol.

3.2. Resveratrol Inhibited the Induction of the Nitric Oxide Pathway by mCRP and LPS

Nitric oxide is a signaling molecule produced by activated microglia via the enzyme iNOS. An excess of nitric oxide production will cause nitrosative and oxidative stress.

mCRP at 50 μg/mL induced a significant increase in nitric oxide production, as detected by nitrite levels in the conditioned media, with a 14-fold increase over basal values. Resveratrol showed a concentration-response inhibitory effect that was statistically significant at the concentration of 50 μM (Figure 2a). LPS induced an approximate 3- and 5-fold increase in nitrite levels over basal levels at the concentration of 0.1 μg/mL and 1 μg/mL, respectively. Resveratrol reduced nitric oxide generation by LPS (Figure 2b).

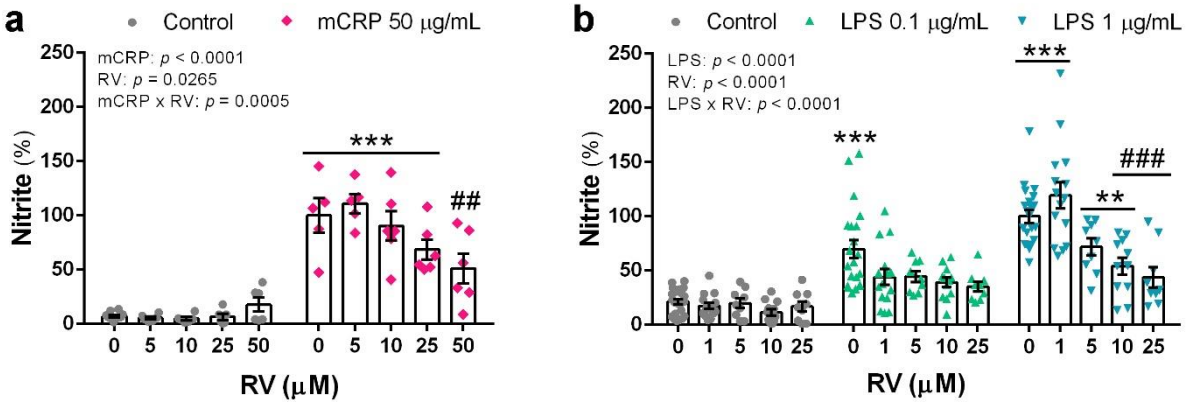

Figure 2. Nitrite levels in the culture media of BV2 microglial cells that indicate nitric oxide generation induced by mCRP (**a**) or LPS (**b**), in the absence or presence of resveratrol. Statistics: two-way ANOVA, significance of factors is indicated in graph; post hoc test, ** $p < 0.01$, *** $p < 0.001$ compared to control, ## $p < 0.01$, ### $p < 0.001$ compared to mCRP or LPS in the absence of resveratrol. Nitrite levels induced by LPS at 1 μg/mL without resveratrol or with resveratrol at 1 μM were higher than the corresponding treatments with LPS at 0.1 μg/mL ($p < 0.05$, not shown). RV, resveratrol.

Protein levels of the iNOS enzyme were determined to confirm the protective action of resveratrol on the nitric oxide pathway. iNOS protein was significantly increased by mCRP at 50 µg/mL and by LPS at 0.1 µg/mL. Resveratrol efficiently inhibited the increase in iNOS levels by mCRP (Figure 3a). However, inhibition of LPS-induced iNOS by resveratrol up to 25 µM did not reach significance, probably due to the high dispersion of the data (Figure 3b).

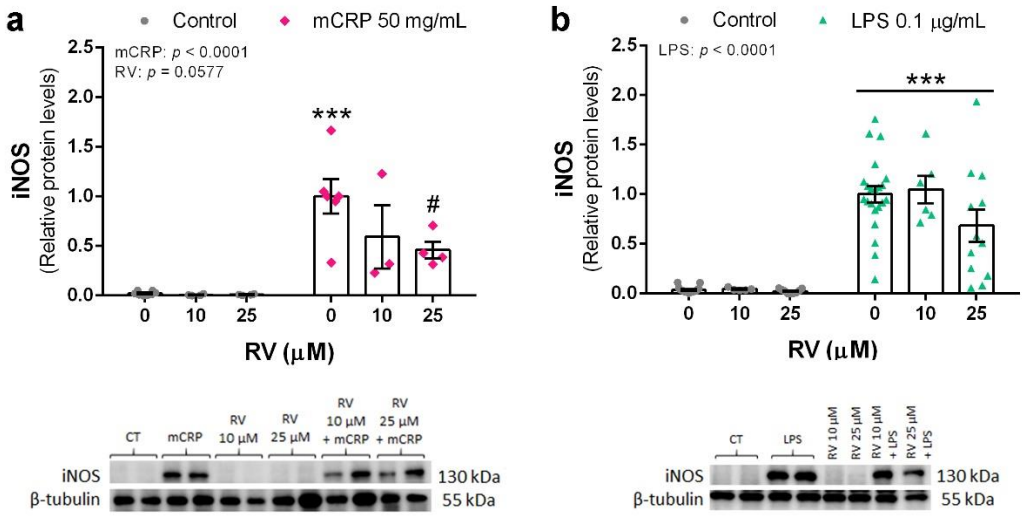

Figure 3. Protein levels of iNOS enzyme in BV2 microglial cells, induced by mCRP (**a**) or LPS (**b**), in the absence or presence of resveratrol. Statistics: two-way ANOVA, significance of factors is indicated in graph; post hoc test, *** $p < 0.001$ compared to control, # $p < 0.05$ compared to mCRP in the absence of resveratrol. Results and representative blots are displayed in the upper and lower panels, respectively. RV, resveratrol.

Therefore, resveratrol was able to inhibit the induction of iNOS and subsequent production of NO, a main mechanism leading to oxidative stress and proinflammatory signaling by activated microglia.

3.3. Resveratrol Inhibited the Induction of the NLRP3 Inflammasome Pathway by mCRP and LPS

NLRP3 is a key sensor inflammasome found in microglia cells. This pathway is activated in response to diverse proinflammatory stimuli, including oxidative stress signals.

Protein levels of NLRP3 in BV2 cells were significantly increased by mCRP at 50 µg/mL and by LPS at 0.1 µg/mL. Co-incubation with resveratrol at 10 µM, or a higher concentration, reduced NLRP3 levels induced by both mCRP (Figure 4a) and LPS (Figure 4b).

These results showed that resveratrol was effective in avoiding the activation of the NLRP3 inflammasome pathway, which can lead to a spiral of neuroinflammation and further oxidative stress.

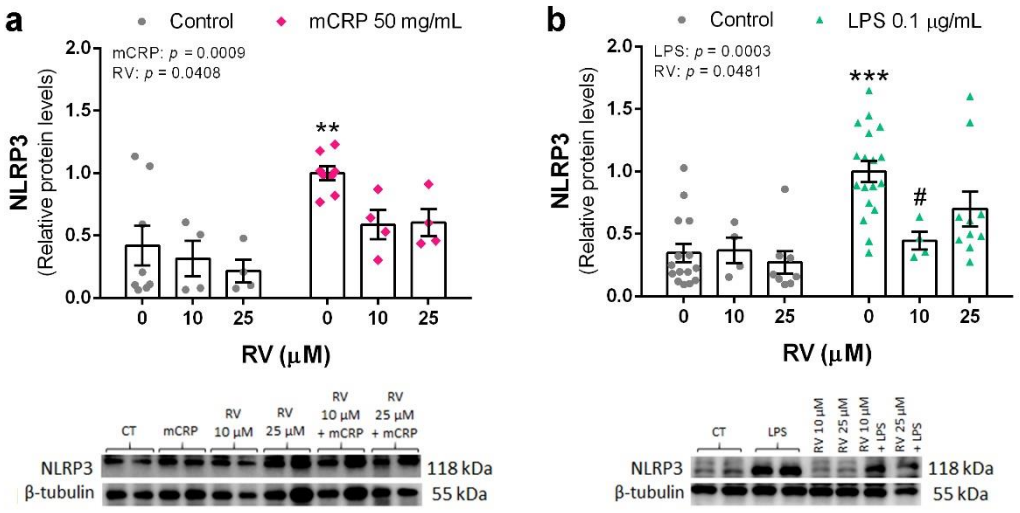

Figure 4. Protein levels of NLRP3 in BV2 microglial cells, induced by mCRP (**a**) or LPS (**b**), in the absence or presence of resveratrol. Statistics: two-way ANOVA, significance of factors is indicated in graph; post hoc test, ** $p < 0.01$, *** $p < 0.001$ compared to control, # $p < 0.05$ compared to LPS in the absence of resveratrol. Results and representative blots are displayed in the upper and lower panels, respectively. RV, resveratrol.

3.4. Resveratrol Inhibited the Activation of NF-κB by mCRP

Next, we studied the central mechanisms of mCRP as a much less characterized proinflammatory agent than LPS. First, we analyzed the nuclear translocation of NF-κB that initiates the transcription of genes involved in inflammation and oxidative stress, among other pathways.

Resveratrol decreased the nuclear content of p65 subunit of NF-κB after mCRP activation in BV2 microglial cells (Figure 5a). p65 and p50 form the most common heterodimers of the NF-κB complex, and increased detection of p65 in the cell nucleus indicates NF-κB activation. Morphological changes of polarization were observed in the mCRP treated microglia that were partially reverted by resveratrol. Confocal microscopy images of a representative cell from each treatment are shown in Figure 5b and images at lower magnification are shown in Figure 5c.

Therefore, the analysis of NF-κB showed that resveratrol protected against its activation and subsequent downstream deleterious mechanisms.

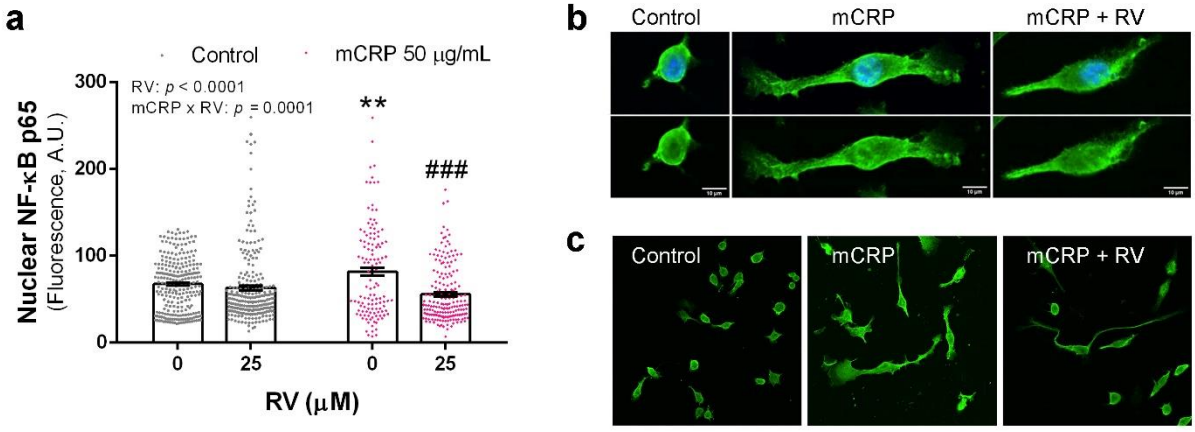

Figure 5. Immunofluorescence detection of NF-κB p65 in BV2 microglial cells. (**a**) Quantification of NF-κB p65 in the nucleus after exposure to mCRP, in the absence or presence of resveratrol. (**b**) Representative confocal images used for analysis. In the upper panel, images show green fluorescence for NF-κB p65 and blue fluorescence for DAPI nuclear staining; in the bottom panel, images of the same cells show green fluorescence. (**c**) Cell cultures stained for NF-κB p65 shown at lower magnification. Statistics in (**a**): two-way ANOVA, significance of factors is indicated in graph; post hoc test, ** $p < 0.01$ compared to control, ### $p < 0.001$ compared to mCRP in the absence of resveratrol. RV, resveratrol, A.U., arbitrary units.

3.5. Resveratrol Inhibited the Induction of Proinflammatory Genes by mCRP

In a second step, we analyzed the changes in the expression of selected proinflammatory genes induced by mCRP and their modulation by co-incubation with resveratrol.

Increased expression of *Nos2*, the gene codifying for iNOS, in mCRP treated BV2 confirmed the activation of the nitric oxide pathway. Resveratrol inhibited iNOS production at the gene level (Figure 6a).

The cyclooxygenase pathway is also activated by mCRP, as shown by increased *Cox2* expression. Similarly to the nitric oxide pathway, resveratrol inhibited cyclooxygenase 2 production and prevented the proinflammatory effects of its enzymatic activity (Figure 6b).

mCRP increased the expression of *Clec7a*, a gene codifying for a pattern recognition receptor in microglia. Interestingly, resveratrol decreases *Clec7a* mRNA levels in mCRP treated BV cells and in control cells. Therefore, resveratrol decreased both, the basal levels and those induced by mCRP, showing a preventive and protective function against downstream inflammatory processes (Figure 6c).

Interleukin 6 is a first line cytokine in the brain and the expression of *Il6* gene was increased by mCRP and inhibited by resveratrol (Figure 6d).

Taken together, resveratrol showed powerful anti-inflammatory properties by inhibiting the transduction of genes that trigger a variety of pathways.

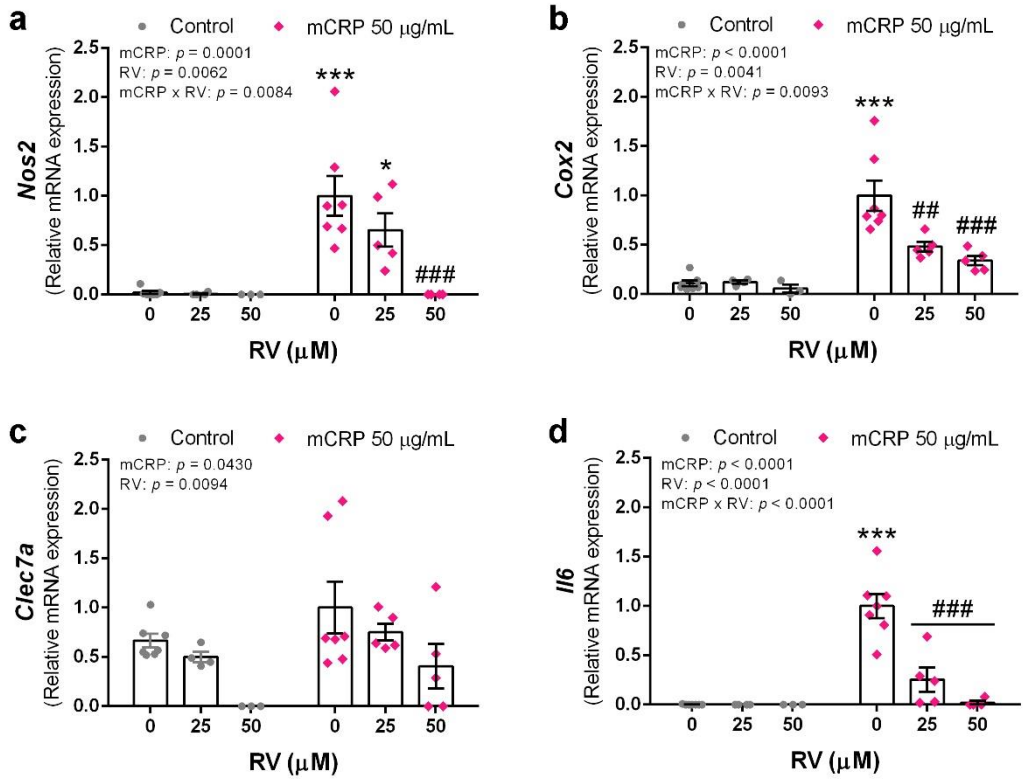

Figure 6. Expression levels of proinflammatory genes in BV2 microglial cells, induced by mCRP in the absence or presence of resveratrol. (**a**) *Nos2*. (**b**) *Cox2*. (**c**) *Clec7a*. (**d**) *Il6*. Statistics: two-way ANOVA, significance of factors is indicated in graph; post hoc test, * $p < 0.05$, *** $p < 0.001$ compared to control, ## $p < 0.01$, ### $p < 0.001$ compared to mCRP in the absence of resveratrol. RV, resveratrol.

3.6. Resveratrol Induced Antioxidant Genes to Protect against mCRP

Finally, we analyzed the changes in the expression of antioxidant and detoxifying genes induced by resveratrol that may protect against mCRP injury in a scenario of intertwined oxidative stress and inflammatory processes.

Resveratrol increased *Sirt1* gene expression as expected, although the effect was lower in the presence of mCRP (Figure 7a). The encoded protein SIRT1 can upregulate antioxidant and anti-inflammatory genes.

Resveratrol induced the expression of *Nfe2l2*, but in this gene the effect was mainly in the cells treated with mCRP. mCRP itself also induced this transducer of antioxidant and detoxifying genes (Figure 7b).

As probe of their protective antioxidant mechanisms, resveratrol induced the expression of the two first line defense genes *Cat* and *Sod2*. mCRP also induced an increase in the expression of both genes (Figures 7c and 7d, respectively). Catalase is widely present in the cytoplasm and superoxide dismutase 2 in the mitochondria.

Therefore, resveratrol induced powerful antioxidant and detoxifying mechanisms.

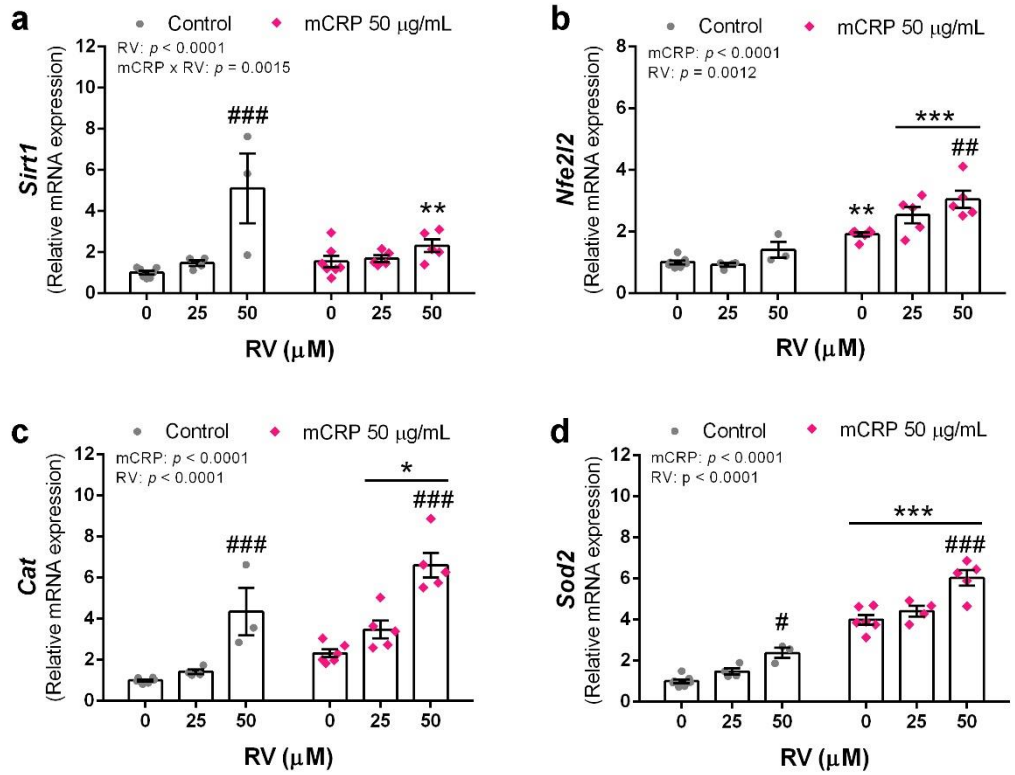

Figure 7. Expression levels of antioxidant genes induced by resveratrol in BV2 microglial cells, either control or mCRP treated. (**a**) *Sirt1*. (**b**) *Nfe2l2*. (**c**) *Cat*. (**d**) *Sod2*. Statistics: two-way ANOVA, significance of factors is indicated in graph; post hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control, ## $p < 0.01$, ### $p < 0.001$ compared to the absence of resveratrol. RV, resveratrol.

3.7. Resveratrol Inhibited Proinflammatory Changes Induced by mCRP in Mixed Glial Cultures

We used primary mixed glial cultures to test the inhibitory effect of resveratrol on microglia activation by mCRP in a physiological setting.

Images of the cultures did not evidence significant morphological changes of astrocytes with mCRP treatment. However, the microglia changed to a larger cell with flat morphology in the presence of mCRP. These changes were greatly reduced by resveratrol. Representative images of glial cultures are shown in Figure 8a.

Analysis of nitrite levels in the conditioned media showed an increase in nitric oxide release by cultures exposed to mCRP that was partially inhibited by resveratrol in a concentration-response effect (Figure 8b).

Similarly, resveratrol significantly inhibited the mCRP-induced increase in the release of the key cytokine IL1ß (Figure 8c).

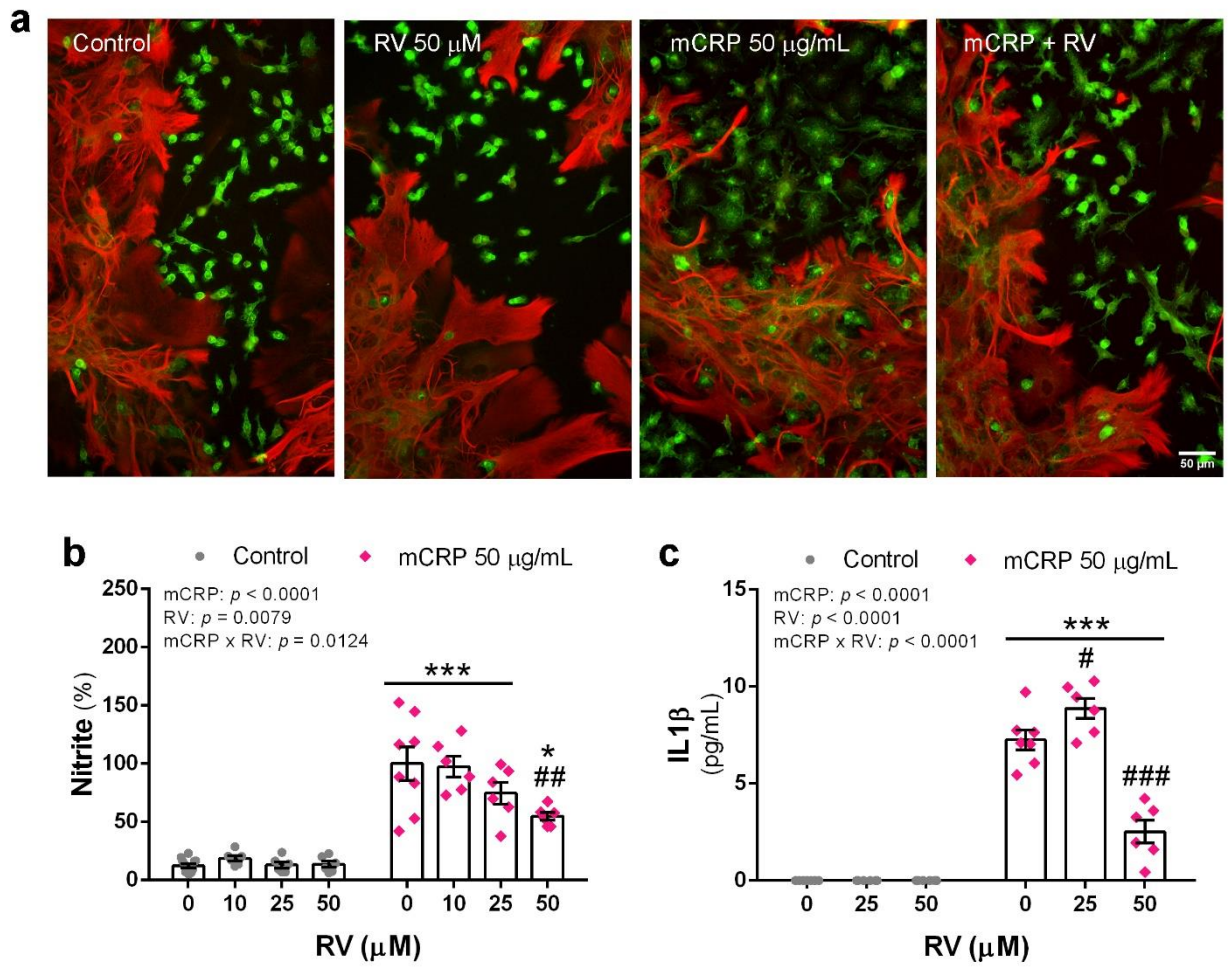

Figure 8. Proinflammatory changes induced by mCRP in primary mixed glial cultures in the presence or absence of resveratrol. (**a**) Microphotographs of cultures showing astrocytes (GFAP, red fluorescence) and microglia (lectin, green fluorescence) submitted to treatments as indicated in the figure. (**b**) Nitrite levels in the culture media indicative of nitric oxide generation. (**c**) IL1ß release to the culture media. Statistics: two-way ANOVA, significance of factors is indicated in graph; post hoc test, * $p < 0.05$, *** $p < 0.001$ compared to control, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to mCRP in the absence of resveratrol. RV, resveratrol.

4. Discussion

Resveratrol has been shown to protect against many harmful agents and conditions, but it had not previously been tested against mCRP. The protective results of resveratrol against mCRP shown here are relevant for possible future therapies in diseases involving this activated harmful form of CRP. Furthermore, the mCRP pathways uncovered here are relevant for the control of neuroinflammatory diseases where it may have a role.

In this study, we showed the protective action of resveratrol in two *in vitro* models of BV2 cells, treated with either LPS or mCRP, which mimic the aberrant activation of microglia associated with AD and other neurodegenerative diseases. LPS is a used *in vivo* model of AD neuroinflammation [61,65]. The LPS recognizing receptor, Toll-like receptor 4 (TLR4), is mainly expressed on microglia. In addition, TLR4 is also activated by amyloid ß, thus sharing downstream pathways with LPS [66]. Therefore, LPS-activated microglial cultures are a reliable model to study AD neuroinflammation in an *in vitro* setting. BV2

microglial cells have been used extensively to characterize the mechanisms of protective agents against LPS. mCRP is a novel and promising agent in the study of proinflammatory mechanisms associated with the onset and progression of AD. In a previous study, we had shown protection of mCRP dementia in the mouse by the anti-inflammatory agent N-[1-(1-Oxopropyl)-4-piperidinyl]-N'-[4-(trifluoromethoxy)phenyl]urea (TPPU) [57]. TPPU is an inhibitor of the soluble epoxide hydrolase enzyme and therefore enhances the concentration of the beneficial epoxyeicosatrienoic acids [67]. Furthermore, we showed that mCRP at the concentration of 100 μg/mL activated the nitric oxide pathway in BV2 microglia which was inhibited by TPPU [57]. Here, we used a lower concentration of 50 μg/mL mCRP and analyzed several inflammatory pathways known to be induced by LPS. Unlike LPS, the sequence of mCRP on microglial signaling is not known. mCRP may interact with the cell through a cholesterol binding sequence (a.a. 35–47) [68] that allows its attachment to the membrane lipids, complement component C1q and other elements [68,69]. However, we found that mCRP induces BV2 polarization to an activated phenotype as does LPS.

Resveratrol inhibited the aberrant activation of the nitric oxide pathway and the NLRP3 inflammasome pathway in both proinflammatory models, mCRP and LPS. The nitric oxide/NOS system plays an important role in many physiological processes. From the different NOS isoforms, iNOS is expressed by activated microglia. *Nos2* is the gene that codifies for the inducible form. This gaseous molecule modulates inflammatory cascades and therefore it may cause neuroinflammation [70]. In addition, excess production of nitric oxide together with insufficient antioxidant defense contribute to the unbalanced redox state associated with AD and other neurological diseases [71,72]. Furthermore, the multiprotein oligomer NLRP3 belongs to the inflammasome family and is a crucial player on the innate immune response. However, its aberrant activation may cause inflammatory damage and contribute to the progression of neurodegenerative diseases, such as AD [73,74]. A third pathway involved in neuroinflammatory processes is the production of prostaglandins and other downstream arachidonic acid metabolites through increased cyclooxigenase activity. From the two enzyme isoforms, COX-1 and COX-2, the latter is more likely involved in neurodegenerative processes due to its higher expression in brain [75,76]. The relevance of this pathway in neurodegeneration is more controversial than that of nitric oxide and the NLRP3 inflammasome. However, resveratrol also counteracted this pathway, as shown by inhibition of *Cox2* transduction.

Remarkably, resveratrol showed powerful protective properties against mCRP by inhibiting the three major signaling pathways discussed above that lead to downstream cascades of proinflammatory and pro-oxidant mediators. For instance, resveratrol counteracted the increase in TNFα cytokine levels and IL6 cytokine gene expression by mCRP. TNFα is involved in innate immunity and inflammation signaling and can cause necrosis or apoptosis. Genetic or pharmacological inhibition of TNFα was proposed to prevent or decrease the progression of AD pathology [77,78]. IL6 can promote a homeostatic or pathological role in the brain depending on the stimulus [79]. IL6 levels in the brain in a setting of neuroinflammation is a proposed target in the fight against AD [80].

Resveratrol protective effects shown here against LPS confirmed previous reports in BV2 microglia challenged with this proinflammatory agent [44–46,81].

We also used primary mixed glial cultures to confirm the resveratrol protection against mCRP as a novel agent in study. We showed a response pattern to mCRP and resveratrol similar to that of BV2 cells. We found a protective response against activation of the iNOS pathway. Furthermore, resveratrol inhibited the increased generation of IL1ß. One pathway inducing this cytokine is the NLRP3 inflammasome [82]. These results confirm that the main target cells of proinflammatory agents are microglia, as shown for LPS in a comparison between mixed cultures and pure microglia cultures [83]. Microglia are the main innate immune cells in the brain [84]. However, astrocytes contribute to the innate immune response. These cells can also release cytokines and other proinflammatory mediators, especially under conditions of sustained neuroinflammation such as in the AD brain [85]. It should be noted that resveratrol was able to normalize the levels of the three main pro-inflammatory cytokines IL1ß, IL6 and TNFα in either primary cultures or BV2 cells.

In the analysis of early signaling mechanisms, we found here that resveratrol increased *Sirt1* expression in the BV2 microglia, which would lead to an increase in the synthesis of the protein SIRT1. This is consistent with the known feature of resveratrol as an activator of SIRT1 [86]. SIRT1 is a deacetylase enzyme that regulates the activity of several transcriptional factors and enzymes involved in cell metabolism, stress defense and survival [87,88]. It may be coupled to another nutrient sensing molecule, AMPK [89]. SIRT1 pathway is considered the main effector of resveratrol benefits in experimental models of AD [90], although this pleiotropic agent can act through several mechanisms [4]. We previously showed an increase in Sirt1 expression parallel to the antioxidant and anti-aging effects in lymphocytes from AD patients treated with resveratrol [33]. We also showed that *Sirt1* overexpression induced cognitive improvement in transgenic AD mice [91] similar to that of resveratrol supplementation [92]. Here, we propose that SIRT1 is the main mediator of antioxidant and anti-inflammatory mechanisms leading to the protection against mCRP and LPS.

Studies reported in N9 microglial cells conclude that SIRT1/AMPK pathway is involved in the protective effect of resveratrol against the activation of the NLRP3 inflammasome and NF-kB by LPS [47]. In BV2, activation of SIRT1 by resveratrol and its inhibition with an antagonist demonstrated the involvement of this deacetylase in the modulation of proinflammatory cytokines induced by LPS [48]. In BV2 microglia challenged with mCRP, SIRT1 induced by resveratrol might modulate both key pathways NLRP3 and NF-κB leading to inhibition of downstream inflammatory targets. SIRT1 may also mediate the antioxidant gene response of resveratrol against mCRP oxidative damage [93]. SIRT1 is an activator of nuclear factor E2-related factor 2 (Nrf2), whose *Nfe2l2* gene expression was shown here to be activated by resveratrol and mCRP. Nrf2 transcriptional activity subsequently induced gene expression of antioxidant genes, as shown by parallel increases in *Cat* and *Sod2* expression in BV2 cells. Interestingly, hub transcription factors such as NF-κB and Nrf2 are stimulated by resveratrol, but also respond to ROS [94] as

shown here for *Nfe2l2* gene expression, in a crosstalk between the mechanisms of this hormetic agent and those of mCRP.

A schematic representation of the proposed protective mechanisms induced by resveratrol against proinflammatory activation of microglial cells is displayed in Figure 9. Results for mCRP are those found in this study. Results for LPS are from this study and previously published data, as referred to above. We speculate that anti-inflammatory mechanisms of resveratrol against both agents, mCRP and LPS, are common.

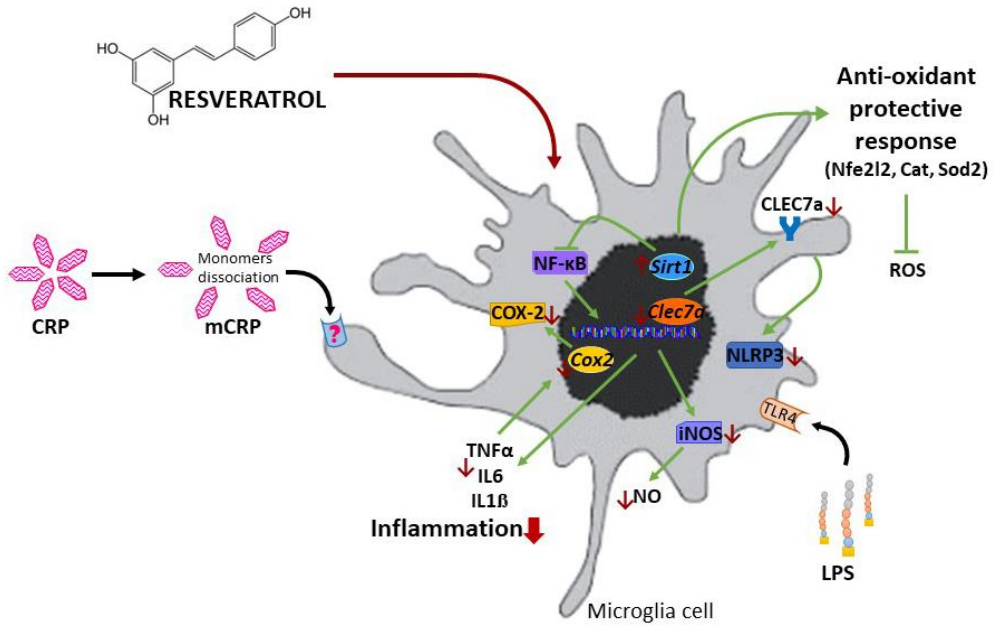

Figure 9. Schematic representation of the protective mechanisms of resveratrol against the proinflammatory agent mCRP and LPS.

We showed molecular targets of resveratrol in activated microglia cells, although pharmacodynamics was not further studied at the subcellular organelle level. Intracellular trafficking of resveratrol to cellular targets in these cells is also unknown. Other authors reported some advances in the analysis of the intracellular trafficking of resveratrol in peripheral cell types [17,95,96]. It is proposed that resveratrol crosses the cell membrane via passive diffusion, endocytosis via lipid rafts, or binding to receptors intro rafts [17,95]. Resveratrol may accumulate primarily in the endosomal−lysosomal system. It would then be released into the cytoplasm to perform its actions on key targets. Early endosomes, late endosomes, lysosome vesicles and other cell organelles can be identified with fluorescent labels [97]. Colocalization studies of these organelles with labeled resveratrol or resveratrol coupled to labeled nanocarriers [98,99] will show the time course and spatial distribution of resveratrol in cellular compartments. We wish to develop new studies using fluorescently tagged resveratrol to follow its entry into cultured cells or the mouse brain using confocal microcopy technologies. Specifically, spinning disk confocal microscopy and infrared laser multiphoton microscopy will be used for *in vitro* and *in vivo* studies, respectively. These studies would reveal subcellular targets in microglia and other brain cells. Despite its low bioavailability *in vivo*,

resveratrol induces undoubted brain benefits, as demonstrated by a large number of preclinical and clinical studies for AD [4,15,100]. Promising results in enhancing stability, pharmacokinetics and pharmacodynamics of resveratrol in *in vitro* and *in vivo* cancer studies were recently critically reviewed [96]. Likewise, improved formulations of resveratrol to avoid its poor bioavailability and pharmacokinetics in brain tissue will improve its neuroprotective potential.

Therefore, the analysis of resveratrol protection in both AD models of inflammation, mCRP and LPS, strengthened the value of this nutriceutical agent. However, all the experimentation is carried out in *in vitro* models. We can speculate on the validity of the findings in humans, although further confirmation in *in vivo* preclinical models is required. Furthermore, there are other inflammatory/oxidative stress mechanisms not analyzed here that may play a role in the benefits of resveratrol on microglia. This is the case of the modulation of nicotinamide adenine dinucleotide phosphate oxidase 2 [101,102] and mitogen-activated protein kinases (ERK1/2, JNK, and p38) [45]. Recent insights into the contributions of resveratrol against persistent neuroinflammation include improving mitochondrial status and glucose metabolism in microglia [47,99,103].

5. Conclusions

Resveratrol protected against the polarization of BV2 microglia into an activated phenotype induced by two critical proinflammatory agents, LPS and mCRP.

The characterization of mCRP proinflammatory and pro-oxidant mechanisms in BV2 microglia showed the activation of the inflammatory/oxidative cascades of nitric oxide, NLRP3 inflammasome and COX-2 in this novel *in vitro* model.

Resveratrol protective mechanisms against mCRP required the modulation of SIRT1, Nrf2, and NF-κB pathways that reduced downstream inflammatory mediators and, most notably, induced antioxidant enzymes.

Resveratrol protective mechanisms against activation to proinflammatory phenotype by mCRP was confirmed in primary mixed glial cultures.

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Chapter 4.
General discussion

4.1. Prenatal and perinatal influence of early-inflammation

One of the aims of this work was to investigate the implications of sEH inhibition and EETs in the brain. To achieve this, we first examined whether the administration of the sEHI TPPU during gestation and lactation to WT female mice mated to heterozygous 5XFAD, could have beneficial effects on the brains of their two-month-old WT and 5XFAD offspring. Maternal pharmacological treatment with the sEH inhibitor TPPU exhibited long-term preventive and protective effects against the development of AD in both WT and 5XFAD mice, confirming the importance of sEH inhibition during early brain development.

To support our initial findings, we investigated whether oxylipins, particularly EETs, played a role in the neurodevelopment of children exposed to low or high levels of environmental Hg contamination. Methylmercury is the predominant form of Hg contamination and is known to be neurotoxic during brain development [214]. Our results suggest that EETs and other oxylipins derived from PUFA can be associated to changes in the neurodevelopment of children at 14 months and 5 years of age. Moreover, prenatal exposure to methylmercury, as indicated by Hg levels in cord blood, appears to influence the levels of these oxylipins.

These results suggest that the risk of developing neurodegenerative diseases, such as AD, is influenced by factors occurring in the early stages of life. They also align with the concept of developmental plasticity, a period when the brain is particularly malleable and sensitive to environmental influences. During this critical period, maternal environmental factors can directly influence the foetus and its developing tissues, predisposing them to be more or less susceptible to diseases later in life [215,216]. This malleable phase allows for several physiological and morphological states to manifest according to environmental conditions, regardless of the genetic profile [217]. Understanding this plasticity is of great importance, as it highlights the potential for early-life interventions to have a long-lasting impact on the resistance of the brain to health and disease.

4.1.1. Perinatal programming and health outcomes

Long-chain PUFAs start accumulating during gestation, sourced from the mother, and continue to do so from dietary intake throughout life [160,161,166]. Oxylipins derived from PUFAs play vital roles in various homeostatic functions [166] and in early imprinting processes, although their specific mechanisms remain underexplored. As previously mentioned, PUFA-derived oxylipins are primarily responsible for the beneficial effects attributed to their precursors. PUFA influence brain function and development, exerting long-lasting effects through metabolic imprinting, potentially accompanied by epigenetic molecular changes.

Maternal DNA methylation patterns can significantly impact gene expression and define epigenetic processes in offspring during development. These methylation imprints can impact in both placental function and neurodevelopment [218,219]. Interestingly,

alterations in DNA methylation patterns have also been observed in aging and neurodegenerative diseases like AD [220].

In our first study, we found that global DNA methylation was increased in 5XFAD mice offspring, and maternal treatment with TPPU normalized these levels to those of WT mice. Subsequently, in our second study, we observed that children prenatally exposed to high Hg exhibited elevated levels of trimethylated H3K27 in placental tissues. Proper regulation of DNA methylation is known to be essential for normal cognitive function [221]. Importantly, the early inflammatory state can influence epigenetic remodelling in the brain, especially in microglia, potentially granting these cells tolerance or susceptibility to future inflammatory insults later in life [222]. Our studies suggest a role for microglia imprinting in the inflammatory and cognitive states during prenatal and perinatal stages, potentially influencing the risk of developing neurodegenerative disorders.

In a study by Gódor-Kacsándi et al. (2013), offspring of pregnant Harlan–Wistar rats fed with a diet supplemented with DHA, EPA, and AA during pregnancy and lactation showed long-term beneficial effects on learning and memory functions. Moreover, the supplemented diet demonstrated long-term neuroprotective effects against future excitotoxic lesions [223]. Similarly, our research found that maternal TPPU treatment preserved learning and memory functions in two-month-old offspring, being able to directly relate a protective imprinting to levels of EETs. Analysis to determine whether an increase in EETs would confer resistance to further future insults holds promise for successful outcomes. This expectation is supported by what we observed in our second study with children regarding the benefice of prenatal higher levels of anti-inflammatory oxylipins, where improvements in mental and psychomotor tests persisted at 14 months and five years of age.

Wendeln et al. (2018) investigated immune memory, particularly its impact on microglia, and its effects on neuropathology. They administered daily intraperitoneal injections of LPS to mice for four consecutive days. Demonstrating that immune memory in the brain was mediated by microglia, they subsequently induced focal ischemia one month after the final injection. Their findings confirmed a long-term modulation of brain immune responses; mice receiving four LPS injections exhibited reduced damage and microglial activation, suggesting an induced state of tolerance or reduced responsiveness [222]. Therefore, immune memory in the brain may play a significant role in shaping the response to future neurological diseases characterized by an inflammatory component.

Regarding to the immune memory, in our second study, we observed a compelling association between prenatal exposure to Hg and the presence of DiHETs in placenta tissue. Remarkably, higher exposure to Hg was associated with increased DiHET levels, which in turn were linked to better working memory in children at the age of five. These levels of DiHET suggests previous elevated levels of the anti-inflammatory compounds EETs. Despite considering Hg as a harmful contaminant, these findings might suggest that Hg can trigger an immune response, by increasing EETs and DiHET. Nonetheless, this potential beneficial effect of Hg appears to be undermined at 14 months of age, as

higher exposure to Hg coincide with increased presence of inflammatory oxylipins, ultimately resulting in poorer psychomotor scale scores. This implies a complex relationship between environmental factors like Hg exposure and biological responses, including immune function and cognitive abilities, in children.

Our research has shown a correlation between oxylipins and cognitive and psychomotor functions from an early age. Additionally, we have observed variations and interactions among these metabolites based on high or low exposure to Hg. In placenta samples with high exposure to Hg, there was an increase in mild anti-inflammatory DiHETs. In the same study, EETs and DiHETs showed a positive correlation with mental scale scores in the children prenatally exposed to low Hg. While there is concern that DiHETs may have an impact on neutrophils that could impair innate immunity [224], it is possible that DiHETs do not possess negative effects for neutrophils. Instead, their lower metabolic activity might result in a lack of the expected positive impact.

4.1.2. Early-inflammation as an AD risk factor

The inflammatory response, when triggered during pregnancy, may initially arise in the mother and subsequently lead to inflammation in either the placenta or the foetus [225]. Prenatal exposure to maternal antibodies or immune alterations can significantly impact foetal development. Specific cytokines or chemokines from maternal blood can play defining roles in brain development at various levels [226].

In the first study, WT female mice matted with 5XFAD males, exhibited low inflammation as expected during pregnancy and breastfeeding, as evidenced by low levels of CRP and TNF-α. Treatment of dams with the sEHI standard agent TPPU during pregnancy and breastfeeding reflected its effects on their offspring, who demonstrated lower levels of the microglial activation marker TREM2. Moreover, Hg exposure can induce inflammatory and autoimmune responses [227]. As expected, in the second study, prenatal exposure to high levels of Hg increased the presence of inflammatory oxylipins in cord blood samples, thus influencing the neurodevelopment of children. Likewise, environmental or genetic factors may influence the inflammatory response in foetuses and new-borns, thereby impacting postnatal brain development [228].

Notably, gestational inflammatory conditions can influence the predisposition of offspring to neurodegenerative disease. Early-inflammation has to be considered as a risk factor for neurodegenerative diseases, especially AD. Nonetheless, it should not be forgotten the posterior role of cumulative life-long life-styles and environmental exposures, conceptualized as the exposome, and the response of the body accordingly to its resilience and resistance [229].

4.1.3. Impact of sex dimorphism on inflammation during early stages of life

Sex dimorphism is of great importance during the development, progression, and clinical manifestation of AD. In fact, women usually exhibit a more pronounced pathology, with higher levels of neuroinflammation, mitochondrial dysfunction, and other pathologic

effects. Variations in sex hormones and changes in the hypothalamic-pituitary-adrenal axis throughout life may contribute to worse outcomes in neurodegenerative diseases in women [230].

In our first study with two-month-old young adult mice, females exhibited higher levels of gliosis and neurodegenerative markers, when compared to males and regardless of genotype. These findings are consistent with what we know about the increased susceptibility of women to AD [230,231]. However, in the second study, there were no gender differences in children at 14 months or at five years of age. This lack of differences was probably due to the early age of the participants and that only cognitive and psychomotor tests were performed.

In the healthy brain, there are intrinsic sex differences that manifest in protein and RNA patterns, which can shape the response to future pathological challenges [232]. During early age, microglia in females present lower inflammatory markers when compared to males. However, as female ages, microglia display higher levels of pro-inflammatory markers, partly attributed to epigenetic modifications [233,234]. This demonstrates the non-static nature of microglial inflammatory responses across the lifespan, along with their sex-specific variations [232].

Machine learning for brain age prediction demonstrated that there is a distinct effect of sex in both brain structure and neuropathological pathways when considering biomarkers, risk factors for neurodegeneration and AD, and the influence of sex [235]. Thus, sex must be considered as a critical factor to understand the development and progression of neurodegenerative diseases, particularly at early ages.

4.2. Innate immunity in the brain and microglia's essential role

The innate immune system has a key role in maintaining brain homeostasis and orchestrating its initial response against infectious organisms, brain injury, and chronic disease [236]. In the brain, microglia and astrocytes are the main cells participating in the immune response, accompanied by infiltrating monocytes, macrophages, and neutrophils [237,238].

Perturbations in the innate immune system during neurodevelopment can have lifelong repercussions in the CNS [239]. Microglia are primary resident immune cells and the most characterized cells in the brain. Moreover, microglia participate in various neurodevelopmental processes [240]. In fact, early-life experiences and environmental factors can imprint different responses in the microglial cells, influencing their response to subsequent immune challenges. In addition, this imprinting may lead to an enhanced microglial reactivity and dysfunction, potentially contributing to neurological disorders such as AD [241–244].

4.2.1. The role of microglia in the neuroinflammatory pathogenesis of AD

Microglia are the key leaders of the inflammatory response. In response to infection, injury or inflammatory stimuli, they release a significant amount of pro-inflammatory factors such as TNF, IL, NO, and ROS [116–118,245]. When the inflammatory response of microglia persists for too long, it can become chronic, potentially leading to synaptic dysfunction, decreased neurogenesis, and eventually neuronal death [246,247]. Consequently, neuroinflammation has been strongly linked to AD and other neurodegenerative diseases [248].

As part of our broader work, we aimed to study neuroinflammation associated with AD, with a particular focus on the involvement of microglia in this process. Therefore, mCRP was used as a tool to understand how microglia orchestrates the inflammatory response when facing pathological insults. While mCRP has been previously associated with AD pathology, little is known about its interaction with the main inflammatory mediators in the brain. This also provided us with an opportunity to unravel how mCRP is able to induce a pro-inflammatory response in microglia and to support its significant role in AD development.

For these studies, murine BV2 microglial cells were employed as it is a well-established microglial cell line, particularly suited for studying inflammatory conditions [249–252]. Additionally, primary mixed glial or microglial cultures were also used to better portray microglia phenotype following mCRP stimulation. Before testing mCRP in BV2 cells, we first used LPS, a well-known bacterial endotoxin, to validate the accuracy of the model. LPS triggered an inflammatory response in BV2 cells, as evidenced by the increased release of TNF-α and the activation of the iNOS and the NLRP3 pathways. Our findings were consistent with previous studies that, after LPS activation, also observed the secretion of other cytokines such as IL-6 and IL-1β, as well as COX2 activation, ROS production, and the upregulation of genes associated with phagocytosis, migration, and morphological changes [245,253,254].

LPS is frequently recognized as an inflammatory agent relevant to AD. While the potential connection between bacterial infection and AD development exists [255], LPS remains an exogenous inflammatory insult to the brain, unless it originates from microbiota dysbiosis or gut disruption [256]. Concurrently, mCRP can be considered an endogenous insult coming from the dissociation of CRP.

Similar to studies with LPS, mCRP induced moderate morphological changes in BV2 cells. In both mixed and microglial primary cultures, microglia exhibited enlarged and flattened cell bodies, indicative of intense activation of the microglial phenotype, likely originating from changes in the homeostasis of the microenvironment [257].

Although previous studies have demonstrated co-localization of mCRP with glial cells in ischemic brain [138], and it has been directly related to dementia onset [148], the pro-inflammatory mechanisms of microglial mCRP-induced activation have been scarcely studied. Through our research, we utilized the BV2 cell line as well as microglial and

mixed primary cultures, employing three distinct *in vitro* models of microglia. We have identified multiple mechanisms through which mCRP initiates the inflammatory response in microglia. This has allowed us to outline a highly plausible signalling cascade (figure 9), which can be extrapolated to understand the mechanisms of other inflammatory agents.

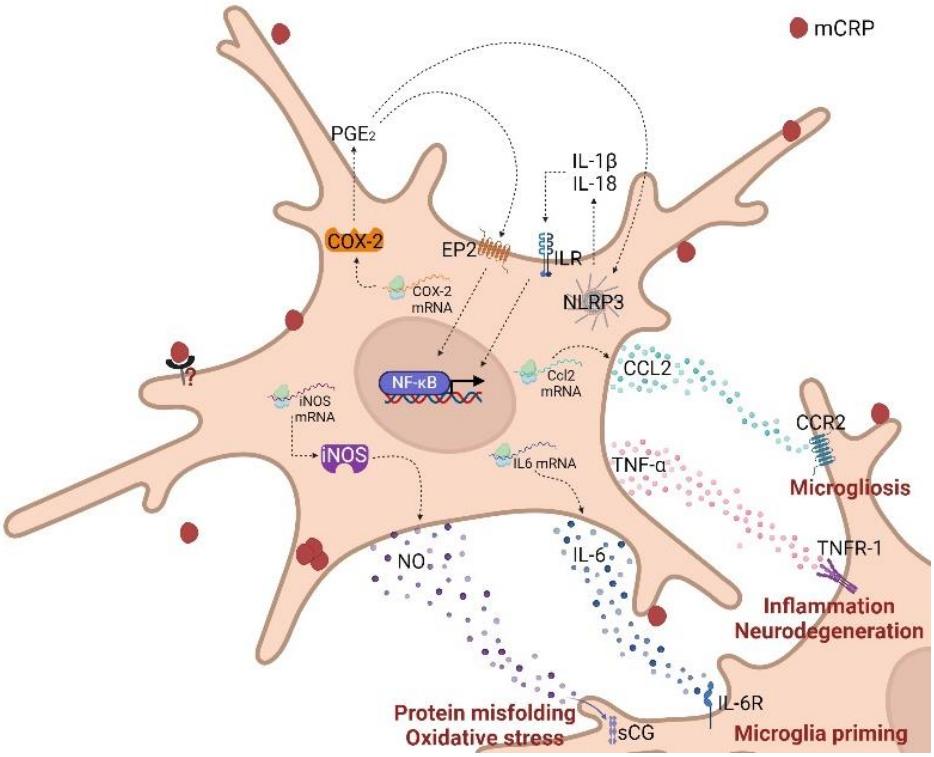

Figure 9. Proposed mechanisms of mCRP-activated inflammatory pathways in a microglial cell. Image taken and edited from the third publication of this thesis. Created with BioRender.com

Through interaction with lipid rafts or potentially an unidentified receptor on the microglial cell membrane, mCRP can interact with several ligands and stimulate NF-κB p65 subunit translocation to the nucleus. Additionally, mCRP can bind to and cross the BV2 cell membrane. This finding provides valuable insight into its mechanisms of action. All this initiates the transcription of various genes involved in key inflammatory pathways such as *Cox2* and *Nos2*, as well as several cytokines including *Il6* or *Ccl2*, and pattern recognition receptors like *Clec7a*. Additionally, NF-κB p65 subunit when translocated to the nucleus can also activate the translation of CRP [137]. There is a generalized inflammatory response from microglia, resulting in the release of NO, which is implicated in various inflammatory conditions including AD [258]. Moreover, the COX2 enzyme enhances the production of the inflammatory oxylipin eicosanoid PGE2, while activation of the NLRP3 inflammasome pathway mediates the release of pro-inflammatory cytokines IL-1β and IL-18 [135]. In addition, there is an increased release of cytokines such as TNF-α, IL-1β and IL-6. All in all, creating an inflammatory pathogenic spiral that leads to increased microglial activation, which in turn exacerbates neuroinflammation.

Thus, after an endogenous pathological insult, microglia can initiate neuroinflammation via several mechanisms, remarking NF-κB p65 subunit translocation to the nucleus and the intensified release of multiple inflammatory cytokines, indicating a propensity for sustaining chronic inflammation. Moreover, we can confirm that it is plausible that a maintained mCRP activation of microglia can induce neuroinflammation and neurodegeneration [259,260].

In most of the *in vitro* studies, a potential limitation arises from the exclusive use of LPS to induce an inflammatory state to microglia, despite the fact that most of brain injuries occur without bacterial infection. Other studies have also studied the response of microglia with interferon-γ or TNF-α, with crucial contribution to neuroinflammation [253], although their role could be considered more about amplifying and sustaining the inflammatory response. Herein, we propose mCRP as an endogenous trigger of inflammation. Moreover, special attention must be paid to microglia, as their excessive activation is directly linked to neurodegeneration. By profiling microglia response to mCRP, we can better understand the involvement of microglia in AD pathology, and this information can be applied to future studies.

4.3. Counteracting inflammation through sEH inhibition in an AD-like environment

4.3.1. Inhibition of sEH in anticipation of AD-like pathology

Given the known benefits of sEHI, our initial study aimed to investigate whether TPPU could yield positive effects during both prenatal and perinatal stages. By extending EETs half-life [198], our goal was to enhance their potent anti-inflammatory properties within the brain [196].

Ghosh et al. (2020) performed gene ontology enrichment analysis in 5XFAD mice and identified differential gene expression, with upregulation in genes related to immune and inflammatory responses. Most of the upregulated inflammatory pathways were counteracted following 2.5 months of TPPU administration, starting at 2 months of age. Notably, TPPU rescued the expression of genes related to complement components C1q and C3, thereby influencing microglia's role in synaptic plasticity [192].

In our research, we studied the effects of maternal administration of TPPU during pregnancy and lactation on their WT or 5XFAD offspring. Having a high metabolic stability, TPPU is known to freely diffuse through membranes, including those in the liver, spleen, heart, and kidney, as well as the BBB [261]. As anticipated, TPPU crossed the placental barrier, reaching the foetus, and exerting its beneficial properties. Consequently, TPPU prevented cognitive and behavioural changes in offspring with a 5XFAD genotype. It also induced neuroprotective epigenetic changes, mitigated p-tau levels, and reduced neuroinflammatory markers, thus demonstrating the beneficial effects of EETs.

Compared to previous studies, our study offered crucial insights into the preventive potential of early-life interventions, highlighting the importance of timing in therapeutic strategies. Notably, it revealed the potential for TPPU to mitigate disease processes before they manifest, providing a base for transgenerational benefits. Additionally, it increased our understanding of neuroinflammation from a developmental perspective, which is critical for brain health and can influence the onset of neurodegenerative diseases.

Furthermore, administration of TPPU during brain development resulted in a long-term downregulation of the sEH gene *Ephx2*. This led to the maintenance of an anti-inflammatory environment in the offspring's brain. GWAS on AD have already identified the EPHX2 gene, which encodes sEH in humans, as being associated with AD risk [262]. Polymorphisms in EPHX2 can either increase or decrease susceptibility to ischemic cell death depending on whether they increase or decrease sEH activity, respectively [263]. Importantly, sEH levels have been found to be higher in AD patient's brain [191].

One may wonder about the potential downsides of systemic sEH enzyme inhibition. Checking the literature, there are only positive outcomes associated with sEHI. Studies have demonstrated their efficacy in treating cardiovascular and liver abnormalities induced by metabolic syndrome in rats [264], in preserving endothelial function in atherosclerotic hearts in both rats and human cells [265], and in reducing inflammation in human adipose tissue [266]. These findings position sEHIs as a safe and promising area of study. It is still necessary to determine whether long-term inhibition of sEH could result in alterations in metabolic and immune homeostasis. Importantly, these potential negative effects may vary depending on factors such as the specific inhibitor used, dosage, treatment duration, and individual patient characteristics. Therefore, exhaustive studies are still needed in the subject.

4.3.2. sEH inhibition against microglia-mediated inflammation

After confirming the beneficial effects of maternal treatment with TPPU in the WT and 5XFAD offspring, we investigated whether the observed benefits of sEHI could be attributed to their impact on microglia. Subsequently, we studied their anti-inflammatory effect in microglia following an inflammatory insult, with mCRP as our inflammatory agent.

TPPU offers numerous beneficial effects and has served as a standard and well-described sEHI, owing to its favourable pharmacokinetics and potency in both rodents and humans [267]. However, over the past years, various classes of new sEHI have been developed. Therefore, we tested *in vitro* the newly synthesized sEHI compounds UB-SCG-55 and UB-SCG-65 with enhanced potency and microsomal stability [268–270], and compared their efficacy to that of TPPU.

Successfully, the protective influence of sEHI against microglial-mediated neuroinflammation detrimental effects were quite significant in both BV2 microglia and primary cultures of microglia. In our study, sEHI were able to diminish the inflammatory response of BV2 microglia after mCRP exposure, by directly decreasing the iNOS and the

NLRP3 inflammasome pathways. Additionally, sEHI decreased *Cox2* transcription. These inhibitions, lead to an observable decrease in NO, TNF-α, IL-6, and Ccl2 release, which play crucial roles in coordinating various aspects of the immune system response to pathological insults, orchestrating the inflammatory response. While all three sEHI managed to reduce the levels of NO, TNF-α and IL-6, the rest of the previously mentioned inflammatory markers and mediators were only diminished by UB-SCG-55. Moreover, this latter sEHI inhibited morphological changes indicative of M1-like phenotype in primary microglia. All things considered, we can confidently confirm that the beneficial effects of sEHI are, either completely or partially, mediated through the modulation of microglial activity, thus unraveling their role in mitigating neuroinflammation in an AD scenario.

UB-SCG-55 anti-inflammatory potency stood out in front of the other sEHI, being this results highly relevant for future pharmacological strategies targeting the sEH enzyme. The sEH enzyme has a molecular weight of 62 kDa and consists of two globular regions connected by a proline-rich linker. It has two distinct domains: the N-terminal domain, with the catalytic site and a hydrophobic pocket, and the C-terminal domain, which is the regulatory site, and participates in the binding of the substrate and in the protein stability. sEHI compounds bind to the N-terminal domain and inhibit sEH activity [197,269]. The potency and therapeutic efficacy of sEHIs are linked to their chemical structure. The structure not only defines their inhibitory potency but also influences other biological properties, such as pharmacokinetics, which can modify their anti-inflammatory potency in cells and organisms. This influence is evident when comparing different SCG agents. Studying the relationship between chemical structure and the beneficial effects of various inhibitors in disease cell models can aid in developing more effective sEHIs.

The structure not only defines their inhibitory potency but also influences other biological properties, such as pharmacokinetics, which can modify their anti-inflammatory potency in cells and organisms. This influence is evident when comparing different SCG agents.

4.3.3. EETs as key players in the fight against neuroinflammation

The potential counteraction of sEHI against neuroinflammation can be attributed to the increase in EETs levels. EETs have been associated with the repression of NF-κB activity [271]. This is significant because the translocation of the p65 subunit of NF-κB to the nucleus triggers the activation of the inflammatory pathways mentioned earlier. We can hypothesize that EETs might operate by interacting with the structure of cell membranes, potentially engaging with ligands, receptors or proteins, and consequently exerting their anti-inflammatory effects in the microglia.

There are limited studies demonstrating the beneficial effects of EETs against an inflammatory agent *in vitro* in macrophages or microglia. Luo et al. (2020) investigated how EETs protected peritoneal macrophages from C57BL/6 mice against the harmful effect of LPS. They observed a reduction in NLRP3 inflammasome caused by a diminution in the levels of IL-1β, ROS, and Ca^{2+} [272]. Similarly, Dai et al. (2015), using the same type

of macrophages, studied the effects of different EETs on the polarization induced by LPS. EETs inhibited NF-κB pathway activation, with 11,12-EETs demonstrating the highest potency, thus indicating differences in the bioactivity of different EETs. Furthermore, 11,12-EETs counteracted the M1-like phenotype after LPS exposure and promoted the polarization of the macrophages to a M2-like phenotype via the upregulation of PPARα/γ expression [273].

With the provided information, it is evident that EETs are responsible for sEHI effects. However, EETs short half-life both *in vivo* and *in vitro* can be challenging. For instance, *in vivo* experiments treating intervertebral disc degeneration with EETs found that their short half-life required multiple injections in rats, making the approach quite impractical [274]. Consequently, sEHIs are a better alternative to overcome this limitation, as they are specifically designed to extend the lifespan of EETs and have a longer half-life compared to EETs, ensuring a more sustained effect. However, there are long-lasting EET analogues that mimic the natural functions of EETs and could be used for direct treatment with EETs.

4.4. The effects of the nutraceutical agent resveratrol on inflammation in microglia

Diet interventions are a promising approach to prevent and delay the progression of neurodegenerative disorders. Healthy dietary patterns are rich in bioactive compounds found in plant foods, known as phytochemicals, which include polyphenolic compounds [275]. Many polyphenols have been proposed to have direct anti-inflammatory effects on microglia [275–277]. The polyphenol RV and its metabolites are among the most studied compounds. However, studies *in vitro* regarding the anti-inflammatory effect of RV in microglia have only demonstrated to be effective against LPS or Aβ activation [278–281]. In our study, the nutraceutical RV was tested for the first time against the mechanisms of microglial aberrant activation associated with mCRP, with LPS serving as a reference inflammatory agent.

In our fourth study, RV showed potent anti-inflammatory effects. In BV2 cells activated with mCRP, RV reduced the translocation of the NF-κB p65 subunit to the nucleus and inhibited the transcription of the pro-inflammatory genes such as *Cox2*, *Clec7a*, and *Il6*. Additionally, RV diminished the increased release of TNF-α, the activation of the iNOS pathway, and the upregulation of the NLRP3 inflammasome pathway after LPS and mCRP stimulation. In primary mixed glial cultures, RV counteracted all the pro-inflammatory effects induced by mCRP, including the activation of the iNOS pathway and the release of IL-1β. Remarkably, studies in LPS-stimulated BV2 demonstrated that polydatin which is a resveratrol derivative, diminished the inflammatory response in the microglia, by decreasing the activation of iNOS and NLRP3 inflammasome pathways and the release of several cytokines [282], suggesting that not only RV has beneficial properties but also its derivatives.

Interestingly, Abraham et al. (2009) studied if RV could have protective effects in mice after LPS administration. Surprisingly, RV protected the inflammatory and cognitive state in aged mice but not in young adults [283] . This suggests that the anti-inflammatory and neuroprotective effects of RV may have a more significant impact with aging, indicating that these effects vary depending on the physiological context. Additionally, RV might enhance a protective imprinting in microglia and immune cells. To advance further, it would be essential to study the epigenetic profile in microglia and its interaction after RV administration.

In terms of RV antioxidant effects on BV2 cells, our investigation revealed an increase in Sirt1 expression after the treatment with this agent. SIRT1 is proposed to mediate antioxidant and anti-inflammatory mechanisms, thereby contributing to the protection against microglia-mediated oxidative response. Remarkably, SIRT1 activates Nrf2, the expression of which was observed to be upregulated by RV and mCRP. Concurrently, Nrf2 triggered increases in the expression of the antioxidant genes Cat and Sod2, a response similarly observed with mCRP in conjunction with RV. These findings suggests an interaction between RV and different inflammatory mechanisms, highlighting the induction of powerful antioxidant and detoxifying processes.

It is known that despite its poor solubility and bioavailability, RV can be absorbed and cross the BBB, having beneficial effects for the CNS [284]. Some *In vitro* studies propose alternative methods to deliver encapsulated RV in nanoparticles or micelles, ensuring its delivery to the brain in higher concentrations and enhancing its potential anti-inflammatory and antioxidant properties [285,286]. However, further *in vivo* research is necessary to confirm the therapeutic efficacy of alternative methods of RV delivery.

4.5. Pharmacological or natural drugs?

If we were to decide which is better between sEHI and RV, it would be challenging to decide. Both have demonstrated to be effective targeting neuroinflammation induced by mCRP. On one hand, pharmacological drugs such as sEHI target a specific enzyme, allowing a precise dosage control and ensuring consistent potency and efficacy. However, they need to be exhaustively tested for safety and efficacy. On the other hand, nutraceuticals such as RV are natural ingredients with usually fewer side effects compared to pharmaceutical drugs. However, they usually have lower bioavailability, being their beneficial effects just apparent over time with consistent integration into a healthy lifestyle. Importantly, these benefits may exhibit multitarget effects [287]. Nevertheless, for greater effectiveness, higher doses of RV and/or other nutraceuticals may be required than those obtained through diet [211–213]. All in all, and despite addressing the same condition, each option offers unique advantages.

4.6. General considerations

After evaluating all the gathered information, it becomes evident that the inhibition of sEH is crucial in understanding the beneficial effects of EETs derived from PUFAs. By

inhibiting sEH, we can accentuate the neuroprotective qualities of EETs, particularly emphasizing their potent anti-inflammatory properties, from prenatal stages onward. Indeed, sEHI have neuroprotective effects that may shape our susceptibility and resilience to both external and internal insults.

To pursue effective treatments for neuroinflammatory diseases, it is of great importance to unravel the complexity of neuroinflammation and its triggers and mediators. mCRP has been proven to be a valuable tool in understanding microglial responses to endogenous inflammatory stimuli, unlocking several avenues for therapeutic interventions. Moreover, it leaves the door open to the study of new biomarkers.

It is essential to emphasize the importance of a healthy lifestyle, with particular emphasis on diet. Through a balanced diet, we can supply ourselves, and potentially our offspring during gestation, with natural bioactive compounds. These compounds contribute to a protective imprinting, enhancing not only our physical health but also our brain reserve, as demonstrated in our studies involving oxylipins and RV.

While it may be implicit, a healthy lifestyle typically leads to healthy aging. Nonetheless, genetic, epigenetic, and other uncontrollable external factors can significantly influence or predispose us to neurodegenerative disorders like AD. Understanding the biological processes implicated in this pathologies, such as neuroinflammation, is of great importance to develop more effective treatment strategies. sEH enzyme inhibition arises as a good pharmacological target to diminish or slow down the neuroinflammatory process, not only in AD but also in all the dementias with an inflammatory basis.

Given the complex aetiology of AD, a combinatory treatment strategy targeting different aspects of the disease pathology simultaneously or sequentially, along with healthy lifestyle interventions, would represent the ultimate success in improving the continuum outcome of AD. Nonetheless, a comprehensive study of every involved factor is what will help us develop the most effective combined treatment.

Chapter 5. Conclusions

This doctoral thesis has made significant contributions to our understanding of neuroinflammation in the AD pathology, particularly focusing on the impact of early-life events on this complex process. Additionally, our research brings new perspectives about the modulation of oxylipins, specifically EETs, and their long lasting beneficial effects. The inhibition of the sEH enzyme and its positive outcomes, particularly focusing in its effects in microglia, stablishes a basis for future early-life preventive therapies in individuals at risk of AD and other neurodegenerative disorders with a neuroinflammatory basis.

The conclusions of this thesis are:

1) Pharmacological maternal intervention with TPPU during pregnancy and lactation, preserved learning and memory in an AD mouse model, suggesting that EETs anti-inflammatory actions can prevent or delay the course of AD.
2) The beneficial effects of TPPU and EETs may be attributed to epigenetic imprinting, as they modulate epigenetic mechanisms during neurodevelopment, leading to improved cognition. This suggests that anti-inflammatory conditions during the gestational period can induce favourable epigenetic changes.
3) Prenatal exposure to Hg induces changes in PUFA metabolites that correlate with postnatal neurodevelopment in children, demonstrating that early-life events can define future health outcomes and influence the predisposition to neurodegenerative diseases.
4) EETs and other oxylipins are notable anti-inflammatory metabolites that can influence neurodevelopmental outcomes in children, may provide resilience against environmental risks of neurodevelopmental damage.
5) The beneficial effects of sEHIs are directly linked to reduced levels of activated microglia, as demonstrated in mice with decreased TREM2 levels and *in vitro* by changes in morphology and reduced activation of inflammatory pathways and modulators.
6) mCRP is a useful tool to study microglia-mediated neuroinflammation, confirming its role in the AD pathology. In the brain, endogenous inflammatory proteins can activate pro-inflammatory pathways that may lead to chronic neuroinflammation
7) New-generation sEHIs are effective in reducing microglial inflammation by decreasing the activation of key inflammatory pathways and the release of inflammatory cytokines, laying the groundwork for designing more refined versions of sEHIs as potential therapeutic agents against neuroinflammation.
8) The protective effects of resveratrol against aberrant microglial inflammatory responses highlight the importance of its antioxidant and anti-inflammatory properties in combating neuroinflammation. By incorporating nutraceuticals into a healthy diet we can improve overall health outcomes and resilience against neurodevelopmental disorders.

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