Shedding light on the early neuroinflammatory process in Alzheimer's disease and Down syndrome

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This Thesis is dedicated to my parents,

Silvia Aguilar Castrejón and *Liborio A. Flores Lagunas*

Abstract

It is well-established that Alzheimer's disease (AD) has a large preclinical stage where neuropathological changes begin to accumulate. Growing evidence indicates that medical treatment at such stages might have a successful impact on delaying or halting the disease. Henceforth, the understanding of AD preclinical stages is of utmost importance.

Some studies suggest that a disease-aggravating neuroinflammatory process occurs at preclinical AD stages. Such scenario is well-illustrated by epidemiological data indicating that anti-inflammatory medications have a sparing effect on AD if administered early enough; highlighting the detrimental role of early neuroinflammation. Little is known about preclinical neuroinflammation in AD; therefore, the main objective of this Thesis was to better understand preclinical neuroinflammation in populations at risk of AD.

Individuals with Down syndrome (DS) represent the largest population at genetic risk of AD. Inflammatory expression and microglial morphology was assessed from fetal to adult stages in the frontal cortex of individuals with DS and age-matched controls. Our investigations revealed increased cytokine expression (IL-10, IL-8, IL-1, IL-6, IL-15, IP-10, MIP-1β, TARC, Eotaxin-3, MDC), intermediate microglial activation and increased rod-like microglial cells before the development of a full-blown AD pathology in DS. A downregulation of inflammatory markers (TNFα, IFNγ, IL-12 and IL-10) along with an increase in dystrophic microglia was observed in older adults with DS. Increased levels of inflammatory cytokines were also detected in DS fetal cortical cells *in-vitro*. Our analyses also indicate that cytokine expression is brain region and sex specific.

Next, we assessed cytokine expression in non-demented elderly individuals with significant AD neuropathology. Cytokine expression between non-demented individuals with amyloid-beta (Aβ+) and without amyloid deposition (Aβ-) revealed that Aβ+ individuals displayed an increased IL-1β, IL-6 and eotaxin-3 levels in the temporal cortex, and higher expression of IL-6 and MCP-1 in the parietal cortex. A trend towards higher levels of IL-1β and MCP-1 was observed in the frontal cortex of Aβ+ individuals. A positive association was found between inflammatory protein expression and the extent of brain amyloid deposition. Importantly, the Aβ+ group displayed a faster rate of cognitive decline than the Aβ- group.

Finally, degeneration of the locus coeruleus (LC) noradrenergic system is an early event in AD. We examined whether early LC degeneration, before the onset of amyloid plaques, had an impact on the early neuroinflammatory process in an APP transgenic rat model. LC demise promoted early cognitive deficits, downregulation of cholinergic synaptic boutons, decreased neurotrophin levels, and deregulation of inflammatory mediators. Increased astroglial and microglial recruitment towards Aβ-burdened neurons and intermediate microglia activation was also observed.

Taken together, our results demonstrate the existence of a neuroinflammatory process associated with incipient AD stages. We have identified key inflammatory molecules which might be involved in driving such process. Therefore, our findings should provide valuable insights for the identification of likely therapeutic windows applying anti-inflammatory strategies at preclinical stages of AD.

Résumé

Il existe une longue phase préclinique dans la maladie d'Alzheimer (MA), au cours de laquelle s'accumulent les lésions neuropathologiques menant à MA. De plus en plus d'études indiquent qu'un traitement médical initié au cours de cette phase pourrait ralentir ou arrêter la maladie. C'est pourquoi il est capital de comprendre ce stade préclinique de la maladie.

Un processus neuro-inflammatoire aggravant la maladie serait présent aux cours des stades précliniques de la MA. Selon des données épidémiologiques, les médicaments anti-inflammatoires ont un effet bénéfique sur la MA s'ils sont administrés *suffisamment tôt* au cours de la maladie, mettant en évidence le rôle préjudiciable de la neuroinflammation précoce, qui reste encore méconnu. Dans ce contexte, l'objectif principal de cette thèse est de mieux comprendre le processus neuro-inflammatoire présent au stade préclinique de la MA.

Les individus atteints du syndrome de Down (SD) représentent la plus large population à risque génétique de développer la MA. L'expression de marqueurs neuro-inflammatoires a été évaluée chez des individus atteint du SD et un groupe témoin, du stade fœtal jusqu'à l'âge adulte, révélant une élévation de l'expression de cytokines (IL-10, IL-8, IL-1, IL-6, IL-15, IP-10, MIP-1β, TARC, Eotaxin-3, MDC), une activation intermédiaire des microglies et une augmentation des rod microglia avant le développement complet de la MA chez les individus atteints du SD. Nous avons observé une régulation négative de marqueurs inflammatoires dans le cerveau (TNFα, IFNγ, IL-12 and IL-10) ainsi qu'une augmentation des microglies dystrophiques chez les individus plus âgés atteints du SD. Des niveaux élevés de cytokines inflammatoires ont également été mesurés *in vitro* dans des cellules fœtales corticales de SD. D'après nos analyses, l'expression des cytokines varie aussi selon la région du cerveau et le sexe.

Nous avons ensuite évalué l'expression de protéines inflammatoires chez des individus sans démence avec une neuropathologie de type MA. Nous avons observé une augmentation des niveaux de IL-1β, IL-6 et eotaxin-3 dans le cortex temporal, et une augmentation des niveaux de IL-6 et MCP-1 dans le cortex pariétal des individus avec dépôts bêta amyloïde (Aβ+), comparés a ceux sans dépôts amyloïdes (Aβ-). Une tendance vers des niveaux plus élevés de IL-1β et MCP-1 a été observé dans le cortex frontal des individus Aβ+. Une association positive a été trouvé entre

l'expression de protéines inflammatoires et l'étendue du dépôt amyloïde. En particulier, le groupe Aβ+ présente un déclin cognitif plus rapide comparé au groupe Aβ-.

La dégénérescence progressive du système noradrénergique du locus coeruleus (LC) est un évènement précoce dans la MA. Nous en avons examiné les premiers stades, avant que le dépôt de plaques amyloïdes n'ait un impact sur le processus neuroinflammatoire précoce, dans un modèle de rat transgénique APP. La dégénérescence du LC entraine des déficits cognitifs, une perte de boutons synaptiques cholinergiques, une régulation négative de l'expression des neurotrophines et une dérégulation des médiateurs inflammatoires. Nous avons aussi observé une élévation du recrutement des cellules astrogliales et microgliales vers les neurones contenant de l'Aβ et l'activation intermédiaire des cellules microgliales.

Dans l'ensemble, nos résultats démontrent l'existence d'un processus neuroinflammatoire associé aux phases naissantes de la MA. Nous avons identifié des molécules inflammatoires clés qui pourraient contribuer à faire avancer ce processus. Par conséquent, nos trouvailles pourraient fournir des indices précieux afin d'identifier une fenêtre thérapeutique propice à l'utilisation de médicaments anti-inflammatoires au stade préclinique de la MA.

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List of Abbreviations

3xTg	Amyloid precursor protein transgenic mouse (APP K670N/M671L; tau P3010L mutations)
5xFAD Tg	Amyloid precursor protein transgenic mouse (APP K670N/M671L, V717I; PS1 M146L, L286V mutations)
AchE	Acetylcholinesterase
AD	Alzheimer's disease
ADAM	A disintigrin and metalloprotease domain
ADCS	Alzheimer's Disease Cooperative Study
ADHD	Attention-deficit hyperactive disorder
ADRDA	Alzheimer's Disease and Related Disorders Association
AICD	APP (amyloid precursor protein) intracellular domain
AIM2	Absent in melanoma-2
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
APA	American Psychiatric Association
APH1	Anterior pharynx defective
APOE	Apolipoprotein E
APP	Amyloid precursor protein
APP Tg	Amyloid precursor protein transgenic
APP-CTF	Amyloid precursor protein C-terminal fragment
APP/PS1 Tg	Amyloid precursor protein transgenic mouse (APP K670N/M671L; PSEN1 L166P mutations)
APS	Alzheimer Progression Score
ATP	Adenosine triphosphate
Aβ	Amyloid-beta
B2M	Beta-2-microglobulin
BACE	β-site amyloid precursor protein cleaving enzyme
BBB	Blood brain barrier
BCSFB	Blood-cerebrospinal fluid barrier

BDNF	Brain-derived growth factor
C1q	Component 1q
C3	Component 3
C99 or βCTF	Carboxy-terminal fragment
CA1	*Cornu ammonis 1*
CAA	Cerebral amyloid angiopathy
CAMCOG	Cambridge Cognitive Examination
CAMDEX-R	Cambridge Examination for Mental Disorders in the Elderly-Revised
cAMP	Cyclic adenosine monophosphate
CANTAB	Cambridge Neuropsychological Automate Test Battery
CARD	Caspase activation and recruitment domain
CCL	C-C motif-ligand
CD	Cluster of differentiation
CDKs	Cyclin-dependent kinases
CERAD	Consortium to establish a registry for Alzheimer's
ChAT	Choline acetyl transferase
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CREB	cAMP response element-binding protein
CSF	Cerebrospinal fluid
CSF1	Colony stimulating factor 1
CTS	Cathepsin
DAB	3,3'-diaminobenzidine
DAMES	Down's Syndrome Attention, Memory, and Executive Function Scales
DAMP	Damage-associated molecular pattern
DBH	Dopamine-Beta-Hydroxylase
DG	Dentate gyrus
DI	Discrimination index
DLB	Dementia with Lewy bodies

DR	Dimensional ratio
DRN	Dorsal raphe nucleus
DS	Down syndrome
DSBI	Down Syndrome Biomarker Initiative
DSM	Diagnostic and Statistical Manual of Mental Disorders
DSMSE	Down Syndrome Mental State Exam
DSP4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FC	Fear conditioning
FCRLS	Fc receptor-like molecules
FDA	Food and Drug Administration
FDG	Fluorodeoxyglucose
FTH1	Ferritin heavy chain 1
FTLD	Frontotemporal lobar degeneration
G-CSF	Granulocyte-colony stimulating factor
GFAP	Glial fibrillary acidic protein
GnRH	Gonadotropin-releasing hormone
GRO	Growth-related oncogene (CXCL1)
GWAS	Genome-wide associated studies
HD	Huntington’s disease
HEXB	Hexosaminidase subunit beta
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
Iba-1	Ionized calcium binding adaptor molecule-1
IDE	Insulin degrading enzyme
IFN	Interferon
IL	Interleukin

INCLUDE	Investigation of Co-occurring conditions across the Lifespan to Understand Down syndrome
iNOS	Inducible nitric oxide synthase
INTREPAD	Impact of Naproxen Treatment in Pre-symptomatic Alzheimer's Disease
IP-10	Interferon gamma-induced protein 10 (CXCL10)
IR	Immunoreactivity
IκBα	Nuclear factor of kappa light polypeptide gene enhancer
L-DOPA	l-3,4-dihydroxyphenylalanine
L-DOPS	L-threo-3,4,-dihydroxyphenylserine
LC	Locus coeruleus
LOAD	Late onset Alzheimer's disease
LPS	Lipopolysaccharide
LRP	Lipoprotein receptor-related protein 1
LRRC33	Leucine-rich-repeat-containing protein 33
LTP	Long-term potentiation
LYZ2	Lysozyme 2
MCI	Mild cognitive impairment
MCP-1	Monocytic chemoattractant protein-1 (CCL2)
MDC	Macrophage-derived chemokine (CCL22)
MHC	Major histocompatibility complex
MIP-1β	Macrophage inflammatory protein-beta (CCL4)
MMP	Matrix metalloprotease
MMSE	Mini-Mental State Examination
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSD	Mesoscale Discovery
NA	Noradrenaline
NBM	Nucleus basalis of Meynert
NCI	Non cognitive impairment
NEP	Neprilysin

NeuN	Neuronal nuclei
NFTs	Neurofibrillary tangles
NFκB	Nuclear factor kappa-light-chain-enhancer of activated
NGF	Nerve growth factor
NGS	Normal goat serum
NIA-AA	National Institute on Aging-Alzheimer's Association
NIA-Reagan	National Institute on Aging-Reagan
NIH	National Institutes of Health
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
NLR	Nucleotide-binding oligomerization domain-like
NLRC-5	NLR Family CARD Domain Containing 5
NLRP	Nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing
NMDA-r	N-methyl-D-aspartate receptor
NOD2	Nucleotide-binding oligomerization domain-containing
NOR	Novel object recognition
NSAIDs	Nonsteroidal anti-inflammatory drugs
NVU	Neurovascular unit
p-Tau	Phosphorylated tau
P2RY12	Purinergic receptor P2Y12
PAMPs	Pathogen-associated molecular patterns
PB	Phosphate buffer
PD	Parkinson's disease
PEN2	Presenilin enhancer
PET	Positron emission tomography
PI3K	Phosphoionositide 3-kinase
PMI	*Post-mortem* interval
PS	Presenilin
PSP	Progressive supranuclear palsy
RAGE	Receptor for advanced glycation endproducts

ROS	Reactive oxygen species
RT-qPCR	Real time quantitative polymerase chain reaction
sAPPα	Soluble amyloid precursor protein alpha
sAPPβ	Soluble amyloid precursor protein beta
SAS	Subarachnoid space
SCD	Subtle/subjective cognitive decline
SNAP	Suspected non-Alzheimer's pathology
SNPs	Single nucleotide polymorphisms
T21	Trisomy 21
TACE	Tumor necrosis factor α-converting enzyme
TARC	Thymus and activation regulated chemokine (CCL17)
TBI	Traumatic brain injury
TBS	Tris-buffered saline
tg	transgenic
Tg2576	Amyloid precursor protein transgenic mouse (APP K670N/M671L mutations)
TgCRND8	Amyloid precursor protein transgenic mouse (APP K670N/M671L/V717F mutations)
TGFBR1	Transforming growth factor beta receptor 1
TH	Tyrosine hydroxylase
TLR	Toll-like receptor
TMEM119	Transmembrane protein 119
TNF	Tumor necrosis factor
T_{regs}	Regulatory T cells
TREM2	Triggering receptor expressed on myeloid cells
TYROBP	Transmembrane immune signaling adaptor
VAChT	Vesicular acetylcholine transporter
VEGF-A	Vascular endothelial growth factor A
wt	Wild type
α7nAChR	Alpha 7 nicotinic receptor

Contribution of Authors

Chapters 2-4 of this doctoral Thesis describe experimental work encompassing three individual publications.

Lisi Flores Aguilar: *LFA* was the leading investigator for all the research studies comprised in this Thesis (Chapters 2-4 and appendices 1-2). LFA significantly contributed to the original ideas behind these studies. LFA conducted the majority of experiments, data quantification, and data analyses. LFA wrote independently the first draft of all manuscripts and exclusively generated all the figures for publication.

A. Claudio Cuello: ACC was the Principal Investigator and responsible of all projects. As my doctoral supervisor, ACC was the main intellectual influence for the studies presented in this Thesis. He contributed with original ideas for experimental work, intellectual guidance and significantly edited the writing of all the final manuscripts.

CHAPTER 2

Neuroinflammation across the lifespan of individuals with Down syndrome: from fetal to adult stages**.**

L Flores Aguilar, MF Iulita, Olivia Kovecses, MD Torres, T. Wisniewski, J Busciglio, AC Cuello. 2019, *submitted.*

M. Florencia Iulita: *MFI* contributed intellectually to the project design and edition of the manuscript.

Olivia Kovecses: *OK* performed IHC in *post-mortem* human brain tissue. She performed the microscopy experiments and analyses of microglia activation in human brains.

Maria D. Torres: *MDT* generated primary cultures from fetal cortical brain tissue and collected the respective conditioned media.

Sarah M. Levi: *SML* performed IHC to assess amyloid and tau pathologies in *post-mortem* human brain tissue.

Thomas Wisniewski: *TW* provided frontal cortex brain tissue and contributed to the generation of the final manuscript.

Jorge Busciglio: *JB* has been the principal collaborator of this study. He provided human cortical brain tissue and the conditioned media of fetal cortical cells. He significantly contributed intellectually to the study design and generation of the final manuscript.

CHAPTER 3

Deregulation of inflammatory markers in the brains of cognitively normal individuals with Alzheimer's neuropathology. **L Flores Aguilar,** MF Iulita, N Tanna, DA Bennett, AC Cuello. 2019, *in preparation for submission*

M Florencia Iulita: *MFI* performed the global inflammatory protein expression and cognitve data analyses of this study. She contributed intellectually to the project and to the edition of the manuscript.

Neil Tanna: *NT* performed troubleshooting experiments (gene expression and IHC) that were essential for this study.

David A Bennett: *DB* was the principal collaborator of this research project. He provided the human frontal, temporal and parietal brain samples along with neuropathological and cognitive data. He contributed significantly to editions of the final manuscript.

CHAPTER 4

Impact of Locus Coeruleus degeneration in a transgenic rat model of the early AD-like amyloid pathology. **L Flores Aguilar,** H Hall, Orciani C, O Kovecses, MK Foret, A Ducatenzeiler, AC Cuello. 2019, *in preparation for submission*

Hélène Hall: *HH* performed the Morris Water Maze test and data analyses. She provided behavioral training. She contributed intellectually to the study design and edited the final manuscript.

Chiara Orciani: *CO* performed WB against pro-NGF, IHC against Iba-1/McSA1/NeuN. She assisted with microscopy experiments.

Olivia Kovecses: *OK* performed IHC against DCX. She performed microscopy experiments and DCX + neuronal count.

Morgan K Foret: *MKF* assisted with immunohistochemistry against DBH in the LC region. She assisted with microscopy imaging.

Adriana Ducatenzeiler: AD performed the genotyping of the rats used in this study. She also assisted with the DSP4 injections.

CHAPTER 1

General Introduction

Preamble

Alzheimer's disease (AD) is a chronic neurodegenerative disease and the most common form of age-related dementia. Neuropathologically, AD is characterized by the extracellular accumulation of amyloid plaques in the brain parenchyma and the intraneuronal accumulation of neurofibrillary tangles composed of pathological tau. It is estimated that by 2030, 70 million people will suffer from AD; thus, one in every 2-3 people over 85 years will develop the disease (McDade *et al.*, 2017). Current treatments are only symptomatic and directed to individuals already at clinical stages of AD, a time when profound brain damage is present. As such, most of clinical trials have been directed towards late stages of AD.

During the past ten years, it has come to light that AD has a long asymptomatic phase where AD-related neuropathological changes start to build up in the brain (Bateman *et al.*, 2012; R. Sperling *et al.*, 2014; G. Wang *et al.*, 2019). Therefore, significant efforts are being put in place to thoroughly characterize the preclinical stages of AD. Along with this, secondary and primary prevention clinical trials have been launched with the aim of preventing or halting the disease before its clinical manifestation (R. Sperling *et al.*, 2014).

A significant challenge lies on the identification of AD at its preclinical stages. Current methodologies cannot unequivocally ascertain whether an individual is in the asymptomatic phase of AD. However, the investigation of populations at risk of AD has offered significant knowledge about AD preclinical stages.

An example of such population are individuals with DS, who given the trisomy of the *APP* gene, which encodes the Amyloid Precursor Protein (APP), will unavoidable develop clinical AD (Lott *et al.*, 2019). Indeed, the predictability in which they develop AD pathology makes them a significant population to study the long asymptomatic phase of AD. In fact, investigation of AD-related neuropathology in the DS population has revealed strikingly similarities. Significant interest has been put in clinically normal individuals with abnormal deposition of amyloid beta, as they are also at increased risk of progression to symptomatic AD (R. Sperling *et al.*, 2014; Sperling *et al.*, 2011). Studies in animal models mimicking some aspects of AD have been an essential towards our understanding of neuropathological changes occurring at early stages of AD. Such models permit specific manipulations that cannot be conducted in the human population.

Taking advantage of the above models, we have investigated pathological changes at incipient stages of AD neuropathology. In particular, we have focused our investigations on characterizing the neuroinflammatory process that accompanies such early stages. Our results revealed that before the onset of a full-blown AD pathology and dementia, inflammation is already present in the human brain.

A century after the discovery of Alzheimer's disease

Alzheimer's disease: "a peculiar serious disease of the cerebral cortex"

In 1906, the German neuropathologist Alois Alzheimer described the first case of "presenile dementia" (Alzheimer, 1907). In his 1906 report entitled "Uber eine eigenartige Erkrankung der Hirnrinde" [On an unusual illness of the cerebral cortex, for an English translation see (Alzheimer *et al.*, 1995)], Alzheimer described, neuropsychologically and neuropathologically, the case of Auguste Deter (Alzheimer, 1907). Auguste D. was a 51-year-old woman who had been placed in Emil Sioli's insane asylum in Frankfurt am Main. Alzheimer reported that Auguste D.'s first symptom was extreme jealousy towards her husband, followed by progressive memory loss, disorientation, visual hallucinations, aggressiveness, and paranoia (Alzheimer, 1907). Auguste D. died four and a half years later after her admission to the asylum. Thereafter, Alzheimer performed a *post-mortem* pathological examination of Auguste's brain. Her brain was highly atrophic and showed vascular arteriosclerotic changes (Alzheimer, 1907). Histological analyses using Bielschowsky's silver stain revealed "*striking changes of the neurofibrils*" which were characterized by their "*unique thickness and capacity for impregnation*" (Figure 1-1) (Alzheimer, 1907). One third to one quarter of neurons in the cerebral cortex displayed these neurofibrillary pathological changes accompanied by disappearance of the upper neuronal layer (Alzheimer, 1907). He further proposed that fibrillar changes were the result of the deposition of a "metabolic substance" inside neurons (Alzheimer, 1907). Moreover, he reported the occurrence of "minute miliary foci" caused by the deposition of a "special substance" in the cerebral cortex (Alzheimer, 1907).

Yet, the presence of brain "foci" was previously noticed as "amas ronds" (round heaps) by Paul Blocq and Georges Marinesco in the brain of an old individual with epilepsy in 1892 (Blocq, 1892). In 1898, Emil Redlich described the presence of "miliare Sklerose" (miliary sclerosis) in two cases of senile dementia and referred to them as "plaques", a term that continues to be used (Redlich, 1898). Blocq, Marinesco and Redlich believed that the plaques represented a modified glial cell and did not further investigate the relevance of these aggregates (Blocq, 1892; Redlich, 1898).

In the same year that Alzheimer communicated his observations, Oskar Fischer described the presence of plaques in 12 of 16 cases of senile dementia and for the first time, he described the neuritic plaque (Figure 1-1) (P. D. D. O. Fischer, 1907). Moreover, contrary to Blocq, Marinesco and Redlich's beliefs, Fischer did not attribute a glial origin to the plaques. He proposed that the presence of plaques and neuritic plaques was associated to a clinical condition named "presbyophrenia", a dementia subtype characterized by disorientation, short-memory impairment, euphoria and confabulations [reviewed in (Goedert, 2009)]. On the other hand, the early presentation of dementia (presenile dementia) followed by a rapid and progressive memory loss accompanied by unique neuropathological changes was characterized as a "special illness" by Alzheimer (Alzheimer, 1907). In 1910, Emil Kraepelin, endorsed this neuropathology as Alzheimer's disease in the *Textbook of Psychiatry* and defined it as a rare form of presenile dementia different from senile dementia (Kraepelin, 1910).

It was not until the 1970's that presenile and senile dementia were encompassed as "Alzheimer's disease" (AD) by Robert Katzman (Katzman, 1976). Thus, it was the complimentary seminal discoveries of Alzheimer and Fischer that lead to the identification of the clinicopathological hallmarks of AD; nowadays regarded as the most common form of age-related dementia.

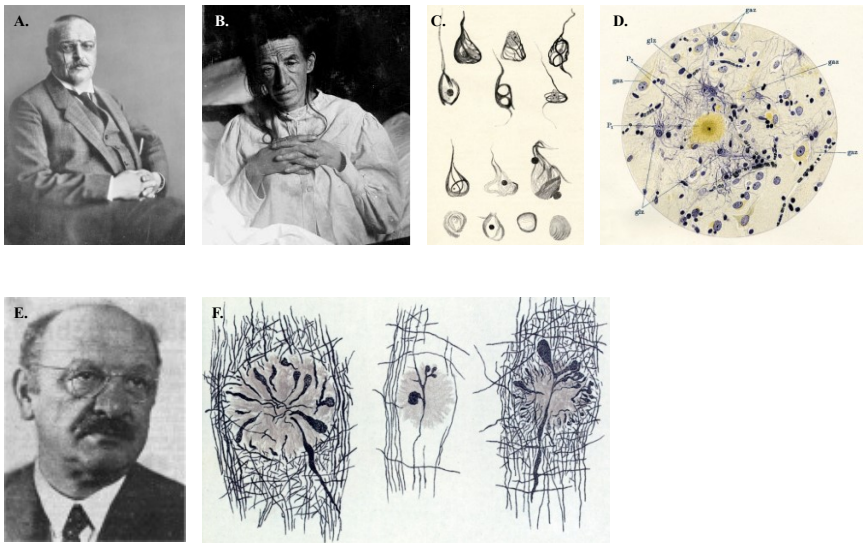

Figure 1- 1. Alois Alzheimer and Oskar Fischer

(A) Alois Alzheimer (June 14, 1864 – December 19, 1915) was a German psychiatrist and neuropathologist. He was the first to describe the neuropathology and clinical presentation of Alzheimer's disease. (B) Auguste Deter (May 16, 1850 – April 8, 1906) was the first patient to be officially diagnosed with Alzheimer's disease. (C-D) Alzheimer's drawings depicting neurofibrillary tangles and amyloid plaques. E) Oskar Fischer (April 12, 1876 – February 28, 1942) was a Czech psychiatrist and neuropathologist who firstly described neuritic plaque formation in Alzheimer's disease brains. (F-G) Fischer's drawings depicting neuritic plaques from the brains of patients with senile dementia. Images from (Dahm, 2006; Goedert, 2009; Maurer *et al.*, 1997) with permission from *Elsevier* and *Oxford University Press*.

Alzheimer's as a major health problem

In 1975, AD was estimated to be the fourth or fifth cause of death in the United States (Katzman, 1976). Until then, AD had been overlooked and research in the field was insufficient. In 2019, an estimated of 5.8 million of Americans had AD making this pathology the sixth cause of death and the leading cause of morbidity and disability in the United States (Alzheimer's Association, 2019). The Alzheimer's Society of Canada reported that half a million of Canadians have been diagnosed with Alzheimer's and 25 000 new cases arise every year. This number is expected to rise by 66 % in 2031.

Even though seminal discoveries have led to the understanding of several aspects of AD, there is currently no cure or preventive treatment for this neuropathology. Therefore, intense research and international collaborations are currently ongoing with the main objective of preventing, slowing or halting this devastating disease.

Clinical diagnosis of Alzheimer's disease

Dementia or major neurocognitive disorder

The term dementia was recently replaced with "major neurocognitive disorder" by the American Psychiatric Association (APA) in the fifth edition of their Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (American Psychiatric Association, 2013). Nevertheless, the term dementia continues to be globally used by clinicians. Dementia is a major cognitive disorder severe enough to interfere with both cognitive function (such as speech, judgement, language, reasoning, planning, and memory) and daily life activities. Different causes of dementia have been identified and associated to specific pathologies and symptoms. Examples of common dementias include AD, vascular dementia, dementia with Lewy bodies (DLB), frontotemporal lobar degeneration (FTLD), Parkinson's disease (PD) dementia, Creutzfeldt-Jakob disease, normal pressure hydrocephalus, and mixed dementia (Alzheimer's Association, 2016). AD accounts for 60% to

80% of all dementia cases, making it the most common form of age-related dementia (Alzheimer's Association, 2016).

Clinical diagnosis of Alzheimer's disease

Diagnosis of AD is mostly based in the criteria established by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA). In 2011, the National Institute on Aging-Alzheimer's Association (NIA-AA) workgroups revised the 1984 AD clinical diagnosis criteria established by the NINCDS-ADRDA.

The revised criteria consists of three terminologies to classify individuals with dementia due to AD: (1) probable AD dementia (amnestic or non-amnestic), (2) possible AD dementia, and (3) probable or possible AD dementia with evidence of the AD pathophysiological process (McKhann *et al.*, 2011). According to the guidelines, individuals with probable AD amnestic dementia display learning and recall impairments accompanied by impairment in at least one other cognitive domain. The non-amnestic presentation is characterized by visuospatial, language and executive deficits. An absence of other dementias or neurological disease is necessary to fit in this category. Possible AD dementia meets the core criteria for AD dementia and is always accompanied by an atypical onset or mixed etiology dementia. The third classification is used when AD biomarkers are positive; however, this classification is only used for research purposes.

Besides the recent developments that allow early detection of possible or probable AD, a definite diagnosis of AD is dependent upon histological *post-mortem* confirmation.

Clinical progression of Alzheimer's disease

Prior to being diagnosed with AD dementia, individuals already present symptoms reflecting an underlying pathophysiological process related to the AD pathology. Such symptoms manifest as low performance in one or more cognitive domains (memory, attention, executive function,

language, visuospatial skills) that is greater than anticipated for someone of the same age and educational background accompanied by longitudinal cognitive decline (Albert *et al.*, 2011; Bennett *et al.*, 2002; Petersen *et al.*, 1999).

This symptomatic predementia phase of AD is termed "mild cognitive impairment (MCI) due to AD" (Albert *et al.*, 2011). Individuals that enter this stage are non-demented and can independently execute normal life tasks with minimal assistance. Episodic memory impairments are the most common in individuals with MCI that progress to AD (Albert *et al.*, 2011). It is important to clarify that several conditions can lead to the development of MCI, thus, when in the clinic, the physician needs to rule out other possible causes of MCI such as vascular, traumatic brain injury and medical causes of cognitive decline (Albert *et al.*, 2011).

It has been estimated that the annual conversion rate from MCI to dementia is 8% to 15%; indeed 38% of individuals with MCI that were followed for more than 5 years developed AD dementia during this period (Albert *et al.*, 2011; Petersen, 2016). Individuals that display mild AD symptoms can carry out most of their daily tasks with a certain degree of assistance and might present mood changes such as anxiety, disinhibition, and irritability (Mega *et al.*, 1996). When individuals enter the moderate stage of dementia (the longest phase), they have difficulties communicating and performing daily tasks. Behavioral and personality changes, such as agitation, apathy, depression, hallucinations and suspiciousness are common at these stages (Albert *et al.*, 2011; Mega *et al.*, 1996). The late stages of Alzheimer's disease are characterized by neuropsychiatric features such as dysphoria, delusions and paranoia (Doody *et al.*, 1995; Mega *et al.*, 1996). As the disease progresses, motor impairments that affect speech, walking, and swallowing lead the patient to be bed-bound (Alzheimer's Association, 2019). In general, life expectancy after AD dementia diagnosis is 4 to 8 years, however in some cases, certain individuals can live 20 years or more (Alzheimer's Association, 2019).

Neuropathological hallmarks of Alzheimer's disease

The major neuropathological hallmarks of AD are the deposition of amyloid plaques in the brain parenchyma and neurofibrillary tangle formation inside the neurons. These two events lead to the

typical brain atrophy observed at late stages of AD. In addition, the AD brain is characterized by the presence of dystrophic neurites, synaptic loss, vascular pathology, and neuroinflammation.

Amyloid plaques

Electron microscopy techniques allowed Robert Terry and Michael Kidd to demonstrate that the plaques described by Alzheimer were composed by an amyloid protein (Kidd, 1964; Terry *et al.*, 1964). Almost a century after the identification of plaques by Alzheimer, in 1984, Glenner and Wong isolated the protein that composed the amyloid plaques from the cerebrovasculature of AD and Down syndrome (DS) brains. This 4kDa protein coined to be the A4 peptide which nowadays is known as the "amyloid-beta" (Aβ) peptide (Glenner *et al.*, 1984a, 1984b). One year later, Colin L. Masters purified Aβ from cortical deposits in AD and DS brains (Masters *et al.*, 1985).

Today we know that amyloid plaques are composed of Aβ fragments mainly of 40-42 amino acids, where the $A\beta_{42}$ fragment is more prone to aggregation (Miller *et al.*, 1993). Such aggregates are widespread in the cortex and hippocampus of individuals with AD and have been classified into 2 groups based on the aggregation state (Figure 1-2, C-D). (1) *Diffuse amyloid plaques* have an amorphous morphology without distinct edges and are commonly not surrounded by astroglia, microglia or dystrophic neurites. These plaques are Congo Red and Thioflavine-S negative. Diffuse plaques are more abundant at preclinical stages of AD; reflecting early stages of Aβ deposition (Dickson *et al.*, 2001). Nevertheless, the presence of diffuse amyloid plaques does not guarantee the occurrence of AD. (2) *Dense core amyloid plaques* are mature plaques that can be labeled with CongoRed and Thioflavin-S dyes. These plaques are commonly surrounded by activated glia and dystrophic neurites are generally present (Serrano-Pozo *et al.*, 2011).

Deposition of amyloid plaques in the AD brain follows an unpredictable progression. Despite this, researchers have conducted histological studies aiming to stage amyloid plaque deposition. The first criteria was proposed by Heiko Braak and Edith Braak in 1991 where Braak proposed three stages of amyloid deposition (Braak *et al.*, 1991). He detected that amyloid deposits first appear in the frontal, temporal and occipital lobes (stage A), followed by isocortical association areas with mild involvement of the hippocampus (stage B) before spreading to all cortical areas including the

sensory and motor cortex and in some cases to the molecular layer of the cerebellum and subcortical nuclei (stage C).

A second more detailed criteria, involving five stages of amyloid deposition was proposed by Thal et. al. (Thal *et al.*, 2002). Stage (1) involves amyloid plaque deposition in isocortical areas, (2) is characterized by deposits in allocortical areas such as the entorhinal cortex, hippocampus, amygdala and the insular and cingulate cortices, (3) amyloid deposits appear in the striatum, basal forebrain cholinergic nuclei, thalamus, hypothalamus and white matter, (4) amyloid deposits occur in some structures in the brainstem such as the substantia nigra, reticular formation of the medulla oblongata, superior and inferior colliculi and the red nucleus, in stage (5) amyloid deposits spread to other areas in the brainstem such as the raphe nuclei, locus coeruleus and the molecular layer of the cerebellum. From these classifications it is clear that amyloid deposition starts in isocortical areas, followed by limbic areas before finally spreading to subcortical regions. Amyloid deposits are spread throughout the six layers of the cortex; however, layers II-V display a higher degree of amyloid pathology.

The deposition of Aβ in the brain cerebrovasculature, known as cerebral amyloid angiopathy (CAA), is a common feature in AD (Figure 1-2, E) and 80% of AD brains display mild CAA at autopsy (Serrano-Pozo *et al.*, 2011). While $A\beta_{42}$ is more commonly present in parenchymal amyloid plaques, $A\beta_{40}$ is preferentially deposited in the cerebral vasculature of AD patients, where the parietal and occipital cortices are mainly affected (Serrano-Pozo *et al.*, 2011). Amyloid can accumulate in the capillaries, arterioles, small arterioles and leptomeningeal vessels, the latter being more affected than parenchymal vessels (Serrano-Pozo *et al.*, 2011).

Neurofibrillary tangles

Tau is a microtubule binding protein normally located within neurons (Weingarten *et al.*, 1975). Six tau isoforms, resulting from alternative splicing, are normally expressed in the Central Nervous System (CNS) (Y. Wang *et al.*, 2016). Intracellular transport, neurite development and cell polarity are common phosphorylation-dependent physiological roles of tau (Mandelkow *et al.*, 1998).

Neurofibrillary tangles (NFTs) in AD were firstly identified by Alzheimer (Alzheimer, 1907). NFTs are formed by paired helical filaments (Kidd, 1963; Terry *et al.*, 1964) of abnormally

misfolded and hyperphosphorylated tau protein (Goedert *et al.*, 1988; Grundke-Iqbal *et al.*, 1986). Hyperphosphorylation of tau in AD might be the consequence of increased kinase activity and deficiencies in phosphatase activation (Y. Wang *et al.*, 2016). As tau pathology progresses, its cellular localization changes and NFT are mainly found in the somatodendritic compartment (Figure 1-2, F-G). At advanced AD stages, tau "ghost" tangles result from the death of NFT-bearing neurons (Mena *et al.*, 1991).

Contrary to amyloid deposition, NFT formation follows a predictable and well-demarked spatiotemporal pattern in AD. Six stages of NFT deposition have been proposed by Braak (Braak *et al.*, 1991). The first NFTs appear in the perirhinal region (stage I) followed by NFTs in the entorhinal cortex, the *cornu ammonis* 1 (CA1) region of the hippocampus and the antero-dorsal nucleus of the thalamus (stage II). Stage III is characterized by accumulation of NFTs in limbic regions such as the hippocampal subiculum. During stage IV, NFTs appear in the amygdala, thalamus, claustrum and to a small extent in isocortical association areas. Spreading to all isocortical areas occurs in stage V and involvement of motor, visual, and sensory areas is evident in the final stage (stage VI). Certain laminae are more affected by NFT pathology. Pyramidal neurons from cortical layers III and V, entorhinal layer II-IV and pyramidal neurons from CA1 and subiculum are preferentially affected by tau pathology (Braak *et al.*, 1991).

Unlike amyloid plaque deposition, the extent of NFT deposition correlates with the degree of cognitive impairment in AD (Arriagada *et al.*, 1992; Bierer *et al.*, 1995; Giannakopoulos *et al.*, 2003; G. K. Wilcock *et al.*, 1982), suggesting that NFT formation might reflect the ongoing neurodegeneration in the AD brain.

Gross brain atrophy

Figure 1-2, A-B illustrates the gross brain atrophy commonly found in the Alzheimer's brain. Seminal studies conducted by Nick Fox and colleagues using MRI have revealed significant increased rates of hippocampal atrophy at presymptomatic and mild stages of AD (Fox *et al.*, 1996; Scahill *et al.*, 2002). In addition, parietal lobe atrophy was observed at all AD stages. The inferiolateral regions of the temporal lobes become atrophic at mild to moderate stages of AD

while frontal lobe atrophy occurs later in the disease. Moreover, dilation of the lateral ventricles becomes apparent at presymptomatic AD stages. The primary sensory, motor and visual areas are relatively spared of atrophic changes. It is also common to find cerebrovascular disease such as lacunar infarcts, microinfarcts and demyelination of the paraventricular white matter (Dickson *et al.*, 2001). Finally, the melanin pigmentation of the locus coeruleus disappears. Besides the gross and obvious macroscopical changes in the AD brain, they are not specific to AD. Histopathological analysis of the brain is still needed for a final AD diagnosis.

Synaptic loss

Electron microscopy studies revealed for the first time synapse loss in biopsies from the frontal, temporal and entorhinal cortices and hippocampus of AD brains (C. A. Davies *et al.*, 1987; Scheff *et al.*, 1990; Scheff *et al.*, 1993a; Scheff *et al.*, 1996; Scheff *et al.*, 1993b). Such synaptic loss correlated strongly with cognitive impairment (Terry *et al.*, 1991), suggesting that loss of synapses can represent the culprit of cognitive deterioration in AD.

Immunohistochemical techniques allowed the detection and quantification of pre and post synaptic markers. Synaptophysin, a presynaptic protein, was reported to be decreased in the hippocampus and association cortices of AD brains, supporting the occurrence of synaptic loss in clinical stages of AD (Masliah *et al.*, 1991). Furthermore, Masliah and colleagues, reported a decrease in synaptophysin in mild AD (Masliah *et al.*, 2001) and in the hippocampus of MCI cases (Scheff *et al.*, 2007), indicating that synaptic loss was already apparent at preclinical AD stages.

The occurrence of synaptic loss has been attributed to amyloid and tau toxicity. Moreover, in the past years, synaptic pruning by microglial cells has been acknowledged as a possible mechanism contributing to synapse loss in transgenic models of the amyloid-like pathology (Hong *et al.*, 2016). Transgenic models that overexpress APP display a decrease in synaptic proteins even before the accumulation of amyloid plaques [for a review see (Bell *et al.*, 2006)] indicating that soluble Aβ oligomers can already promote the loss of synapses.

Indeed, soluble Aβ oligomers have been confirmed as the most toxic species of Aβ surpassing the toxicity of amyloid plaques (Haass *et al.*, 2007; Walsh *et al.*, 2002). Supporting this concept, other

studies found a strong correlation between soluble cortical Aβ levels and cognitive impairment (McLean *et al.*, 1999). Multiple hypotheses have been proposed to explain how Aβ oligomers promote cognitive deficits. For example, Aβ oligomers can cause disruption of long-term potentiation (LTP) formation; thus promoting cognitive deficiencies in AD-like transgenic models (Selkoe, 2002). Furthermore, recent studies indicate that toxic Aβ oligomers lead to upregulation of complement factors that promote pathological synapse pruning by microglial cells (Hong *et al.*, 2016). Therefore, before the development of a full-blown AD pathology, microglial activation might contribute to the progression of AD, a proposition discussed later in this chapter. Besides Aβ acting as a toxic agent, pathological tau can also promote synapse loss well before the formation of tau aggregates as revealed in a transgenic tau rodent model (Yoshiyama *et al.*, 2007).

Preferential loss of cholinergic input in the AD brain were identified in the mid 1970's by (P. Davies *et al.*, 1976) and (Bowen *et al.*, 1976). Transgenic models have also revealed the vulnerability of certain neurotransmitter systems to the progression of the amyloid pathology (Bell *et al.*, 2006). Cholinergic boutons are the first to be affected followed by a loss of glutamatergic terminals and finally by GABAergic boutons (Bell *et al.*, 2006). A paradoxical upregulation of cortical cholinergic boutons precedes the development of amyloid plaques in an APP transgenic model (Wong *et al.*, 1999), suggesting that a compensatory mechanism might be taking place before the frank loss of synapses. Supporting this, increases in choline acetylcholine transferase (ChAT) activity (DeKosky *et al.*, 2002) and hippocampal activation (Dickerson *et al.*, 2005) have been observed prior to the onset of AD.

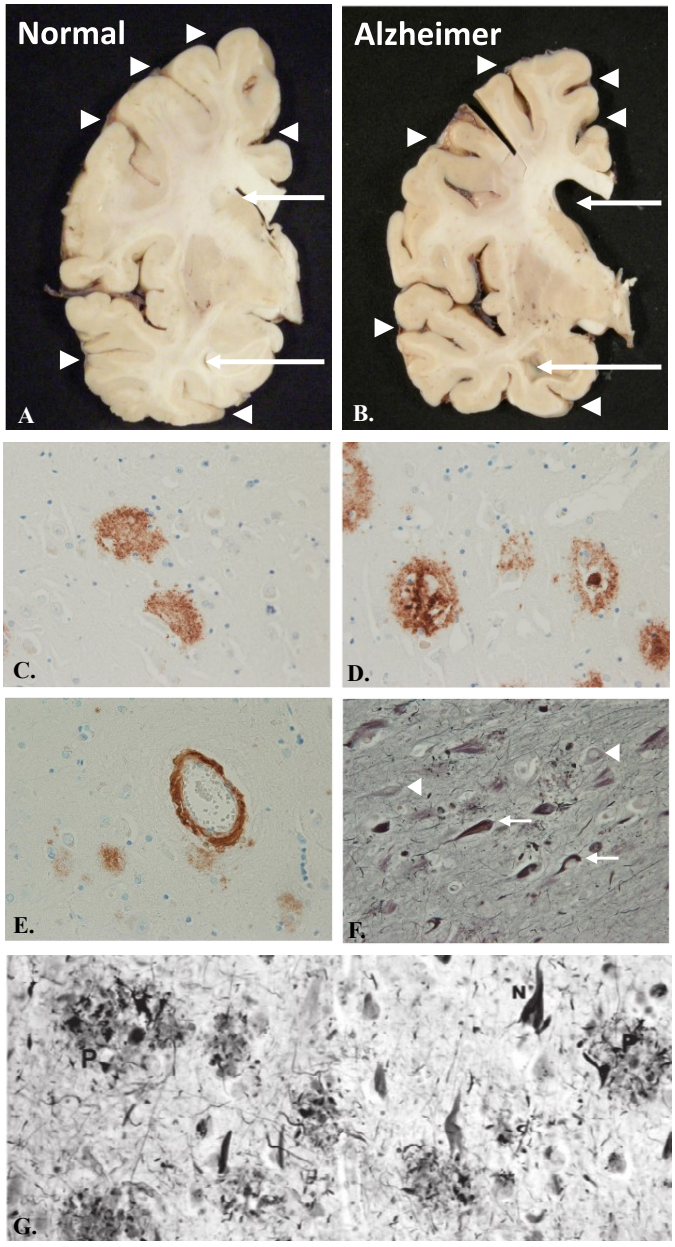

Figure 1- 2. Alzheimer's disease neuropathological hallmarks

(A-B) Alzheimer's disease brains display gross alterations. The arrowheads indicate widening of sulcal spaces and narrowing of brain gyri in the Alzheimer's brain compared to a normal brain. In Alzheimer's disease the frontal and temporal horns of the lateral ventricles are enlarged (arrows). (C) Diffuse and dense core (D) amyloid plaques as revealed by immunohistochemistry against Aβ. (E) Cerebral amyloid angiopathy (F) Bielschowsky silver impregnation allows for the detection of neurofibrillary tangles (arrows) and extracellular ghost tangles (asterisk). (G) [P] Neuritic plaque and [N] neurofibrillary tangle as revealed by modified Bielschowsky silver impregnation. Images from (Dickson *et al.*, 2001; Wippold *et al.*, 2008) with permission from *Springer Natur*e and the *American Society of Neuroradiology*.

APP processing and Aβ generation

The Aβ peptide originates from regulated proteolysis of the Amyloid Precursor Protein. The *APP* gene was identified and mapped to chromosome 21 in 1987 (J. Kang *et al.*, 1987; Tanzi *et al.*, 1987). APP is a transmembrane type I glycoprotein with its amino terminal in the lumen or extracellular space and its carboxyl terminal in the cytoplasm. Alternative splicing of the *APP* gene yields three isoforms, APP751, APP770, and APP695. The latter being expressed mainly in neurons. This protein is evolutionary conserved across different species indicating a common but presently unresolved main physiological role [for a review see (Nhan *et al.*, 2015)].

APP processing is regulated by the activity of α, β, and γ-secretases. Several members of the ADAM (disintegrin and metalloprotease) family can act as α secretases such as ADAM9, ADAM10 and ADAM17 (also known as tumor necrosis factor α-converting enzyme, TACE) (Fahrenholz *et al.*, 2000; Lammich *et al.*, 1999). On the other hand, BACE-1 (β-site APP cleaving enzyme, or Asp-2 and memapsin-2) is the only β-secretase involved in APP processing (Vassar *et al.*, 1999). Finally, γ-secretase is a macromolecular protein complex consisting of presenilin (PS), presenilin enhancer (PEN2), nicastrin, and anterior pharynx defective (APH1) (Nhan *et al.*, 2015).

Two main pathways are responsible for APP processing: the amyloidogenic pathway, where Aβ is generated and the non-amyloidogenic pathway, where other peptides, but not Aβ, are produced. The non-amyloidogenic pathway involves APP sequential cleavage by α and γ-secretases (Figure 1-3, A). First, α-secretase cleaves APP in the middle of the Aβ region, releasing the soluble ectodomain named soluble-APPα (sAPPα) and a membrane-bound intracellular C-terminal fragment known as the APP-CTF fragment (α-CTF or C83). Thereafter, γ-secretase cleaves the carboxyl terminal fragment generating the p3 peptide and the APP intracellular domain (AICD or C59) (Nhan *et al.*, 2015).

Generation of Aβ involves the sequential cleavage by β and γ-secretases (Figure 1-3, B). First, BACE-1 cleaves between residues 671 and 672, generating the N-terminus of Aβ and releasing a part of the APP ectodomain (sAPPβ). The generated carboxy-terminal fragment (C99 or βCTF) is then cleaved by γ-secretase); this last step generates the Aβ peptide and the AICD fragment. Secreted forms of Aβ have a tendency to aggregate and form the so-called amyloid plaques.

Mounting evidence from AD and DS brains along with transgenic animal models of the amyloid-like pathology indicates that the accumulation of intraneuronal Aβ precedes its extracellular deposition (Gouras *et al.*, 2010; Gouras *et al.*, 2000; LaFerla *et al.*, 2007; Lemere *et al.*, 1996; Mori *et al.*, 2002). Given that Aβ production can occur in every cellular site where APP, BACE-1 and γ-secretase are expressed, intracellular Aβ release occurs in several cell compartments. Aβ accumulation has been reported to occur in endosomes, exosomes, the trans-golgi network and the endoplasmic reticulum (Haass *et al.*, 2007; LaFerla *et al.*, 2007). Several lines of research also indicate that Aβ can be uptaken from the extracellular pool via α7nAch receptors, low density lipoprotein receptor-related protein 1 (LRP) or receptor for advanced glycation endproducts (RAGE); thus enhancing its intracellular accumulation (LaFerla *et al.*, 2007). LRP and RAGE are also involved in Aβ vascular clearance across the blood brain barrier (BBB). Clearance of Aβ is mediated by different cell types, enzymes, and clearance systems in the brain [reviewed in (Zuroff *et al.*, 2017)]. For example, the insulin degrading enzyme (IDE) (Mukherjee *et al.*, 2000) and neprilysin (NEP) (Kanemitsu *et al.*, 2003) can degrade soluble $A\beta_{40}$ and $A\beta_{42}$ in the extracellular space. Intracellular degradation within lysosomes, the ubiquitin-proteasome system and autophagy, can also contribute to clearance of Aβ (Zuroff *et al.*, 2017). Aβ can also be phagocytized and catabolized by monocytic cells (El Khoury *et al.*, 2007; Koronyo-Hamaoui *et al.*, 2009; Simard *et al.*, 2006). Finally, clearance by the glymphatic system might be an alternate mechanism to clear Aβ from the brain to the periphery (Iliff *et al.*, 2012). Failure in any of the above mechanisms and the rate of Aβ clearance (D. E. Kang *et al.*, 2000; Zlokovic *et al.*, 2000) may contribute to amyloid deposition and development and/or progression of AD.

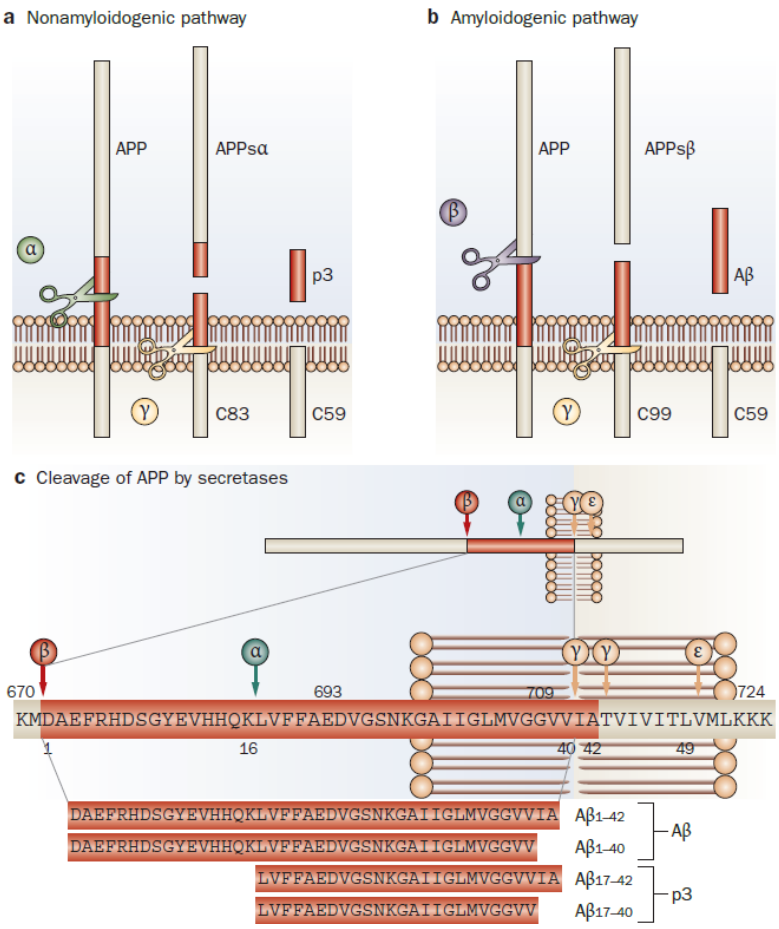

Figure 1- 3. Processing of the Amyloid Precursor Protein

(A) In the non-amyloidogenic pathway, the APP is sequentially cleaved by α and γ-secretase generating sAPPα, the p3 and AICD (C59) fragments. (B) In the amyloidogenic pathway, APP is cleaved by β and γ-secretase generating the Aβ peptide, sAPPβ and the AICD fragment. (C) Scheme depicting the amino acid residues where α, β, and γ secretases cleave APP. β-secretase can cleave at two different sites, generating Aβ and p3 peptides of differing lengths. s = soluble; AICD = APP intracellular domain; C = carboxy-terminal. Image from (De Strooper *et al.*, 2010) with permission from *Springer Nature*.

The amyloid hypothesis

The notion that the A4 peptide (or Aβ) could be the underlying cause of AD was suggested by Glenner and Wong (Glenner *et al.*, 1984a, 1984b). However, it was not until the discovery that the development of AD was favored by certain mutations in *APP*, that the so-called "amyloid-hypothesis" was proposed by John Hardy (J. A. Hardy *et al.*, 1992) and revisited 10 years later by Hardy and Selkoe (J. Hardy *et al.*, 2002). The amyloid hypothesis poses Aβ pathological accumulation as the etiological factor that drives a cascade of events, such as synaptic loss, NFT formation, and inflammation, that eventually lead to the development of AD. It was also hypothesized that this abnormal accumulation arises from an improper balance between the production and clearance of Aβ (J. Hardy *et al.*, 2002; D. E. Kang *et al.*, 2000). The rationale behind the amyloid hypothesis is based on distinct scenarios where Aβ overproduction leads to the development of genetic or sporadic AD.

Given triplication of chromosome 21 and hence triplication of *APP*, most individuals with DS will develop AD neuropathology and dementia by age 40-60 [for a review see (Lott *et al.*, 2019)]. Indeed, individuals with DS display Aβ accumulation since early in their lifespan. This is followed by the appearance of NFT by age 30-40, and by 40-years old, individuals with DS develop an overt AD pathology. The chronological protein deposition in DS highly suggests that Aβ pathology occurs before NFT formation. Moreover, individuals with DS without full triplication of *APP* do not develop AD but display the DS phenotype (Doran *et al.*, 2017; Prasher *et al.*, 1998). On the other hand, individuals with mini-duplications of the *APP* gene do not have DS but develop AD in their 50's (Rovelet-Lecrux *et al.*, 2006). Therefore, APP overexpression leads to the development of AD.

The first *APP* mutation was discovered in 1990 (Levy *et al.*, 1990) in a Dutch family suffering from an autosomal dominant form of cerebral amyloidosis mainly characterized by extensive amyloid deposition in the leptomeninges (Wattendorff *et al.*, 1982). The discovery that mutations in the *APP* gene (within the Aβ sequence or very near to the sites cleaved by α, β, or γ-secretases) or in PS, lead to early onset Alzheimer's disease further supported the role of APP in the development of AD (Selkoe *et al.*, 2016). Notably, in 2012 a protective mutation was described. The A673T mutation in the second Aβ amino acid decreases APP cleavage by β-secretase (Jonsson *et al.*, 2012). Carriers of the protective mutation have a lower risk of developing AD as well as

age-related cognitive decline (Jonsson *et al.*, 2012). Notably, centenarians carrying the mutation do not display amyloid plaques (Jonsson *et al.*, 2012), further supporting the central role of Aβ in the development of AD.

One can argue that this evidence only applies to the genetic forms of AD, accounting for less than ~1% of total AD cases. Is there evidence linking APP overproduction with the development of AD in the general population? The discovery of risk factors that promote the aggregation or accumulation of Aβ in the general population support the validity of the amyloid hypothesis. Individuals homozygous for the ε4 allele of the Apolipoprotein E (APOE) have a 20-fold increased risk of developing AD, making ApoE4 a major AD risk factor (Corder *et al.*, 1993; Poirier *et al.*, 1993; Strittmatter *et al.*, 1993). Indeed, the ε4 allele is present in more than half of sporadic AD cases. This isoform leads to Aβ aggregation and impaired clearance, leading to an increase in Aβ toxic effects [(for a detailed review of ApoE4 and AD, see (C. C. Liu *et al.*, 2013)].

Almost 30 years have passed since the formulation of the amyloid hypothesis and is yet, it is not universally accepted. The main critique is that NFT pathology correlates better with cognitive impairment than the number of amyloid plaques. Hardy and Selkoe have revisited the hypothesis and proposed, based on a large body of evidence, that the most toxic amyloid species are Aβ oligomers rather than plaques (J. Hardy *et al.*, 2002). In fact, as mentioned earlier, Aβ oligomers correlate better with synaptic dysfunction and cognitive decline. Moreover, studies in transgenic animal models support the notion that Aβ pathology can promote tau phosphorylation and pre-tangle formation, accelerating the progression of the AD-like pathology (Gotz *et al.*, 2001; Jin *et al.*, 2011; Lewis *et al.*, 2001) .

The general failure of clinical trials directed towards diminishing Aβ in the brain have been disappointing and have called into question whether Aβ is the main culprit of AD. The counterargument to this critique is that clinical trials have been conducted in individuals at advanced stages of AD, most likely when not only Aβ is the main driver of the disease. The A4 study (Anti-Amyloid Treatment in Asymptomatic Alzheimer's Disease, www.adcs.org/Studies/A4.aspx) led by Reisa Sperling, is a secondary prevention clinical trial in Phase III (R. A. Sperling *et al.*, 2014). Administration of a monoclonal antibody against the mid part of the Aβ peptide (solanezumab, Eli Lilly and Company) is currently being administered to cognitively normal individuals (65-85 years old) that display brain amyloid accumulation; thus,

making them a population at risk of progression to AD (as discussed later in this chapter). The trial will finish in December 2020.

Whether the trial succeeds or fails, the development of therapeutic drugs targeting other pathological mechanisms than amyloid is an unmet need. It is likely that combinational therapies in AD will be more effective given the broader spectrum of pathological mechanisms that can be targeted.

Pre-clinical stages of Alzheimer's disease: the starting point

Autopsy, imaging and fluid biomarker studies indicate that one third of old cognitively healthy individuals display AD pathology (Bennett *et al.*, 2006; De Meyer *et al.*, 2010; Fagan *et al.*, 2009; J. C. Morris *et al.*, 1996). Henceforth, a proportion of this population might be at the preclinical stages of AD. The understanding and identification of AD pre-clinical stages is of uttermost importance; similar to cancer, diabetes, or cardiovascular disease, the early detection of AD, might be the key to prevent its progression.

The investigation of genetically at risk and older cohorts, has revealed a temporal evolution of pathological changes that might be reflective of preclinical AD stages (Jack *et al.*, 2013). As amyloid accumulates in the brain, its cerebrospinal fluid (CSF) amyloid levels decrease. Indeed, the first detectable abnormality is a drop in CSF $A\beta_{42}$ levels (Skoog *et al.*, 2003; Strozyk *et al.*, 2003; Tapiola *et al.*, 2009) followed by an increase in brain Aβ binding as revealed by positron emission tomography (PET) (Jack *et al.*, 2008; Jack *et al.*, 2009). Then, increased tau and p-tau levels in the CSF become detectable (Buerger *et al.*, 2006; Tapiola *et al.*, 2009) and correlate to neuronal loss and NFT formation (Jack *et al.*, 2013). This is followed by brain hypometabolism in the posterior cingulate, precuneus, and temporoparietal cortices as measured by fluorodeoxyglucose 18F-PET (FDG-PET) uptake (Iturria-Medina *et al.*, 2016; Sperling *et al.*, 2011). Moreover, magnetic resonance imaging studies indicate the existence of brain atrophy, affecting primarily the lateral temporal, posterior cingulate and medial parietal cortices and hippocampus (Sperling *et al.*, 2011). Figure 1-4 illustrates the hypothetical model of AD biomarkers.

The time between the detection of Aβ abnormalities and onset of cognitive decline has not yet been established; however, some studies suggest a duration of at least 15-30 years (Bateman *et al.*, 2012; Masters *et al.*, 2015; R. Sperling *et al.*, 2014; Villemagne *et al.*, 2013; G. Wang *et al.*, 2019). It is important to note that this hypothetical model is based only on biomarkers that reflect some of the underlying AD pathophysiological processes. Development of new or more sensitive technologies/assays will allow the identification of earlier pathological changes in the AD continuum.

Individuals with MCI are more likely to progress to AD if they display positivity for CSF AD biomarkers mentioned before (Hansson *et al.*, 2006) . Notably, CSF biomarkers continue to be abnormal in clinical stages of AD, and in fact the International Working Group criteria for Alzheimer's disease proposed a "CSF Alzheimer signature": low CSF $A\beta_{42}$ and high tau or p-tau, for the diagnosis and follow up of individuals with AD (Dubois *et al.*, 2014).

Based on the hypothetical model discussed above, the NIA-AA has proposed some guidelines to describe the preclinical AD stage in research settings (Sperling *et al.*, 2011): Stage 1 is defined by asymptomatic cerebral amyloidosis in the form of elevated brain Aβ levels and/or low CSF $A\beta_{42}$. Neurodegeneration markers or cognitive decline are negative at this stage. In stage 2, individuals display amyloidosis and the presence of one or more markers of neurodegeneration such as increased CSF tau or p-tau, hypometabolism, and cortical thinning/grey matter loss. Stage 3 comprises individuals that are approaching the MCI stage. They are positive for $A\beta_{42}$ and neurodegeneration biomarkers and might present subtle/subjective cognitive decline (SCD) (Acosta-Baena *et al.*, 2011; Bateman *et al.*, 2012). While this decline is still in the "normal range", a decline related to their own baseline cognitive levels is apparent. The proportion of conversion from SCD to MCI is 24.5 % within a period of 4.1 years and 11% to AD within a period of 4.8 years (Mitchell *et al.*, 2014).

Two additional categories, stage 0 and suspected non-Alzheimer's pathology (SNAP) were further introduced by Jack Jr and colleagues (Jack *et al.*, 2012). Stage 0 comprises individuals with no biomarker evidence of AD pathology nor cognitive impairment. SNAP includes individuals without positive markers of Aβ accumulation but positive for one or more markers of neurodegeneration. Some individuals with SNAP can display cognitive decline. The proportion of

cognitively normally individuals at each stage is approximately 40%-50% in Stage 0, 10%-15% in Stage 1, 15% in Stage 2, and 25% as SNAP (Jack *et al.*, 2012; R. Sperling *et al.*, 2014).

Will cognitively normal individuals displaying AD biomarker positivity develop AD in the future? Today, the current preclinical CSF AD biomarker signature cannot predict with certainty whether an individual will develop AD. However, Aβ+ (Aβ positive) individuals are indeed at an increased risk for cognitive decline and are more likely to progress to MCI; the presence of a neurodegeneration biomarker augments the progression rate to AD (Knopman *et al.*, 2012; Lim *et al.*, 2014a; Lim *et al.*, 2014b; Vos *et al.*, 2013).

While AD biomarkers reflect an ongoing pathological process, *post-mortem* histological analyses can reveal more faithfully certain processes that cannot be measured in fluids or with imaging techniques. *Post-mortem* brain examination of cognitively normal individuals has revealed that those harboring AD pathology already display pathological changes that are commonly found in AD brains. However, it cannot be ascertained that these individuals would have developed AD had they lived longer. A deregulation in pre and post-synaptic proteins (Scheff *et al.*, 2016; Zolochevska *et al.*, 2018), deregulation of the nerve-growth factor (NGF) metabolic pathway (Pentz *et al.*, 2019, *submitted*) and oxidative stress (Scheff *et al.*, 2016) have been observed in cognitively normal individuals with AD pathology. The early presence of oxidative stress underlines the occurrence of early inflammatory processes in individuals at risk of AD, a proposition further examined in Chapter 3 of this Thesis.

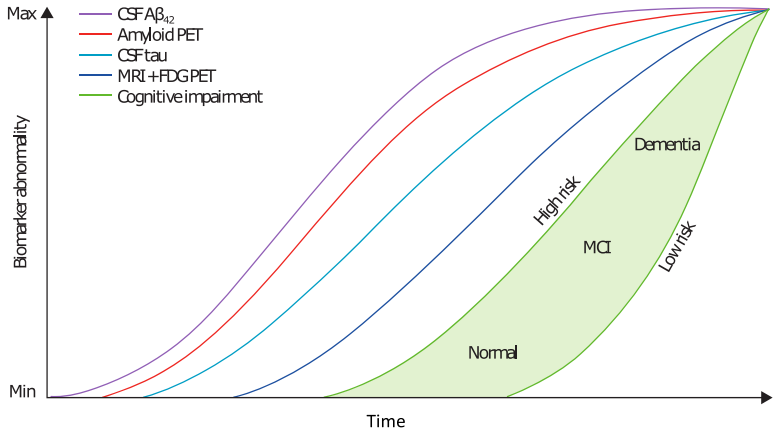

Figure 1- 4. Hypothetical model of dynamic biomarkers of Alzheimer's disease

Aβ measured in CSF or PET amyloid imaging becomes abnormal first. This is followed by CSF tau abnormalities. FDG PET and MRI (measuring brain atrophy) are the last biomarkers to become abnormal but occur shortly before the appearance of cognitive impairment. Note that individuals with a high risk of cognitive impairment due to AD neuropathology display a curve shifted to the left in the time axis. People with low-risk might have a protective profile that allows them to maintain normal cognitive function even when they have accumulated substantial AD neuropathology. Aβ = amyloid-β; CSF = cerebrospinal fluid, PET = positron emission tomography; FDG = fluorodeoxyglucose; MCI = mild cognitive impairment. Image reprinted from the Lancet (Jack *et al.*, 2013) and adapted with permission from *Elsevier*.

Current treatments for AD

Today, there is no treatment that can prevent, slow or halt AD. The current treatments available are only symptomatic. Given the marked neurotransmitter deficits in AD, the administration of "enhancing-cognition" agents have been beneficial in reducing the symptoms that accompany the development of AD. In this line, acetylcholinesterase (AchE) inhibitors are widely used in mild to severe cases of AD. Current AchE inhibitors include donepezil, rivastigmine and galantamine. The second compound used is memantine which is believed to prevent glutamate toxicity and is indicated in moderate to severe AD. A combination of memantine with an AchE inhibitor is well tolerated and beneficial usually at late stages of AD. Psychotropic agents are administered for behavioral changes ascribed to the failure of noradrenergic, serotonergic and dopaminergic neurotransmitter systems.

Neurotransmitter deficits in Alzheimer's disease

Current therapeutic targets for the treatment of AD are major enzymes or receptors involved in neurotransmitter homeostasis reflecting common neurotransmitter alterations in AD.

The cholinergic system is involved in higher brain functions such as learning, memory, and attention. It is therefore not surprising that the cholinergic network fails in AD. Deficits in ChAT, the enzyme that synthesizes acetylcholine from acetyl-CoA and choline, were firstly described in the AD brain in the late 1970's (Bowen *et al.*, 1976; P. Davies *et al.*, 1976; Etienne *et al.*, 1986; E. K. Perry *et al.*, 1981; E. K. Perry *et al.*, 1977). Notably, cholinergic deficits correlated with ante mortem cognitive deficits (Etienne *et al.*, 1986; E. K. Perry *et al.*, 1981). The neuronal cell bodies composing the nucleus basalis of Meynert (NBM) are the main origin of cortical cholinergic innervation. The NBM becomes atrophic in AD (Pearson *et al.*, 1983; Whitehouse *et al.*, 1982) and neuronal loss is apparent at late stages of AD (Whitehouse *et al.*, 1982).

Supporting the cholinergic involvement in AD, administration of a muscarinic receptor antagonist, scopolamine to young individuals and non-human primates promoted cognitive impairments (Bartus *et al.*, 1976; Drachman *et al.*, 1974). Furthermore, the administration of a cholinesterase inhibitor reversed such cognitive deficits. These observations led Bartus and colleagues to

formulate the *cholinergic hypothesis of memory dysfunction*, which poses that cognitive and behavioral impairments stem from deficits in the cholinergic system (Bartus *et al.*, 1982).

The above findings promoted the development of a "cholinergic therapy" based on cholinesterase inhibitors to prevent the breakdown of acetylcholine and potentiate cholinergic transmission. In 1993, tacrine, the first cholinesterase inhibitor was approved (Summers *et al.*, 1986). Given the toxic side effects of tacrine, a second generation of cholinesterase inhibitors were developed between 1999 and 2001 (galantamine, rivastigmine, and donepezil). Cholinergic treatments are mainly symptomatic and do not improve cognitive impairments. Interestingly, recent studies have shown that donepezil treatment in people with prodromal AD is associated with less cortical thinning and brain atrophy (Cavedo *et al.*, 2016; Cavedo *et al.*, 2017). Furthermore, the rate of hippocampal atrophy was reduced by 45% in the prodromal AD population and in individuals with AD (Dubois *et al.*, 2015; Hashimoto *et al.*, 2005). While the mechanism explaining these effects is not well-understood, these observations suggest that cholinergic treatment might have neuroprotective effects. Nowadays, the administration of acetylcholinesterase inhibitors continues to be the first line of therapy worldwide. The significance of the cholinergic deficit in the neurobiology and treatment of AD has been recently reviewed by "the cholinergic working group" (Hampel *et al.*, 2018; Hampel *et al.*, 2019) .

Given the frank loss of pyramidal neurons, the glutamatergic system is severely compromised in AD. Glutamate, the main excitatory neurotransmitter in the CNS, is highly involved in learning and memory (Collingridge *et al.*, 1983; Curtis *et al.*, 1960; Hayashi, 1952; R. G. Morris, 2013). Glutamatergic deregulation might be an early event in AD as revealed by studies of Bell and colleagues (Bell *et al.*, 2007). A paradoxical upregulation of glutamatergic synaptic boutons has been reported in the cortex of MCI individuals followed by a depletion of such boutons after clinical AD presentation (Bell *et al.*, 2007). This goes in line with decreased glutamate brain levels at advanced stages of AD (P. T. Francis *et al.*, 1985; Lowe *et al.*, 1990). Further studies suggest that clearance of glutamate is affected in AD and results in excitotoxicity (Paul T. Francis, 2003). Memantine, a non-competitive N-methyl-D-aspartate receptor (NMDA-r) receptor antagonist, is thought to alleviate the toxicity of glutamate release.

Less studied are the contribution of monoaminergic systems to the development of AD. However, deficits in serotonergic, dopaminergic, histaminergic, melatonergic and noradrenergic system

metabolites have been reported to occur in AD [reviewed in (Simic *et al.*, 2017; Trillo *et al.*, 2013)]. Notably, solid evidence suggests that NFT pathology might start in the locus coeruleus (LC) (Braak *et al.*, 2011), the main source of noradrenaline in the brain. The LC is severely affected in AD and has been regarded as "ground zero" in the AD continuum.

The Locus coeruleus: main source of noradrenaline in the mammalian brain

Historical overview

In 1904, the German chemist Friedrich Stolz artificially synthetized noradrenaline (NA) (Stolz, 1904) without being aware of its important physiological roles. During the mid 1900's, scientists all over the world including Walter B. Cannon (collaborating with Bacq and Rosenblueth), Peter Holtz, and Ulf von Euler were investigating the effects of "sympathins", today known as adrenaline and NA. It was Ulf von Euler who identified that NA was the main neurotransmitter in the sympathetic nervous system (Euler, 1946; Von Euler, 1946). The discovery of the catecholaminergic pathway by Blaschko (Blaschko, 1939, 1957; Blaschko *et al.*, 1937) and its occurrence *in-vivo* noted by Sidney Udenfriend led to the isolation and characterization of the enzymes involved in the synthesis of NA (Lovenberg *et al.*, 1962; Nagatsu *et al.*, 1964; Udenfriend *et al.*, 1953). Tyrosine hydroxylase (TH) is the rate-limiting enzyme which converts tyrosine into l-3,4-dihydroxyphenylalanine (L-DOPA). L-DOPA is transformed into dopamine by L-decarboxylase. Finally, Dopamine-Beta-Hydroxylase (DBH), mediates the conversion of dopamine into NA (Levin *et al.*, 1960).

A pioneer in catecholamine research in the CNS was Marthe Vogt. Vogt measured for the first time the distribution of NA and adrenaline in the dog CNS and further demonstrated that these molecules could be modulated by drugs and anesthesia (Vogt, 1954). She was the first scientist to suggest that NA and adrenaline, besides their role as transmitters at vasomotor endings, could act as neurotransmitters in the brain:

"It might be tempting to assign to the cerebral sympathin (NA and adrenaline) a transmitter role like that which we assign to the sympathin found in the sympathetic ganglia and their post-ganglionic fibres" (Vogt, 1954).

The development of fluorescent techniques to visualize catecholamines, pioneered by Bengt Falck and Nils-Åke Hillarp, in the early 1960's (Falck *et al.*, 1982; Falck *et al.*, 1961), allowed scientists around the world to map the catecholaminergic nuclei in the brain. A. Carlsson, B. Falck, N.A.

Hillarp, A. Dahlstrom, K. Fuxe, N.E. Anden, and, K. Larsson among others, demonstrated that catecholamines accumulate in neuronal synaptic terminals and to a lesser extent in their cell bodies. Their description of numerous aminergic cell groups and terminals agreed with previous biochemical reports mapping the content and distribution of brain catecholamines. According to the nomenclature proposed by Dahlstroem and Fuxe, 6 noradrenergic nuclei can be distinguished in the brain (Figure 1-5, A) (Dahlstroem *et al.*, 1964). A2, A4, and A6 are dorsally located and A1, A5, and A7 are located in the ventral part of the brain. From these, A1, A2, A5 and A7 send projections to the spinal cord, brainstem, hypothalamus and basal telencephalon while A4 and A6 project to the cortex, thalamus, cerebellum and spinal cord [reviewed in (Simic *et al.*, 2017)]. A6 is the main source of NA in the brain and is also known as the LC.

The discovery of the LC dates back to the 18th century. It was perhaps because of its melanin containing neurons that the LC did not escape the eye of the French neuroanatomist, Felix Vicq d'Azyr, when he first described this structure [for a review of his scientific contributions see (Tubbs *et al.*, 2011)]. Indeed, the name LC was coined by Joseph and Karl Wenzel in 1812 and is derived from the Latin "blue place", alluding to the color of melanin in this region which is visible to the naked-eye (Swanson, 2014).

In 1962, Arvid Carlsson, using fluorescence histological techniques developed by Falck and Hillarp, described that the LC was composed of monoaminergic neurons (Carlsson *et al.*, 1962). The following work by Dahlstrom and Fuxe established the noradrenergic nature of LC neurons (Dahlstroem *et al.*, 1964).

The LC is located deep in the pons lining the fourth ventricle and is composed of ~ 40 000 to 50 000 large multipolar and small fusiform neurons in the human (Baker *et al.*, 1989; German *et al.*, 1988) and ~ 4 000 neurons in the rat (Goldman *et al.*, 1981). Despite of being a small structure, the LC innervates the brain widely and it is the sole source of NA for the hippocampus and cortex (Figure 1-5, A-B) [for a review see (Schwarz *et al.*, 2015)]. Given its extensive innervation, the LC regulates diverse physiological mechanisms such as arousal, response to stress, attention, memory and learning (Berridge *et al.*, 2003; Sara, 2009). Thus, affections in the LC likely contribute to disruption of physiological homeostasis.

Locus coeruleus degeneration in Alzheimer's disease

In 1978, Lysia Forno, presented a seminal abstract to the VIII International Conference organized by the American Association of Neuropathologists (Forno, 1978). Her work described the occurrence of LC nerve cell loss in 25 cases of early and late onset AD (Forno, 1978). She reported that cases of early onset AD had a greater LC cell loss than those with late onset. Moreover, she described the presence of paired helical filaments in the LC (Forno, 1978). Forno attributed the neuronal loss in the LC to the diseased cortex of AD cases. She finalized her abstract by stating: "*It is as yet not clear how the nerve cell degeneration in the LC is reflected in the symptomatology of AD*", an event that still is not well-understood.

Following Forno's reports, several investigators confirmed her findings. In 1982, William Bondareff reported that LC degeneration was greater in younger and highly demented cases of AD than older AD cases (Bondareff *et al.*, 1982). He reported an 80% loss of LC neurons in cases of early onset AD and a 20% loss in older cases (Bondareff *et al.*, 1982) . In 1983, Leslie Iversen conducted the first immunohistochemical assessment of LC neurons in AD and control brains (Iversen *et al.*, 1983). He utilized DBH as a specific noradrenergic marker to label LC neurons and reported that AD cases had a 60% neuronal loss in the LC that was accompanied by a drop of approximately the same magnitude in cortical NA levels (Iversen *et al.*, 1983). Although brain NA levels are reduced in AD, possible compensatory mechanisms have been reported. LC neurons exhibit increased expression of TH and abnormal sprouting has been proposed as a mechanism to explain the normal to elevated levels of noradrenergic receptors (NET and α_2-AR) in the LC and hippocampus of AD cases (Szot *et al.*, 2006) . In agreement with this, elevated NA levels or its metabolites (reflecting an increase in NA turnover) have been reported in *post-mortem* AD brains (Hoogendijk *et al.*, 1999; Palmer *et al.*, 1987a) and CSF samples (Elrod *et al.*, 1997) (Tohgi *et al.*, 1992) which negatively correlate to the number of NFT in AD (Palmer *et al.*, 1987b).

Further research indicates that neuronal loss in the LC is greater to that found in the NBM, and that it correlates better with disease duration (Zarow *et al.*, 2003). Notably, a subgroup of individuals with AD but without major NBM degeneration already displayed LC neuronal loss (G. K. Wilcock *et al.*, 1988), indicating a higher susceptibility and degeneration of the noradrenergic

nucleus over the cholinergic one. However, this view has been challenged by a series of investigations reporting that the LC and NBM display the same extent of neuronal loss (Arendt *et al.*, 2015; Lyness *et al.*, 2003; Mann *et al.*, 1984).

Degeneration of the LC is not specific to AD. Individuals with DS, PD, progressive supranuclear palsy (PSP) and dementia pugilistica present significant neuronal LC loss (Mann, 1983). Nevertheless, in AD, degeneration of LC neurons follows a region-specific pattern, with cell loss occurring in rostrocaudal and dorsoventral neurons (German *et al.*, 1992). Thus, degeneration of cortical-projecting LC neurons is a hallmark of AD.

One of the key contributors to LC degeneration might be the deposition of NFT within LC neurons. While Braak and colleagues proposed that NFT formation appeared first in the entorhinal cortex, a new neuropathological study assessing NFT formation in brains from individuals below 30 years old revealed the appearance of abnormally hyperphosphorylated tau in the LC (Braak *et al.*, 1991). Henceforth, it has been proposed that NFT formation does not begin in the entorhinal cortex but rather in the LC. Further studies have shown that pathological tau accumulation in the LC negatively correlates with cognitive status, as assessed by the Mini-Mental State Examination (MMSE) (Grudzien *et al.*, 2007).

Thus, solid evidence indicates that the LC might be one of the earliest sites accumulating AD-associated pathology. But, when does it start degenerating? Comprehensive stereological analyses revealed that LC neuron number is already reduced by 30% at preclinical AD stages (Figure 1-5, C) (Kelly *et al.*, 2017). Moreover, while LC neuronal loss occurs during MCI, volumetric shrinkage is already detectable before the appearance of cognitive impairments. Such shrinkage is reflected by the reduction in LC volume by 8.4% for each unit increment in the Braak score, starting at Braak score 0 (Theofilas *et al.*, 2017). These observations clearly indicate that LC demise occurs early in the progression of AD. Furthermore, neuronal microarray analyses in the LC of MCI and AD cases revealed a reduction in genes regulating redox homeostasis, mitochondrial respiration and neuritic/structural plasticity (Kelly *et al.*, 2017). Further analyses indicated an association between LC neuronal number and cognitive impairments (Kelly *et al.*, 2017).

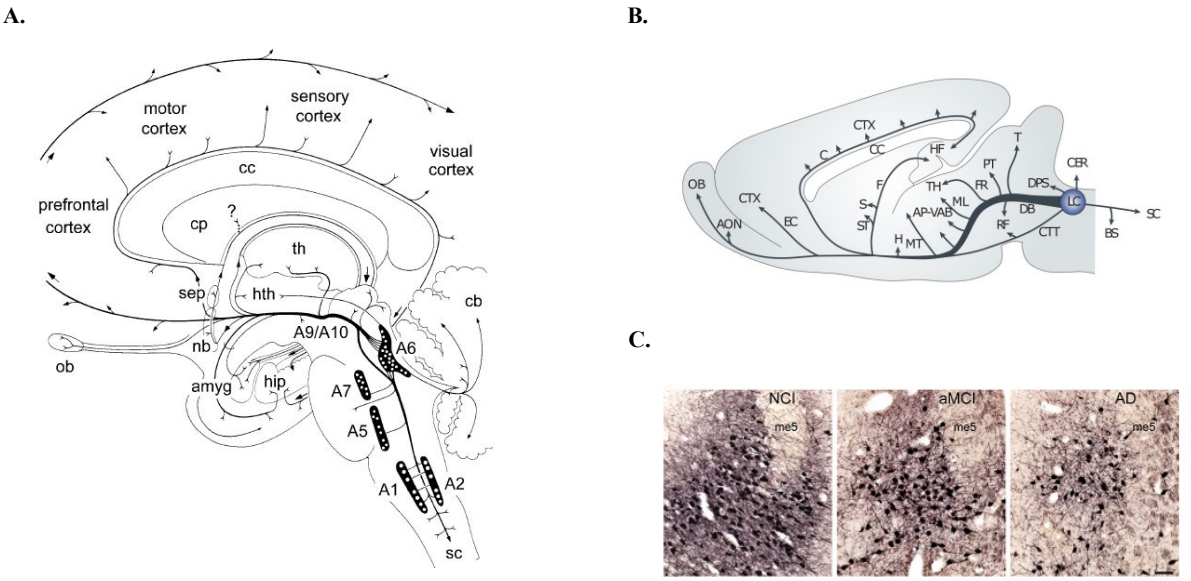

Figure 1- 5. Locus coeruleus innervation in the human and rat brain

(A) The human locus coeruleus or A6 is composed of ~ 40 000 – 60 000 neurons and is located in the brainstem lining the fourth ventricle. Note the wide innervation of the locus coeruleus throughout the cerebral cortex, cerebellum and brainstem. The locus coeruleus gives raise to the ascending and descending noradrenergic bundles and dorsal periventricular pathways. The dorsal medullary (A2) and lateral tegmental (A1, A5, A7) noradrenergic cell groups are also illustrated. A9/10 depict the substantia nigria and ventral tegmental area, respectively. Amygdala = amyg; cerebellum = cb; corpus callosum = cc; caudate and putamen = cp; hippocampus = hip; hypothalamus = hth; nucleus basalis = nb; olfactory bulb = ob; medial septum/diagonal band = sep; spinal cord = sc; thalamus = th; ? = sparse noradrenergic innervation to putamen and caudate, its origin(s) are unclear. (B) The rat locus coeruleus is composed of ~ 4 000 neurons. Note, that as in the human, it also innervates extensively the brain. AON = anterior olfactory nucleus; AP-VAB = ansa peduncularis–ventral amygdaloid bundle system; BS = brainstem nuclei; C = cingulum; CC = corpus callosum; CER = cerebellum; CTX = cortex; DB = dorsal bundle; DPS = dorsal periventricular system; F = fornix; F = frontal cortex; FR = fasiculus retroflexus; H = hypothalamus; HF = hippocampal formation; ML = medial lemiscus; MT = mamillothalamic tract; OB = olfactory bulb; PT = pretectal area; RF = reticular formation; S = septum; SC = spinal cord; T = tectum; TH = thalamus. (C) Tyrosine hydroxylase immuhistochemistry in the human locus coeruleus from NCI, aMCI and AD individuals. The aMCI group has a ~ 30% of locus coeruleus neuronal loss while the AD group displays a 50% loss of locus coeruleus neurons as compared to the NCI group. NCI = Non-cognitively impaired; aMCI = amnestic mild cognitive impairment; AD = Alzheimer's disease. Images adapted from (Kelly *et al.*, 2017; Marien *et al.*, 2004; Sara, 2009) with permission from *Springer Nature* and *Elsevier*.

Noradrenaline as a neuromodulator

Volume transmission, a non-synaptic chemical signalling, allows the diffusion of neuromodulators into the extracellular space. This type of signaling allows molecules to reach non-neuronal and neuronal targets in a paracrine manner. Noradrenergic varicosities may release NA to the extracellular space and thus allow NA to signal on glial cells, blood vessels and neurons via α and β adrenoreceptors (Szabadi, 2013). NA can promote neurogenesis and promote the release of neurotrophins such as brain-derived growth factor (BDNF) and NGF (Marien *et al.*, 2004). Moreover, studies have shown that BDNF colocalizes with noradrenergic terminals and LC neurons (Fawcett *et al.*, 1998). Henceforth, it has been proposed that NA release exerts neuroprotective effects that might have a positive impact in mediating cognitive reserve (Robertson, 2013).

NA can also act as an anti-inflammatory molecule in the CNS by decreasing inflammatory gene expression in astrocytes, microglial and neuronal cells (Galea *et al.*, 1999; Madrigal *et al.*, 2006; Minghetti *et al.*, 1997) [reviewed in (Feinstein *et al.*, 2016)]. Moreover, NA downregulated inflammatory marker expression in microglial cells *in vitro* previously incubated with Aβ (Heneka *et al.*, 2010).

LC denervation in animal models of the amyloid-like or tau-like pathologies results in increased neurodegeneration, cognitive deficits, increased microglial activation and upregulation of inflammatory markers (Chalermpalanupap *et al.*, 2018; Choudhary *et al.*, 2018; Heneka *et al.*, 2002; Heneka *et al.*, 2003; Heneka *et al.*, 2010; Heneka *et al.*, 2006; Hurko *et al.*, 2010; Jardanhazi-Kurutz *et al.*, 2010; Jardanhazi-Kurutz *et al.*, 2011; Kalinin *et al.*, 2007; Oikawa *et al.*, 2010; Pugh *et al.*, 2007). Therefore, the loss of NA in AD could interfere with NA's anti-inflammatory properties leading to worsening of the pathology.

The study of the LC noradrenergic system in animal models has been mainly centered on the late stages of amyloid and tau deposition. Less studied is the impact of LC degeneration at the very early stages of the amyloid pathology which we have furthered addressed in Chapter 4 of this Thesis.

Flamma, phlogosis: inflammation

The word immunity is derived from the latin "*immunitas*" a term referring to the protection that Roman senators had against legal prosecution. Therefore, since ancient times, immunity has been related to protection of disease. The main function of the immune system is to protect against infectious diseases and toxic or foreign molecules that disrupt physiological homeostasis. The orchestrated mechanism by which immune cells and signalling molecules exert their protective functions is called the immune response. A common result of immune responses is inflammation. Normally, inflammation serves a protective role controlling infections or noxious agents; however, when uncontrolled, it can promote tissue injury and/or inflammatory diseases.

The Egyptian medical texts as well as the Greek Hippocratic writings used the hieroglyphic or word, respectively, "fire" to refer to inflammation. While the Egyptians believed that inflammation was a superficial heat originating outside the body, the Greeks attributed it to the influx of blood to tissues that did not contain it. Notably, they thought that the degree of inflammation correlated inversely to the ease of healing [for a historical review see (Smith, 1978)]. In the 1st century A.D., the roman Cornelius Celsius described the four cardinal signs of inflammation: rubor (redness), tumor (swelling), calor (heat) and, dolor (pain). Later, Virchow introduced the 5th cardinal sign: functio laesa (loss of function). It was John Hunter, an English surgeon and anatomist, who firstly proposed in the 18th century that inflammation was a beneficial response to injury (Jarcho, 1970). In 1884, Elie Metchnikoff, a Russian zoologist, discovered a fundamental part of the immune response: phagocytosis (Metchnikoff, 1892). While studying the digestive system of starfish larvae and introducing into them dyes and splinters, he observed that amoeboid cells "*move toward, englobe and destroy the noxious agent*" (Metchnikoff, 1892). He was also a proponent that inflammation was mediated by blood vessels: "*through the blood circulation, the organism at any moment can dispatch to the menaced locale a considerable number of phagocytes to stop the illness*" (Metchnikoff, 1892). He furthered named phagocytes "*the defenders of the organism*" and attributed phagocytes a protective role (Metchnikoff, 1892).

What Metchnikoff was describing at the time is known today as the "innate immune response" (natural or native immunity). The innate immune system provides the early and first line of defense against noxious agents. It comprises a variety of cell types and mechanisms that are already in place before exposure to a microbe or molecules released by injured cells. The main components

of the innate immune system are: (1) physical and chemical barriers such as epithelial cells and anti-microbial molecules, (2) phagocytic cells (macrophages and neutrophils) and natural killer cells, (3) blood proteins such as the complement system, and (4) cytokines, which regulate the activity and communication between immune cells (Abbas, 2010b). Phagocytic macrophages express cell surface and cytosolic receptors such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs) that can recognize and bind common molecular patterns expressed by foreign agents/microbes (Abbas, 2010a). Upon recognition, the macrophage engulfs the toxic material and promotes the secretion of cytokines that will enhance vasodilation, and migration of other immune cells that will promote a local inflammatory response (Abbas, 2010a). If the innate immune response is not successful, other mechanisms have evolved to further maintain tissue homeostasis. In contrast to the innate immune system, the adaptive immune system exquisitely recognizes particular substances known as antigens (Abbas, 2010b). The main players of the adaptive immune response are T and B lymphocytes, the latter being involved in secreting antibodies against specific antigens. The adaptive immunity is the second line of immune response and has the advantage of generating "immunological memory". Therefore, proliferating lymphocytes will differentiate into memory cells which will respond rapidly against a successive infection with the same pathogen (Abbas, 2010b).

The innate and adaptive immune responses are not independent of each other, and an intertwined communication is necessary for a successful inflammatory response. Following a pro-inflammatory response, resolution of inflammation must take place to avoid its chronic activation that can lead to maladaptive immunity.

Resolution of inflammation is a regulated response that aims to eliminate the injurious agents that triggered the inflammatory response (Fullerton *et al.*, 2016). This is followed by catabolism of inflammatory mediators, suspension of pro-inflammatory molecule synthesis and leukocyte recruitment, and clearance of immune cells either by drainage or cell death (Fullerton *et al.*, 2016). The resolving process is mediated by pro-resolution mediators such as lipids (lipoxins, resolvins, maresins, protectins), proteins (galectin 1, annexin A1, cehemerin 15), gaseous mediators (CO, H_2S), signalling pathways (NF-κB, CDKs, PI3K, ERK1 and ERK2, cAMP), and intracellular regulators (LRRC33, CD180, microRNAs) (Fullerton *et al.*, 2016). It has been proposed that following the resolution process, a post-resolution mechanism occurs. The post-resolution response involves the migration of monocyte-derived cells to the initial site of injury. Such

migration ensures the generation of memory B and T cells and induces immune tolerance by generating regulatory T cells (T_{reg}) (Newson *et al.*, 2014). Therefore, the resolution and post-resolution processes might represent a bridge between innate and adaptive immunity. Frustrated attempts of resolution might lead to a failed post-resolution mechanism which taken together, can lead to the development of chronic inflammatory diseases.

Neuroinflammation

The notion that the brain is an immune privileged organ stems from Sir Peter Medawar's seminal discoveries in the 1940's (Medawar, 1948). Medawar showed that while experimentally implanted skin grafts in the periphery provoked an immune response leading to graft rejection, when implanted into the CNS the graft did not promote rejection (Medawar, 1948). On the other hand, when the tissue graft was first implanted in the periphery and then a second graft was introduced in the brain parenchyma, a peripheral immune response was elicited leading to rejection of the brain graft (Medawar, 1948). Thus, the lack of a stereotypical immune response in the brain has led to the proposition that the brain is an immune-privileged site.

Prior evidence supporting the immune-privilege of the brain came from Paul Ehrlich (Ehrlich, 1885), his student Edwin Goldmann (Goldmann, 1909) and Lewandowsky (Lewandowsky, 1900) in the late 1800's and early 1900's when they described the existence of a compartmentalization between the brain and the body: the hematoencephalic barrier or the blood-brain barrier (BBB) as coined by Lewandowsky [for a historical review about the BBB see (Liddelow, 2011).

The adult CNS has three anatomical barriers (Abbott *et al.*, 2010; Engelhardt *et al.*, 2017):

1. *The arachnoid barrier* is located at the brain surface between the dura mater and the CSF-drained subarachnoid space (SAS). This barrier is impermeable to fluids and separates the arteries, veins and lymphatics in the dura from the SAS.

2. *The blood-CSF barrier* (BCSFB) separates the CSF from fenestrated microvessels. It is established by choroid plexus CSF-producing ependymal cells joined by tight junctions on its apical surface (facing the ventricles); thus, limiting the passage of molecules between

the cells. However, the fenestrated endothelium allows for a rapid flow of water to aid the production of CSF. Immune cells such as macrophages and dendritic cells lie within the stroma of the choroid plexus.

3. *The BBB* is located at the level of cerebral blood capillaries. Such endothelial cells are connected by tight junctions and form a barrier that regulates the transfer of molecules from the blood to the brain parenchyma. As a result of its extensive microvascular bed, the BBB lies proximal to individual neurons and associates to other non-neuronal cells forming the neurovascular unit. The neurovascular unit is composed by the capillary endothelium partially enveloped by pericytes or smooth muscle cells. Astroglial cells are also part of the neurovascular unit (NVU) and send foot processes that surround the capillary network. Further to it, astrocytes maintain close contact with neurons which allows the regulation of the vascular tone and blood flow.

The main functions of these endothelial barriers are to tightly regulate the exchange of substances between the blood and the brain and to ensure the homeostasis of the brain parenchyma. Therefore, the BBB keeps out toxins, controls molecular traffic and ion homeostasis, limits the trafficking of proteins, separates the central and peripheral neurotransmitter pools, and allows immune surveillance (Abbott *et al.*, 2010). Notably, it has been proposed that immune T-cell surveillance in the CNS takes place in the choroid plexus (Louveau *et al.*, 2015). The rediscovery of the brain lymphatic system has recently revealed that immune cells traffic along lymphatic vessels located in the cribriform plate and the dura matter (Andres *et al.*, 1987; Louveau *et al.*, 2015).

While the brain does not show a myelomonocytic response to injuries, leukocytes can cross the vascular barriers in a regulated manner by induction of adhesion molecules, matrix-metalloproteinases and release of chemoattractants (Engelhardt, 2008; Engelhardt *et al.*, 2005; Ransohoff *et al.*, 2015). Therefore, the exquisite regulation of immune cell trafficking in the brain differs from that in the periphery. This further supports the notion that the brain is a relatively immune-privileged site in the sense that while immune activation is present, it is tightly regulated. Nevertheless, the occurrence of chronic inflammatory diseases might lead to uncontrolled invasion of the brain parenchyma by peripheral immune cells.

Neuroinflammation might occur independently of BBB compromise. While almost every cell in the brain can mount an inflammatory response, microglia, astroglia and neurons are the main players in the CNS.

Microglial cells: resident tissue macrophages of the CNS

Microglial cells were first recognized by Nissl back in the late 1800's, and he named them "Stabchenzellen" or rod cells. Nissl classified them as reactive neuroglia and further suggested that they had migratory and phagocytic properties (Nissl, 1899). Santiago Ramon y Cajal described them as the third element in nervous tissue, differing from neurons and astrocytes (Ramon y Cajal, 1897). It was not until 1919 when Pio del Rio-Hortega discovered that the "third element" were indeed two different cells: oligodendrocytes and microglia [for an English translation of del Rio-Hortega's 1919 articles see (Sierra *et al.*, 2016)]. Moreover, he suggested that microglial cells had a mesodermal origin and he further recognized their phagocytic activity, the latter also was proposed by Nissl (Sierra *et al.*, 2016).

Microglial cells are derived from a single wave of yolk sac erythromyeloid progenitors which migrate to the CNS at embryonic day 9 and differentiate into embryonic microglia (Ginhoux *et al.*, 2010) . At this stage, microglia display an amoeboid morphology before transitioning to their well-known ramified morphology (Ginhoux *et al.*, 2010). During development, microglia are required for the correct wiring of the brain: they phagocytose apoptotic neurons from the developing CNS, provide trophic support to developing neurons, promote vascular guidance and branching, and neuronal circuit maturation by pruning synapses [for a review see (Kierdorf *et al.*, 2017)].

In the adult brain, under physiological conditions, microglia display a small soma and elongated processes with secondary branching and lamellipodia (Sierra *et al.*, 2016). Microglia express neurotransmitter receptors that allow them to constantly sense neuronal activity. They further maintain synaptic plasticity and promote dendritic spine remodeling during learning tasks (Kierdorf *et al.*, 2017). Two-photon microscopy has shown that microglial processes are highly motile and continuously survey their environment (Nimmerjahn *et al.*, 2005). Microglia's "sensome", consisting of pattern- recognition receptors, cytokine and chemokine receptors,

purinergic receptors and extracellular matrix (ECM) receptors allows them to sense small changes in their microenvironment (Hickman *et al.*, 2013).

Microglial activation has been described since the days of del Rio-Hortega's (Sierra *et al.*, 2016). Upon sensing a noxious stimulus, microglial cells become hypertrophic by enlarging their somas and thickening their processes, and they finally adopt an amoeboid phenotype which resembles the morphology of peripheral monocytes (Figure 1-6) (Stence *et al.*, 2001; Streit *et al.*, 2014). Activated cells migrate to the site of injury where they initiate a neuroinflammatory response by releasing cytokines, chemoattractants, complement proteins, matrix metalloproteases (MMPs), reactive oxygen species (ROS) and nitric oxide. When microglial cells are fully activated (amoeboid) they can exert their phagocytic activities.

Chronic neuroinflammation provokes constant activation of microglia which in turn promotes neurotoxicity. Dystrophic microglia, displaying spheroid inclusions and fragmentation are commonly present in neurodegenerative diseases (Figure 1-6) (Streit *et al.*, 2014). It is likely that dystrophic microglia in a neuroinflammatory milieu are unable to maintain their physiological role and in turn, contribute to further exacerbate the neuroinflammatory profile and neurodegeneration.

Previous studies have aimed to classify microglia into proinflammatory M1 cells or anti-inflammatory M2 cells. Such distinction is based on the common classification of macrophages into M1 or M2 cells. The proinflammatory or classical activated phenotype and is associated to an increase in proinflammatory cytokines such as IL-1β, IL-12, IL-6, TNFα, decreased IL-10 levels, and phagocytosis (D. M. Wilcock, 2012). The M2 phenotype, or alternatively activated are biased towards an anti-inflammatory and tissue resolution response. The M2a phenotype is induced by IL-4 and IL-13 and characterized by increased levels of arginase-1, IL-1Ra, the chitinase like protein YM1, and decreased IL-12 levels (D. M. Wilcock, 2012). Such phenotype has been associated to tissue repair (D. M. Wilcock, 2012). The M2b phenotype is activated by immune complexes, TLR activation or ligands to the IL-1R, and by an increased expression of IL-10, IL-1β, TNFα, IL-6, CD86 (D. M. Wilcock, 2012). The M2c phenotype is induced by IL-10 and is characterized by increased levels of TGFβ, IL-10, and low IL-12 levels (D. M. Wilcock, 2012). It should be noted that transcriptomic analyses have revealed that microglial cells can express M1 and M2 markers under different inflammatory stimuli and in the context of different

neurodegenerative diseases (Dubbelaar *et al.*, 2018). Therefore, the M1 and M2 classification system must be taken with caution as it is outdated and requires revision.

Notably, singe-cell RNA sequencing has revealed that homeostatic microglia are maintained by TGF-β1 signaling and are characterized by the expression of *P2ry12*, *Tmem119*, *Fcrls*, *Hexb*, and *Tgfbr1* (Butovsky *et al.*, 2014). In contrast, a subset of microglial cells has been found in neurodegenerative diseases and has been associated to loss of TGFβ1 signaling (Keren-Shaul *et al.*, 2017). Such microglia have been termed disease-associated microglia (DAM) or microglia of the neurodegenerative disease. The DAM signature is associated to a decrease in Cc3cr1, P2ry12, and Tmem119 and to an increase in *Tyrobp*, *Trem2*, *Csf1*, *Ccl6*, *Ctsb*, *Ctsd*, *Lyz2*, *Fth1*, *B2m*, *Apoe* among others (Keren-Shaul *et al.*, 2017). Such observations reflect the diversity of gene expression in microglial cells under neuroinflammatory conditions and support the notion that the M1/M2 classification is rather simplistic.

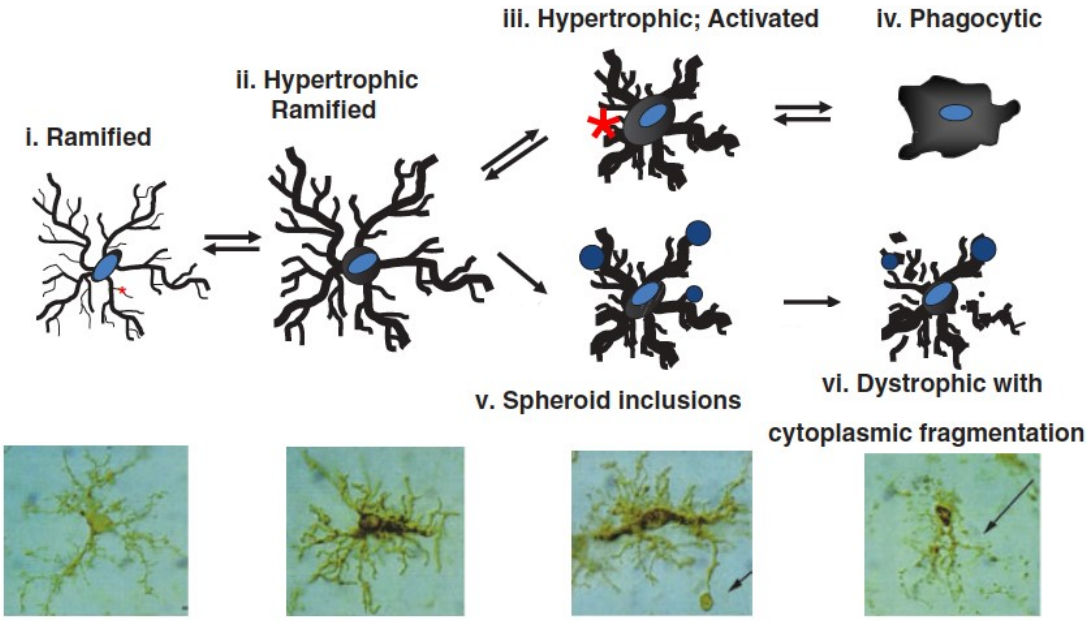

Figure 1- 6. Microglia activation states in the human brain

Upon sensing a noxious stimulus surveying microglia (i) become hypertrophic, displaying thicker processes and enlarged soma (ii). Microglia can retrieve their processes (intermediate activation) (iii) and adopt a phagocytic amoeboid morphology reflecting a full activation state (iv). During chronic activation, microglia become dystrophic and display spheroid swellings (v) and fragmentation (vi). * denotes enlarged cells bodies and thickened processes. The micrographs show activated and dystrophic microglia as seen in the human brain. Image from (Raj *et al.*, 2014) with permission from *John Wiley and Sons*.

Astroglia: the star-shaped glial cells

Astroglial cells are the most numerous CNS cells, comprising up to 40% of the total mammalian brain cells (Herculano-Houzel, 2014). Astrocytes have primary branches stemming from their cell soma which give rise to finer branchlets from where leaflets emanate (Khakh *et al.*, 2015; Ramon y Cajal, 1909). Astrocyte morphology varies between gray and white matter regions, from which two main morphologies can be identified: protoplasmic and fibrous astrocytes. Protoplasmic astrocytes are located in the gray matter and exhibit numerous branches distributed in a globoid shape (Ramon y Cajal, 1909). On the other hand fibrous astrocytes localize to the white matter and display long fiber-like processes (Ramon y Cajal, 1909). Astrocytes have shown a high degree of molecular diversity (Khakh *et al.*, 2015) but is still yet not well-understood if these variations have an impact on their physiological responses. Astrocytic cells cover the entire CNS in a contiguous and non-overlapping manner. This anatomical organization is due to the formation of gap-junctions between them at the tip of their branches, thus allowing for a constant communication across long distances.

In the healthy CNS, astrocytes are involved in structural and neurotrophic support, neurotransmitter clearance, ion homeostasis, synapse formation and removal, and in neurovascular coupling by formation of the astrocytic end-feet (Sofroniew *et al.*, 2010). In response to CNS damage or disease, astrocytes become reactive and display a wide variety of morphological and biochemical changes, reflecting the extent of the insult. Upon sensing a noxious stimulus, astroglial cells upregulate the expression of glial fibrillary acidic protein (GFAP) and become hypertrophic while preserving their individual physical domain (Wilhelmsson *et al.*, 2006). In severe cases of astrogliosis, they proliferate and migrate to the site of injury where they overlap their processes in order to border injured tissue, forming the so-called glial scar (Sofroniew, 2009; Sofroniew *et al.*, 2010).

Upon induction of neuroinflammation or ischemia in the mouse brain, gene profiling studies revealed that two different types of astrocytes were induced: A1 and A2. Neuroinflammation promoted the A1 astrocyte in which harmful genes for synapses were upregulated, such as those involved in the complement cascade and inflammatory cytokines (Liddelow *et al.*, 2017a). On the contrary, ischemia promoted the generation of A2 astrocytes, which had an upregulation of

neurotrophic factors. Thus, A1 astrocytes might be harmful while A2 might have a reparative role. Interestingly, microglial activation and further release of IL-1α, TNFα and the complement subunit 1q (C1q) can promote the generation of A1 astrocytes (Liddelow *et al.*, 2017b). A1 astrocytes further lose their physiological role as reflected by loss of their neurotrophic support and deregulation of their synapse formation/remodeling function (Liddelow *et al.*, 2017b). The occurrence of A1 astrocytes has been reported in several neurodegenerative diseases such as AD, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), PD and Huntington's disease (HD) (Sofroniew *et al.*, 2010).

Neurons

Perhaps the most overlooked cells contributing to an immune response in the CNS are the neurons. Neurons express several receptors for chemokines and cytokines as well as pattern-recognition receptors, suggesting that they have the necessary machinery to orchestrate an immune response (Bajetto *et al.*, 2002). Furthermore, certain neuronal neurotransmitters such as NA (Feinstein *et al.*, 2016), acetylcholine (L. Li *et al.*, 2019), GABA (Crowley *et al.*, 2016) and endocannabinoids (Xu *et al.*, 2015) might modulate the neuroinflammatory response.

Upregulation of neuronal iNOS, pro-inflammatory cytokines, and COX-2, has been reported in AD brains and rodent AD models (Ferretti *et al.*, 2012b; Hanzel *et al.*, 2014; Heneka *et al.*, 2001; Hoozemans *et al.*, 2001; Mulet *et al.*, 2017). Moreover, noxious stimuli such as Aβ accumulation (Kaushal *et al.*, 2015) and spinal cord injury (de Rivero Vaccari *et al.*, 2008) promote the activation of the neuronal inflammasome that culminates in the release of IL-1.

Furthermore, neurons release immunomodulators and thus maintain a constant communication with non-neuronal cells. One of the most studied neuronal cytokines regulating the communication between neurons and microglial cells is fractalkine/CX3CL1. Under physiological conditions neuronal fractalkine signals through cognate receptors located mostly in microglial cells (Finneran *et al.*, 2019). This regulated signaling is thought to maintain microglia in a quiescent state by downregulating the expression of microglial pro-inflammatory mediators (Finneran *et al.*, 2019). Fractalkine signalling has been reported to be altered in several neurodegenerative diseases such

as AD, ALS, PD and stroke. While deficiency of fractalkine might be detrimental in some scenarios by promoting chronic microglial activation, it can also be beneficial if the microglial response is well-regulated. In sum, through the regulation of immunomodulators, neurons can directly influence the neuroinflammatory response.

Neuroinflammation in Alzheimer's disease

The first suggestion of the occurrence of inflammation in AD brains was proposed by Oskar Fischer. In his 1907 and 1910 publications, Fischer suggested that the deposition of a *peculiar foreign substance* in the cerebral cortex was inducing a local inflammatory response leading to amyloid plaque formation and nerve regeneration (O. Fischer, 1912; P. D. D. O. Fischer, 1907). However, Fischer could not find any substantial data supporting this hypothesis and posed himself the question "However, where is then the inflammatory reaction?" ("Aber–wo bleibt dann die entzndliche Reaktion?'').

It wasn't until the 1980's that the occurrence of neuroinflammation in AD started to be acknowledged. The presence of complement proteins (C1q, C3 and C4) in amyloid plaques was firstly described by Piet Eikelenboom (Eikelenboom *et al.*, 1982), followed by the McGeers reporting the presence of the terminal membrane attack complex in amyloid plaques (McGeer *et al.*, 1989a, 1989b). Moreover, the presence of activated microglia surrounding amyloid plaques was also described (McGeer *et al.*, 1988; McGeer *et al.*, 1987). Subsequently, W. Sue T. Griffin reported that microglia surrounding amyloid plaques in AD and DS brains were immunoreactive for IL-1β (Griffin *et al.*, 1989) and Joseph Rogers showed that the Aβ peptide was able to bind to complement receptors (Rogers *et al.*, 1992). The presence of complement proteins, activated microglia surrounding plaques and expressing IL-1β in AD and DS brains strongly suggested the presence of an inflammatory component in AD. These observations opened a new window to a new research avenue in the AD field.

Today, we know that deposition of amyloid plaques and NFT is accompanied by a chronic neuroinflammatory process characterized by activation of glial cells and immune complexes (Akiyama *et al.*, 2000; Heneka *et al.*, 2015; Wyss-Coray *et al.*, 2012). Molecular, biochemical and immunohistological techniques have allowed the identification of a plethora of inflammatory

mediators in the AD brain. Upregulation of potent inflammatory cytokine pathways, activation of the complement cascade, upregulation of adhesion molecules, protein nitration, and oxidative stress are some examples of the overt inflammatory reaction in the AD brain [for a classical review see (Akiyama *et al.*, 2000)]. The ample interaction and activation of different immune pathways in AD poses a challenge in detecting the starting point of neuroinflammation.

The involvement of the immune system as an etiological factor to AD is further supported by the identification of immune AD risk genes. Genome-wide associated studies (GWAS) in AD brain tissue have identified the presence of genetic loci and variants associated with the inflammatory response (Guerreiro *et al.*, 2013a; Karch *et al.*, 2015). From all the identified genes, CD33 and TREM2 have been the most studied. CD33 is a receptor expressed on myeloid and microglial cells and its activation leads to monocytic inhibition and decreased phagocytosis (Crocker *et al.*, 1997). Increased CD33 levels have been detected in AD brains and positively correlate with cognitive decline (Karch *et al.*, 2012). Single nucleotide polymorphisms (SNPs) in CD33 have been shown to lower the risk of developing AD (Hollingworth *et al.*, 2011; Naj *et al.*, 2011). Such reduced risk might result from increased Aβ clearance as mutation carriers show a decrease in Aβ deposition. Missense mutations in the microglial receptor TREM2, lead to impaired microglial phagocytosis and an increased risk for developing late onset Alzheimer's disease (LOAD) risk (Guerreiro *et al.*, 2013b; Jonsson *et al.*, 2013). Furthermore, mutation carriers have extensive brain neurodegeneration when compared to AD-noncarriers (Rajagopalan *et al.*, 2013). While TREM2 mutations are rare and CD33 SNPs confer a low risk of AD, they support the idea that pathways leading to impaired phagocytosis might pose a central role in disease pathogenesis.

Late-stage inflammation in Alzheimer's disease

Restoration of tissue homeostasis is the final step of the inflammatory response (Fullerton *et al.*, 2016). Phagocytic microglia at final stages of the inflammatory response contribute to the resolution of inflammation by neutralizing the inflammatory agents. While little is known about this process in the CNS, several resolving mediators such as lipids, anti-inflammatory cytokines and peripheral immune cells have been involved in this mechanism (Bazan, 2018; Fullerton *et al.*, 2016; Schwartz *et al.*, 2014). The presence of chronic inflammation is indicative of a failed resolving response. Indeed, resolution of inflammation is highly altered in AD (X. Wang *et al.*,

2015). It is plausible that a chronic loop between Aβ production and inflammatory responses will lead to aberrant microglial activation and possible dysfunction affecting its phagocytic and resolving properties. It has been proposed that extravasation of peripheral immune cells such as macrophages, might play a role in effectively phagocytizing pathological protein aggregates and therefore positively contribute to halting the inflammatory response (Naert *et al.*, 2011; Simard *et al.*, 2006; Theriault *et al.*, 2015). In line with this, Eikelenboom and colleagues reported that inflammation in AD brains wanes with aging, further indicating a possible attempt of the CNS to restore homeostasis (Hoozemans *et al.*, 2011). Unfortunately, the accrual accumulation of toxic molecules and overt neurodegeneration at clinical stages of AD make the restoration of tissue homeostasis unfeasible. Figure 1-7 illustrates the late neuroinflammatory process in AD.

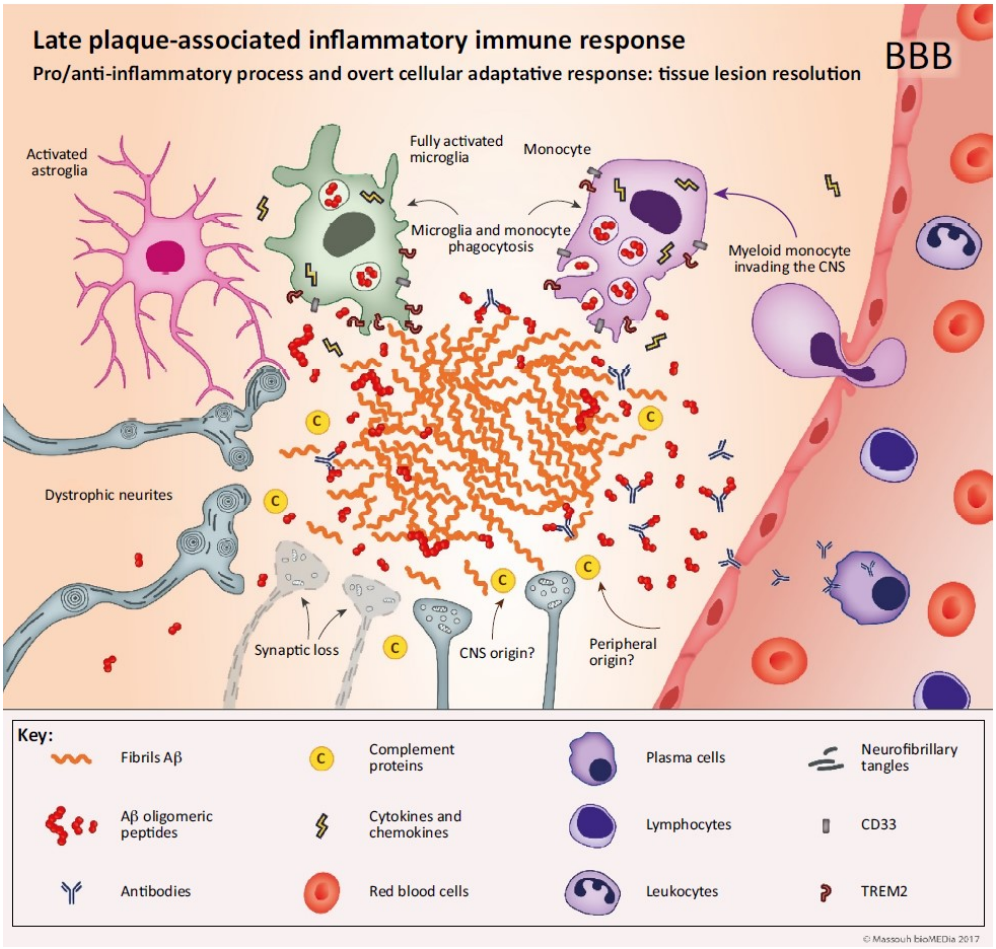

Figure 1- 7. Late plaque-associated neuroinflammation

The late inflammatory process in Alzheimer's disease is characterized by an overt-inflammatory reaction comprising activated microglia and astroglia surrounding amyloid plaques and CNS invasion by peripheral immune cells. In addition, an upregulation of cytokines, complement proteins and immunoglobulins can be found in Alzheimer's brains. At this stage, degeneration of synaptic elements and occurrence of dystrophic neurites is common. BBB = blood brain barrier. Image from (Cuello, 2017) with permission from *Elsevier*.

Neuroinflammation as a therapeutic target in Alzheimer's disease: timing is everything

Population-based, incidence and case-control epidemiological studies revealed that cognitively healthy individuals that were under a long treatment with non-steroidal anti-inflammatory drugs (NSAIDs) had a reduced risk of developing AD (Etminan *et al.*, 2003; in 't Veld *et al.*, 2002; McGeer *et al.*, 2013; Szekely *et al.*, 2004; Vlad *et al.*, 2008). These studies demonstrated that significant sparing of AD occurs only if administration is performed prior to the onset of cognitive symptoms with a treatment duration longer than two years. Furthermore, NSAIDs with high COX-1/COX-2 inhibitory ratio are more effective in reducing the risk of AD while COX-2 inhibitors are associated to worsening of the disease (McGeer *et al.*, 2013). Evidence from transgenic APP mouse models has also revealed that anti-inflammatory treatment can rescue cognitive deficits, ameliorate amyloid pathology and reduce microglial activation (McGeer *et al.*, 2013).

The potential role of NSAIDs as therapeutic agents in AD led to the design of anti-inflammatory clinical trials [(reviewed in (Heneka *et al.*, 2015)]. The outcome of clinical trials has not been as beneficial as expected, while some degree of success was achieved in certain cohorts (Rogers *et al.*, 1993; Scharf *et al.*, 1999), the majority of them failed to demonstrate significative changes in cognitive outcomes.

It has been proposed that the success of anti-inflammatory clinical trials lies on the selection of the target population, the anti-inflammatory compound used, and the duration of the treatment. Neutral effects or worsening of the disease has been observed when anti-inflammatories are administered at clinical AD stages (Aisen *et al.*, 2000; Lyketsos *et al.*, 2007; Martin *et al.*, 2008; Simons *et al.*, 2002). Several researchers have argued that NSAID administration at late stages of AD could interfere with the possible resolving nature of inflammation occurring and thus worsen the pathology (Cuello, 2017; Rogers, 2018). Furthermore, it is unlikely that anti-inflammatory treatment (or other treatments) could halt AD or show a beneficial outcome when severe brain damage is already present. Therefore, as the epidemiological evidence revealed, administration of NSAIDs at the most prodromal stages of AD could be more effective. Such beneficial effect at the earliest stages of AD suggests the occurrence of an early neuroinflammatory process which might be disease aggravating and amenable to therapeutic intervention. This proposition is discussed later in this Chapter.

The Impact of Naproxen Treatment in Pre-symptomatic Alzheimer's Disease (INTREPAD) led by John Breitner, was a 2-year double-blind placebo controlled prevention trial that examined the effects of naproxen on slowing the progression of AD in cognitively healthy older individuals (mean age 63 years old) with a family history of AD (Meyer *et al.*, 2019a; Meyer *et al.*, 2019b). Two daily doses (220 mg per dose) were administered for over a period of two years. Cognitive and neurosensory performance, neuroimaging markers (brain structural and functional MRI) and CSF biomarkers ($A\beta_{42}$, t-tau, and p-tau) were assessed. The primary outcome was the rate of change in a multimodal composite pre-symptomatic Alzheimer Progression Score (APS), which incorporates all the measured parameters. INTREPAD did not reach the primary outcome measure. Moreover, adverse effects of naproxen such as dyspnea, constipation, hypertension and petechiae were noted.

While the failure of this trial led to questioning the use of NSAIDs for the treatment of AD, some limitations of this study need to be discussed before reaching definitive conclusions. The main limitation of the INTREPAD trial was that it was underpowered thus leading to the possibility of having a type II error (false positive). The second limitation was that the APS baseline levels, CSF tau levels and CSF- $A\beta_{42}$ levels where higher in the naproxen treated group, therefore this imbalance could have had affected the main primary outcome of the trial.

The failure of anti-inflammatory clinical trials clearly reflects the lack of knowledge of the longitudinal evolution of neuroinflammation in AD. Henceforth, a comprehensive study of the evolution of neuroinflammation along the continuum of AD is urgently needed. This issue has been addressed in Chapter 2 of this Thesis.

Preclinical brain inflammation in Alzheimer's disease

As individuals with MCI already display an overt amyloid pathology and accumulation of NFT, it is thus not surprising that an established neuroinflammatory profile is already detectable in CSF and *post-mortem* brain tissue (Bruno *et al.*, 2009; Craig-Schapiro *et al.*, 2011; Galimberti *et al.*, 2006; Hu *et al.*, 2010; Janelidze *et al.*, 2018; Parachikova *et al.*, 2007). Further to it, microglial activation has been reported to occur before the onset of degeneration in AD (Sheng *et al.*, 1997). Notably, imaging studies have detected the activation of astroglia prior to the onset of clinical

symptoms in individuals at risk of AD (Rodriguez-Vieitez *et al.*, 2016). In line with this, astroglia activation and upregulation of IL-1β is already detectable in fetuses and neonates with DS (Griffin *et al.*, 1989), further suggesting the early occurrence of neuroinflammation in populations at risk of AD. Upregulation of COX-2 early in AD and in young individuals with DS has been reported (Hoozemans *et al.*, 2001; Mulet *et al.*, 2017). Notably, upregulation of oxidative stress markers is detected in early AD and in DS (Nunomura *et al.*, 2001; Nunomura *et al.*, 2000).

Does neuroinflammation occurs before MCI? Given that it is not possible to ascertain if a cognitively healthy individual with AD biomarker positivity will develop AD, the study of neuroinflammation at preclinical stages of AD is challenging. The use of transgenic animal models mimicking some aspects of Alzheimer's disease has been an indispensable tool to address and study silent stages of the amyloid-like pathology. Indeed, an increase in inflammatory cytokines, intermediate microglial activation and migration towards Aβ-burdened neurons has been associated to the intraneuronal accumulation of Aβ in rodent models of the human-like amyloid pathology (Figure 1-8) (Ferretti *et al.*, 2012a; Hanzel *et al.*, 2014; McAlpine *et al.*, 2009). This "*amyloid plaque-independent*" neuroinflammatory process is disease aggravating as anti-inflammatory treatment diminishes the amyloid pathology, rescues cognitive deficits and downregulates inflammatory markers (Cavanagh *et al.*, 2018; Ferretti *et al.*, 2012a).

At these early stages of amyloid accumulation, the first inflammatory agents are thought to be the neurons as inflammatory marker expression is localized to the neuronal compartment (Hanzel *et al.*, 2014). If we part from the premise that intraneuronal accumulation of toxic Aβ oligomers is one of the first pathological events in AD, and that neurons can sense this noxious stimulus and mount an inflammatory response against it, then it is reasonable that Aβ pathological accumulation might unleash a series of neuronal-driven inflammatory events.

Henceforth, mounting evidence suggests that neuroinflammation might occur at the very early stages of AD and that this incipient process might be amenable to therapeutic intervention. Translation of studies in animal models to the human population is challenging. However, some insights can be gained by investigating the neuroinflammatory profile in populations at risk of developing AD or in cognitively healthy individuals harboring incipient AD pathology. Such propositions are discussed in Chapters 2 and 3 of this Thesis.

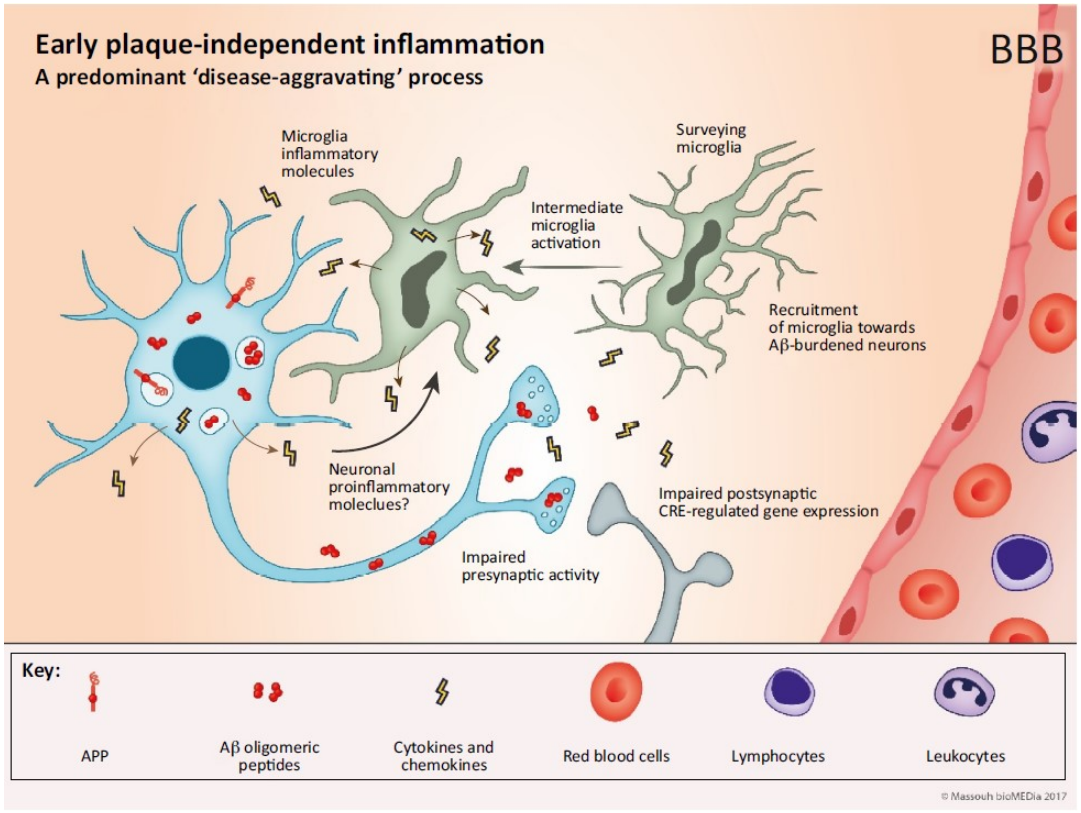

Figure 1- 8. Early neuroinflammation in the amyloid-like pathology

The study of transgenic models of the amyloid-like pathology has revealed the presence of an early neuroinflammatory process characterized by intermediate activation of microglia and their migration to Aβ-burdened neurons. Note that at these early stages the neurons upregulate the production of cytokines. BBB = blood brain barrier. Image from (Cuello, 2017) with permission from *Elsevier*.

Alzheimer's disease in Down syndrome

Intense research is being conducted in order to understand the asymptomatic phases of AD. The characterization of these early stages in populations at risk of developing AD is currently one of the major goals in AD research. Individuals with DS represent the largest group with genetically determined AD and therefore the study of AD preclinical stages in this population might shed light on the earliest pathological mechanisms leading to AD.

In 2013, the Alzheimer's Disease Cooperative Study (ADCS) supported by the National Institutes of Health launched the Down Syndrome Biomarker Initiative (DSBI). The main objective of the DSBI is to identify longitudinal AD biomarkers in the DS population. The ultimate aim of this project is to develop therapies targeting dementia in DS and in the general population. More recently, the NIH has launched INCLUDE (INvestigation of Co-occurring conditions across the Lifespan to Understand Down syndromE), a second project that aims to study various pathologies associated to trisomy 21 across the lifespan of individuals with DS. Moreover, the INCLUDE project aims to actively recruit individuals with DS for ongoing and future clinical trials both within and outside the field of AD research. These are exciting times for DS research; the understanding of underlying pathologies in individuals with DS might hold the clue to the cure of several diseases such as cancer and AD.

Historical overview of Down syndrome

The recognition of DS physical traits has been observed in figurines from ancient cultures from Mexico (1500 B.C – 500 A.D), Peru (1200-1500 A.D.), Colombia and Ecuador (500 B.C.) as well as in paintings from the 15th and 16th centuries (Starbuck, 2011). In 1838, Jean-Etienne-Dominique Esquirol, a physician working under Pinel at the Salpetriere Hospital, published the first psychiatric handbook entitled "*Des maladies mentales considerees sous les rapports medical, hygienique et medico-legal*" (Mental Disease: medical, health/hygiene and medical-legal considerations). In this handbook, under a section covering "idiocy" he was the first to describe some DS physical traits: "oblique eye fissures, epicanthic eye-folds, flat nasal bridge, protruding tongue, short stature with no neck, malformed limbs and mental retardation" [for a reprint of the

original report see (Esquirol, 1976)]. Years later, in 1848, Edouard Seguin gave a detailed description of the nose, open mouth, tongue's morphology and the susceptibility of the lungs to infection. He further communicated that "*despite profound idiocy, the good kids (referring to those with DS) were able to learn basic things*". He elaborated in the "mental pathology" of individuals with DS and named it "furfuraceous cretinism" (E. Seguin, 1846; E. Seguin, 1856, 1866). In 1866, the British physician, John Langdon Haydon Down, made the first clear distinction between trisomy 21 and other mental conditions (Down, 1995). In his "observations on an ethnic classification of idiots" scientific communication, he described the clinical aspects of his patients at the Royal Earlswood Asylum for Idiots:

''The Mongolian type of idiocy occurs in more than ten per cent of the cases which are presented to me. They are always congenital idiots and never result from accidents after uterine life. They are, for the most part, instances of degeneracy arising from tuberculosis in the parents. They have considerable power of imitation, even bordering on being mimics. They are humorous and a lively sense of the ridiculous often colors their mimicry. This faculty of imitation can be cultivated to a very great extent and a practical direction given to the results obtained. They are usually able to speak; the speech is thick and indistinct but may be improved very greatly by a well-directed scheme of tongue gymnastics. The coordinating faculty is abnormal, but not so defective that it cannot be strengthened. By systematic training, considerable manipulative power may be obtained. The circulation is feeble and however much advance is made intellectually in the summer, some amount of retrogression may be expected in the winter. Mental and physical capabilities are, in fact, directly as the temperature. The improvement which training effects in them is greatly in excess of what would be predicted if one did not know the characteristics of the type. The life expectancy, however, is far below the average, and the tendency is to tuberculosis which I believe to be the hereditary origin of the degeneracy.''

Given the English popularity of racial classification at the time, and the resemblance of them to people from Mongolia, he referred to individuals with DS as "Mongoloids", an obsolete term that evolved to "Down's syndrome" (Allen *et al.*, 1961) or "Trisomy 21", perhaps the latter being more accurate.

Down syndrome arises from triplication of chromosome 21

As outlined above, Down attributed trisomy 21 to maternal tuberculosis. Following Down's observations, different aetiologies for DS were proposed and included uterine exhaustion, consanguinity, syphilis, fetal or endocrine dysfunction, alcoholism, and social position. In the time that Theophilus Painter suggested that humans had 45 to 48 chromosomes (Painter, 1921), Charles B. Davenport (1932), proposed that intellectual disabilities might be the result of chromosomal irregularities (Davenport, 1932) and sent a tissue sample from an individual with DS to Painter. Painter did not detect any chromosomal irregularities in the sample. At that time, Petrus J. Waardenburg theorized that DS might be caused by nondisjunction of the chromosomes (Allen, 1974).

Joe H. Tjio's and Albert Levan's report that humans have 46 chromosomes opened a new avenue in genetics research (Tjio, 1978). As a result of this, Jerome Lejeune, a French geneticist and pediatrician, discovered that individuals with DS have 47 chromosomes, with an extra chromosome 21 (Lejeune *et al.*, 1959). His findings were further confirmed by three independent groups (Fraccaro *et al.*, 1960; Jacobs *et al.*, 1959) and today, is well-established that DS results from triplication of chromosome 21.

Approximately 95% of DS cases arise from chromosomal non-disjunction during meiosis. ~3-4 % of DS cases result from translocation of chromosome 21 to other chromosomes, usually 14 or 15 and leads to partial trisomy 21 (Kazemi *et al.*, 2016). This percentage of cases are considered as "familial" DS as translocations can be inherited. A less common form of DS, accountable for ~1-2 % , of the cases is caused by abnormal cell division after fertilization resulting in mosaicism where a third copy of chromosome 21 is present in some set of cells (Kazemi *et al.*, 2016).

Two hypotheses have been proposed to explain the mechanism by how triplication of chromosome 21 causes DS. The first one is the "*gene dosage-effect*" hypothesis which poses that elevated expression of dosage-sensitive chromosome 21 genes (protein or non-protein coding) leads to DS. In fact, gene expression analyses from lymphoblastoid cells from individuals with DS have shown that 29% of the expressed transcripts in chromosome 21 display a dosage effect with 1.5 overexpression and 7% are amplified (over 1.5 overexpression) (Ait Yahya-Graison *et al.*, 2007). Interestingly, 56% of the genes are compensated and 15% are variable between individuals with

DS (Ait Yahya-Graison *et al.*, 2007). Therefore, overexpressed genes are likely to contribute to DS features. The other proposition is known as the "*amplified developmental instability*" hypothesis (Ait Yahya-Graison *et al.*, 2007). It proposes that the characteristic features of DS arise from non-specific disturbances in chromosome balance leading to genetic instability and disruption of homeostasis. Both hypotheses are not mutually exclusive.

Epidemiology

DS is the most common genetic cause of learning disabilities and currently affects 5.8 million people worldwide (Ballard *et al.*, 2016). The probability of having a child with DS increases with maternal age and in general, 1 in 650-1000 births results in a child with DS (Lott *et al.*, 2010). The Public Health Agency of Canada has reported that between 2005 and 2013, the birth prevalence of Down syndrome averaged 15.8 per 10, 000 births (Public Health Agency of Canada, 2017). Around 60 years ago, 45% of individuals with DS survived the first year of life and only 40% lived till their 5th year. Over the past 30 years, life expectancy has doubled and an individual with DS can expect to live into their sixties (Lott *et al.*, 2019). The main reason explaining the increased life expectancy is that nowadays individuals with DS receive increased medical care and concurrent disorders/pathologies can be treated.

A wide arrange of clinical conditions are associated with DS. The most common congenital abnormality is congenital cardiac disease, present in half of the DS population. Gastrointestinal tract abnormalities and hematologic disorders such as lymphoblastic and myeloid leukaemia are also common in individuals with DS. Trisomy 21 patients also commonly present hearing loss, ophthalmological problems, periodontal disease, higher frequency of obesity, juvenile rheumatoid arthritis-like arthropathy, diabetes mellitus, hypothyroidism, sleep apnea, and skin conditions (Roizen *et al.*, 2003). As expected, neurological and neurodevelopmental abnormalities are present in DS and will be detailed below.

Neurodevelopmental and neurological abnormalities in Down syndrome

The brains of individuals with DS are 20% smaller when compared to the general population. This reduced weight is the result of gross anatomical abnormalities. Absolute volumes of temporal and frontal lobes along with the hippocampus, are smaller than the median compared to the euploid population (Lott, 2012). However, when adjusted by overall brain volume, the smaller brain volumes are not significantly different to the non-DS population (Pinter *et al.*, 2001). Nevertheless, the smaller volumes are still reflective of brain underdevelopment. Notably, individuals with DS have a larger parahippocampal gyrus and the parietal cortex does not display any abnormalities (Kesslak *et al.*, 1994; Pinter *et al.*, 2001). Moreover, the brain is brachycephalic with a reduced number of cerebral sulci and a small cerebellum (Coyle *et al.*, 1986; Wisniewski, 1990). Cerebellar hypoplasia might account for hypotonia, motor coordination problems, and speech disturbances which are commonly observed in the DS population (Lott, 2012). Gross brain abnormalities are depicted in Figure 1-9.

Microscopical abnormalities include a reduced number of neurons, abnormal neuronal distribution mainly in cortical layers II and IV and decreased neurogenesis (Contestabile *et al.*, 2007; Golden *et al.*, 1994; Ross *et al.*, 1984; Schmidt-Sidor *et al.*, 1990). Interestingly, children with DS display greater dendritic branching and length than euploid individuals (Becker *et al.*, 1991). However, a decrease in dendritic arborization is apparent later in life (Becker *et al.*, 1991). These alterations result in impaired neuronal connectivity most likely affecting their cognitive abilities. *In-vitro* analyses have revealed increased apoptotic activity in DS cells (Guidi *et al.*, 2008), suggesting that it might contribute to brain abnormalities.

These neurodevelopmental abnormalities are also reflected in the common cognitive deficits observed in the DS population (Figure 1-10). Notably, there is a broad heterogeneity in cognitive abilities in individuals with DS most likely due to allelic variation, gene-environment interactions, or genetic imbalance. Overall, verbal short-term memory, language abilities, morphosyntax, and explicit long-term memory are impaired in individuals with DS [reviewed in (Lott *et al.*, 2010)]. On the contrary, associative learning, visuospatial short-term memory, and implicit long-term memory are usually preserved (Lott *et al.*, 2010). Cognitive impairments worsen with age as the

individual with DS is unable to consolidate information. Further cognitive deficits appear in those individuals that develop dementia of the Alzheimer's type.

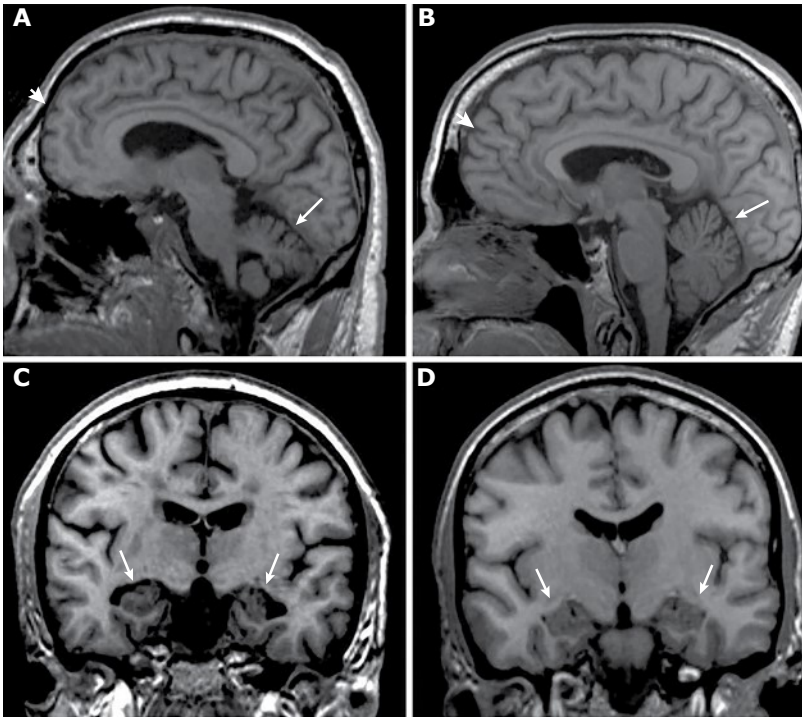

Figure 1- 9. Structural brain changes in an adult with Down syndrome

Sagittal MRI sections from a Down syndrome (A) and non-Down syndrome individual (B). Note the cerebellum hypoplasia (arrows) and frontal cortex structural differences (arrowheads) in the individual with Down syndrome (age = 46). Ventricular dilation and hippocampal atrophy (arrows) can be noted in coronal sections from a Down syndrome individual (C) as compared to an individual without Down syndrome (D). Image from (Lott *et al.*, 2019) with permission from *Springer Nature*.

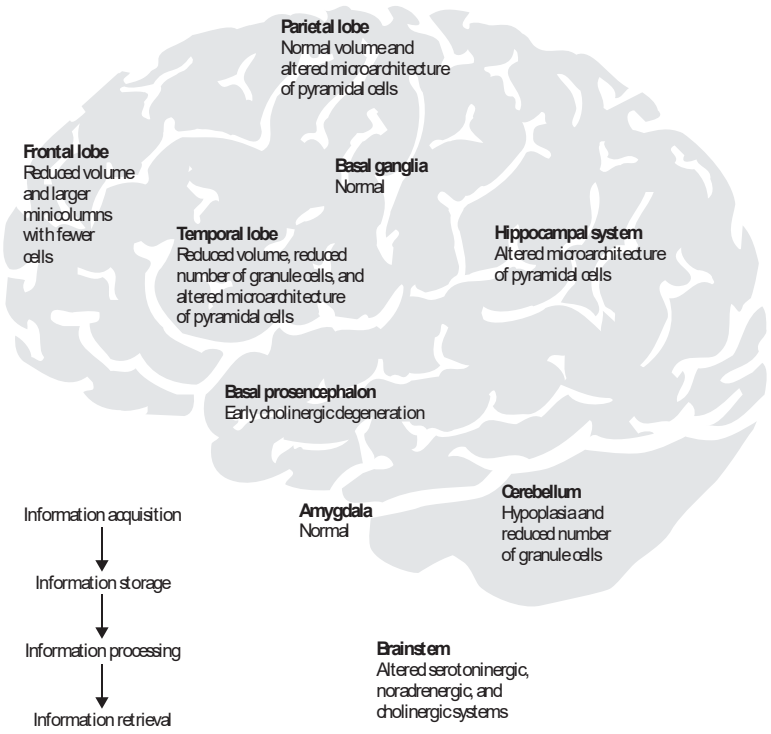

Figure 1- 10 Neurodevelopmental brain abnormalities in Down syndrome

Regional abnormalities contribute to cognitive deficits in DS. The flow diagram represents the stages of flow information related to learning and memory that are altered in DS. Reprinted from the Lancet (Lott *et al.*, 2010) with permission from *Elsevier*.

Alzheimer's disease in Down syndrome: "a sort of precipitate senility"

In 1876, Fraser and Mitchell made the first association between DS and dementia (Fraser, 1876). They examined 62 cases and noted "*In not a few instances however, death was attributed to nothing more definite than general decay- a sort of precipitate senility*". They also proposed that maternal age could be a possible cause of DS. The presence of amyloid plaques in DS brains was revealed in following reports by Friedrich Struwe, Bertrand and Koffas (Bertrand I, 1949; Struwe, 1929). However, it was the seminal work of George A. Jervis, that provided the evidence of the occurrence of AD in DS (Jervis, 1948).

Jervis reported the clinical and neuropathological findings in 3 individuals with DS aged 35, 42 and 47 years (Jervis, 1948). He proposed that the personality changes observed in these individuals (irritability, depression, apathy, and aggressiveness) were the result of intellectual and emotional deterioration (Jervis, 1948). He further proposed to classify this deterioration as early senile dementia (Jervis, 1948). His neuropathological analyses demonstrated the presence of plaques and neurofibrillary tangles in DS brains. His findings suggested a link between senile dementia and AD neuropathological changes in DS brains. He further suggested that the study of DS could hold some clues to the etiological factors leading to AD, a proposition that nowadays is actively pursued:

"Since mongoloid patients show a marked tendency to develop this type of reaction (senile dementia), it is suggested that the study of it offers some information which may contribute to a better understanding of the cause of senile dementia"(Jervis, 1948).

Alzheimer's dementia in Down syndrome

The diagnosis of dementia in individuals with DS has been challenging given the significant variations in baseline cognitive deficits that are present in this population. A number of cognitive tests generally used to detect AD dementia in the general population have been adapted to the DS population. However, one third of the older DS population is unable to perform these tests. Given test complexity and reliance of some tests in language abilities, these tests are not completely accurate. Modified versions of verbal learning and memory scales have been included in the

Cambridge Neuropsychological Automate Test Battery (CANTAB) (Ballard *et al.*, 2016). Further tests to detect dementia in the DS population include the Down's Syndrome Attention, Memory, and Executive Function Scales (DAMES), the Down Syndrome Mental State Exam (DSMSE) , the Cambridge Cognitive Examination (CAMCOG)-DS, and the Cambridge Examination for Mental Disorders in the Elderly-Revised (CAMDEX-R) (Ballard *et al.*, 2016; Strydom *et al.*, 2018).

The variability of results obtained with different cognitive tests, has led to variation in the reported prevalence of AD in DS. Some have reported a 5% prevalence of AD in individuals with DS under the age of 40 (Tyrrell *et al.*, 2001). As in AD, the risk of dementia in DS increases with aging. As such, some studies estimated that the prevalence of AD in individuals with DS aged 40-49 was 55% and this increased to 68% - 88% in older individuals with DS (Ballard *et al.*, 2016). Further reports indicate that the prevalence of AD in 50 year old individuals with DS is 23% and this increments to 45% at age 55 (Holland *et al.*, 2000; McCarron *et al.*, 2017).

Diagnosis of AD in the DS population is usually done at late AD stages. Therefore, detection of MCI in the DS population is even more challenging. Some tests have been adapted to detect earlier cognitive impairments due to AD; however, a consensus has not been universally reached. Of note, coupled with cognitive tests, assessment by the clinician following the individual with DS has proven to be the best method to detect early signs of cognitive decline in the DS population.

Early signs of cognitive decline in DS include language and executive function impairments, the latter reflecting frontal lobe dysfunction (Lott *et al.*, 2010). Early behavioral changes are more common in the DS population than in other forms of AD. Apathy, depression and impulsivity are common in the early phases of dementia; reflecting impairments in executive function (Ball *et al.*, 2008; F. Lai *et al.*, 1989; Lautarescu *et al.*, 2017). Furthermore, the appearance of seizures in the old DS population is highly suggestive of the development of AD (Lott *et al.*, 2010).

Certain risk factors might promote and potentiate the development of AD in DS. As in the general population, individuals with DS bearing the ApoE4 allele have a higher risk of developing AD (Deb *et al.*, 2000). It has also been shown that individuals with DS harboring the ApoE4 allele have a higher deposition of Aβ (Hyman *et al.*, 1995). On the other hand, some individuals with DS do not develop AD-dementia. While this is intriguing given their genetic disposition, some factors such as the level of education, early stimulation, allelic variation and cognitive reserve might

confer resilience to the development of a full-blown dementia. The identification of these factors is of great interest for the prevention of dementia in DS as well as in the general population.

Alzheimer's-related neuropathology in Down syndrome

Following Struwe, Bertrand and Koffas, and Jervais reports, authoritative studies have comprehensively analyzed and validated the occurrence of amyloid plaques and NFT in the brains of individuals with DS (Lemere *et al.*, 1996; Mann *et al.*, 1989; Mori *et al.*, 2002; Wisniewski *et al.*, 1985). Individuals with full trisomy 21 develop all the spectrum of AD neuropathology. Soluble Aβ has been detected in fetuses at the second trimester of gestation (Teller *et al.*, 1996). Indeed, *in-vitro* studies have shown that Aβ can be isolated from DS fetal cortical neurons (Busciglio *et al.*, 2002). Intraneuronal accumulation has been reported to occur in the entorhinal cortex and hippocampal pyramidal neurons of very young infants with DS (~ 1 year old) (Gyure *et al.*, 2001). This is followed by deposition of diffuse amyloid plaques composed of $A\beta_{42}$ in young individuals with DS (Gyure *et al.*, 2001; Lemere *et al.*, 1996; Mori *et al.*, 2002). $A\beta_{40}$ deposition occurs later, at around age 30, along with the formation of dystrophic neurites (Lemere *et al.*, 1996). By age 30, the presence of mature amyloid plaques is common in individuals with DS (Lemere *et al.*, 1996). Further to it, individuals with DS also display CAA at advanced stages of AD (55 years old) (Head *et al.*, 2017).

PET studies have revealed that the striatum is the first region of Aβ deposition in the DS brain; being detectable after 35 years of age (Handen *et al.*, 2012). Imaging studies have shown that following Aβ striatal deposition, Aβ spreads to neocortical areas following the sequence: (i) Aβ pathology spreads to the dorsal prefrontal cortex and anterior cingulate cortex; (ii) Aβ is then deposited in the ventral prefrontal cortex and some areas of the parietal lobe; (iii) this is followed by Aβ deposition in the lateral temporal cortex and the rest of the parietal lobe; (iv) Aβ aggregates appear in motor and sensory areas and spread to (v) the visual cortex, premotor cortex and the rest of the temporal lobe; (vi) the occipital lobe and, (vii) the thalamus and parahippocampal cortex; (viii) the amygdala is the last site of Aβ accumulation (Annus *et al.*, 2016).

NFT pathology appears usually at 35 years of age and shows a progressive spreading. The entorhinal cortex, hippocampus and subcortical regions (LC and the dorsal raphe nucleus, DRN) are the first sites were tau pathology is detected (Davidson *et al.*, 2018). Later, tau pathology spreads to neocortical areas and is well-established in individuals aged ~55 years (Davidson *et al.*, 2018; Wisniewski *et al.*, 1985). Therefore, tau pathology follows a similar spreading pattern as the one encountered in AD.

In sum, AD neuropathology follows a continuum in DS with intraneuronal Aβ accumulation appearing first, followed by deposition of amyloid plaques and lastly by the occurrence of NFT pathology (Figure 1-11).

Individuals with DS also display other neuropathological hallmarks of AD such as neurotransmitter system degeneration (Godridge *et al.*, 1987; Mann *et al.*, 1985; Reynolds *et al.*, 1985; Yates *et al.*, 1981; Yates *et al.*, 1980), neurotrophic factor deregulation (Iulita *et al.*, 2014), oxidative stress, and neuroinflammation [for a review see (D. M. Wilcock *et al.*, 2013)]. Nevertheless; such events have been poorly characterized along the continuum of AD in DS.

As outlined throughout this section, the continuum of the AD pathology in DS mimics some aspects of AD in the general and genetic populations at risk of developing AD. Therefore, as individuals with DS are the largest population at genetic risk of AD, they represent a valuable group to study the most silent stages of AD. Given that AD is the main medical problem in the DS population, great efforts are needed to ascertain the best possible strategies to treat AD in the DS context.

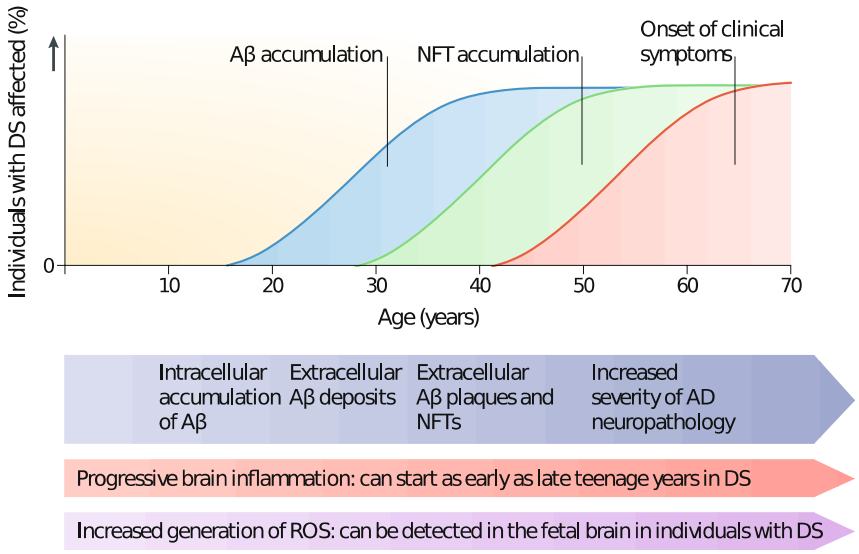

Figure 1- 11. Continuum of AD neuropathology in Down syndrome

Scheme reflecting AD neuropathological changes across the lifespan of individuals with DS. Amyloid pathology starts in the form of intraneuronal Aβ with appearance of amyloid plaques during young adulthood. This is followed by deposition of NFT leading to a full-blown AD pathology by age 40. Brain inflammation and oxidative stress might be early events in the AD continuum in DS. Image from (Lott *et al.*, 2019) with permission from *Springer Nature*.

Thesis Objectives and Rationale

It is becoming clear that early therapeutic intervention might be necessary to halt or prevent the progression of AD. The understanding of the neuropathological events occurring at AD-asymptomatic stages should offer a window for identifying potential targets amenable to promising therapeutic strategies. As discussed in the Introduction of this Thesis, the early neuroinflammatory process might play an important role in the progression and the development of AD asymptomatic stages. Therefore, the main goal of this Thesis was to study the neuroinflammatory process at asymptomatic stages of AD. Toward this objective, the following evidence has been taken into consideration:

1. The fact that individuals with DS represent the largest population at genetic-risk of developing AD, henceforth, they represent a valuable population to study early inflammatory events at preclinical stages of AD and their evolution across different stages towards the clinical presentation of AD.

2. One-third of cognitively normal individuals display brain AD neuropathology; hence a part of this population might be at the earliest stages of AD. This is an ideal population to study the possible association of incipient development of AD neuropathology and brain inflammation even before the onset of cognitive deficits.

3. Early degeneration of the locus coeruleus (LC) in AD might facilitate AD neuropathology by the loss of its CNS anti-inflammatory role. Nevertheless, the effects of LC degeneration have been mainly studied in transgenic animals mimicking the human-like amyloid pathology at late stages of amyloid deposition. As transgenic animals mimic some aspects of the early human-like amyloid pathology, the study of LC degeneration before overt amyloid deposition should reveal new aspects related to the impact of noradrenergic loss in the evolution of the AD pathology.

Based on this evidence, we have generated the following hypotheses:

Hypothesis I: A differential neuroinflammatory profile occurs across the lifespan of individuals with DS, where brain inflammation is exacerbated at preclinical stages of AD and wanes at late stages of AD in DS.

Hypothesis II: The presence of incipient AD neuropathology in cognitively healthy individuals is associated to an increase in pro-inflammatory mediators.

Hypothesis III: LC noradrenergic denervation will accelerate the early neuroinflammatory process and neuropathological changes before the accumulation of amyloid plaques in a transgenic rodent model of the amyloid-like pathology.

Chapters 2, 3, and 4 of this Thesis will cover the testing and outcome of the above hypotheses.

CHAPTER 2

Evolution of neuroinflammation across the lifespan of individuals with Down syndrome

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Under review, March 2020

Abstract

Epidemiological and experimental studies suggest that a disease-aggravating neuroinflammatory process is present at preclinical stages of Alzheimer's disease. Nevertheless, the characterization of neuroinflammation along the Alzheimer's continuum is an unmet need. Given that individuals with Down syndrome are at increased genetic risk of Alzheimer's disease and therefore develop the spectrum of Alzheimer's neuropathology in a uniform manner, they constitute an important population to study the evolution of neuroinflammation across the Alzheimer's continuum. Therefore, in this cross-sectional study, we characterized the brain inflammatory profile across the lifespan of individuals with Down syndrome.

Microglial morphology and inflammatory cytokine expression were analyzed by immunohistochemistry and MesoScale immunoassays in frontal cortex from fetuses to adults with Down syndrome and controls (16 gestational weeks to 64 years), totalling 127 cases. The inflammatory profile in mixed fetal primary cultures and hippocampus of adults with Down syndrome, as well as the effects of sex on cytokine expression were also analyzed.

Microglial soma enlargement, process thickening and retraction, were observed early, in the frontal cortex of children and young adults with Down syndrome before the development of full-blown Alzheimer's pathology. At this stage, the brains from young adults also displayed increased numbers of rod-like microglia. Changes in microglia morphology were accompanied by increased levels of IL-8 and IL-10 in children with Down syndrome (1-10 years; Down syndrome n=5, controls n=10) and higher levels of IL-1β, IL-1α, IL-6, IL-8, IL-10, IL-15, eotaxin-3, IP-10, MDC, and MIP-1β in young adults with Down syndrome compared to euploid cases (13-25 years, Down syndrome n=6, controls n=24). Notably, increased cytokine expression was also found in the conditioned media of mixed cortical primary cultures from second trimester foetuses with Down syndrome.

In contrast, older adults with Down syndrome (39-68 years, Down syndrome n=22, controls n=16) displayed a cerebral inflammatory signature associated to a defective resolution and waning of inflammation characterized by reduced levels of IL-10, IL-12p40, IFNγ and TNFα concurring with a highly dystrophic microglial phenotype and rod-like microglia aligning to neurons harboring tau

pathology. Moreover, sex stratification analyses revealed that females with Down syndrome displayed increased IL-6 and IL-8 levels compared to males with Down syndrome.

Our findings indicate the presence of an early and evolving neuroinflammatory phenotype across the lifespan in Down syndrome, a knowledge that is relevant for the discovery of new targets and for the design of anti-inflammatory trials against Alzheimer's disease in this population.

Introduction

Down syndrome or trisomy 21 (T21) is a genetic disorder that arises from complete or partial triplication of chromosome 21 (Lejeune *et al.*, 1959). As the Amyloid Precursor Protein (*APP*) gene is encoded on this chromosome, adults with Down syndrome are at a high genetic-risk of developing early-onset dementia due to Alzheimer's disease (Ballard *et al.*, 2016).

Individuals with Down syndrome display Alzheimer's neuropathological features since early in life. Soluble accumulation of amyloid beta (Aβ) can be detected in the brains of fetuses with Down syndrome as early as 15-21 weeks of gestational age (Busciglio *et al.*, 2002; Teller *et al.*, 1996) and intraneuronal Aβ is present before the development of amyloid plaques in young individuals with Down syndrome (Busciglio *et al.*, 2002; Gyure *et al.*, 2001; Mori *et al.*, 2002). This is followed by the formation of diffuse amyloid plaques during childhood (8-12 years old) (Gyure *et al.*, 2001; Lemere *et al.*, 1996; Mori *et al.*, 2002) and later by mature amyloid plaques, which are commonly present in 30 year-old individuals with Down syndrome (Handen *et al.*, 2012), followed by abnormal tau phosphorylation (Davidson *et al.*, 2018). By 40-60 years of age, a full-blown Alzheimer's neuropathology, comprised by widespread accumulation of mature amyloid plaques and neurofibrillary tangles, is present throughout Down syndrome brains (Wisniewski *et al.*, 1985). Furthermore, neurodegeneration (Godridge *et al.*, 1987; S. H. Kim *et al.*, 2001; Mann *et al.*, 1985; Martin *et al.*, 2014; Reynolds *et al.*, 1985; Reynolds *et al.*, 1988; Yates *et al.*, 1981; Yates *et al.*, 1980), cerebral oxidative stress (Busciglio *et al.*, 2002; Busciglio *et al.*, 1995; Cenini *et al.*, 2012; Di Domenico *et al.*, 2014), neurotrophic factor deregulation (Iulita *et al.*, 2014), white matter degeneration (Olmos-Serrano *et al.*, 2016; Powell *et al.*, 2014; Romano *et al.*, 2018), and cerebral amyloid angiopathy (Head *et al.*, 2017), have also been reported to occur in Down syndrome brains.

It has been widely acknowledged that neuroinflammation plays an important role in the development and progression of Alzheimer's disease in the general population (Akiyama *et al.*, 2000). However, little is known about the evolution of neuroinflammation in Alzheimer's disease and few investigations have examined the occurrence of neuroinflammation in Down syndrome. Microglial and astrocytic activation, increased inflammatory gene expression, formation of immune complexes, and cerebral oxidative stress have been reported to occur at different ages in

Down syndrome brains (Eikelenboom *et al.*, 1982; Griffin *et al.*, 1989; Head *et al.*, 2001; McGeer *et al.*, 1988; McGeer *et al.*, 1987; Stoltzner *et al.*, 2000; Wilcock *et al.*, 2015; Xue *et al.*, 2011). However, a detailed analysis of microglial activation and brain inflammatory cytokine expression across the lifespan of individuals with Down syndrome is lacking. Such information might reveal shared pathological pathways between individuals with Down syndrome and Alzheimer's disease in the general population, assisting to the identification of potential therapeutic targets.

With the objective of achieving a comprehensive understanding of neuroinflammation in Down syndrome we gathered the largest cohort of *post-mortem* Down syndrome brains to investigate microglial morphological changes and inflammatory cytokine expression before the occurrence of overt Alzheimer's neuropathology, in frontal cortex tissue from fetal to adult stages. We complemented these findings with analyses of mixed primary cultures from fetal Down syndrome cortex. Our results indicate that young individuals with Down syndrome have a heightened neuroinflammatory profile in the frontal cortex characterized by microglia soma enlargement, process thickening and retraction, and an increase in "rod-like" microglia along with an upregulation of key inflammatory cytokines. In contrast, even though older adults with Down syndrome also show an upregulation of inflammatory mediators, the inflammatory signature was characterized by a downregulation of pro-resolving cytokines and the presence of highly dystrophic microglia; indicative of a failure in inflammation resolution. Our results revealed differential neuroinflammatory responses across the lifespan of individuals with Down syndrome, supporting the concept of early and late neuroinflammatory processes as it has been hypothesized for Alzheimer's disease (Cuello, 2017).

Methods

Human brain tissue

Available *post-mortem* fixed and frozen brain tissue from the frontal cortex individuals with Down syndrome (T21) and age-matched controls (euploids) were obtained from the brain banks at the Alzheimer's disease Research Centre, University of California Irvine, New York University School of Medicine, and the NIH Neurobiobank (University of Maryland and Pittsburgh's University), totaling 102 samples. Additionally, hippocampal frozen tissue was available for 25 older adult cases (NIH Neurobiobank). The leading cause of death of the control subjects was accidental. No major neuropathological abnormalities were reported for the control subjects. Cause of death and *post-mortem* pathological reports are available upon request. The age range for the cortical fetal samples was 18-23 weeks of gestational age and 1-68 years for the cortical and hippocampal samples. Confirmation of full trisomy 21 was performed at the brain repositories. Demographic information is detailed in Supplementary Tables 2-1-4. This project has been approved by the McGill University Research Ethics Board.

Brain tissue block processing for immunohistochemistry

Formaldehyde-fixed brain tissue blocks were stored in cryoprotectant solution composed of ethylene glycol and sucrose in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Then, tissue blocks were placed overnight in a 15% sucrose solution in PBS at 4°C. When brains sank, they were transferred to a 30% sucrose solution in PBS and later cut into 40-µm-thick sections using a freezing microtome (Leica SMA 200R). Sections were stored in cryoprotectant solution at -20°C until processed for immunohistochemistry.

Microglial detection

Brain tissue sections were washed with PBS prior to quenching endogenous peroxidase activity with 3% H_2O_2 and 10% methanol in PBS for 30 minutes at room temperature. To prevent

nonspecific antibody binding, tissue sections were blocked with 10 % normal goat serum (NGS) in PBS-T (0.2% Triton X-100) for 1 hour. Sections were incubated at 4°C overnight with rabbit anti-Iba1 to detect microglia (1:8000, Wako). For Iba-1 and PHF-1 double immunostaining (1:1000, provided by Peter Davies, Albert Einstein College of Medicine, NY) to detect microglia and phosphorylated tau at serine residues 396 and 404, antigen retrieval (30 minutes in Tris-EDTA pH 9.0 at 90°C) was performed before adding the blocking solution. Sections were washed and incubated with either of the following secondary antibodies in 5% NGS in PBS-T for 1 hour at room temperature: goat anti-mouse-IgG (1:100, MB Biochemicals) and biotinylated goat anti-rabbit-IgG (1:200, Vector Laboratories). Signal amplification was performed with either mouse anti-HRP monoclonal antibody (1:30) pre-incubated with 5 μg/mL horseradish peroxidase (HRP) (MAP kit, MediMabs) or Vectastain Elite ABC-HRP kit (Vector Laboratories). Sections were incubated in 0.06 % DAB (3,3′-diaminobenzidine) (Sigma-Aldrich) and then developed with 0.01% hydrogen peroxide (Sigma-Aldrich). For the double immunostaining (Iba-1/PHF-1), Iba-1 immunoreactivity was revealed by further incubating the developed PHF-1 sections with a secondary goat anti-mouse antibody and MAP-HRP. Immunoreactivity was visualized using the VECTOR SG substrate kit (Vector Laboratories). Sections were washed and mounted in gelatin-coated coverslips, air-dried, dehydrated in ascending alcohol concentrations. Sections immunolabelled with Iba-1 were rehydrated in a descending series of alcohol concentrations and counterstained with 0.25% cresyl violet (Sigma-Aldrich) for 2.5 minutes followed by acetic acid (1:400) in 95% ethanol for 4 minutes. Then, Iba-1-labelled sections were dehydrated in an ascending series of alcohols. Finally, all sections were cleared with xylene and coverslipped using Entellan mounting medium (EMD, Millipore).

Amyloid-beta and phosphorylated tau immunohistochemistry

Immunohistochemistry was conducted as previously described in a total of 3 sections per case. mouse McSA1 monoclonal antibody was applied to detect human Aβ (1: 4000, MediMabs) (Grant *et al.*, 2000), and mouse anti-AT8 (1:250, Thermo Fischer Scientific) to detect phosphorylated tau at both serine 202 and threonine 205.

Quantification of microglial morphological changes

Z-stack images of cortical lamina III (LIII) and lamina V (LV) were taken using a microscope equipped with an AxioCam 506 color digital camera (Carl Zeiss, Germany). Four brain sections were imaged, and 4 images were captured per cortical lamina per brain section. Image analysis was done using the software ImageJ (NIH, Bethesda, Maryland).

Microglial morphological changes were assessed by calculating the dimensional ratio (DR). The DR is defined by dividing the total microglial area by the total microglial length. Activated microglia display an amoeboid morphology characterized by increased soma area and process retraction. Therefore, an increase in the DR is reflective of changes in microglial morphological state. The DR was calculated as previously described (Pimentel *et al.*, 2015). Briefly, using a macroinstruction algorithm, Iba-1 immunoreactive structures were isolated from cresyl violet counterstain using a color deconvolution plug-in (H&E 2). Then, images were converted to 8-bit grayscale and sharpened (unsharp mask, radius=5, mask = 0.6). The "despeckle filter" was applied to the images to remove some noise generated by the Unsharp filter. The images were then converted to a binary file using the auto threshold option (triangle method: 0,192). To further decrease background noise, the "Despeckle", "Close-" and "Remove outliers" filters were applied. The total area covered by Iba-1 per micrograph was calculated using the resulting binary file. In order to measure the total length of microglial processes, binary files were then skeletonized using the Skeletonize (2D/3D) function.

Microglial cell count was performed in the above mentioned Iba-1 immunolabeled sections. Only non-dystrophic microglia were counted in micrographs from cases of children, young adults, and adults with Down syndrome and controls as microglial cell fragments could not be attributed to a particular cell body. A total of 18 micrographs from LIII and LV were analyzed per case. The demographics for these analyses are illustrated in Supplementary Table 2-3.

Rod microglia quantification

Iba-1 immunolabeled sections from young adults and adults were scanned using an Olympus VS120 slide scanner. A counting frame (3 mm × 1 mm) was vertically positioned spanning lamina

VI to lamina II-III. In this study, rod microglia were morphologically defined as elongated microglial cells with considerable process retraction, generally but not exclusively perpendicular to the pial surface as described in previous publications (Au *et al.*, 2017; Bachstetter *et al.*, 2017; Bachstetter *et al.*, 2015; Sierra *et al.*, 2016; Taylor *et al.*, 2014). Rod microglia were manually quantified in 4 counting frames per section by an experimenter blinded to the genotype. A total of 4 brain sections were quantified per case. The average number of rod microglia per case was quantified. Demographic information for these cases is available in Supplementary Table 2-4.

Inflammatory cytokine expression analyses

Sample preparation

50 mg of frozen brain tissue were sonicated in ice-cooled lysis buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween-20, 5 µl per mg of tissue) with 2x phosphatase (PhosSTOP, Roche) and protease inhibitors (cOmplete Mini, Roche). Homogenates were centrifuged at 13 000 rpm for 45 minutes at 4°C. Supernatants were aliquoted to avoid repeated freeze and thaw cycles and stored at -80°C until analysis. Protein concentrations were measured using the DC™ Protein Assay Kit (BioRad).

Assessment of inflammatory cytokine expression

Inflammatory protein expression in *post-mortem* brain tissue was measured using multiplex immunoarrays (MesoScale Discovery). Three different plates were customized to measure an array of 20 cytokines (Supplementary Table 2-5). On the day of the assays, samples were thawed on ice and their concentration was normalized to 5 µg/µl in lysis buffer with 1% blocker A (MesoScale Discovery) and 1x of protease and phosphatase inhibitors (Roche) as previously described (Chen *et al.*, 2016). Calibrators and samples were prepared according to the manufacturer's recommendations and loaded in duplicates. Electrochemiluminiscence signal reflecting inflammatory protein expression was measured using a SECTOR Imager 6000 reader (MesoScale Discovery) and processed using the Discovery Workbench 4.0 software. For those analytes below

the lower limit of detection, half of the lower limit of detection was considered as the minimum detectable concentration as advised by MesoScale.

Cytokine quantification in the conditioned media of fetal cortical cells

Mixed primary cultures were established from the frontal cortex of Down syndrome fetuses and controls as previously described (Busciglio *et al.*, 2002). Undiluted conditioned media was analyzed by Eve Technologies (Eve Technologies, Inc) using a bead-based multiplex assay for 42 inflammation-related proteins (Human Cytokine Array / Chemokine Array 42-Plex with IL-18 (HD42, Eve Technologies, Inc) (Supplementary Table 2-6). Quantification of MCP-1 (Abcam) was performed by ELISA, following manufacturer's recommendations.

Statistical analysis

Data was analyzed with GraphPad Prism 8. Data normality was analyzed with the Shapiro-Wilk normality test. For two-group comparisons, two-tailed Student's t-tests and two-tailed Mann-Whitney tests were employed according to data distribution. When appropriate, one-way ANOVA or Kruskal-Wallis tests were used followed by Tukey or Dunn's post-hoc tests, respectively, with appropriate multiple comparison adjustment. Categorical variables were assessed by performing Fisher's exact test. Sex differences on inflammatory protein expression were assessed using a Two-Way ANOVA followed by Tukey's test corrected for multiple comparisons. Extreme values, defined as three times the interquartile range, were excluded from the analyses. Significance was set at $P < 0.05$.

Data availability

The data supporting the findings of these studies are available from the corresponding author upon reasonable request.

Results

Microglia morphology and inflammatory cytokine expression were analyzed in a total of 127 *post-mortem* brains ranging from fetuses, children, young adults and older adults with Down syndrome and age-matched controls. The demographics of the study population are described in Supplementary Tables 2-1-4. For the microglial morphological analyses, children in the Down syndrome group were younger than the controls (1 ± 0 vs 4.66 ± 2.06 years-old). No demographical differences were found between groups when analyzing rod microglia. For inflammatory protein expression analyses, no differences were found in *post-mortem* interval (PMI) between groups except for the young adult group, in which the PMI was longer in individuals with Down syndrome (21 ± 5.21 hours) compared to controls (14.63 ± 7 hours). No significant differences were found in the frequency of females or males between each age group.

Altered microglial phenotype and increased brain cytokine expression in infants with Down syndrome

Little is known about alterations in cerebral inflammatory protein expression in fetuses and children with Down syndrome. To fill this gap, we first analyzed the levels of key inflammatory markers in the frontal cortex of fetuses with Down syndrome and euploid controls. Fetal Down syndrome brain homogenates did not display significant differences in the concentration of inflammatory markers when compared to the euploid group. However, a trend towards higher levels of IL-6 and IL-15 was observed (Supplementary Figure 2-1).

We expanded the analysis of inflammatory markers to mixed fetal cortical primary cultures. A significant increase in the levels of the interleukins IL-1α, IL-4, IL-6, IL-7, IL-10, IL-12 and in the chemokines IL-8, MDC, MIP1β, GROα and G-CSF was observed in the conditioned media from Down syndrome fetal cortical cells (Supplementary Figure 2-2), indicating that inflammatory changes are detectable at the cellular level at this early age.

In agreement with the increased inflammatory expression in Down syndrome fetal cortical cells, we found that microglial cells from children with Down syndrome (1 – 10 years) displayed early and significant morphological changes (Figure 2-1A-D) before Aβ deposition or tau

hyperphosphorylation (Supplementary Table 2-7), as indicated by an increase in the dimensional ratio as compared to controls (Figure 2-1E). At this stage, no changes in in microglial density (Figure 2-1F) were noted.

Inflammatory protein analyses further revealed that IL-8 and IL-10 levels were significantly higher, while VEGF-A was lower in the frontal cortex of children with Down syndrome (Figure 2-1 G-I). IL-1β, IL-13, MDC and IL-15 showed a trend towards higher expression in this group (Figure 2-1J-M). Thus, individuals with Down syndrome exhibit early changes in neuroinflammatory markers and microglia morphology before the overt development of Alzheimer's pathology.

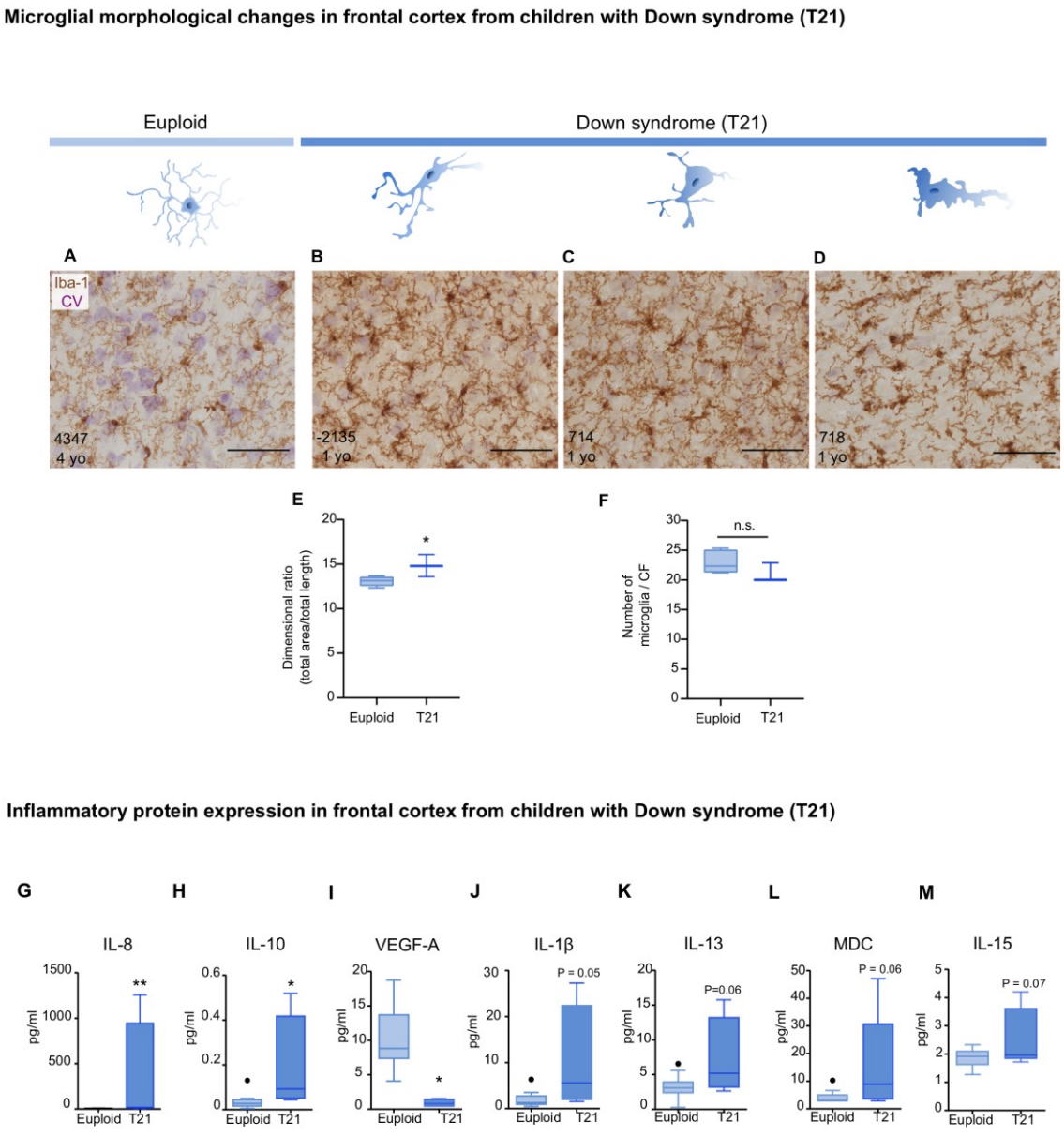

Figure 2- 1. Microglial morphological changes and increased inflammatory mediators in the frontal cortex of children with Down syndrome.

Upper panel: (A-F) Microglial cells were detected by immunohistochemistry against the monocytic marker Iba-1 (brown). Cresyl violet (CV) staining was used to identify cortical laminae. (A-D) Representative micrographs of control (A) and Down syndrome (B-D) cases (1 -10 years). (A) Microglia from a non-Down syndrome case displays thin and ramified processes which are indicative of a surveying state. (B-D) Down syndrome microglia from infants show thickening of the processes and soma enlargement, characteristic of intermediate activation. (E) Microglia from children with Down syndrome had a higher dimensional ratio than their age matched controls

revealing an intermediate activation state, t (6) = 2.83, *P = 0.02*, Student two-tailed t-test. (F) No significant differences were found in the number of microglia between T21 and euploids t (6) = 1.50; *P = 0.18*, Student two-tailed t-test. Euploid *n* = 6, T21 *n* = 3. *Lower panel:* increased levels (pg/ml) of (G) IL-8 (U = 0, *P = 0.002*) and (H) IL-10 (U = 4, *P = 0.03*) and decreased levels of (I) VEGFA (t (12) = 3.764, *P = 0.002*) were observed in the frontal cortex of children with Down syndrome (1 – 13 years), as examined by a multispot protein electrochemiluminescent assay. A trend towards an increase of (J) IL-1β; U = 6; *P = 0.05* (K) IL-13; t (12) = 2.02, *P = 0.06*; (L) MDC; U = 9, *P = 0.06* and (M*)* IL-15; t (13) = 1.97, *P = 0.07*, was also noted in the Down syndrome group. (G, H, J, L) two-tailed Mann-Whitney test and (I, K, M), two-tailed Student t-test. Data is displayed in boxplots where the median is represented by the horizontal line, the whiskers represent the 25th and 75th percentiles. Full circles represent outliers according to Tukey's rule. Euploid *n* = 10, T21 *n* = 5. Scale bar = 50µm. * *P < 0.05,* ** *P < 0.01*

Enhanced neuroinflammation in young adults with Down syndrome

To further elucidate the evolution of neuroinflammation in Down syndrome as Alzheimer's disease neuropathology progresses, we next examined the frontal cortex of individuals with Down syndrome aged 13 to 25 years. Microglial cells from young adults with Down syndrome displayed soma enlargement and retraction of processes (Figure 2A-D) with a trend towards higher dimensional ratio values (Figure 2-2E), accompanied by sparse amyloid plaques and phosphorylated tau in 50% of the cases (Supplementary Figure 2-3, Supplementary Table 2-7).

Moreover, a heightened neuroinflammatory profile was found in young adults with Down syndrome compared to children. Besides IL-8 upregulation, there was an increase in proinflammatory molecules involved in the acute immune response such as IL-1β, IL-1α, IL-6 and IL-15 and the leukocyte chemoattractants eotaxin-3, IP-10, MDC, and MIP-1β (Figure 2-2G-O).

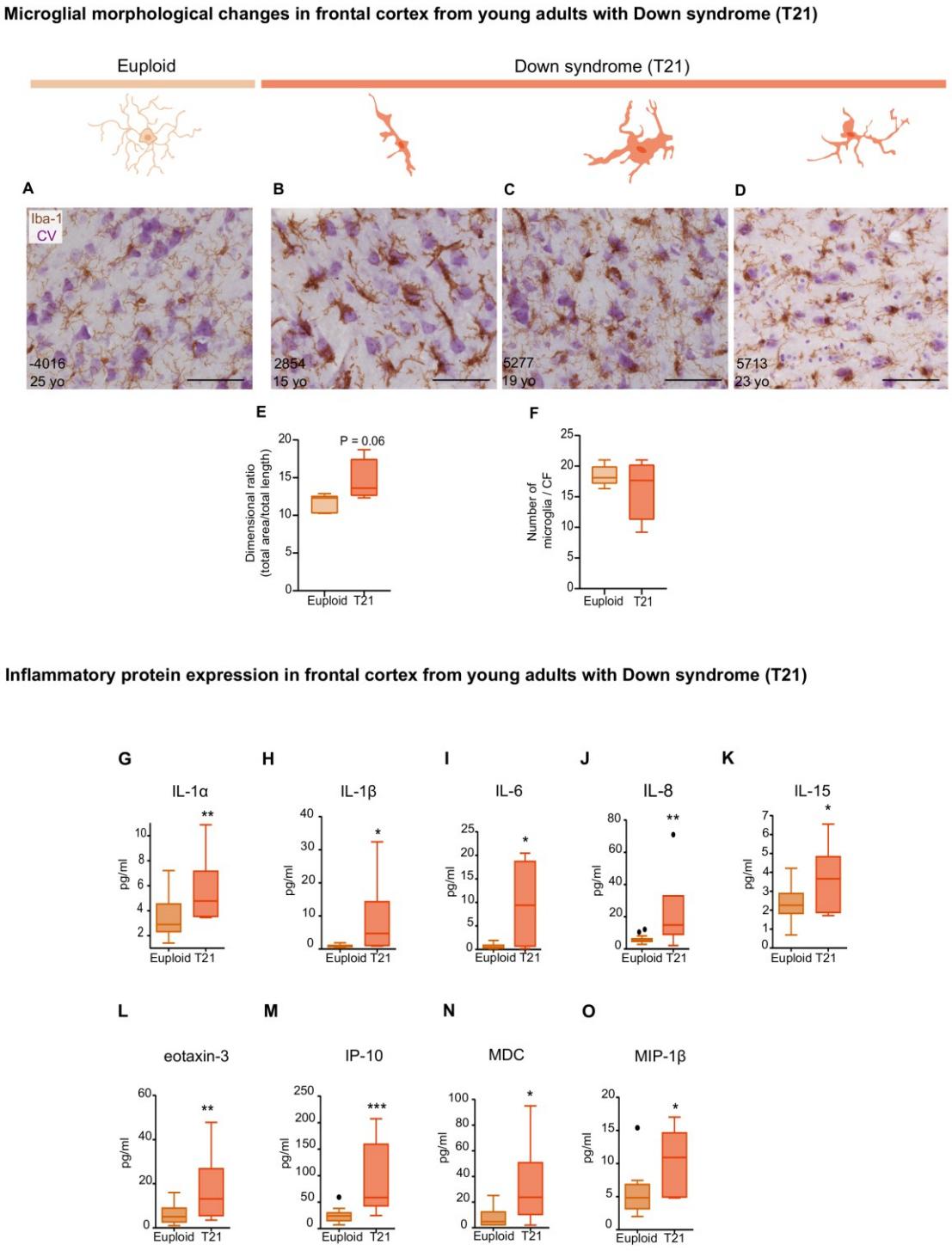

Figure 2- 2. The frontal cortex of young adults with Down syndrome displays an exacerbated inflammatory profile.

Upper panel: (A-D) Microglial cells from young individuals (15 – 25 years) as revealed by immunohistochemistry against Iba-1 and cresyl violet (CV) counterstain. (A) Ramified microglia from a non-Down syndrome young adult (B-D) Down syndrome cases show enlarged microglia and process retraction. (E) A trend towards higher DR in the Down syndrome group was observed

(U = 3, *P = 0.06*), two-tailed Mann-Whitney test (F) The density of microglia remained unchanged at this age, (t (8) = 0.94, *P = 0.37*), Student two-tailed t-test. Euploid *n* = 7, T21 *n* = 4. *Lower panel:* Multispot protein electrochemiluminescent assays revealed that young adults with Down syndrome (13 – 27 years old) have an upregulation of inflammatory markers (pg/ml) before the development of a full-blown Alzheimer's disease pathology (G-O). (G) U= 29, *P = 0.03*; (H) U= 24, *P = 0.01*; (I) U = 25, *P = 0.02*; (J) t (24) = 3.13, *P = 0.004*; (K) t (27) = 2.49, *P = 0.01*; (L) t (28) = 3.22, *P = 0.003*; (M) t (27) = 4.51, *P = 0.0001*, (N) U=25, *P = 0.01*; (O) U = 25, *P = 0.02*. (G-I, N, O), two-tailed Mann Whitney; (J-M), two-tailed Student t-test. Data is displayed in boxplots where the median is represented by the horizontal line, the whiskers represent the 25th and 75th percentiles. Full circles represent outliers according to Tukey's rule. Euploid *n* = 24, T21 *n* = 6. Scale bar = 50µm. * *P < 0.05,* ** *P < 0.01,* *** *P < 0.001*

Neuroinflammation wanes with age in Down syndrome

As reference of advanced stages of Alzheimer's disease pathology, we then investigated the inflammatory status in the frontal cortex of older adults with Down syndrome (39-64 years old). Microglia from older adults with Down syndrome were highly dystrophic, displaying spheroidal swellings, process beading and fragmentation (Figure 2-3A-D). We also observed a significant increase in the dimensional ratio in this age group accompanied by a decrease in the number of intact microglia (Figure 2-3E-F), possibly reflecting the overt Alzheimer's disease pathology at this stage (Supplementary Figure 2-3, Supplementary Table 2-7).

Most inflammatory markers that were upregulated in the young adult Down syndrome group were also upregulated in the frontal cortex of older adults with Down syndrome as revealed by increases in IL-1β, IL-6, IL-15, IL-8, eotaxin-3, IP-10, MDC, MIP-1β, and a decrease of VEGF-A (Figure 2-3G-U). Moreover, chemoattractants such as MCP-1 and TARC were also upregulated in the frontal cortex of older adults with Down syndrome. Surprisingly, our analyses also revealed a downregulation of IFNγ, IL-12p40, IL-10 and TNFα in the older adult Down syndrome frontal cortex at advanced stages of Alzheimer's disease pathology (Figure 2-3R-U).

Given that the hippocampus is highly vulnerable to Alzheimer's disease neuropathology we further analyzed inflammatory protein expression in hippocampal samples available to us for this age group (Supplementary Table 2-2). Although the frontal cortex and hippocampus shared common elevations in IL-1β, IL-6, IL-15, eotaxin-3, MCP-1, MIP-1β, and TARC accompanied by lower levels of VEGF-A in adult Down syndrome brains (Figure 2-4), a differential expression of certain inflammatory markers was observed between these regions. While an upregulation of the chemoattractants IP-10 and MDC was solely observed in the Down syndrome frontal cortex (Figure 2-3L, N), higher levels of IL-1α and IL-4 were exclusively upregulated in the Down syndrome hippocampus (Figure 2-4A, C).

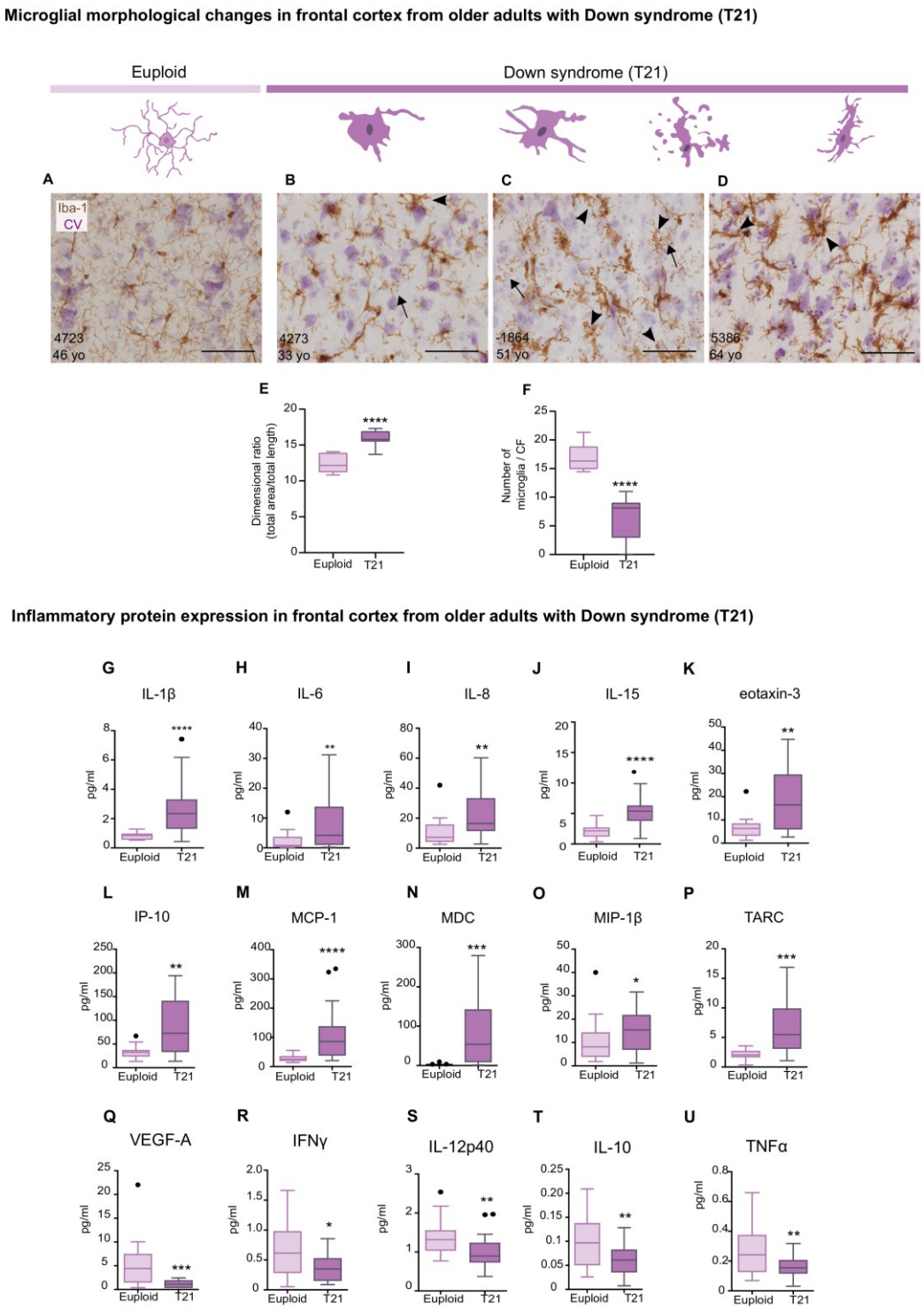

Figure 2- 3. Dystrophic microglia and waning of neuroinflammation in older adults with Down syndrome.

Upper panel: (A-D) Representative photomicrographs of morphologically activated and dystrophic microglia from Down syndrome adult cases (30 – 64 years) as revealed by immunohistochemical assessment of Iba-1 and cresyl violet as counterstain. (B-D) Dystrophic microglia display spheroidal swellings and process beading (arrowheads), and fragmentation

(arrows). (E) An increase in the DR was noted in the Down syndrome cases; t (15) = 5.79, $P < 0.0001$, Student two-tailed t-test (F) The number of non-fragmented microglia is lower in the Down syndrome-adult group; t (15) = 6.89, $P < 0.0001$, Student two-tailed t-test. Euploid $n = 10$, T21 $n = 8$. *Lower panel:* (G-U) Analysis of frontal cortex homogenates by multispot protein electrochemiluminescent assays revealed an increase in inflammatory expression (pg/ml) in (G-P) along with a decrease of inflammatory mediators in adults with Down syndrome (39 – 68 years) (Q-U). (G) U = 35, $P < 0.0001$; (H) U = 82, $P = 0.01$; (I) U = 72, $P = 0.005$; (J) t (37) = 4.73, $P < 0.0001$; (K) U = 70, $P = 0.008$; (L) t (34) = 3.48, $P = 0.001$; (M) U = 34, $P < 0.0001$; (N) U = 51.5, $P = 0.0004$; (O) U = 117, $P = 0.04$; (P) U = 57, $P = 0.0005$; (Q) U = 63.5, $P = 0.0004$; (R) t (36) = 2.59, $P = 0.01$; (S) U = 74, $P = 0.001$; (T) t (36) = 2.87, $P < 0.006$; (U) t (35) = 2.72, $P = 0.009$. (G-I, K, M-P, R) two-tailed Mann Whitney tests; (J, L, R, T, U), two-tailed Student t-test. Data is displayed in boxplots where the median is represented by the horizontal line, the whiskers represent the 25th and 75th percentiles. Full circles represent outliers according to Tukey's rule. Euploid $n = 16$, T21 $n = 22$. Scale bar = 50µm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

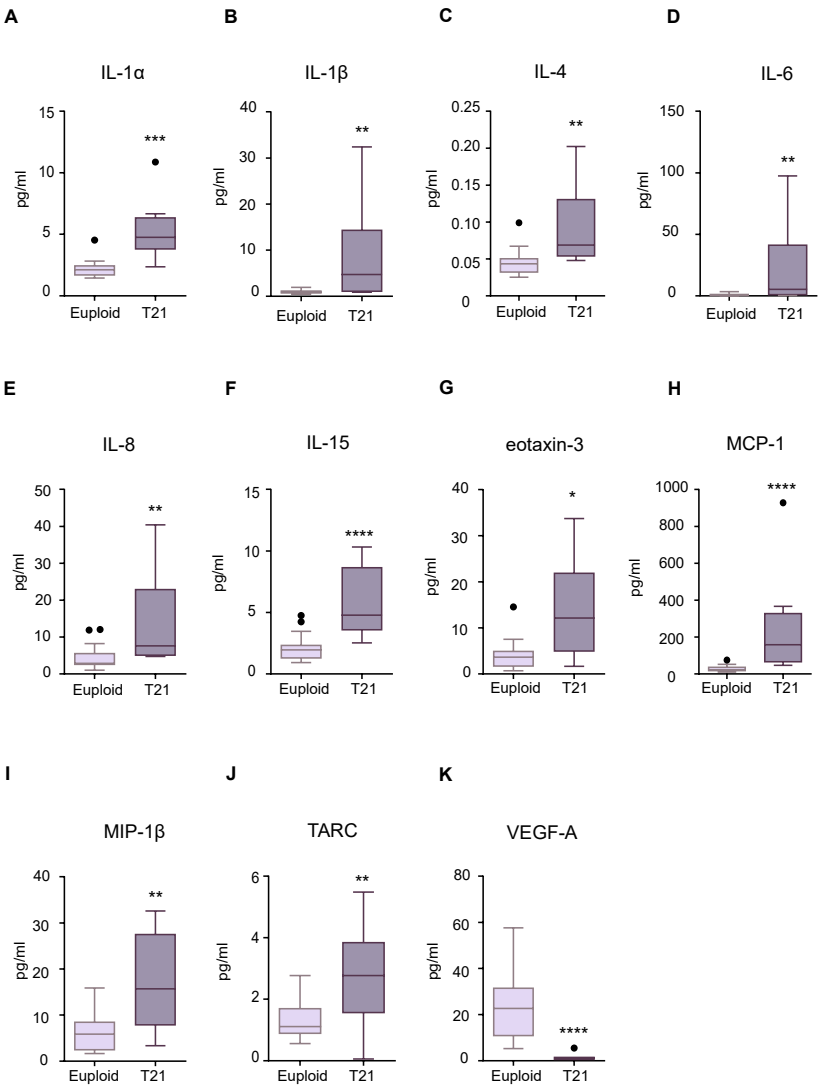

Figure 2- 4. Differential expression of inflammatory markers in the hippocampus of older adults with Down syndrome.

Multispot protein analyses revealed an inflammatory signature which is specific to the hippocampal region in adults with Down syndrome (41 – 66 years). (A) U = 7, *P = 0.0001*; (B) U = 17, *P = 0.007*; (C) U = 16, *P = 0.001*; (D) U = 24, *P = 0.009*; (E) U = 19, *P = 0.006*; (F) U = 9, *P = 0.0001*; (G) U= 22, *P = 0.01*; (H) U = 4, *P < 0.0001*; (I) t (23) = 3.74, *P = 0.001*; (J) t (24) = 3.25, *P = 0.003*; (K) U = 1, *P < 0.0001.* (A-H, K), two-tailed Mann Whitney tests; (I-J), two-tailed Student t-test. Data is displayed in boxplots where the median is represented by the horizontal line, the whiskers represent the 25th and 75th percentiles. Full circles represent outliers according to Tukey's rule. Euploid *n* = 17, T21 *n* = 8. * *P < 0.05, ** P < 0.01, *** P < 0.001, ****P < 0.0001*

Occurrence of rod-like microglia in Down syndrome brains

While analyzing microglia morphology we noticed the occurrence of a distinct, rod-shaped phenotype of microglia in the brains of young adults and adults with Down syndrome (Figure 2-5). As previously reported in other neuropathologies including Alzheimer's disease (Bachstetter *et al.*, 2015), rod microglia were also found forming trains along cortical layers (Figure 2-5A). We furthered quantified the number of rod microglia in the brains of young adults and adults with Down syndrome and compared it to euploid age-matched controls. As illustrated in Figure 2-5B, the brains of young adults and adults with Down syndrome displayed a remarkable increase in rod-microglia as compared to euploid brains. As exemplified in Figure 2-5C, we also observed that some rod microglial cells were near and aligned to neurons bearing tau neurofibrillary tangles.

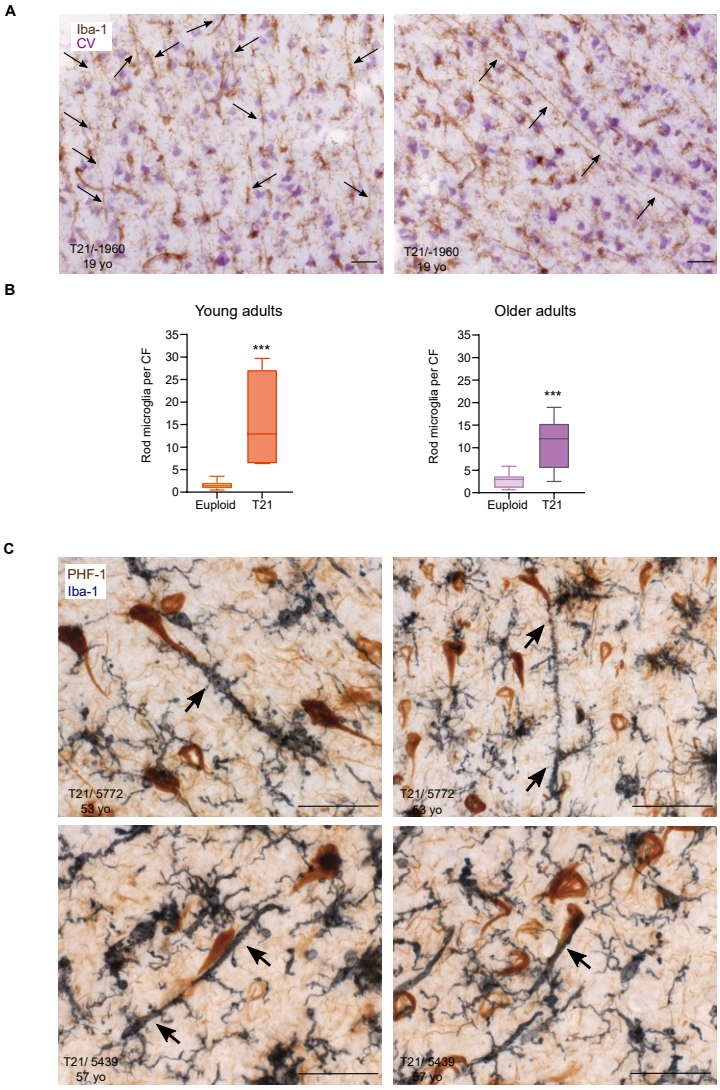

Figure 2- 5. Rod shaped microglia in young and adult Down syndrome brains.

(A) Micrographs depicting rod microglia (arrows) in the frontal cortex of a 19-year-old individual with Down syndrome (left) and a train of rod microglial cells (right) as revealed by immunohistochemistry (IHC) against the monocytic marker Iba-1 (brown). Cresyl violet (CV) was used as a counterstain. (B) Increased number of rod microglial cells in the brains of young adults ($t(14) = 4.57$; $P = 0.0004$) and older adults with Down syndrome ($t(19) = 4.54$; $P = 0.0002$) as compared to euploid controls. Student two-tailed t-test. Euploid young adults $n = 12$, T21 young adults $n = 4$; euploid adults $n = 10$, T21 adults $n = 11$. (C) IHC against Iba-1 (blue) and pathological tau as revealed by PHF-1 (brown) in the frontal cortex of a 53-year-old (upper panel) and a 57-year-old (bottom panel) individual with Down syndrome. Note the close alignment of rod microglia to PHF+ neurons. Data is displayed in boxplots where the median is represented by the horizontal line, the whiskers represent the 25th and 75th percentiles. Scale bar = 50µm. *** $P < 0.001$

Females with Down syndrome display heightened brain inflammatory protein expression

We next investigated whether inflammatory protein expression is affected by sex and genotype in the adult population. We found that IL-6 expression levels were dependent on sex and genotype. Post-hoc analyses revealed that females with Down syndrome had higher levels of IL-6 than males with Down syndrome as illustrated in Figure 2-6A. Furthermore, females in general (T21 and euploid) had higher levels of IL-8 and Eotaxin-3 than males; however, females with Down syndrome had higher levels of IL-8 than males with Down syndrome (Figure 2-6B, C).

Neuroinflammation peaks before the development of an overt Alzheimer's pathology in Down syndrome

Given the differential expression of inflammatory markers across age intervals we were interested to elucidate if the neuroinflammatory process peaked at a certain age in Down syndrome. To account for aging effects on inflammatory protein expression, each Down syndrome group was normalized against its own age-matched control group. Our results indicate that IL-6 is significantly more upregulated in young adults with Down syndrome (~15-fold change with respect to euploid individuals) as compared to the Down syndrome older adult population (~ 4-fold change) (Figure 2-7A). Instead, the monocytic chemoattractant MCP-1 (CCL2) was found significantly more upregulated in the older adult Down syndrome population in relation to children with Down syndrome (Figure 2-7B). Moreover, a drop in the expression levels of IL-10, TNFα, and IFNγ was observed in the older adults with Down syndrome when compared to children with Down syndrome (Figure 2-7C-E).

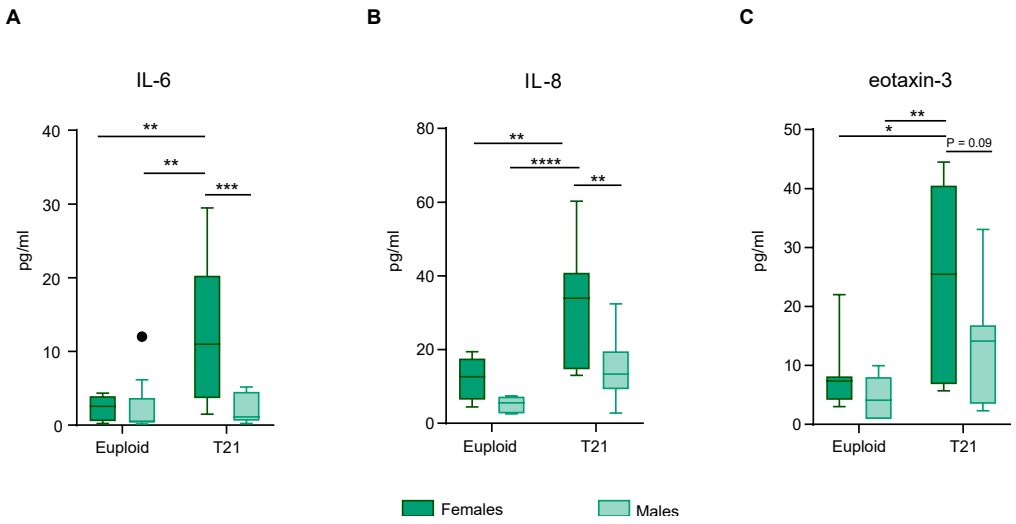

Figure 2- 6. Adult females with Down syndrome display a more pronounced cortical inflammatory profile than males with Down syndrome.

(A) T21 females have higher IL-6 levels when compared to T21 males (39 – 68 years). Two-way ANOVA, effect of genotype, $F_{(1, 30)}= 7.03$, *P = 0.01,* effect of sex, $F_{(1, 30)}= 7.79$, *P = 0.009*, sex × genotype interaction, $F_{(1, 30)}= 8.13$ *P = 0.007* followed by Tukey's post-hoc test (T21 females vs T21 males, *P = 0.0009*; T21 females vs euploid males *P = 0.001* and T21 females vs euploid females, *P = 0.005*, euploid females vs euploid males *P > 0.9999*). (B) A significant increase in IL-8 levels was found in T21 females when compared to T21 males. Two-way ANOVA, effect of genotype, $F_{(1, 29)} = 15$, *P = 0.0005,* effect of sex, $F_{(1, 29)} = 10$, *P = 0.003*, sex × genotype interaction, $F_{(1, 29)} = 1.7$ *P = 0.20* followed by Tukey's post-hoc test (T21 females vs T21 males, *P = 0.004*, T21 females vs euploid males, *P < 0.0001*, and T21 females vs euploid females; *P = 0.009*). (C) T21 females had higher levels of Eotaxin-3 when compared to female and male euploids. Two-way ANOVA, effect of genotype, $F_{(1, 31)} = 10.85$, *P = 0.002,* effect of sex, $F_{(1, 31)} = 4.34$, *P = 0.04*, sex × genotype interaction, $F_{(1, 31)} = 0.99$, *P = 0.32* followed by Tukey's post-hoc test (T21 females vs euploid females, *P = 0.02*; T21 females vs euploid males, *P = 0.003)*. Data is displayed in boxplots where the median is represented by the horizontal line, the whiskers represent the 25th and 75th percentiles. Full circles represent outliers according to Tukey's rule. Euploid males *n* = 9, euploid females *n* = 7, T21 males *n* = 12, T21 females *n* = 10. * *P < 0.05,* ** *P < 0.01,* *** *P < 0.001,* ****P < 0.0001*

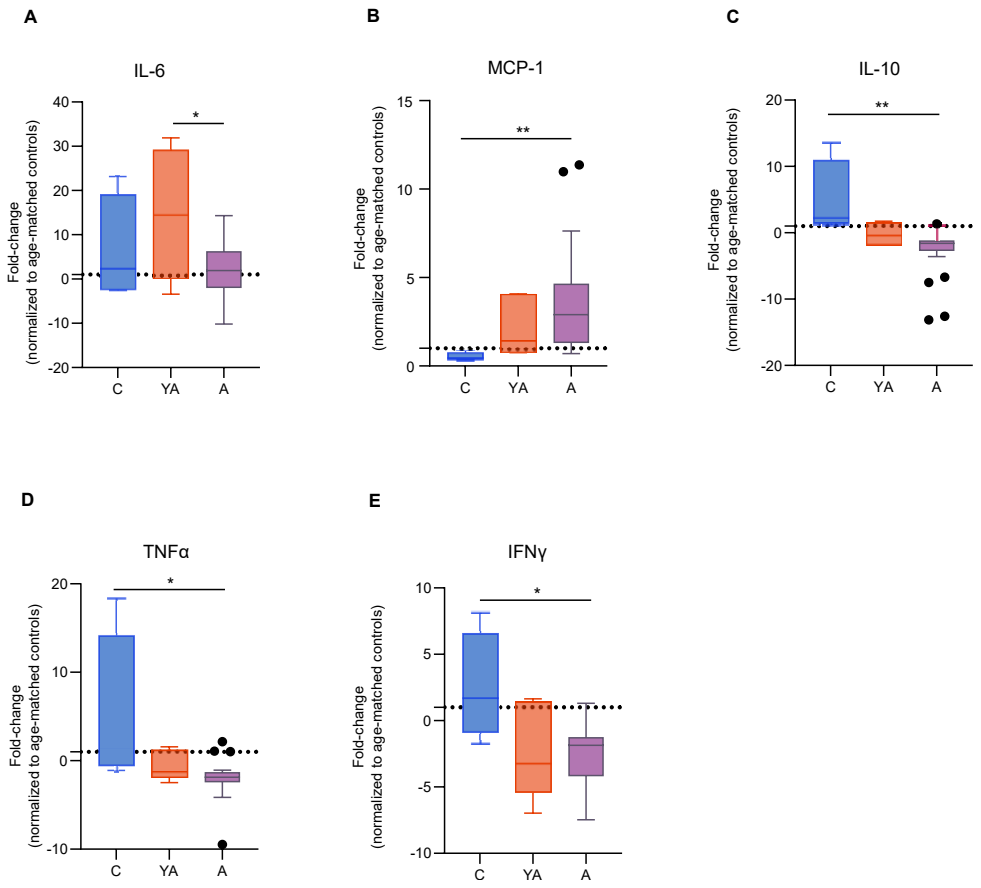

Figure 2- 7. Neuroinflammation is exacerbated before the development of an overt Alzheimer's disease pathology in Down syndrome.

(A) IL-6 levels where significantly higher in young adults (13 – 27 years) with Down syndrome than in older adults with Down syndrome (39 – 68 years). One-way ANOVA, $F_{(2, 27)} = 4.34$, $P = 0.02$ followed by Tukey's post-hoc test (young Down syndrome adults vs Down syndrome adults $P = 0.01$). (B) The Down syndrome adult group showed higher levels of MCP-1 when compared to children with Down syndrome (1 – 10 years). Kruskal-Wallis test, MCP-1 H = 9.36, $P = 0.009$ followed by Dunn's post-hoc test (T21 children vs T21 adults, $P = 0.007$). (C-E) IL-10, TNFα and IFNγ expression levels were higher in children with Down syndrome than in the Down syndrome adult group. Kruskal-Wallis test followed by Dunn's post-hoc test, IL-10 H = 10.64, $P = 0.004$, T21 children vs T21 adults, $P = 0.004$; TNFα H = 9.01, $P = 0.01$, T21 children vs T21 adults, $P = 0.01$; IFNγ H = 6.84, $P = 0.03$, T21 children vs T21 adults, $P = 0.02$. Data is displayed in boxplots where the median is represented by the horizontal line, the whiskers represent the 25th and 75th percentiles. Full circles represent outliers according to Tukey's rule. The fold change was calculated by normalizing each age group to its own age-matched control. Dotted line represents age-matched control levels. C = children, YA = young adults, A = adults. T21 children, $n = 4$; T21 young adults, $n = 6$; T21 adults, $n = 20$. * $P < 0.05$, ** $P < 0.01$

Discussion

This is the first comprehensive study to evaluate the progression of neuroinflammation across the lifespan of individuals with Down syndrome, one of the largest populations at genetic risk of Alzheimer's disease. Here, we report that young individuals with Down syndrome (age range 1-25 years) displayed an exacerbated and progressive brain inflammatory process characterized by microglial intermediate activation, increase in rod microglia, and increased levels of inflammatory mediators before the occurrence of overt Alzheimer's disease pathology. In turn, concomitant with the development of a full-blown Alzheimer's disease pathology, older adults with Down syndrome displayed highly dystrophic microglia accompanied by an inflammatory signature indicative of a failure in resolution of inflammation processes.

Thus, our findings support the concept of a dual neuroinflammatory scenario, in which key inflammatory mediators are exacerbated before the occurrence of overt Alzheimer's disease pathology and wane after its presentation, as schematically summarized in Figure 2-8. Such observations suggest that anti-inflammatory therapeutic interventions should be most beneficial at the earliest Alzheimer's disease stages, when the inflammatory process is likely of a "disease-aggravating" nature (Cuello, 2017). Indeed, epidemiological studies indicate that NSAIDs administered at presumable preclinical Alzheimer's disease stages diminish the risk of developing Alzheimer's disease-dementia, whereas administration at symptomatic stages has no effects or even worsens the disease (Cuello, 2017; McGeer *et al.*, 2013). Moreover, it is well supported that anti-inflammatory treatments in individuals with underlying systemic inflammation have a sparing effect on Alzheimer's disease (McGeer *et al.*, 2013). In consequence, as individuals with Down syndrome display increased systemic inflammation and immune cell activation (Iulita *et al.*, 2016; Sullivan *et al.*, 2017; Waugh *et al.*, 2019), they represent a significant population which could benefit from preventive anti-inflammatory treatment. The origin of early neuroinflammation in Down syndrome brains has not been fully elucidated. However, it is known that early intraneuronal Aβ accumulation can unleash a disease-aggravating neuroinflammatory response in animal models of Alzheimer's disease-like amyloid pathology (Ferretti *et al.*, 2012; Hanzel *et al.*, 2014; Janelsins *et al.*, 2005). The link between Aβ and inflammation is further strengthened by the observation that in such Alzheimer's disease models, early anti-inflammatory treatment ameliorates Aβ-induced pathology and rescues cognitive deficits, highlighting the detrimental nature of early

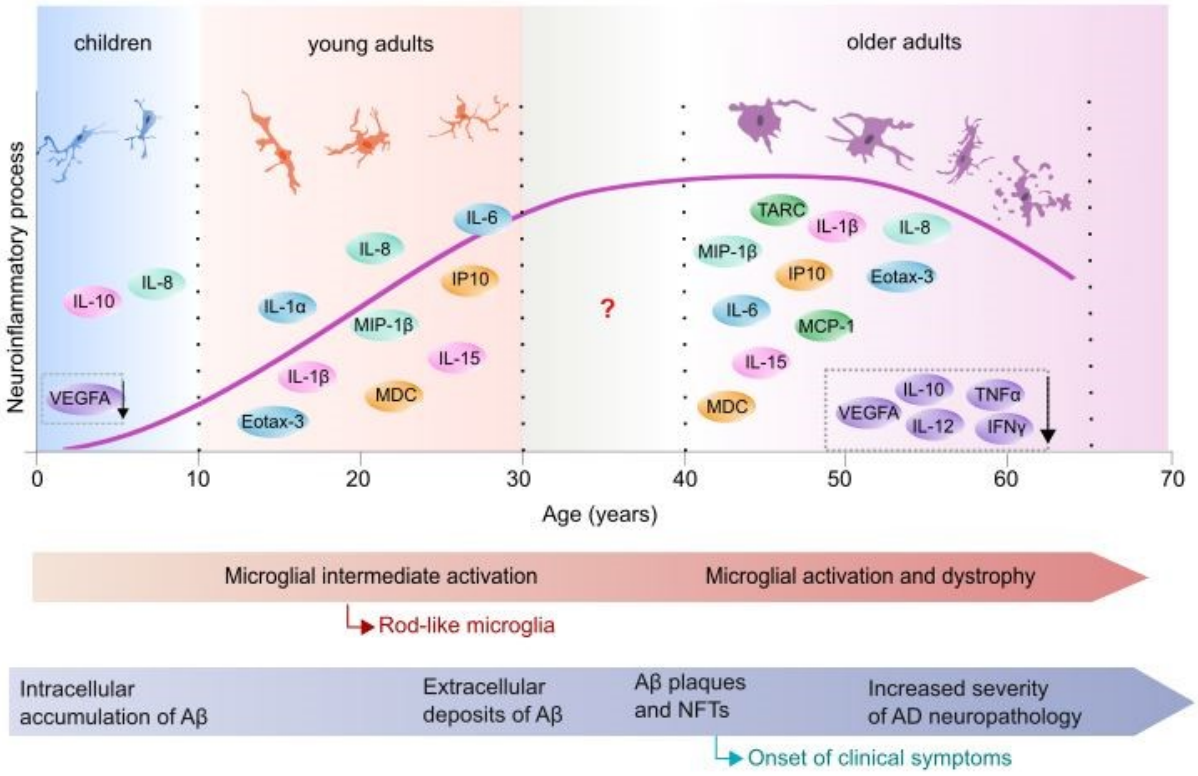

Figure 2- 8. Schematic representation of the evolution of brain inflammation across the lifespan in Down syndrome.

Early microglial morphological changes in process thickening and retraction as well as increases in rod microglia accompanied by a heightened neuroinflammatory profile was observed in children and young individuals with Down syndrome, at stages when limited or no Alzheimer's disease pathology is present. The late inflammatory process in Down syndrome is characterized by an increase in dystrophic microglia and chronic neuroinflammation along with a decrease in inflammatory marker expression possibly reflecting an impairment in the resolution of inflammation. Modified image from (Lott *et al.*, 2019) with permission from SpringerNature

neuroinflammation (Ferretti *et al.*, 2012; McAlpine *et al.*, 2009).detrimental nature of early neuroinflammation (Ferretti *et al.*, 2012; McAlpine *et al.*, 2009). Therefore, we postulate that the presence of intraneuronal Aβ in young individuals with Down syndrome is likely a key driver of neuroinflammation in this population.

In line with this, our analyses in primary fetal Down syndrome mixed cortical cultures, revealed a marked upregulation of inflammatory mediators. While fetal brain homogenates did not reveal changes in inflammatory markers, the results with the fetal cultures highlight that important inflammatory alterations may be occurring at the cellular level at very early stages. Indeed, microglia from children with Down syndrome displayed an intermediate activation state characterized by soma enlargement, process thickening and retraction; a phenotype associated to the intraneuronal accumulation of Aβ in animal models of Alzheimer's disease-like pathology (Ferretti *et al.*, 2012; Hanzel *et al.*, 2014). Thus, reactive microglia at preclinical Alzheimer's disease stages in Down syndrome would contribute to a heightened neuroinflammatory profile.

Moreover, we observed that brains of children with Down syndrome showed a marked upregulation of IL-10 and IL-8. A detrimental effect of these cytokines is indicated by prior studies showing that high levels of IL-10 promote cognitive deficits and impair microglial Aβ phagocytosis in animal models (Chakrabarty *et al.*, 2015; Guillot-Sestier *et al.*, 2015). Therefore, increased IL-10 levels in the brains of children with Down syndrome would contribute to worsen Aβ pathology. IL-8, a potent chemoattractant of neutrophils (Hammond *et al.*, 1995), is elevated in the CSF of individuals with mild cognitive impairment (MCI) and Alzheimer's disease (Galimberti *et al.*, 2006), indicating its involvement also in the general population. Moreover, the annual change in IL-8 plasma levels plasma has been shown to predict prospective cognitive decline in adults with Down syndrome without symptomatic manifestation of Alzheimer's disease (Iulita *et al.*, 2016). Notably, IL-8 was the only cytokine chronically overexpressed in Down syndrome across all the age brackets analyzed; thus, its chronic upregulation could feed forward an innate immune response in Down syndrome brains. Additionally, infants and adults with Down syndrome displayed a downregulation of VEGF-A. Low levels of VEGF have been associated to a deficiency in neural perfusion and motoneuron degeneration (Oosthuyse *et al.*, 2001), impairments which are also observed in the Down syndrome population (Gokcora *et al.*, 1999; Gupta *et al.*, 2011; Kao *et al.*, 1993; Watson-Scales *et al.*, 2018). Decreased VEGF levels in Down

syndrome might be attributed to overexpression of VEGF negative regulators encoded in chromosome 21 (Arron *et al.*, 2006; Y. M. Kim *et al.*, 2002).

Interestingly, a different set of inflammatory mediators was detected in the frontal cortex of young individuals with Down syndrome (13-25 years). At this stage, upregulation of cytokines involved in the acute inflammatory response such as IL-1 and IL-6 would likely accelerate Alzheimer's neuropathology by potentiating neuroinflammation and Alzheimer's disease pathology (Akiyama *et al.*, 2000). Our analyses also revealed that IL-6 levels are highest in young Down syndrome brains compared to all other age brackets. This significant finding supports the concept that younger individuals with Down syndrome have a heightened neuroinflammatory response before the establishment of overt Alzheimer's disease pathology.

Further to it, we found increased levels of MIP-1β, IL-15 and IP-10, cytokines found in Alzheimer's disease astrocytes under inflammatory clinical conditions (Gomez-Nicola *et al.*, 2008; Krauthausen *et al.*, 2015; M. Li *et al.*, 2017; Saikali *et al.*, 2010; Xia *et al.*, 2000). Moreover, IL-15 levels are increased in the CSF of Aβ-positive non-cognitively impaired (NCI) and MCI individuals, again suggesting their likely CNS upregulation in the general population (Janelidze *et al.*, 2018). CSF and plasma IP-10 levels rise before the onset of Alzheimer's disease and its deregulation in early MCI could contribute to vascular impairments (Galimberti *et al.*, 2006; Iturria-Medina *et al.*, 2016). Thus, upregulation of these cytokines could indicate the occurrence of astroglial activation and early vascular impairments in Down syndrome brains well before clinical Alzheimer's disease; as previously reported in fetuses and neonates with Down syndrome and familial Alzheimer's disease (Griffin *et al.*, 1989; Rodriguez-Vieitez *et al.*, 2016). Finally, an increase in the immune cell recruiters, eotaxin-3 and MDC, was also noted in the brains of young adults with Down syndrome, as also found in other CNS disorders (Columba-Cabezas *et al.*, 2002; Craig-Schapiro *et al.*, 2011; Galimberti *et al.*, 2008; Huber *et al.*, 2018; Niven *et al.*, 2015; Ogilvie *et al.*, 2003).

Moreover, for the first time we identified and report the occurrence of rod microglia in the young Down syndrome brain. Such microglia phenotype was found increased at young and adult stages in Down syndrome. Rod microglia were firstly described by Nissl in the early 1900's in *post-mortem* brain tissue from paralytic individuals; however, their function remains elusive. Rod microglia display a polarized shape consisting of retraction of planal processes, and extension of

polar processes; such characteristics give rod microglia their thin and elongated morphology (Taylor *et al.*, 2014). The occurrence of rod microglia has been reported in several neurological conditions (Boche *et al.*, 2013; Sapp *et al.*, 2001; Sierra *et al.*, 2016; Wierzba-Bobrowicz *et al.*, 2002) including Alzheimer's disease (Bachstetter *et al.*, 2015) and formation of "trains" of rod microglial cells along neuronal processes has been observed during the initial phase of CNS injury (Au *et al.*, 2017; Rojas *et al.*, 2014; Tam *et al.*, 2014; Taylor *et al.*, 2014; Ziebell *et al.*, 2012). Such close alignment to injured axons has prompted the hypothesis that rod microglia might contribute to synaptic stripping (Au *et al.*, 2017). If such is the case, early synaptic stripping in Down syndrome might facilitate synaptic and cognitive deficits. Interestingly, our observations would suggest that brains with less dystrophic and amoeboid microglia display instead an increase in rod microglial cells; alterations which might be characteristic of Alzheimer's disease pathologies. Transgenic Alzheimer's disease rodent models with tau pathology and models of tauopathy have been found to display rod-shaped microglia (Adaikkan *et al.*, 2019; Malcolm *et al.*, 2019) while amyloid models are devoid of such morphological phenotype. Furthermore, rod microglial cells have been encountered close to axons with abnormal tau accumulation in Alzheimer's disease brains (Bachstetter *et al.*, 2015; Odawara *et al.*, 1995). Such observations would indicate that rod microglia might be associated to the occurrence of tau pathology in Down syndrome and Alzheimer's disease, aligning to injured axons with tau aggregation, as we have observed in the Down syndrome brain. Additional in-depth investigations are required to establish the phenotype and function of rod microglia in physiological and pathological conditions.

The brains of older adults with Down syndrome displayed highly dystrophic and activated microglia as previously reported (Xue *et al.*, 2011) along with an increase in rod microglia; further indicating an inadequate termination of neuroinflammation in this group. Concordantly, the brains of older adults with Down syndrome displayed waning of neuroinflammation and a defective tissue resolution response, characterized by low levels of IFNγ, IL-10, IL-12p40, and TNFα. Such profile, in the context of a prolonged chronic inflammation, indicates a failure in the resolution of inflammation as some of these molecules have been recently regarded to be required for a successful inflammation-resolution response (Schwartz *et al.*, 2014; Wang *et al.*, 2015). In contrast, MCP-1 (CCL2) was exclusively upregulated in older adults with Down syndrome. MCP-1 is a monocytic chemoattractant that might mediate monocytic infiltration into the CNS in Alzheimer's disease (El Khoury *et al.*, 2007; Simard *et al.*, 2006; Theriault *et al.*, 2015). Its

increased upregulation at late stages of the neuroinflammatory response in Down syndrome might play an important role in the resolution of inflammation by phagocytosing toxic protein aggregates and cellular debris (Theriault *et al.*, 2015; Wang *et al.*, 2015). Notably, the upregulation of TARC, a cytokine implicated on maintaining the surveying or resting state of microglia (Fulle *et al.*, 2018), could reflect a compensatory mechanism to mitigate chronic brain inflammation. The above findings would explain the failure of anti-inflammatory therapies at Alzheimer's disease clinical stages.

Besides the temporal regulation of inflammation in the Down syndrome brain, we also observed a distinct neuroinflammatory profile between the frontal cortex and hippocampus of older adults with Down syndrome. The hippocampal inflammatory signature was similar to that of younger Down syndrome adults. As such, the hippocampus of older adults with Down syndrome did not display a downregulation of IFNγ, IL-10, IL-12p40, and TNFα as noticed in the frontal cortex. It is likely that these regional differences are associated to the extent of Aβ deposition in the Down syndrome brain as imaging studies have found fibrillar Aβ deposition firstly in the frontal cortex than in the hippocampus (Annus *et al.*, 2016; Davidson *et al.*, 2018).

Furthermore, this report provides the first evidence of an impact of sex on inflammatory protein expression in the Down syndrome brain. Females with Down syndrome displayed higher IL-6 and IL-8 brain levels compared to males. Although few studies have investigated the occurrence of sex differences in Down syndrome, there is evidence that females with Down syndrome display higher tau burden than males with Down syndrome (Raghavan *et al.*, 1994) and a faster rate of cognitive decline after menopause (Patel *et al.*, 2001). The loss of estrogens during menopause has been partly associated to higher Alzheimer's disease risk in women (Alzheimer's, 2014). Estrogens can exert anti-inflammatory actions by disrupting IL-6 and IL-8 transcription factors (R. Li *et al.*, 2000; Liu *et al.*, 2005; Luo *et al.*, 2016; Reed *et al.*, 2004; Roebuck, 1999; Vegeto *et al.*, 2006; Villa *et al.*, 2015). Therefore, the loss of estrogens in females with Down syndrome might contribute to an increased CNS inflammatory response.

The findings of this study must be considered within its limitations. Although this report represents the most comprehensive cross-sectional analysis of inflammatory protein expression and microglia morphology done so far in the largest cohort of Down syndrome brains, the difficulties in obtaining well-preserved *post-mortem* brain tissue led to limited sample size in some groups. Another

limitation was the lack of available relevant neuropsychological data for most cases to indicate the presence or absence of symptomatic Alzheimer's disease, particularly in the older cases. Furthermore, given the paucity of available information we cannot ascertain if comorbidities or medications affected the ongoing neuroinflammatory process in each case. Of note, besides the progressive buildup of Alzheimer's disease pathology in Down syndrome brains, some genes encoded in chromosome 21 could also have a significant impact on the development of brain and peripheral inflammation (Iulita *et al.*, 2016; Sullivan *et al.*, 2017; Waugh *et al.*, 2019). However, as emphasized by Wilcock and Head, the differential cerebral inflammatory profile observed by us and others across different ages underscores that other mechanisms besides the triplication of inflammatory genes might be involved (Wilcock *et al.*, 2015). To best investigate the role of Alzheimer's disease pathology in this dysregulation, it would be of utmost importance to analyze inflammatory protein expression in individuals with Down syndrome lacking the APP triplication (Prasher *et al.*, 1998).

In conclusion, analyses of cerebral inflammatory protein expression in Down syndrome brains revealed a temporal and spatial regulation of neuroinflammation across the lifespan of individuals with Down syndrome. In light of the current failures of anti-inflammatory clinical trials conducted in populations at risk of Alzheimer's disease, unraveling the nature and dynamics of the neuroinflammatory response along the Alzheimer's disease continuum is of utmost clinical significance. Epidemiological and preclinical data strongly suggest that the beneficial outcomes of anti-inflammatory treatment in Alzheimer's disease result from early intervention, most likely at preclinical stages (McGeer *et al.*, 2013). The predictability of Alzheimer's disease evolution in Down syndrome as well as their heightened inflammatory profile, make individuals with Down syndrome an important population to be considered for anti-inflammatory clinical trials. The recent identification of diagnostic biomarkers to detect prodromal and clinical Alzheimer's disease in Down syndrome (Fortea *et al.*, 2018) might aid in the recruitment of potential candidates for such trials and in the evaluation of primary outcomes. Our study also invites to take into consideration the effect of sex on anti-inflammatory clinical trial design. The inclusion of individuals with Down syndrome in clinical trials would strongly contribute to improve treatment options for Alzheimer's disease in Down syndrome.

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Competing interests

The authors have no competing interests.

CHAPTER 2

Supplemental Tables and Figures

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Under review, March 2020

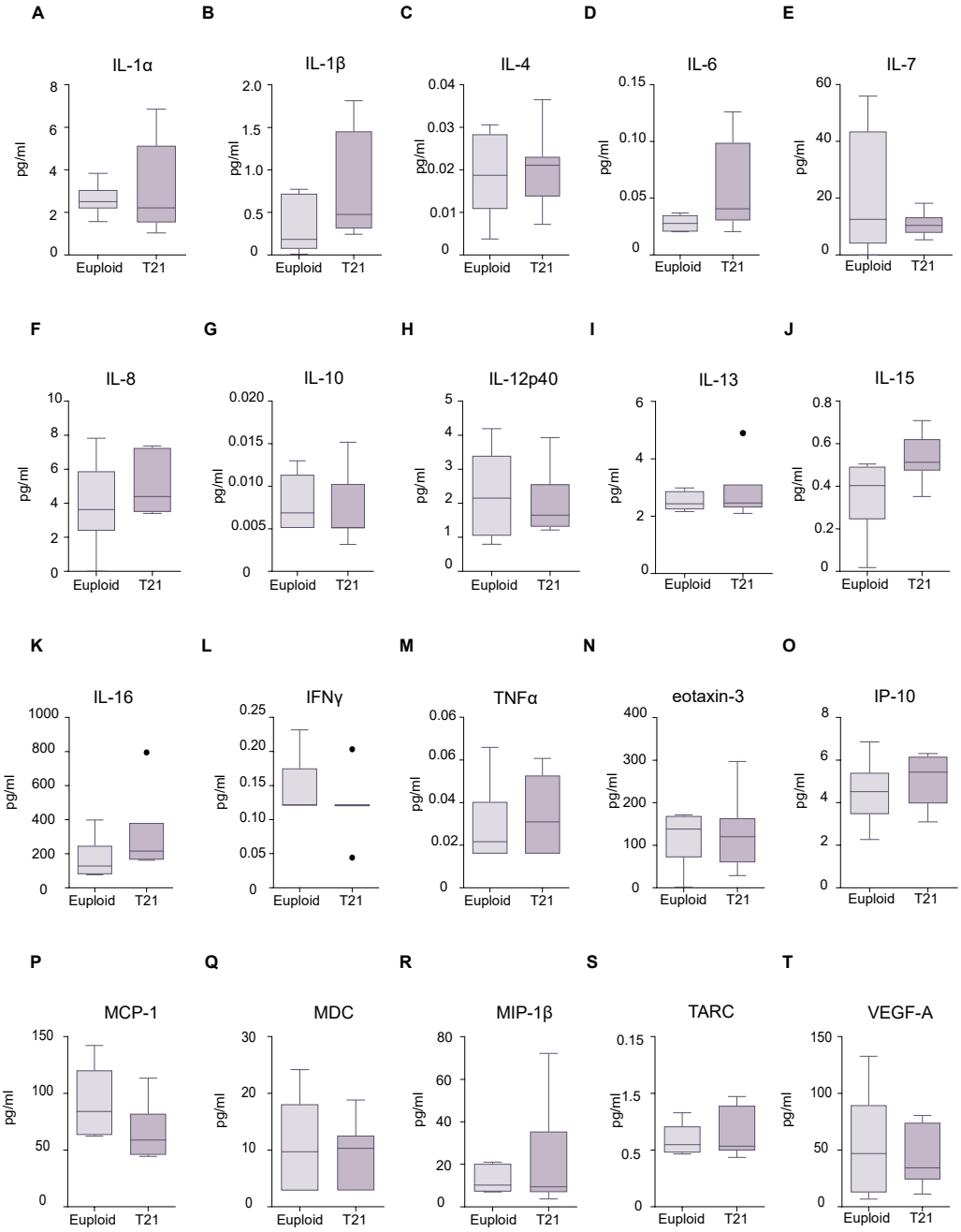

Figure 2-S 1. Fetuses with Down syndrome do not show an altered neuroinflammatory profile.

Multispot protein analyses of the frontal cortex of fetuses did not reveal differences in inflammatory marker expression (pg/ml) between the Down syndrome and euploid fetal groups. (**A**) t (11) = 0.49, *P = 0.62*; (**B**) U = 10, *P* = 0.13; (**C**) t (11) = 0.20, *P = 0.37;* (**D**) U = 6.5, *P = 0.06*; (**E**) t (11) = 1.21, *P = 0.24*; (**F**) t (10) = 0.88, *P = 0.39*; (**G**) t (11) = 0.17, *P = 0.86;* (**H**) t (11) = .36, *P = 0.71*; (**I**) U = 16, *P = 0.*87; (**J**) t (11) = 2.003, *P = 0.07*; (**K**)U = 9, *P = 0.10*; (**L**) U

= 15, *P = 0.41*; (**M**)U = 16, *P = 0.50*; (**N**) t (11) = 0.27, *P = 0.78*; (**O**) t (11) = 0.57, *P = 0.57;* (**P**) t (11) = 1.464, *P = 0.17;* (**Q**) t (11) = .47, *P = 0.64;* (**R**) *U= 21, p >0.99;* (**S**) t (11) = 0.66, *P = 0.51;* (**T**) t (10) = 0.47, *P = 0.64*.(**A, C, E-H, J, N-Q, S, T**) two-tailed Student t-test; (**B, D, I, K-M, R**), two-tailed Mann Whitney tests. Data is displayed in boxplots where the median is represented by the horizontal line, the whiskers represent the 25^{th} and 75^{th} percentiles. Full circles represent outliers according to Tukey's rule. Euploid $n = 7$, T21 $n = 7$

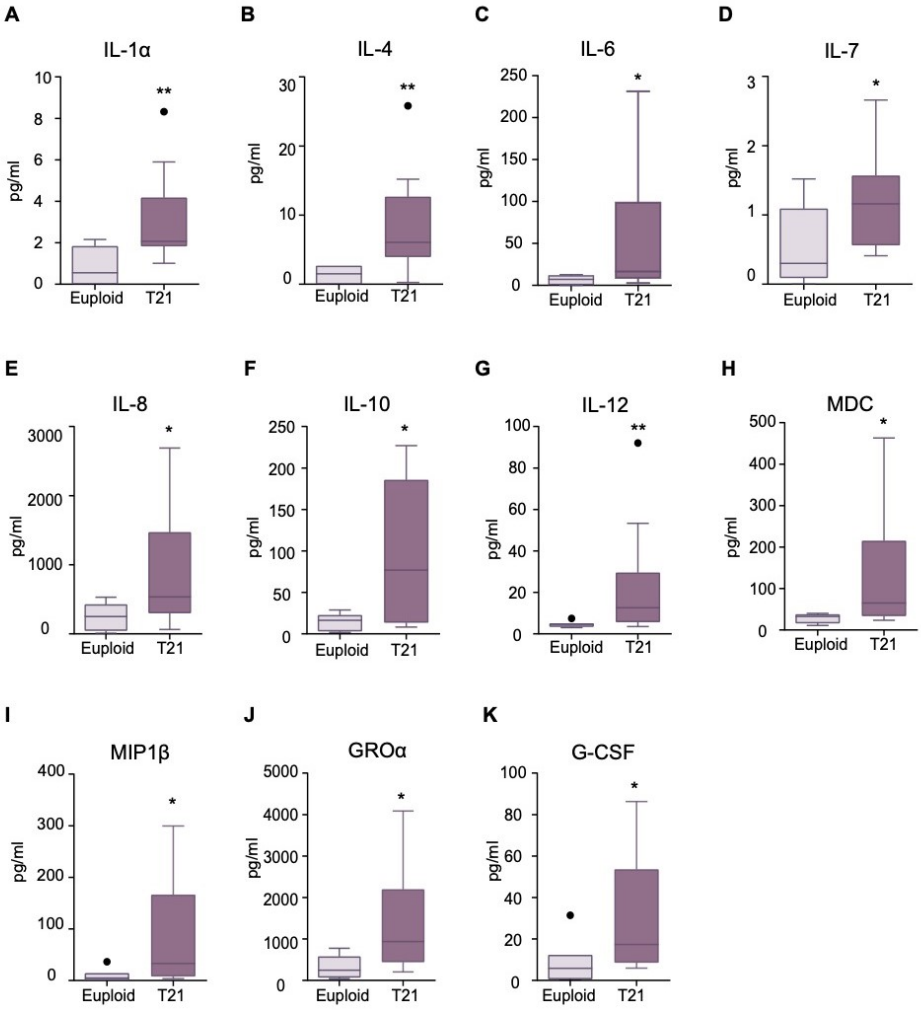

Figure 2-S 2. Human Down syndrome fetal cortical cells display a marked neuroinflammatory profile.

(**A-K**) Increased levels of soluble cytokines (pg/ml) in the conditioned media of second trimester Down syndrome fetal cortical cells measured by a multiplex protein electrochemiluminescent assay. (**A**) U = 11, *P = 0.007*; (**B**) U = 7, *P = 0.004*; (**C**) U = 9, *P = 0.02*; (**D**) t (17) = 2.134, *P = 0.04*;(**E**) U = 13, *P = 0.04*; (**F**) U = 11, *P = 0 .04*; (**G**) U = *12, P = 0 .009;* (**H**) U = 12.5, *P = 0*.02; (**I**) U = 11, *P = 0 .01*; (**J**) U = 9, *P = 0.01*;(**K**) U = 12, *P = 0.01*. (**A-C, E-K**) two-tailed Mann Whitney test, (**D**) two-tailed Student t-test. Data is displayed in boxplots where the median is represented by the horizontal line, the whiskers represent the 25th and 75th percentiles. Full circles represent outliers according to Tukey's rule. Euploid *n* = 7, T21 *n* = 7. * *P* < *0.05,* ** *P* < *0.01*

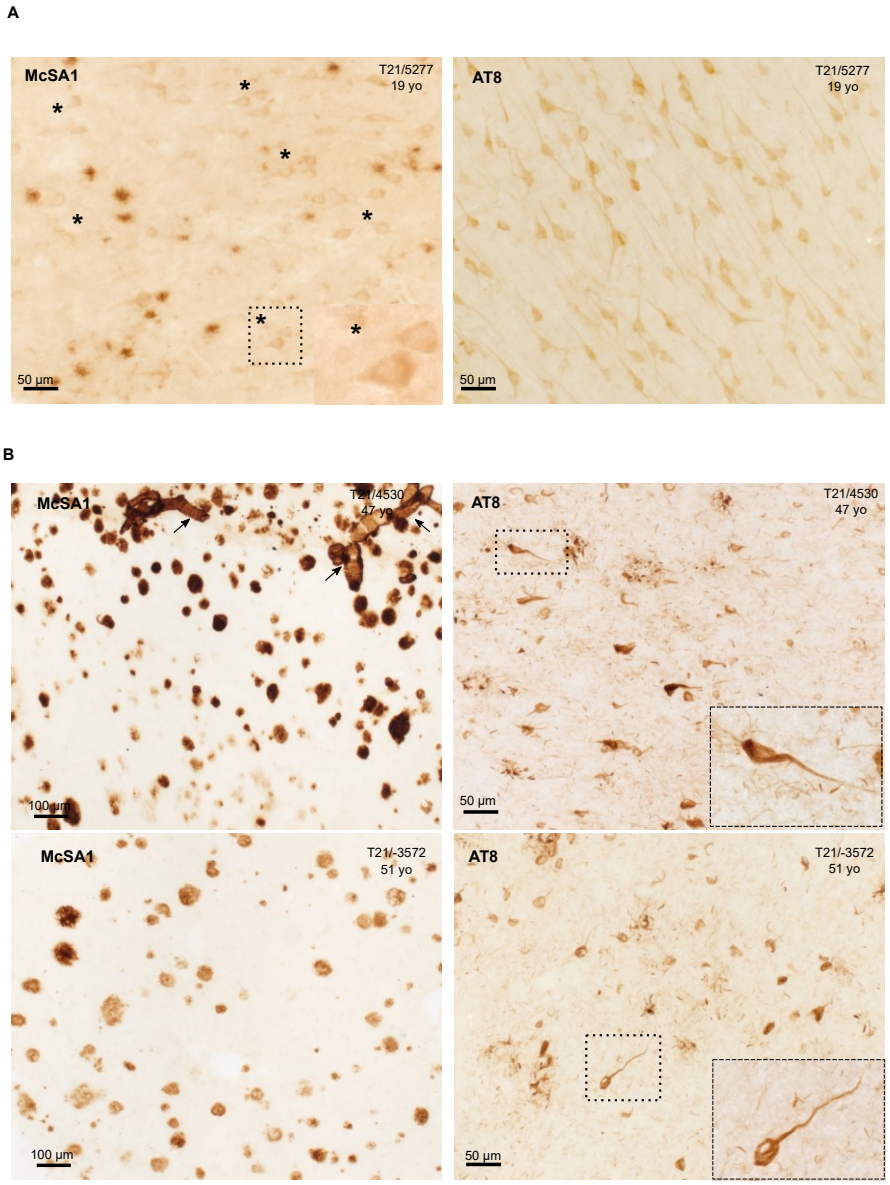

Figure 2-S 3. Amyloid and tau pathologies in the frontal cortex of individuals with Down syndrome.

Representative micrographs depicting amyloid (left panel) and tau pathology (right panel) as revealed by immunohistochemistry (IHC) with McSA1 and AT8 antibodies in the frontal cortex of individuals with Down syndrome. (**A**) Note the presence of intraneuronal amyloid beta (asterisks and inset), sparse amyloid plaques, and early pathological tau accumulation (right panel) in a 19-year-old individual with Down syndrome. (**B**) A full-blown Alzheimer's pathology is present in the brains of older adults with Down syndrome as revealed by the presence of amyloid plaques (left panel) and advanced tau pathology (right panel). Insets depict AT8-IR neurons including ghost-like tau pathology (lower panel inset). Note the presence of cerebral amyloid angiopathy (arrows).

Supplemental Table 2-S 1. Demographics of the studied population: frontal cortical samples from fetuses, children, young adults and adults

		Euploid	T21	*P-value*
Fetuses		*n* = 7	*n*=7	
Age	Range (gestational weeks)	18-20	16-23	
	Mean ± SD	18.83 ± 0.75	20.29 ± 2.69	*0.22*
Sex	(F/M)	6/0	6/1	*0.33*
PMI (h)	Range	1-3	1-5	
	Mean ± SD	2 ± 0.63	2.85 ± 1.57	*0.37*
Children		*n* = 10	*n* = 5	
Age (y)	Range	1-10	1-10	
	Mean ± SD	3.9 ± 3.10	3.2 ± 3.89	*0.41*
Sex	(F/M)	5/5	2/3	*>0.99*
PMI (h)	Range	11-22	11-28	
	Mean ± SD	16.6 ± 3.77	17 ± 6.74	*0.88*
Young adults		*n* = 24	*n* = 6	
Age (h)	Range	13-27	13-25	
	Mean ± SD	20.5 ± 4.46	19.5 ± 4.72	*0.63*
Sex	(F/M)	9/15	0/6	*0.14*
PMI (h)	Range	5-28	14-26	
	Mean ± SD	14.63 ± 7.00	21 ± 5.21	***0.03****
Adults		*n* = 16	*n* = 22	
Age (y)	Range	42-68	39-64	
	Mean ± SD	50.65 ± 9.03	53.82 ± 8.10	*0.23*
Sex	(F/M)	7/9	10/12	*>0.99*
PMI (h)	Range	3-24	3-72	
	Mean ± SD	12.97 ± 5.99	17.56 ± 16.18	*0.57*

Abbreviations: y, years; PMI, *post-mortem* interval; SD, standard deviation; h, hours; F, female; M, male. **P < 0.05*, Mann-Whitney test.

Supplemental Table 2-S 2. Demographics of the studied population: hippocampal samples from adults

		Euploid	T21	*P-value*
Adults		*n* = 17	*n* = 8	
Age (y)	Range	41-66	41-64	
	Mean ± SD	49.83 ± 8.15	54.50 ± 6.45	*0.28*
Sex	(F/M)	6/11	2/6	*>0.99*
PMI (h)	Range	6-25	3-25	
	Mean ± SD	15.65 ± 4.83	16.50 ± 8.26	*0.74*

Abbreviations: y, years; PMI, *post-mortem* interval; SD, standard deviation; h, hours; F, female; M, male

Supplemental Table 2-S 3. Demographics of the cases utilized for microglial morphological analyses

		Euploid	T21	*P-value*
Children		*n* = 5	*n* = 3	
Age (y)	Range	2-8	1	
	Mean ± SD	4.40 ± 2.19	1 ± 0	***0.04****
Sex	(F/M)	1/4	1/2	*>0.99*
PMI (y)	Range	14-19	12-28	
	Mean ± SD	16.20 ± 1.92	19 ± 8.18	*0.47*
Young adults		*n* = 6	*n* = 4	
Age (y)	Range	16-25	15-25	
	Mean ± SD	21.50 ± 3.50	19.50 ± 4.12	*0..43*
Sex	(F/M)	2/4	0/4	*0.46*
PMI (h)	Range	5-37	14-26	
	Mean ± SD	18.83 ± 11.02	19 ± 6.00	*0.97*
Adults		*n* = 9	*n* = 8	
Age (y)	Range	30-57	33-64	
	Mean ± SD	43.22 ± 9.29	49.13 ± 9.71	*0.22*
Sex	(F/M)	3/6	4/4	*0.63*
PMI (h)	Range	7-23	3-36	
	Mean ± SD	15.00 ± 4.74	15.13 ± 10.99	*0.97*

Abbreviations: Y, years; PMI, *post-mortem* interval; SD, standard deviation; H, hours; F, female; M, male. **P* < *0.05*, Student t-test, t = 2.970, df = 7

Supplemental Table 2-S 4. Demographics of the cases utilized for rod microglial analyses

		Euploid	T21	*P-value*
Young adults		*n* = 12	*n* = 4	
Age (y)	Range	16-27	15-25	
	Mean ± SD	22.08 ± 3.31	20.50 ± 4.43	*0.45*
Sex	(F/M)	6/6	0/5	*0.10*
PMI(h)	Range	5-37	14-24	
	Mean ± SD	15.33 ± 8.67	18.50 ± 5.26	*0.50*
Adults		*n* = 10	*n* = 11	
Age (y)	Range	30-58	33-64	
	Mean ± SD	44.60 ± 9.48	49.00 ± 8.30	*0.27*
Sex	(F/M)	3/7	6/5	*0.38*
PMI (h)	Range	7-24	3-36	
	Mean ± SD	16.20 ± 5.24	16.00 ± 10.30	*0.95*

Abbreviations: Y, years; PMI, *post-mortem* interval; SD, standard deviation; H, hours; F, female; M, male.

Supplemental Table 2-S 5. Customized human multispot inflammatory panels and analytes

Panel	Analytes
C-proinflammatory panel	IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, TNFα, IFNγ
C-cytokine panel	IL-1α, IL-5, IL-7, IL-12p40, IL-15, IL-16, VEGFA
C-chemokine panel	Eotaxin-3, IP-10, MIP-1β, MCP-1, MDC, TARC

Abbreviations: c, customized; IL, interleukin; TNF, Tumor necrosis factor; IFN; VEGF, vascular endothelial growth factor; IP, interferon-inducible protein; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; TARC, thymus and activation-regulated chemokine.

Supplemental Table 2-S 6. Human Cytokine/Chemokine array 42-plex HD42

Analytes
EGF, Eotaxin-1, FGF-2, Flt-3L, Fractalkine, G-CSF, GM-CSF, GROα, GROβ, IFNα, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, IL-18, MCP-3, MDC, MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, sCD40L, (TGF)α, TNFα, TNFβ, and VEGF-A.

Abbreviations: EGF, endothelial growth factor; FGF, fibroblast growth factor; Flt-3L *FMS-like tyrosine kinase 3* ligand; G-CSF, Granulocyte-Colony Stimulating Factor; GM-CSF, Granulocyte Macrophage-Colony Stimulating Factor; GRO, growth-related oncogene; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; TGF, transforming growth factor

Supplemental Table 2-S 7. Demographics of the cases utilized for Alzheimer's disease neuropathology analysis

	Age (y)	Aβ-IR	p-tau-IR	CAA
Children				
-2135	1	0	0	Absent
714	1	0	0	Absent
718	1	0	0	Absent
Young adults				
-2854	15	0	0	Absent
5277	19	+	~	Absent
-1960	19	0	0	Absent
5713	25	~	~	Absent
Adults				
4273	33	++	0	Absent
5005	39	++	+	Absent
4530	47	+++	+++	Positive
4870	51	+++	+++	Positive
-1864	51	+++	++	Positive
-3572	51	++	++	Absent
5439	57	+++	+++	Absent
5386	64	+++	+++	Positive

Alzheimer's neuropathology was assessed using McSA1 and AT8 antibodies to detect Aβ and pathological tau, respectively, in the frontal cortex of Down syndrome cases. Presence of Aβ in cerebral blood vessels was considered as cerebral amyloid angiopathy (CAA). 0 = absent, ~ = barely present, + = sporadically present, ++, regularly present, and +++ = consistently present.

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Connecting Text: Chapter 2 to 3

In Chapter 2 we have investigated the expression of brain neuroinflammatory markers across the lifespan of individuals with DS. Our studies have demonstrated an increase in inflammatory markers in the frontal cortex of children and young adults with DS when compared to age-matched controls. Older adults with DS also displayed an increase in inflammatory markers, however, inflammatory molecules associated to the resolution of inflammation process were downregulated. We have also reported a progressive activation of microglial cells. Importantly, microglial cells are intermediately activated in young DS brains before the development of a full-blown AD pathology. An interesting finding from this study, was the differential expression of inflammatory markers between the frontal cortex and the hippocampus, suggesting that brain inflammation might evolve in a region-specific manner. Finally, we have shown that females with DS display increased levels of certain inflammatory molecules when compared to males with DS, underscoring the effect of sex on inflammatory protein expression. Our results suggest the dynamic nature of neuroinflammation across different stages of AD in DS and significantly contribute towards our understanding of neuroinflammation at preclinical stages of AD.

To establish whether such an early neuroinflammatory process is present in the general population, we have investigated brain inflammatory protein expression in *post-mortem* brain tissue from a well-characterized cohort of clinically normal individuals with and without abnormal amyloid beta deposition. Towards this aim, we have investigated cytokine expression in the temporal, parietal, and frontal cortices of non-demented individuals. We have furthered assessed if non-demented individuals with abnormal amyloid beta deposition displayed subtle cognitive deficits. The results of this investigation will be presented in the next chapter of this Thesis.

CHAPTER 3

Upregulation of cerebral inflammatory markers and cognitive decline in non-demented elderly individuals with incipient Alzheimer's pathology

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To be submitted

Abstract

Epidemiological studies suggest that early, anti-inflammatory treatment reduces the risk of Alzheimer's disease (AD). Nevertheless, clinical trials of anti-inflammatory drugs administered at different AD symptomatic stages have failed to demonstrate efficacy. Such observations reflect the complexity of the neuroinflammatory process in AD and the lack of understanding of such process at early, preclinical stages. Therefore, in this study, we investigated the neuroinflammatory profile in the brains of elderly individuals with no symptomatic manifestation of AD belonging to a highly clinically and pathologically characterized cohort. An array of twenty inflammatory cytokines was analyzed with a sensitive multispot immunoassay in frozen temporal, parietal, and frontal cortices of individuals classified according to the presence or absence of cerebral amyloid-beta (Aβ) deposition examined *post-mortem*. Individuals grouped as amyloid positive (Aβ+) had higher levels of IL-1β, IL-6 and eotaxin-3 in the temporal cortex accompanied by an increase in MCP-1 and IL-1β in the parietal cortex, and a trend towards higher levels of IL-1β and MCP-1 in the frontal cortex. Most inflammatory markers showed a positive association with global cortical amyloid burden. Moreover, while individuals with amyloid deposition were clinically classified as healthy, non-demented, they had cognitive deficits in perceptual speed and a faster rate of cognitive decline in such cognitive domain as compared to control subjects. Overall, our results indicate that elderly individuals with AD neuropathology but no symptomatic manifestation of dementia exhibit signs of neuroinflammation and cognitive decline, compared to amyloid-negative subjects. Such observations suggest that neuroinflammation might be an early event in the AD continuum and that stratification by amyloid positivity should be considered when evaluating anti-inflammatory approaches for AD.

Introduction

A large body of evidence indicates that neuroinflammation plays an important role in the development and progression of Alzheimer's disease (AD) (Akiyama *et al.*, 2000; Wyss-Coray *et al.*, 2012). Specifically, epidemiological studies revealed that individuals under long-term administration of anti-inflammatories have a reduced risk of developing symptomatic AD (McGeer *et al.*, 2013; Vlad *et al.*, 2008). Such beneficial effect becomes apparent if the treatment is started early enough in the AD continuum and has a duration of minimum two years (McGeer *et al.*, 2013). In contrast, clinical trials using anti-inflammatory medications at different AD symptomatic stages have failed to demonstrate efficacy (Heneka *et al.*, 2015; Meyer *et al.*, 2019). The benefit of early anti-inflammatory treatment suggests that a detrimental neuroinflammatory process is active at pre-clinical AD stages. Indeed, studies in transgenic models of the amyloid-like pathology have demonstrated the presence of an early neuroinflammatory process which occurs before the development of amyloid plaques and is concomitant with the intraneuronal accumulation of Aβ oligomers (Ferretti *et al.*, 2012b; Hanzel *et al.*, 2014). Furthermore, anti-inflammatory treatment at these early stages rescues cognitive deficits and diminishes AD-like pathology (Cavanagh *et al.*, 2018; Ferretti *et al.*, 2012a; Janelsins *et al.*, 2005; McAlpine *et al.*, 2009). Such observations strengthen the concept that brain inflammation has a disease-aggravating nature at incipient stages of amyloid accumulation (Cuello, 2017), although evidence from human studies remains scarce. The lack of understanding of the evolution of neuroinflammatory processes along the continuum of AD in clinical cohorts, specifically at incipient stages, poses a major drawback for reconsidering anti-inflammatory approaches in future AD clinical trials.

In the general population, one-third of older non-cognitively impaired individuals (NCI) display brain AD neuropathology (Bennett *et al.*, 2006; De Meyer *et al.*, 2010; Fagan *et al.*, 2009; Morris *et al.*, 1996). Given that aging is the major risk factor for AD and that AD has a long, pre-symptomatic phase where neuropathology starts to 'silently' build-up; then, it is expected that a proportion of NCI individuals with AD neuropathology should be at the earliest, asymptomatic stages of AD.

Towards elucidating whether incipient AD pathology is associated to increased brain inflammation, we analyzed proinflammatory cytokine expression in the temporal, parietal and frontal cortices of a well-clinically and neuropathologically characterized cohort of non-demented

elderly grouped according to the presence or absence of cerebral amyloid beta (Aβ) pathology. Moreover, we further investigated whether these individuals also displayed subtle cognitive deficits as compared to individuals without Aβ pathology. Our results indicate that non-demented individuals harboring amyloid pathology display an increase in inflammatory markers accompanied by cognitive decline, strengthening the evidence for an early, detrimental neuroinflammatory process of potential therapeutic relevance.

Methods

Brain samples were obtained from old clergy members and community-dwelling persons involved in the Religious Orders Study (ROS) and Rush Memory and Aging Project (MAP) (Bennett *et al.*, 2006). Briefly, ROSMAP comprises community cohorts of aging and AD that enroll cognitively healthy individuals who agree to an annual detailed cognitive evaluation and brain donation at the time of death. For this study, we randomly selected *post-mortem* brain tissue from the temporal (n = 28), parietal (n = 26), and frontal (n = 27) cortices from individuals under age 80 who had been clinically diagnosed with no cognitive impairment (NCI) at the last neuropsychological evaluation before death (J. A. Schneider *et al.*, 2007). We excluded cases at older ages to avoid for potentially confounding effects of 'SuperAgers' resilient to AD pathology.

Neuropathological examination and phenotype classification

Neuropathological examination of each case was performed by an experienced pathologist blinded to the clinical diagnosis at the Rush Alzheimer's disease Center. A total of 8 brain regions were analyzed (hippocampus, entorhinal cortex, midfrontal cortex, inferior temporal, angular gyrus, calcarine cortex, anterior cingulate cortex and superior frontal cortex). Aβ values for each brain region represent the percentage covered by 4G8 immunoreactivity in 1-2 sections per brain region (Bennett *et al.*, 2006). The global Aβ value represents an average of all cortical areas analyzed. Neurofibrillary tangle burden was determined by immunohistochemistry against phosphorylated tau (AT8), final values represent cortical AT8 density per mm^2 (Bennett *et al.*, 2006). NCI individuals were segregated into two groups depending on the presence or absence of Aβ, globally and in each specific cortical area (temporal, parietal and frontal). Thus, those who had zero amyloid immunoreactivity in each cortical area (Supplemental tables 3-S1 – S3) were classified as Aβ- whereas those harboring Aβ deposition were classified as Aβ+. Additional neuropathological and genetic information was available, including the modified Consortium to Establish a Registry for Alzheimer's disease (CERAD), modified National Institute on Aging (NIA)-Reagan, Braak scores, and APOE genotype, examined as previously described (Bennett *et al.*, 2006). Further demographic details are illustrated in Supplemental Tables 3-S1-S4. This project has been approved by the McGill University Research Ethics Board.

Inflammatory protein expression

50 mg of frozen brain tissue were homogenized in ice-cooled lysis buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween-20, 5 µl per mg of tissue) with 2x phosphatase (PhosStop, Roche) and protease inhibitors (Complete Mini, Roche). Lysed samples were centrifuged at 13 000 rpm for 45 minutes at 4°C. To avoid repeated freeze and thaw cycles, supernatants were aliquoted and stored at -80°C. Protein concentration was measured using the DC™ Protein Assay Kit (BioRad).
Inflammatory protein expression was measured in brain homogenates using an electrochemiluminescence-linked multiplex immunoassay (Meso-Scale Discovery). Three different plates were customized to measure the following inflammatory proteins: Interleukin (IL)-1β, IL-4, IL-6, IL-8, IL-10, IL-13, Tumor Necrosis Factor (TNF)α, Interferon (IFN)γ, (Plate 1), IL-1α, IL-5, IL-7, IL-12p40, IL-15, IL-16, Vascular Endothelial Growth Factor (VEGF)-A, (Plate 2) and Eotaxin-3, Interferon-inducible Protein (IP)-10, Macrophage Inflammatory Protein (MIP)-1β, Monocyte Chemoattractant Protein (MCP)-1, Macrophage-Derived Chemokine (MDC) , Thymus and Activation-Regulated Chemokine (TARC). Samples were normalized to 3 µg/µl in lysis buffer with 1% blocker A (Meso-Scale Discovery) and 1x of protease and phosphatase inhibitors. Samples were loaded in duplicates according to the manufacturer's recommendations and averaged for analysis. Signal was measured with a SECTOR Imager 6000 reader (Meso-Scale Discovery) and data was processed with the Discovery Workbench 4.0 software. VEGF-A signal was below the lower limit of detection and consequently not analyzed in this study.

Cognitive assessment

Global cognitive function was calculated using a battery of 19 cognitive tests to assess five domains of cognitive function: i) episodic memory (word list memory, recall and recognition, east Boston story immediate and delated, and logical memory Ia immediate and IIa delayed tests), ii) semantic memory (Boston naming, verbal fluency, and reading tests), iii) working memory (digit span forward and backward, and digit ordering tests), iv) perceptual speed (symbol digits modality, number comparison, and stroop color naming and reading), and v) visuospatial ability (judgement of line orientation, and standard progressive matrices tests). A composite score of each cognitive domain was obtained by converting raw scores to z-scores using the baseline mean and standard

deviation of all persons in the studies. The z-scores were averaged to yield a final composite score representing global cognitive function (Bennett *et al.*, 2006).

Estimated slopes from random effects model for each cognitive domain: A linear mixed effects model with each cognitive domain (episodic memory, semantic memory, working memory, perceptual speed, and visuospatial ability) as the longitudinal outcome was utilized to estimate the person-specific rate of change in each cognitive domain over time (random slope) (De Jager *et al.*, 2012). The mean time of follow-up for the cognitive evaluation was 5.7 ± 3.6 years (interquartile range: 3 – 9 years). The statistical model controls for age at baseline, sex and years of formal education.

Cognitive differences on domain-specific cognition and the rate of cognitive decline were analyzed within the NCI population. As various brain regions are involved in the different cognitive domains that were assessed, we stratified the NCI population according to their global Aβ deposition score. Thus, those with positive values of global Aβ deposition were classified as gAβ + and those without Aβ deposition were classified as gAβ-.

Statistical analysis

Data was analyzed with Graph Pad Prism 8. Data is presented in boxplots where the median is represented by the horizontal line and the whiskers represent the 25th and 75th percentiles. Outliers are represented according to the Tukey method. Two-tailed Student's or Mann-Whitney tests were used for two-group comparisons, according to data normality analysis. For three-group comparisons, one-way ANOVA or Kruskal-Wallis tests were used followed by Tukey or Dunn's post-hoc tests, respectively. Fisher's exact test was used to assess differences on sex, APOE genotype, Braak score, CERAD and NIA-Reagan criteria. Pearson correlation coefficient was used for correlation analyses. For correlation analyses, data that did not follow a normal distribution was log-transformed. Significance was set at $p < 0.05$.

Results

Inflammatory protein expression was analyzed in the temporal, parietal, and frontal cortices of individuals aged 66 to 80 years with no symptomatic manifestation of AD, grouped by the presence or absence of Aβ pathology (Aβ+ and Aβ-, respectively) in each cortical brain area. When analyzed

per cortical region, no significant differences in age, *post-mortem* interval, sex, tau pathology, Braak score, frequency of ApoE4 genotype, years of education, and global cognitive score were observed between groups (Supplemental Tables 3-S1-S3). Although both subpopulations were clinically and neuropsychologically classified as NCI, the Aβ+ groups met criteria for a neuropathological diagnosis of AD according to modified CERAD and NIA-Reagan scores (Supplemental Tables 3-S1-S4).

Differential upregulation of inflammatory mediators in the temporal, parietal, and frontal cortices of non-demented individuals with Aβ pathology

Recent imaging studies suggest that early amyloid deposition is frequently detected in the inferior temporal and fusiform gyrus, followed by deposition in parietal and frontal regions (Grothe *et al.*, 2017). To establish whether neuroinflammation has a differential expression across different cortical areas, we analyzed inflammatory markers in the temporal, parietal, and frontal cortices separately.

In the temporal cortex, IL-1β ($p = 0.02$), IL-6 ($p = 0.01$) and eotaxin-3 ($p = 0.001$) were significantly elevated in Aβ+ individuals when compared to cognitively normal individuals without Aβ deposition (Figure 3-1 A-C). Furthermore, the expression levels of these three cytokines positively correlated to the extent of Aβ deposition in this brain region (Figure 3-1 D-F).

Then, we assessed cytokine expression in the parietal cortex. Individuals with Aβ positivity in the parietal cortex displayed higher levels of the pro-inflammatory molecules IL-1β ($p = 0.02$) and MCP-1 ($p = 0.04$) (Figure 3-2 A-B). Notably, Aβ deposition positively correlated with IL-1β but not with MCP-1 levels (Figure 3-2 B-C).

Changes in inflammatory protein expression followed a similar pattern in the frontal cortex, revealing trends toward higher levels of IL-1β, IL-6, and MCP-1 in non-demented individuals with Aβ deposition ($p = 0.05$, $p = 0.06$, $p = 0.05$; respectively) (Figure 3-3 A-C). Finally, only IL-1β levels positively correlated with Aβ deposition (Figure 3-3 D-F).

Protein expression levels of cytokines that did not differ between Aβ- and Aβ+ individuals for each cortical region are illustrated in Supplemental Figures 3-S1-S3.

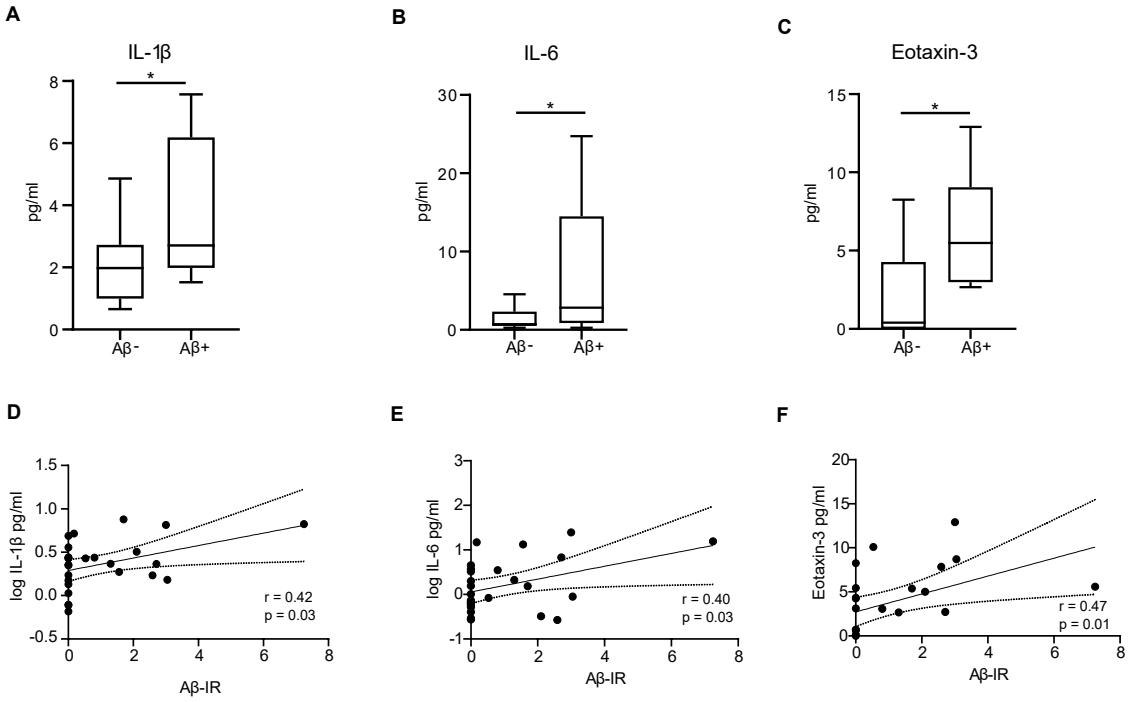

Figure 3- 1. Upregulation of inflammatory markers and their association with Aβ deposition in the temporal cortex of Aβ+ individuals.

Protein expression assessed by electrochemiluminescence analyses revealed increased levels of (A) IL-1β, two-tailed Student t-test; t(24) = 2.403, *p = 0.02*; Aβ-, n = 14, Aβ+, n = 12; (B) IL-6, two-tailed Student t-test; t(24) = 2.56, *p = 0.01*; Aβ-, n = 14, Aβ+, n = 12 and (C) eotaxin-3, two-tailed Student t-test; t(22) = 3.594, *p = 0.001*; Aβ-, n = 14, Aβ+, n = 10, in the temporal cortex of non-demented individuals with Aβ deposition as compared to individuals without Aβ deposition in this brain region. (D-F) Association between Aβ deposition in temporal cortex and cytokine levels in the same brain region by Pearson r analysis. Data is displayed in boxplots where the median is represented by the horizontal line and the whiskers represent the 25th and 75th percentiles. *$p < 0.05$

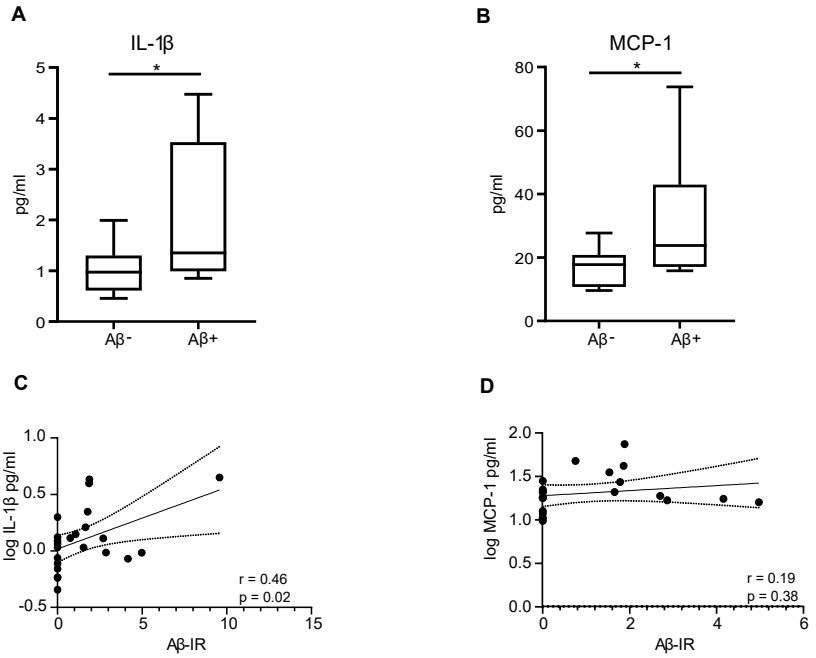

Figure 3- 2. Increased expression of inflammatory markers and their association with Aβ deposition in the parietal cortex of Aβ+ individuals.

Protein expression assessed by electrochemiluminescence analyses revealed increased levels of (A) IL-1β, two-tailed Student t-test; t(22) = 2.46, *p = 0.02*; Aβ-, n = 12, Aβ+, n = 12; (B) MCP-1, two-tailed Mann Whitney test; U = 29, *p = 0.04*; Aβ-, n = 12, Aβ+, n = 10, in the temporal cortex of non-demented individuals with Aβ deposition as compared to individuals without Aβ deposition in this brain region. (C-D) Association between Aβ deposition in the parietal cortex and cytokine levels in the same brain region by Pearson r analysis. Data is displayed in boxplots where the median is represented by the horizontal line and the whiskers represent the 25th and 75th percentiles. *$p < 0.05$.

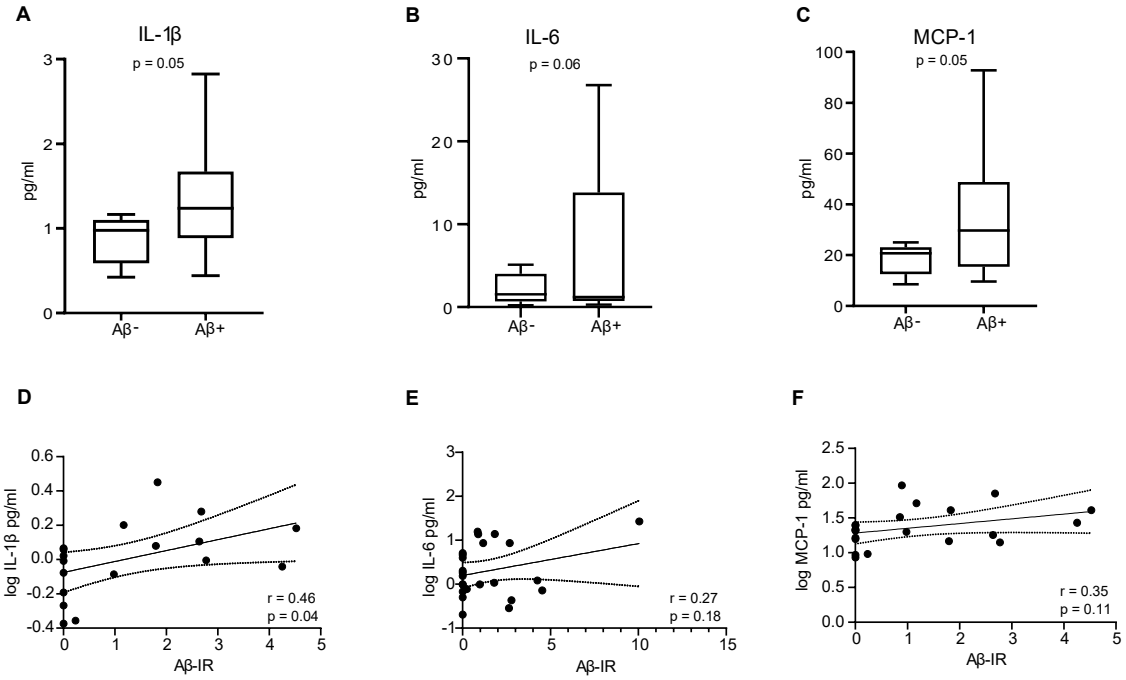

Figure 3- 3. Expression of inflammatory markers and their association with Aβ deposition in the frontal cortex of Aβ+ individuals

Protein expression assessed by electrochemiluminescence analyses revealed a trend towards increased levels of (A) IL-1β, two-tailed Student t-test; t(17) = 2.028, *p = 0.058*; Aβ-, n = 9, Aβ+, n = 10; (B) IL-6, two-tailed Student t-test; t(22) = 1.981, *p = 0.06*; Aβ-, n = 11, Aβ+, n = 13 and (C) MCP-1, two-tailed Student t-test; t(19) = 2.067, *p = 0.052*; Aβ-, n = 9, Aβ+, n = 12, in the frontal cortex of non-demented individuals with Aβ deposition as compared to individuals without Aβ deposition in this brain region. (D-F) Association between Aβ deposition in the frontal cortex and cytokine levels in the same brain region by Pearson r analysis. Data is displayed in boxplots where the median is represented by the horizontal line and the whiskers represent the 25th and 75th percentiles.

Neuropathological and inflammatory differences between cortical regions of non-demented individuals with and without Aβ deposition

Given the differential inflammatory profiles observed in the temporal, parietal and frontal cortices analyzed, we examined whether there were differences in Aβ and tau deposition between these brain regions.

No significant differences in Aβ deposition were observed between the temporal, frontal and parietal cortices of Aβ+ individuals who exhibited a differential pattern of inflammatory markers between these regions (Kruskal-Wallis test, H = 0.496, $p = 0.78$) (Figure 3-4A). In contrast, As illustrated in Figure 3-4B regional differences in p-tau deposition were encountered as the temporal cortex displayed higher levels of p-tau than the parietal and frontal cortices (one-way ANOVA, $p < 0.0001$).

Given that each brain cortical region displayed a differential inflammatory and pathological signature we assessed if inflammatory marker expression differed between the temporal, parietal, and frontal cortices of non-demented individuals with Aβ deposition. To control for possible normal variations in regional cytokine expression, each cytokine was normalized to the cytokine levels of individuals without Aβ deposition in each brain region. Then, the fold-change expression was calculated for every cortical region. The temporal cortex displayed increased expression of eotaxin-3 than the parietal ($p = 0.003$) and frontal ($p = 0.003$) cortices (Figure 3-4C). MCP-1 fold-change expression was increased in the parietal region compared to the temporal cortex ($p = 0.01$) (Figure 3-4D). The fold-change expression of IL-1β and IL-6 did not differ between cortical regions. (Figure 3-4 E-F).

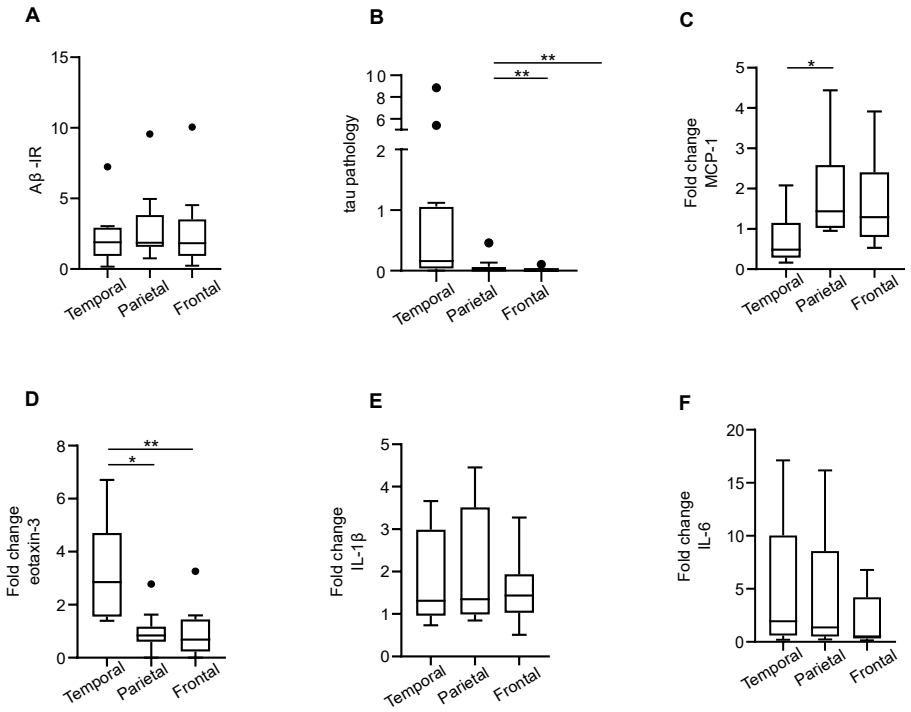

Figure 3- 4. Aβ deposition, tau deposition, and inflammatory protein expression in the temporal, parietal, and frontal cortices of non-demented individuals with and without Aβ pathology.

(A) Aβ deposition did not differ between the temporal, parietal, and frontal cortices of non-demented individuals harboring AD pathology, Kruskal-Wallis test, H = 0.496, $p = 0.78$. (B) The temporal cortex of Aβ+ individuals displayed increased tau pathology when compared to the parietal and frontal cortices, Kruskal-Wallis test, H = 14.94, $p < 0.0006$ followed by Dunn's multiple comparisons test. (C) The parietal cortex displayed higher levels of MCP-1 than the temporal cortex; Kruskal-Wallis test, H = 9.54, $p = 0.008$ followed by Dunn's multiple comparisons test. (D) Eotaxin-3 levels were highest in the temporal cortex compared to the parietal and frontal cortices; Kruskal-Wallis test, H = 14.19, $p = 0.0008$ followed by Dunn's multiple comparisons test. (E) IL-1β, did not differ between cortical areas; one-way ANOVA, $F_{2,31} = 0.55$, $p = 0.58$, and (F) IL-6; one-way ANOVA, $F_{2,31} = 1.4$, $p = 0.26$. Data is displayed in boxplots where the median is represented by the horizontal line and the whiskers represent the 25th and 75th percentiles. Full circles represent outliers according to the Tukey method. *$p < 0.05$, **$p < 0.01$.

Non-demented individuals with higher Aβ deposition display faster cognitive decline

Given that non-demented individuals with amyloid pathology displayed an increase in brain inflammatory proteins, we next assessed whether such population also displayed deficits in five cognitive domains (episodic memory, semantic memory, working memory, perceptual speed, and visuospatial ability). As illustrated in Figure 3-5, gAβ+ individuals displayed deficits in perceptual speed ($p = 0.01$). We then analyzed the person-specific rate of change (i.e. cognitive decline slope) in episodic memory, semantic memory, working memory, perceptual speed, and visuospatial ability were compared between gAβ+ and gAβ-. As illustrated in Figure 3-6, while episodic memory, working memory, semantic memory and visuospatial ability were not altered, a higher rate of cognitive decline in perceptual speed ($p = 0.03$) was observed in individuals with Aβ deposition. Notably, the ApoE4 allele was enriched in cognitively normal individuals with higher Aβ deposition ($p = 0.03$) (Supplemental Table 3-S4).

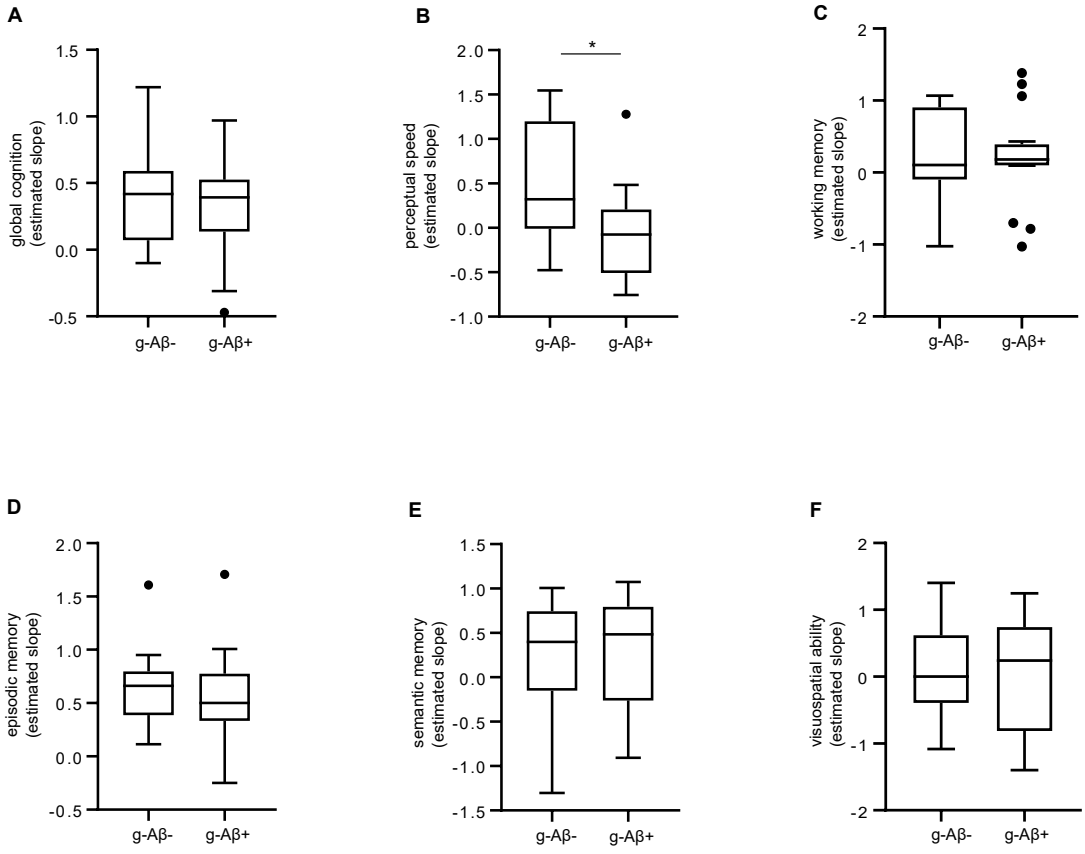

Figure 3- 5. Aβ+ individuals display cognitive deficits in perceptual speed

Cognitive scores in (A) global cognition t(28) = 0.81, $p = 0.42$, (B) perceptual speed, t(28) = 2.68, $p = 0.01$; (C) working memory, t(28) = 0.18, $p = 0.85$; (D) episodic memory, U = 98, $p = 0.57$; (E) semantic memory, t(28) = 0.12, $p = 0.90$; (F) visuospatial ability, t(28) = 0.18, p = 0.85. Data is displayed in boxplots where the median is represented by the horizontal line and the whiskers represent the 25th and 75th percentiles; (A-C, E-F) two-tailed Student t-test; (D) two-tailed Mann-Whitney test; Aβ-, n = 14, Aβ+, n = 16; g = global. Full circles represent outliers. *$p < 0.05$.

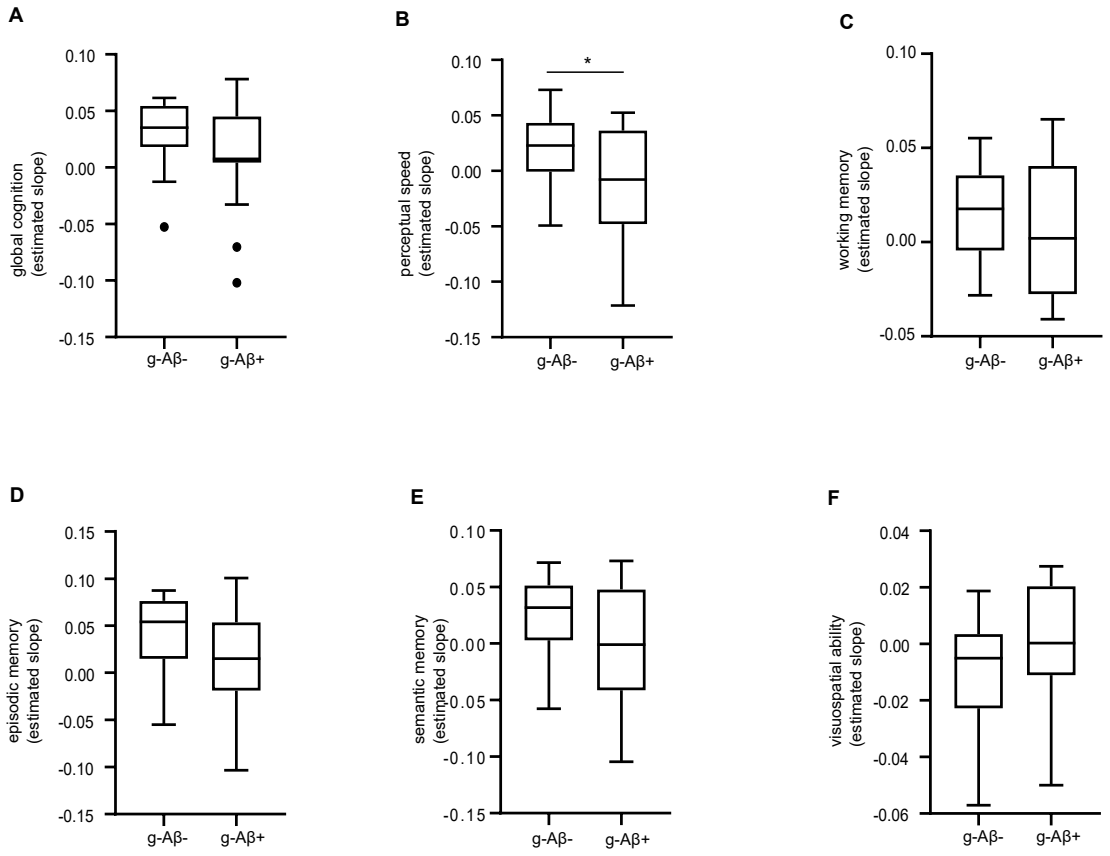

Figure 3- 6. Non-demented individuals with Aβ deposition display a faster cognitive decline than non-demented individuals without Aβ pathology

(A-F) Estimated slopes reflecting the person-specific rate of cognitive decline in (A) global cognition U = 71, $p = 0.23$, (B) perceptual speed, t(26) = 2.15, $p = 0.04$; (C) working memory, t(26) = 0.90, $p = 0.37$; (D) episodic memory, t(26) = 1.20, $p = 0.23$; (E) semantic memory, t(26) = 1.32, $p = 0.19$; (F) visuospatial ability, t(26) = 1.27, $p = 0.21$, within a mean follow up period of 5.7 ± 3.6 years. The mean time of follow-up was 5.7 ± 3.6 years (interquartile range: 3 – 9 years). Data is displayed in boxplots where the median is represented by the horizontal line and the whiskers represent the 25th and 75th percentiles; (A) two-tailed Mann-Whitney test;(B-F) two-tailed Student t-test; Aβ-, n = 13, Aβ+, n = 15; g = global. Full circles represent outliers. *$p < 0.05$.

Discussion

Inflammatory brain expression has been widely documented in *post-mortem* brain tissue from individuals with AD. However, evidence from transgenic models mimicking the very earliest stages of AD indicate that neuroinflammation is present before overt amyloid deposition concomitant with early cognitive deficits (Ferretti *et al.*, 2012b; Hanzel *et al.*, 2014). Such observations have not been investigated in human *post-mortem* brain tissue with incipient AD pathology. Therefore, we took advantage of a well clinically and pathologically characterized cohort of non-demented individuals displaying early signs of AD neuropathology and investigated inflammatory protein expression in different brain regions affected in AD.

Our investigations indicate that neuroinflammation is present throughout different brain regions in elderly individuals clinically defined as healthy, non-demented, but with sufficient cortical Aβ deposition. We have found that the pattern of brain inflammatory markers differs across cortical brain regions and that such pattern is associated with regional Aβ burden. Moreover, non-demented individuals with Aβ pathology had an overall increase in IL-1β in the three regions analyzed. Thus, raising the possibility that IL-1β might be one of the earliest inflammatory markers associated to the accumulation of AD neuropathology in older adults. Furthermore, non-demented individuals with Aβ deposition displayed deficits in perceptual speed compared to subjects without Aβ deposition, suggesting that these individuals could have been going through an active, disease-aggravating stage, such as the preclinical stage of AD (Figure 3-7).

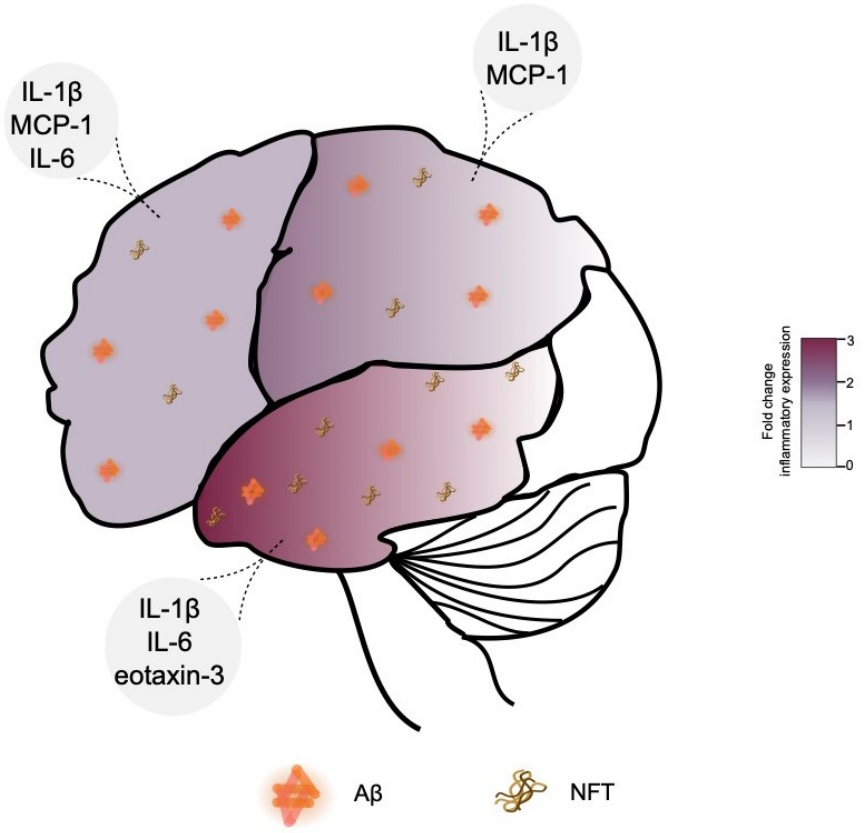

Figure 3- 7. Scheme depicting the differential upregulation of cytokines, Aβ pathology, and tau deposition in the temporal, parietal, and frontal cortices of cognitively normal individuals with Aβ pathology.

The average fold change of inflammatory molecule expression per brain region was calculated. Briefly, NCI individuals with Aβ deposition displayed an increase of IL-1β (~1.8 fold change), IL-6 (~3.4 fold change) , and eotaxin-3 (~3 fold change) in the temporal cortex, an increase in IL-1β (~2 fold change) and MCP-1 (~1.8 fold change) in the parietal cortex and similar trends revealing higher levels of IL-1β (~1.5 fold change), IL-6 (~1.8 fold change) , and MCP-1 (~1.7 fold change) in the frontal cortex as compared to individuals without Aβ deposition. Tau pathology was highest in the temporal cortex as compared to the parietal and frontal cortices from Aβ+ individuals. Individuals with Aβ pathology also showed cognitive deficits in perceptual speed.

Few studies have examined the occurrence of pathological events in the brains of cognitively normal individuals harboring AD neuropathology. Scheff and colleagues have reported a decrease in pre and post-synaptic proteins, and an increase in oxidative stress markers within the hippocampus of non-demented individuals with AD neuropathology (Scheff *et al.*, 2016). Notably, some oxidative stress markers were negatively associated to synaptic protein expression (Scheff *et al.*, 2016). Other studies have shown that nerve growth factor, a neurotrophic factor essential for the phenotypic maintenance of basal forebrain cholinergic neurons, is deregulated in elderly, cognitively normal individuals with AD pathology (Pentz *et al.*, 2019, *submitted*). In addition, further evidence from De Jager and colleagues indicates increased DNA methylation pattern in regions that harbor AD susceptibility alleles in AD brains (De Jager *et al.*, 2014). Notably, a differential DNA methylation pattern is already detectable in non-demented individuals with a neuropathologic diagnosis of AD (De Jager *et al.*, 2014). A study in line with the reported global DNA hypomethylation concurrent to the intraneuronal accumulation of Aβ in an APP mouse model (Do Carmo *et al.*, 2016). Henceforth, the above observations would suggest that elderly cognitively normal individuals harboring AD pathology might indeed be at the earliest, clinically asymptomatic stages of AD.

To our knowledge, this is the first study analyzing cytokine brain expression between non-demented individuals with or without Aβ accumulation in a well-characterized population-based cohort. When analyzing specific brain regions, higher levels of IL-1β were found in the temporal, parietal, and frontal cortices of non-demented individuals harboring Aβ accumulation, further supporting the central role of IL-1β at such early stages of amyloid accumulation. Notably, we observed that IL-1β levels were positively correlated to Aβ burden in the temporal, parietal and frontal cortices.

An early involvement of IL-1β in the AD continuum is also suggested by its upregulation in the brains of fetuses, neonates, and young adults with DS (Flores-Aguilar *et al.*, 2020, *under review*; Griffin *et al.*, 1989), and by its presence in diffuse amyloid plaques in AD brains (Rozemuller *et al.*, 1989). Maturation of IL-1β into its active form is regulated by caspase-1. Notably, caspase-1 is overexpressed at prodromal and clinical stages of AD (Heneka *et al.*, 2013). An early upregulation of IL-1β has also been reported in a transgenic rat model of amyloidosis before the overt deposition of amyloid plaques (Hanzel *et al.*, 2014). At this early, pre-plaque stage, IL-1β expression is restricted to the neuronal compartment, indicating that neurons, instead of microglia,

could be early players of the neuroinflammatory cascade at incipient stages of the amyloid pathology (Hanzel *et al.*, 2014). The concept of an early and late inflammatory process and the involvement of neurons versus microglia has been discussed and described in a comprehensive review (Cuello, 2017).

The consequences of elevated IL-1β levels could be several. IL-1β overexpression can promote the synthesis and processing of APP (Goldgaber *et al.*, 1989; Liao *et al.*, 2004; Rogers *et al.*, 1999; Sheng *et al.*, 1996), induce tau hyperphosphorylation (Griffin *et al.*, 2006; Li *et al.*, 2003), and can modulate hippocampal long-term potentiation (LTP) (H. Schneider *et al.*, 1998). Thus, its upregulation in AD might contribute to potentiate AD neuropathology and cognitive deficits. Moreover, IL-1β can increase the activity and expression of acetylcholinesterase and might further exacerbate cholinergic deficits in AD (Li *et al.*, 2000). Overexpression of IL-1β also leads to the translation of other potent inflammatory cytokines such as IL-6 (Basu *et al.*, 2004). In our study, IL-6 levels where increased in the temporal cortex of non-demented individuals with Aβ pathology. Similar to IL-1β, IL-6 is also present in diffuse amyloid plaques in AD brains (Hull *et al.*, 1996) and can enhance APP expression (Ringheim *et al.*, 1998), tau hyperphosphorylation (Quintanilla *et al.*, 2004), and cognitive deficits in mice (Campbell *et al.*, 1997). Importantly, genetic variations of the IL-6 and IL-1β genes have been associated to an increased risk of AD [reviewed in (McGeer *et al.*, 2001)].

The chemokine eotaxin-3 was also elevated in the temporal cortex of AD-asymptomatic individuals with Aβ pathology. *In-vitro* studies have shown that eotaxin-3 can act as an antagonist of the MCP-1 (CCL2) receptor, CCR2 and synergize with the chemokine MCP-1 to promote monocyte extravasation (Ogilvie *et al.*, 2003). Increased eotaxin-3 levels have been reported in the CSF of individuals with AD dementia and can distinguish AD from other neurodegenerative diseases (Craig-Schapiro *et al.*, 2011; Hu *et al.*, 2010; Westin *et al.*, 2012). Moreover, eotaxin-3 levels correlate with CSF tau pathology in AD (Craig-Schapiro *et al.*, 2011). Notably, in our studies, eotaxin-3 was only upregulated in the temporal cortex of NCI individuals with Aβ pathology, and such region displayed higher levels of tau pathology than the parietal and frontal cortices. Such observations suggest an association between increased eotaxin-3 levels and tau pathology. The role of eotaxin-3 in AD, and its relationship with tau pathology awaits further investigations.

Increased levels of IL-1β and MCP-1 were noted in the parietal cortex of individuals with Aβ. MCP-1 is a potent monocyte chemoattractant and its upregulation in transgenic models of AD was suggested to contribute to peripheral monocyte infiltration into the brain (El Khoury *et al.*, 2007; Naert *et al.*, 2011). MCP-1 upregulation has been reported in AD brains and in body fluids (Galimberti *et al.*, 2006; Sokolova *et al.*, 2009). Interestingly, baseline MCP-1 CSF levels in individuals with MCI, have been associated to a faster cognitive decline (Westin *et al.*, 2012). Notably, MCP-1 neuronal levels are already upregulated before the development of amyloid plaques in a transgenic rat model of the AD like pathology (Welikovitch *et al.*, 2019, *submitted*); indicating its early involvement in the amyloid pathology.

A trend towards higher levels of IL-1β, IL-6, and MCP-1 levels was observed in the frontal cortex of Aβ + individuals, indicating that the frontal cortex might be at an earlier neuroinflammatory state than the temporal and parietal cortices. Such differential neuroinflammatory process might reflect the extent of Aβ deposition as Aβ deposits are seen first in the temporal region than in parietal or frontal regions (Grothe *et al.*, 2017). Parachikova and colleagues have reported a differential inflammatory gene expression in the hippocampus and frontal cortex of individuals with mild AD in which the frontal cortex displays a higher number of inflammatory markers than the hippocampus (Parachikova *et al.*, 2007). In addition, individuals with DS also display a differential CNS inflammatory profile, in which the frontal cortex might be at an advanced neuroinflammatory state than the hippocampus (Flores-Aguilar *et al.*, 2020, *under review*). Interestingly, imaging studies in the DS population have shown that amyloid deposition affects firstly the cortex than the hippocampus (Annus *et al.*, 2016). Henceforth, our results support the notion that neuroinflammation is an early disease-aggravating and evolving phenomenon throughout the AD continuum which might be reflective of the extent of AD neuropathology in each brain region, particularly at preclinical stages.

We further investigated whether non-demented individuals with global Aβ pathology displayed early deficits in cognitive domains. Our analyses indicate that perceptual speed differs between non-demented individuals with and without global Aβ pathology. Moreover, when examining the person-specific rate of cognitive change over time, AD-asymptomatic individuals with global Aβ pathology displayed an increased rate of cognitive decline in perceptual speed compared to non-demented subjects without amyloid deposition; highlighting that such cognitive domain might be disrupted early in the AD continuum.

Our studies also underscore that global cognitive scores, while useful to account for cognitive impairments in a comprehensive manner, might mask underlying subtle cognitive deficits which only become apparent by assessing a person's specific rate of decline. Our results are in line with previous studies reporting the occurrence of cognitive impairments in NCI individuals with Aβ pathology [(Bennett *et al.*, 2012; Bilgel *et al.*, 2018; Boyle *et al.*, 2013; Petersen *et al.*, 2016; Villemagne *et al.*, 2011) for a review see (Weintraub *et al.*, 2018)]. Moreover, the ApoE4 allele was overrepresented in non-demented with Aβ deposition. Such finding suggests that these individuals had an increased risk of AD (Corder *et al.*, 1993; Poirier *et al.*, 1993; Strittmatter *et al.*, 1993) and supports the association of ApoE4 with amyloid deposition and cognitive decline (Hyman *et al.*, 1996; Kantarci *et al.*, 2012).

Inflammatory markers have been investigated in body fluids at preclinical AD stages in the Swedish BioFINDER cohort (Janelidze *et al.*, 2018). In such population, cognitively normal individuals with pathologic Aβ values in the CSF displayed an upregulation of inflammatory and cerebrovascular markers as compared to NCI individuals with normal CSF Aβ values (Janelidze *et al.*, 2018). Of note, the levels of these cytokines continued to be elevated in the CSF of MCI and AD individuals and were associated with cortical thinning, longitudinal decline, and risk of AD. (Janelidze *et al.*, 2018). Similar to our findings, the ApoE4 genotype was enriched in NCI Aβ + individuals (Janelidze *et al.*, 2018).

Evidence from populations at genetic risk of AD indicates that brain inflammation might be present at the initial stages of the AD continuum. A significant study by Rodriguez-Vieitez and colleagues, in a population of autosomal dominant AD mutation carriers, revealed an increase in astroglial activation ~ 17 years before the onset of cognitive symptoms which steadily declined with disease progression (Rodriguez-Vieitez *et al.*, 2016). Furthermore, in individuals with Down syndrome (DS), the major population at genetic-risk of AD, neuroinflammation is exacerbated at incipient stages of AD neuropathology and wanes after the development of a full-blown AD neuropathology (Flores-Aguilar *et al.,* 2020*, under review;* Wilcock *et al.,* 2015). Such observations underscore that neuroinflammation at preclinical and prodromal stages of AD might contribute to disease progression.

It is important to note that some individuals harboring high levels of AD pathology do not develop symptomatic AD nor pathological features of AD such as synaptic dysfunction. Lue and colleagues have investigated the expression of the membrane attack complex (C5b-9) and major

histocompatibility complex II (MHC-II) in the brains of elder individuals with or without AD pathology, and in AD brains (Lue *et al.*, 1996). No differences on expression levels were found between NCI individuals with and without pathology (Lue *et al.*, 1996). A peculiarity from this study was that the brains from elders with AD pathology displayed an increase in synaptophysin levels, and increased brain weight than those without AD pathology (Lue *et al.*, 1996). The authors concluded that this population might have been at the preclinical stages of AD and that increased inflammation might be necessary to impact synapse loss and progress to dementia (Lue *et al.*, 1996). While part of these assumptions might hold true, current evidence suggests that the brains analyzed in (Lue *et al.*, 1996) were resilient to AD.

AD-resilient brains do not display neuronal or synaptic loss, and have decreased microglia and astroglia activation, display an anti-inflammatory and pro-resolution cytokine signature, decreased MCP-1 and MIP-1α levels, and increased neurotrophic factor expression when compared to AD brains (Barroeta-Espar *et al.*, 2019; Perez-Nievas *et al.*, 2013). The brains of the so-called "SuperAgers" also offer some insights regarding the detrimental role of neuroinflammation. SuperAgers are individuals over 80 years old that display cognitive performance comparable or better than individuals aged 50 – 65 years; they also display lower levels of microglial activation, comparable to those found in young individuals (Gefen *et al.*, 2019).

Overall, these observations in resilient AD and SuperAgers brains, further support the notion that the presence of neuroinflammation might be an essential driving factor for the development of AD. This concept is additionally supported by our finding that NCI individuals with Aβ pathology and upregulation of cerebral inflammatory markers have cognitive deficits and a faster rate of cognitive decline. A thorough characterization and understanding of preclinical neuroinflammation in populations at risk of AD as well as in the general population is needed to identify the most adequate therapeutic window to anti-inflammatory therapy and to the correct design of anti-inflammatory clinical trials in AD.

Our study needs to be considered with its limitations. First, although *post-mortem* pathological analysis of AD-asymptomatic individuals indicated the presence of pathology meeting AD criteria, we cannot ascertain whether these individuals would have progressed through the AD continuum and developed AD symptoms had they lived longer. However, the faster cognitive decline in non-demented individuals with high Aβ deposition and the higher proportion of ApoE4 individuals in this population, would suggest that these individuals might have indeed been at the earliest stages

of AD. Second, in order to study preclinical stages of AD and to avoid confounding factors (such as the presence of Super-Agers), we conducted our investigations in NCI individuals under age 80. Such criteria limited the number of available samples from the ROS and ROSMAP cohorts. Of note, the narrow sample analyzed in this study could have limited the detection of subtle cognitive deficiencies in other cognitive domains. Finally, we have segregated our population by the extent of Aβ deposition. Besides being a highly selected sample, we acknowledge that there is not a clear "cut-off" value between what represents healthy and AD-related Aβ deposition.

In sum, this study demonstrates that amyloid deposition in the brains of non-demented individuals is associated to an increased inflammatory cytokine expression in the brain. Moreover, each brain region analyzed (temporal, parietal, and frontal cortices) displayed a differential inflammatory profile between each other, suggesting that neuroinflammation evolves in a region-specific manner. Finally, cognitive domain-specific deficits along with a faster rate of cognitive decline was observed in non-demented individuals with amyloid deposition and increased inflammatory expression.

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CHAPTER 3

Supplemental Figures and Tables

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To be submitted

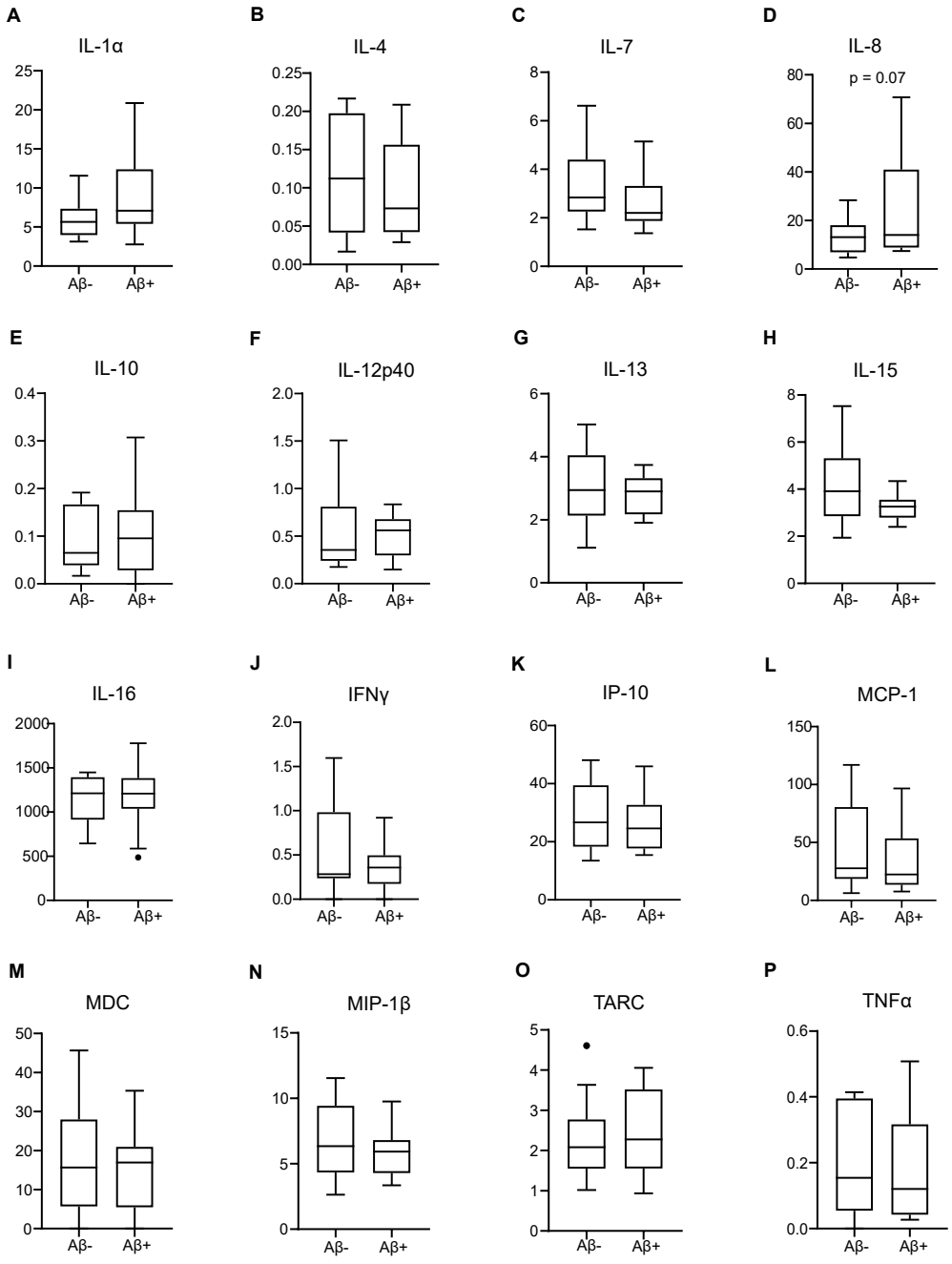

Figure 3-S 1. Expression levels of inflammatory markers in the temporal cortex of individuals with and without Aβ deposition.

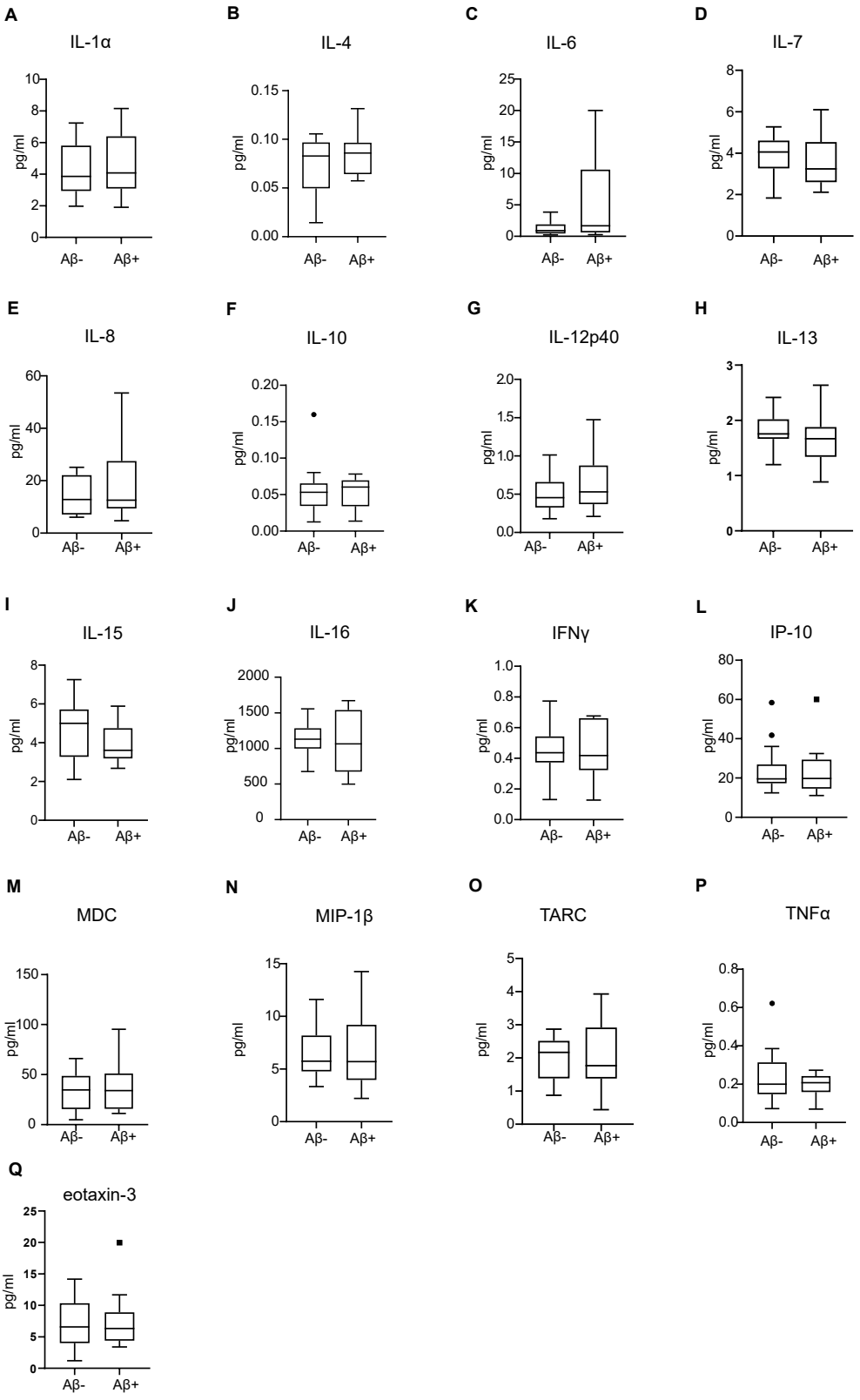

Figure 3-S 2. Expression levels of inflammatory markers in the parietal cortex of individuals with and without Aβ deposition

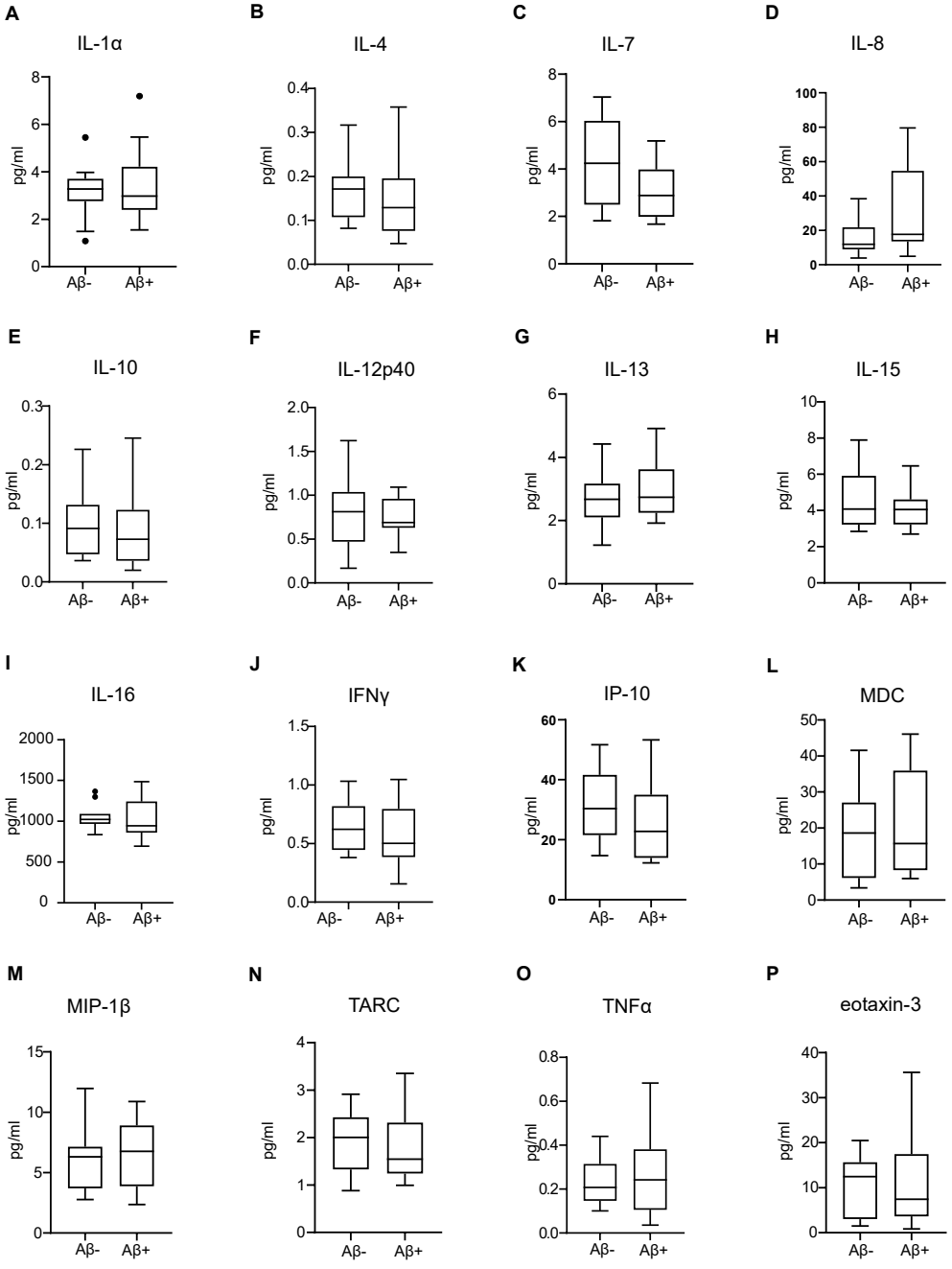

Figure 3-S 3. Expression levels of inflammatory markers in the frontal cortex of individuals with and without Aβ deposition

Supplemental Table 3-S 1. Demographics of the studied population: temporal cortical samples from NCI individuals

		Aβ -	Aβ +	p-value
		n=16	n=12	
Aβ-IR	mean ± SD	0	2.23 ± 1.84	
Age	range (years)	66.21-79.53	69.65-79.63	
	mean ± SD	75.10 ± 4.01	76.19 ± 3.16	0.44
Sex	(F/M)	7/9	7/5	0.70
PMI	range (hours)	3-18.67	2.5-29.58	
	mean ± SD	10.18 ± 5.36	8.56 ± 8.82	0.08
Global cognition	mean ± SD	0.33 ± 0.39	0.38 ± 0.32	0.25
Years of education	mean ± SD	17.88 ± 3.89	18 ± 3.19	0.92
ApoE ε4 allele		0	3 (25%)	0.05
APOE allele distribution	ε 2/2 ε 2/3 ε 2/4 ε 3/3 ε 3/4 ε 4/4	0 4 (25%) 0 12 (75%) 0 0	0 0 0 9 (75%) 3 (25%) 0	
Braak score	0 – II III - IV	9 (60 %) 6 (40 %)	4 (33.33%) 8 (66.67%)	0.25
Braak score distribution	0 I II III IV V VI	1 (6.25%) 5 (31.25%) 4 (25%) 5 (31.25%) 1 (6.25%) 0 0	0 3 (25%) 1 (8.33%) 4 (33.33%) 4 (33.33%) 0 0	
CERAD	possible or no AD probable or definite AD	16 (100 %) 0	3 (25%) 9 (75%)	**<0.0001**
CERAD distribution	no AD possible AD probable AD definite AD	15 (93.75%) 1 (6.25%) 0 0	3 (25%) 0 8 (66.67%) 1 (8.33%)	
NIA-Reagan	low or no likelihood intermediate/high likelihood	16 (100%) 0	5 (41.66%) 7 (58.33%)	**0.0007**
NIA-Reagan distribution	no likelihood low likelihood intermediate likelihood high likelihood	1 (6.25%) 15 (93.75%) 0 0	0 5 (41.66%) 7 (58.33%) 0	
p-tau	mean ± SD	1.24 ± 3.12	1.43 ± 2.77	0.46

Abbreviations: PMI = *post-mortem* interval, Aβ = amyloid beta, F = female, M = male, IR = immunoreactivity, SD = standard deviation. Data are presented as mean ± SD

Supplemental Table 3-S 2. Demographics of the studied population: parietal cortical samples from NCI individuals

		Aβ -	Aβ +	p-value
		n = 15	n = 12	
Aβ-IR	mean ± SD	0	2.90 ± 2.43	
Age	range (years)	67.37-79.53	69.65-79.63	
	mean ± SD	75.99± 3.26	76.28 ± 3.21	0.71
Sex	(F/M)	6/9	7/5	0.44
PMI	range	2.5-18.67	2.5-29.58	
	mean ± SD	9.35 ± 5.53	9.16 ± 8.62	0.37
Global cognition	mean ± SD	0.38 ± 0.36	0.36 ± 0.35	0.86
Years of education	mean ± SD	17.07 ± 3.91	18.67 ± 2.60	0.55
ApoE ε4 allele		1 (6.67%)	3 (25 %)	0.29
APOE allele distribution	ε 2/2 ε 2/3 ε 2/4 ε 3/3 ε 3/4 ε 4/4	0 3 (20 %) 0 11 (73.33 %) 1 (6.66 %) 0	0 0 0 9 (75%) 3 (25%) 0	
Braak score	0 – II III - IV	10 (66.67 %) 5 (33.33 %)	4 (33.33%) 8 (66.67%)	0.12
Braak score distribution	0 I II III IV V VI	1 (6.66 %) 6 (40 %) 3 (20 %) 4 (26.66 %) 1 (6.66 %) 0 0	0 2 (16.66 %) 2 (16.66 %) 4 (33.33%) 4 (33.33%) 0 0	
CERAD	possible or no AD probable or definite AD	15 (100 %) 0	3 (25%) 9 (75%)	**<0.0001**
CERAD distribution	no AD possible AD probable AD definite AD	14 (93.33 %) 1 (6.66 %) 0 0	2 (16.66 %) 1 (8.33 %) 8 (66.67%) 1 (8.33%)	
NIA-Reagan	low or no likelihood intermediate/high likelihood	15 (100%) 0	5 (41.66%) 7 (58.33%)	**0.0009**
NIA-Reagan distribution	no likelihood low likelihood intermediate likelihood high likelihood	1 (6.66%) 14 (93.33%) 0 0	0 5 (41.66%) 7 (58.33%) 0	
p-tau	mean ± SD	0.006 ± .01	0.05 ± .13	.85

Abbreviations: PMI = *post-mortem* interval, Aβ = amyloid beta, F = female, M = male, IR = immunoreactivity, SD = standard deviation. Data are presented as mean ± SD

Supplemental Table 3-S 3. Demographics of the studied population: frontal cortical samples from NCI individuals

		Aβ -	Aβ +	p-value
		n=13	n=13	
Aβ-IR	mean ± SD	0	2.66 ± 2.57	
Age	range (years)	72.25-79.53	69.65-79.63	
	mean ± SD	76.97± 1.97	75.74 ± 3.37	0.27
Sex	(F/M)	5/8	5/8	>.9999
PMI	range	2.5-18.22	2.5-29.58	
	mean ± SD	8.97 ± 5.17	9.86 ± 8.28	0.71
Global cognition	mean ± SD	0.40 ± 0.37	0.33 ± 0.38	0.60
Years of education	mean ± SD	17 ± 3.58	18.54 ± 2.60	0.59
ApoE ε4 allele		1 (7.69%)	3 (23.08 %)	0.59
APOE allele distribution	ε 2/2	0	0	
	ε 2/3	2 (15.38 %)	1 (7.69 %)	
	ε 2/4	0	0	
	ε 3/3	10 (76.92 %)	9 (69.23 %)	
	ε 3/4	1 (7.69 %)	3 (23.07 %)	
	ε 4/4	0	0	
Braak score	0 – II	9 (69.23 %)	5 (38.46 %)	0.23
	III - IV	4 (30.77 %)	8 (61.54 %)	
Braak score distribution	0	1 (7.69 %)	0	
	I	5 (38.46 %)	2 (15.38 %)	
	II	3 (23.07 %)	3 (23.07 %)	
	III	3 (23.07 %)	5 (38.46 %)	
	IV	1 (7.69 %)	3 (23.07 %)	
	V	0	0	
	VI	0	0	
CERAD	possible or no AD	13 (100 %)	4 (30.77 %)	**0.0005**
	probable or definite AD	0	9 (69.23 %)	
CERAD distribution	no AD	12 (92.31 %)	3 (23.1%)	
	possible AD	1 (7.69 %)	1 (7.69 %)	
	probable AD	0	8 (61.54 %)	
	definite AD	0	1 (7.69 %)	
NIA-Reagan	low or no likelihood	13 (100%)	7 (53.85%)	**0.01**
	intermediate/high likelihood	0	6 (46.15%)	
NIA-Reagan distribution	no likelihood	1 (7.69%)	0	
	low likelihood	12 (92.30%)	7 (53.84%)	
	intermediate likelihood	0	6 (46.15%)	
	high likelihood	0	0	
p-tau	mean ± SD	0.06 ± 0.20	0.01 ± .02	0.94

Abbreviations: PMI = *post-mortem* interval, Aβ = amyloid beta, F = female, M = male, IR = immunoreactivity, SD = standard deviation. Data are presented as mean ± SD

Supplemental Table 3-S 4. Demographics of all the study cases with and without global Aβ pathology

		gAβ -	gAβ +	p-value
		n = 14	n = 16	
Aβ-IR	mean ± SD	0	1.962 ± 1.94	
Age	range (years)	66.21 – 79.53	67.37 – 79.63	
	mean ± SD	75.83 ± 3.60	75.32 ± 3.70	0.82
Sex	(F/M)	6/8	9/7	0.71
PMI	range	3 - 29	2.5 – 29.58	
	mean ± SD	11.56 ± 7.23	8.748 ± 7.80	0.07
Cognition global	mean ± SD	0.4162 ± 0.360	0.3103 ± 0.35	0.42
Years of education	mean ± SD	18.29 ± 3.83	17.56 ± 3.24	0.58
ApoE ε4 allele		0	5 (31.25 %)	**0.04**
APOE allele distribution	ε 2/2	0	0	
	ε 2/3	2 (14.28 %)	2 (12.5 %)	
	ε 2/4	0	0	
	ε 3/3	12 (85.71%)	9 (56.25 %)	
	ε 3/4	0	5 (31.25 %)	
	ε 4/4	0	0	
Braak score	0 – II	9 (64.29 %)	7 (43.75 %)	0.29
	III - IV	5 (35.71 %)	9 (56.25 %)	
Braak score distribution	0	1 (7.14 %)	0	
	I	4 (28.57 %)	4 (25%)	
	II	4 (28.57 %)	3 (18.75 %)	
	III	4 (28.57 %)	5 (31.25 %)	
	IV	1 (7.14 %)	4 (25 %)	
	V	0	0	
	VI	0	0	
CERAD	possible or no AD	14 (100 %)	6 (37.5 %)	**0.0003**
	probable or definite AD	0	10 (62.5 %)	
CERAD distribution	no AD	13 (92.86 %)	5 (31.25 %)	
	possible AD	1 (7.14 %)	1 (6.25 %)	
	probable AD	0	9 (56.25 %)	
	definite AD	0	1 (6.25 %)	
NIA-Reagan	low or no likelihood	14 (100%)	9 (56.25%)	
	intermediate/high likelihood	0	7 (43.75%)	
NIA-Reagan distribution	no likelihood	1 (7.14%)	0	**0.007**
	low likelihood	13 (92.85%)	9 (56.25%)	
	intermediate likelihood	0	7 (43.75%)	
	high likelihood	0	0	
p-tau	mean ± SD	1.94 ± 2.31	1.27 ± 1.27	0.91

Abbreviations: PMI = *post-mortem* interval, g = global, Aβ = amyloid beta, F = female, M = male, IR = immunoreactivity, SD = standard deviation. Data are presented as mean ± SD

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Connecting Text: Chapter 3 to 4

In previous chapters we have demonstrated an increased expression of inflammatory cytokines associated to incipient stages of AD pathology in populations at risk of AD. Degeneration of certain neurotransmitter systems might also contribute to such early inflammatory process. It has been proposed that the noradrenergic neurons that compose the locus coeruleus (LC) degenerate early in the curse of AD. Moreover, noradrenaline has been acknowledged to have anti-inflammatory properties in the CNS. Therefore, we have examined if early degeneration of the LC-noradrenergic system had an impact on AD pathogenesis and neuroinflammation at stages preceding amyloid plaque formation in our APP rat model of AD-like amyloidosis.

CHAPTER 4

Locus coeruleus degeneration aggravates and accelerates early neuroinflammation in a rat model of the AD-like amyloid pathology

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Abstract

The locus coeruleus (LC), is the main source of noradrenaline (NA) in the mammalian brain, and degenerates early in the Alzheimer's disease (AD) continuum. Recent studies suggest that, at late stages of the amyloid pathology, LC-pathological alterations affect AD progression by interfering with the neuromodulator and anti-inflammatory properties of NA. Transgenic rodent models of the amyloid-like pathology display a disease-aggravating neuroinflammatory process before the development of amyloid plaques. The impact of LC degeneration at the earliest stages of amyloidosis on the AD-like pathology is not well understood. To assess the impact of early NA demise the LC was lesioned in the McGill-R-Thy1-APP transgenic rat (APP tg) by administering *N*-(2-chloroethyl)-*N*-ethyl-bromo-benzylamine (DSP4) before amyloid plaque deposition. Three months post-treatment, rats displayed a decrease in brain noradrenergic innervation. LC lesion in APP tg rats promoted cognitive deficits in the novel object recognition and fear conditioning tests. Moreover, APP tg-DSP4 treated rats displayed a decrease in hippocampal cholinergic innervation and neurotrophin expression. Further to this, an increase in microglial and astroglial recruitment towards amyloid-beta burdened neurons was found in APP tg-DSP4 treated rats. This was accompanied by microglial morphological alterations indicative of an intermediate activation state and a deregulation in cytokine expression. Our results indicate that early LC demise potentiates the early neuroinflammatory process, cognitive impairments, cholinergic deficits, and neurotrophin deregulation at the earliest stages of the human-like amyloidosis.

Introduction

The locus coeruleus (LC) noradrenergic nucleus is one of the earliest brain structures to degenerate in Alzheimer's disease (AD) (Bondareff *et al.*, 1982; Forno, 1978; Iversen *et al.*, 1983). The LC is the major source of noradrenaline (NA) in the mammalian brain and is involved in arousal, response to stress, alertness, and higher cognitive functions such as learning and memory (Sara, 2009). Stereological analyses have revealed a ~ 30% and ~ 50% of neuronal loss in the LC of individuals with Mild Cognitive Impairment (MCI) and AD, respectively (Kelly *et al.*, 2017). Such neuronal degeneration is accompanied by a drop in NA brain levels in AD brains (Iversen *et al.*, 1983; Storga *et al.*, 1996). In addition, Braak and colleagues have shown that the LC is one of the earliest sites displaying tau pathological accumulation (Braak *et al.*, 2011). Further studies have shown that starting at Braak stage 0, the LC displays a volume reduction of 8.4% with every increasing unit in the Braak score (Theofilas *et al.*, 2017). These observations suggest that LC neuronal shrinkage occurs well before frank neuronal loss in AD (Theofilas *et al.*, 2017) underscoring that LC degeneration occurs at the very early pathological stages of AD.

Besides its role as a classical neurotransmitter, NA can act as an anti-inflammatory molecule in the CNS [for a review see (Feinstein *et al.*, 2016)]. In transgenic models of the AD-like pathology, NA depletion potentiates the inflammatory response by favoring glial activation and upregulation of proinflammatory cytokines (Heneka *et al.*, 2010; Heneka *et al.*, 2006; Jardanhazi-Kurutz *et al.*, 2010; Jardanhazi-Kurutz *et al.*, 2011). Furthermore, an increase in amyloid beta (Aβ) and a modest increase in tau phosphorylation levels have been reported after NA depletion in transgenic models of both amyloid-like (Heneka *et al.*, 2010; Heneka *et al.*, 2006; Jardanhazi-Kurutz *et al.*, 2010; Kalinin *et al.*, 2007) and tau-like pathologies (Chalermpalanupap *et al.*, 2018), respectively. These pathological changes are often accompanied by cognitive deficits (Chalermpalanupap *et al.*, 2018; Heneka *et al.*, 2006; Jardanhazi-Kurutz *et al.*, 2010).

Evidence from transgenic models of the amyloid-like pathology indicates that brain inflammation occurs well-before the deposition of amyloid plaques and is associated with intraneuronal Aβ accumulation (Ferretti *et al.*, 2012a; Ferretti *et al.*, 2012b; Hanzel *et al.*, 2014; McAlpine *et al.*, 2009). Such plaque-independent neuroinflammation is disease-aggravating as anti-inflammatory treatment can rescue cognitive deficits and ameliorate AD-like pathogenesis (Cavanagh *et al.*,

2018; Ferretti *et al.*, 2012a; McAlpine *et al.*, 2009). In humans, epidemiological data suggests that cognitively healthy individuals under a long treatment with anti-inflammatories have a reduced risk of developing AD (McGeer *et al.*, 2013). Moreover, populations at risk of AD, such as individuals with Down syndrome (DS) already display an increase in inflammatory markers well-before the onset of cognitive deficits (Flores-Aguilar *et al.*, 2020, *under review*; Griffin *et al.*, 1989; Janelidze *et al.*, 2018; Rodriguez-Vieitez *et al.*, 2016; Wilcock *et al.*, 2015). The above would suggest that early brain inflammation at incipient stages of AD could be detrimental, but amenable to therapeutic intervention (Cuello, 2017).

The impact of LC-noradrenergic degeneration at the earliest stages of AD is not well-understood. Given that the LC degenerates early in AD and that NA can acts as a neuromodulator, we hypothesized that the loss of NA at the earliest stages of the amyloid pathology should aggravate and accelerate AD-pathogenesis. Therefore, in this study, we investigated whether LC degeneration at the earliest stages of the amyloid-like pathology has an impact on the early neuroinflammatory profile as well as on AD pathogenesis. Towards this goal, we utilized the McGill-R-Thy1-APP tg rats, a transgenic model which displays a continuum of the human-like amyloid pathology in which intraneuronal Aβ burden precedes the overt deposition of amyloid plaques (pre-plaque stage) (Leon *et al.*, 2010). Therefore, the LC-noradrenergic system was denervated at the pre-plaque stage of the amyloid-like pathology. By using biochemical, histological and behavioral approaches we have investigated the effects of LC lesion on neuroinflammation, neurotrophin expression, cholinergic marker expression, and cognition. Here we report that LC demise at incipient stages of the amyloid-like pathology aggravates and accelerates AD-like neuropathological changes and cognitive deficits in the McGill-R-Thy1-APP tg rat.

Materials and Methods

Animals

Female and male McGill-R-Thy1-APP homozygous transgenic rats and their wild-type (wt) littermates were used in this study. McGill-R-Thy-APP rats carry the human APP751 transgene with the Swedish and Indiana mutations (Mullan *et al.*, 1992; Murrell *et al.*, 1991). Genotype was determined by quantitative polymerase chain reaction (qPCR) as described in (Leon *et al.*, 2010). Animals were 4 months old at the start of the experiments and underwent cognitive testing at 7 months of age. Animals were housed in a controlled environment (12 hours light/dark cycle, *ad libitum* access to water and standard rodent diet). Procedures were approved by the McGill Animal Care Committee, following the guidelines of the Canadian Council on Animal Care.

Experimental groups and LC lesion

The LC NA system of wild type and transgenic McGill-R-Thy1-APP rats was lesioned with DSP4 [N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine]; a selective neurotoxin for the LC NA system) (Ross *et al.*, 2015). The toxin was intraperitoneally delivered (50mg/kg followed by a 40mg/kg injection one week after) to pre-plaque (4 months old) transgenic and wild type rats (tg, n=10; wt, n=10). Saline was intraperitoneally injected as a control (tg, n=10; wt, n=10).

Behavioral testing

Novel Object Recognition: The Novel Object Recognition (NOR) test was performed as previously described (Hall *et al.*, 2018; Iulita *et al.*, 2014). Briefly, animals were habituated to the testing arena (80cm × 80 cm × 45 cm) for 3 minutes. Following habituation, animals were familiarized with 5 objects differing in shape and color for 2 minutes. 30 minutes after, a familiar object was replaced by a new object, allowing the animal to explore the new object. The time spent exploring each object was recorded by the experimenter. The discrimination index (DI) was calculated as a measure of discriminative behavior. The DI is defined as the difference in exploring the new

location/object and the total time exploring the familiar objects and dividing this measure by the total time exploring the new location/object and familiar objects (Antunes *et al.*, 2012).

Fear Conditioning: The fear conditioning (FC) test was conducted as previously described (Iulita *et al.*, 2014). The testing chamber was connected to a weight transducer allowing tracking of movement (Panlab, Spain). Coconut extract was used to scent the chamber. The chamber was cleaned with 70% ethanol between animal testing. On day 1, animals were habituated to the testing chamber for 5 minutes (habituation). On day 2, animals were placed back in the chamber for 90 seconds and a tone was presented for 30 sec (75 dB, 5kHz) followed by a 2-second foot shock (0.75mA) (conditioning). Animals recovered in the chamber for 2 minutes. On day 3, the chamber was decorated with visual cues placed on each wall. At the end of day 3, the cues were removed, and the chamber was scented with peppermint extract overnight. On day 4, the animals were placed in the chamber for 2 minutes and 30 seconds and then three 30-second consecutive tones were presented separated by a 30-second pause. The chamber was cleaned with acetic acid 1% between animals. Freezing behavior was measured in each testing session (Freezing v1.3.01, Panlab). Freezing was defined as 2 seconds or more of immobility. Results are presented as percentage time of freezing behavior.

Tissue collection

After behavioral testing, rats were deeply anesthetized with equithesin (6.5 mg chloral hydrate and 3 mg sodium pentobarbital/100g body weight). Animals were transcardially perfused with ice-cold saline pH 7.4, for two minutes. The brain was removed and was dissected into left and right hemispheres. The hippocampus and cortex were dissected from the right hemisphere, snap-frozen and stored at -80°C for further biochemical analyses. The left hemisphere was post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, phosphate-buffered) for 24 h and transferred to 30% sucrose in 0.1 M PB and stored at 4°C. Then, the left hemispheres were cut into 40µm coronal sections with a freezing microtome (Leica SM 2000R; Germany). Free floating sections were stored in cryoprotectant (ethylene glycol and sucrose in PBS, pH 7.4)

Immunohistochemistry

Immunohistochemical analysis was performed in free-floating brain sections. To quench endogenous peroxidase activity, sections were incubated in 0.3 H_2O_2 in PBS for 20 minutes, washed in PBS-T (0.01 M phosphate-buffered saline, 0.2% Triton X-100) and blocked with 10% normal goat serum (NGS) in PBS-T. Then, sections were incubated at 4°C overnight with the mouse McSA1 anti-Aβ monoclonal antibody (1:4000, MediMabs) (Grant *et al.*, 2000), mouse anti-DBH (1:1 for noradrenergic varicosities and 1:50 for LC neurons, MediMabs) (Mazzoni *et al.*, 1991), mouse anti-VAChT (1:8000, Synaptic Systems), mouse anti-NeuN (1: 10000, Millipore), rabbit anti-Iba1 (1: 4000, Wako) and rabbit anti-GFAP (1: 8000, Sigma). Sections were washed and incubated with anti-mouse or anti-rabbit secondary antibodies for 1 hr in 5% NGS-PBST and then incubated for 1 hr with a mouse anti-peroxidase monoclonal antibody (1:30) pre-incubated with horseradish peroxidase (5 µg/ml) in PBS (MAP kit, Medimabs), or with the VECTASTAIN Elite ABC HRP kit (Vector Laboratories). Sections were incubated in 0.06 % DAB (3,3′-diaminobenzidine) (Sigma) and then developed with 0.01% hydrogen peroxide (Sigma). For the double immunostainings, NeuN or McSA1 immunoreactivity was revealed by further incubating the developed Iba-1 sections with a secondary goat anti-mouse and MAP-HRP. Immunoreactivity was visualized using the VECTOR SG substrate kit (Vector Laboratories). Sections were washed and mounted in gelatin-coated coverslips, air-dried, dehydrated in ascending alcohols, cleared with xylene and cover-slipped using Entellan mounting medium (EMD, Millipore).

Quantification of noradrenergic and cholinergic varicosities

Images were captured using a Zeiss Axio Imager M2 microscope equipped with a Zeiss Axiocam 506 color digital camera coupled to the Zeiss ZEN 2 software. For the noradrenergic counts, images were from the parietal cortex (lamina IV-V) and hippocampus (dentate gyrus, DG and CA1) were acquired. Cholinergic varicosities were quantified in the CA1 and DG. In each section, three images per region were captured for a total of 3 sections per animal (AP -2.8 mm, -3.3 mm, -3.8 mm). Bouton quantification was done with ImageJ (National Institutes of Health, Bethesda, Maryland) as previously described (Iulita *et al.*, 2017). Briefly, files were binarized and immunoreactive material was subtracted from background using optical density and size-exclusion

criteria. The algorithm “triangle” was used to threshold the optical density. For the noradrenergic count, the “despeckle’” and “watershed” filters were applied after optical density thresholding. Immunoreactive structures of a size ranging from 0.3 μm^2 to 5 μm^2 and larger than 0.1 μm^2 for the noradrenergic and cholinergic varicosities, respectively were recorded. The final counts are expressed as a density of DBH or VAChT immunoreactivity in an imaging field of 315 × 252.44 μm.

Amyloid-beta quantification

Cortical samples were homogenized in 10% (w/v) of cold TBS buffer (150 mM NaCl, 50 mM Tris / HCl, 5 mM EDTA, pH 7.6) containing a protease inhibitor cocktail (cOmplete, Mini; Roche) and ultracentrifuged at 100 000g for 1 hour at 4°C. Supernatants were collected and stored at -80°C. On the day of the assay, supernatants were thawed, and Aβ38, Aβ40 and Aβ42 levels were measured with a V-PLEX Aβ peptide panel 1 (6E10) kit (Meso Scale Discovery, USA) according to manufacturer’s instructions. Signal was read in a SECTOR Imager 6000 reader (Meso Scale Discovery, USA).

Quantification of microglia and astroglia recruitment to CA1

Brightfield digital images of Iba-1/NeuN and Iba-1/McSA1 immunolabeled sections were acquired with a Zeiss Axio Imager M2 microscope equipped with a Zeiss Axiocam 506 color digital camera using the 20x objective. Two images per section and a total of 3 sections per animal were captured (AP -2.8 mm, -3.3 mm, -3.8 mm). Microglia and astroglia recruitment towards the CA1 neuronal layer were measured in two counting frames (100 × 200 μm) randomly centered in CA1. Iba-1 or GFAP positive cells were counted by an investigator blinded to treatment and genotype. The average number of microglia per counting frame was calculated.

Microglial morphological assessment

Z-stack images of Iba-1/NeuN and Iba-1/McSA1 immunolabeled sections in the CA1 region of the hippocampus were captured using a microscope equipped with AxioCam 506 color digital camera (Carl Zeiss, Germany) using a 40x objective. Three brain sections were imaged, and 3 images were captured per brain section. Image analysis was done using the software ImageJ (NIH, Bethesda, Maryland). Briefly, Iba-1 immunoreactive structures were isolated from NeuN/McSA1 immunoreactive neurons using the color deconvolution plug-in "H & E 2". Then, images were despeckled and the threshold was set using the "triangle" algorithm. To further decrease background noise the "Despeckle", "Close-" and "Remove outliers" filters were applied. The total area covered by Iba-1 per micrograph was calculated by the software. To measure the total length of microglial processes and cell body, binary files were skeletonized using the Skeletonize (2D/3D) function.

Electrochemiluminescence-linked immunoassay

Cortical samples were homogenized in 8% (w/v) of lysis buffer (20mM Tris-HCl, 0.5% nonidet p-40, 150mM, pH 7.5) containing a protease inhibitor cocktail (cOmplete, Mini; Roche) and ultracentrifuged at 13 000 rpm for 40 minutes at 4°C. Supernatants were collected and stored at -80°C. On the day of the assay, supernatants were thawed and interferon (IFN)-γ, Interleukin (IL)-1β, IL-4, IL-5, IL-6, IL-10, IL-13, CXCL1 and Tumor Necrosis Factor (TNF)-α levels were analyzed with the V-Plex Proinflammatory Panel 2 (Meso Scale Discovery, USA) following manufacturer's instructions. Signal was read in a SECTOR Imager 6000 reader (Meso Scale Discovery, USA). Protein concentrations were measured using the DC™ Protein Assay Kit (BioRad, USA). IFN γ, IL-5 and IL-4 were under the detection levels and thus were not considered in the analyses.

RNA extraction and quantitative real time PCR

RNA extraction was performed with the RNeasy Mini Kit (Qiagen) following manufacturer's instructions. Reverse transcription was done in a total of .8 µg of total RNA using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-rad, USA) according to manufacturer's indications. For qRT-PCR reactions, 10 µM of the forward and reverse primers were used. Real-time PCR was performed in a 10µl reaction containing 2 µl of cDNA with iQSYBR® Green (BioRad, USA) in a Bio-Rad iCycler. The cycling conditions were as follows: 95 °C for 15 min, followed by 40 cycles of 10 s at 95 °C, 30 s at 58 °C, and 15 s at 72 °C. The fold change gene expression is expressed relatively to the wt-saline group using the 2^ (- delta delta CT) method with HPRT and β-actin as housekeeping genes. The primer sequences were: BDNF (recognizing all BDNF transcripts), forward primer sequence 5' TGGATGCCGCAAACA 3', reverse primer sequence 5' CCGGGACTTTCTCCAGGACT 3'; HPRT forward primer sequence 5' CAGGCCAGACTTTGTTGGAT 3', reverse primer sequence 5' TCCACTTTCGCTGATGACAC 3'; β-actin forward primer sequence 5' AGGCATCCTGACCCTGAAG 3', reverse primer sequence 5' GCTCATTGTAGAAAGTGTGG 3'.

Immunoblotting

Cortical samples were sonicated in lysis buffer (Cell Signaling, USA) containing a protease inhibitor cocktail (cOmplete mini, Roche). Homogenates were centrifuged at 13 000 rpm for 45 minutes at 4 °C. Supernatants were collected and protein concentration was determined (DC™ Protein Assay kit, BioRad). 40 ug of protein were loaded on 12% sodium dodecyl sufate (SDS)-polyacrylamide gels and semi-dry transferred to PVDF membranes (BioRad). Membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) for 1 hour at room temperature. Following blocking, membranes were incubated with rabbit anti-proNGF (1:2000, Alomone) in TBS-T overnight at 4°C or with mouse anti-GAPDH (1:15 000, Millipore, USA) for 1 hour at room temperature. The next day the membranes were incubated for 1 hour with peroxidase-conjugated secondary anti-rabbit antibody or anti-mouse antibody (1:15000, Jackson Immunoresearch) in TBS-T at room temperature. Western blots were developed

with an enhanced chemiluminescence substrate (Western Lightning® Plus-ECL, PerkinElmer Inc.) and imaged with Amersham Imager 600 (GE Healthcare). The intensity of the immunoreactive band at 27kDa (pro-NGF) was quantified with TotalLab CLIQS Software (TotalLab, UK) and the densitometry values were normalized to GAPDH content and to an internal control loaded to each gel. Final densitometry values are expressed as the fold change relative to the control group (wt-saline).

Statistical analysis

Data was analyzed with Graph Pad Prism 8. Data is displayed as mean $\pm$ SEM. For multiple group comparisons, two-way analysis of variance was performed followed by Tukey's multiple comparisons test. Two-tailed Student's t-test was performed for two-group comparisons. Statistical significance was set at $p < 0.05$.

Results

Noradrenergic lesion

To evaluate the lesion caused by DSP4 administration, noradrenergic varicosities were quantified in the cerebral cortex and hippocampus. Wt-DSP4 treated rats showed a 91% decrease in noradrenergic varicosities in the cortex accompanied by a reduction of 76% and 79% in CA1 and DG respectively, as compared to wt-saline treated rats. In tg APP rats, noradrenergic varicosities were reduced by 83% in the cortex and by 52%, and 63% in CA1, and DG respectively following DSP4 treatment as compared to tg-saline rats (Figure 4-1B). Four months after DSP4 treatment, DSP4-treated animals, wt and tg, also displayed a marked loss of DBH immunoreactivity in the LC (Figure 4-1C). Therefore, DSP4 treatment provoked a marked loss of noradrenergic varicosities in both, wt and APP tg rats.

LC lesion at the pre-plaque stage of the amyloid-like pathology promoted and potentiated cognitive deficits

We further assessed if early degeneration of the LC could contribute to the development of cognitive impairments before the development of a full-blown plaque-like pathology. Wt and APP tg rats were evaluated 3 months after toxin/saline administration in the fear conditioning and novel object recognition cognitive tests.

In the contextual fear conditioning task, DSP4 and saline-treated tg rats showed a decrease in freezing behavior as compared to the wt groups (Figure 4-2B). Following the context recall test, fear-associated memory retrieval was evaluated in the cued recall task. This test revealed that saline-treated APP tg rats were impaired (% freezing = 73.83 $\pm$ 5.34) when compared to saline (% freezing = 91.33 $\pm$ 2.23, $p = 0.02$) (Figure 4-2C). Furthermore, cognitive deficits were further exacerbated in the APP tg rats with LC lesion (% freezing = 32.33 $\pm$ 5.7) when compared to saline-treated tg rats ($p < .0001$); suggesting that deficits in the LC noradrenergic transmission exacerbated pre-existing cognitive deficits (Figure 4-2C).

In the novel object recognition task, tg rats treated with DSP4 displayed working memory deficits (DI =0.09 ± .11) when compared to saline treated tg rats (DI = 0.38 ± 0.08, $p = 0.04$) (Figure 4-2D). Taken together, these results support that loss of LC noradrenergic innervation promotes and aggravates cognitive deficits in tg rats at the pre-plaque stage of the amyloid-like pathology.

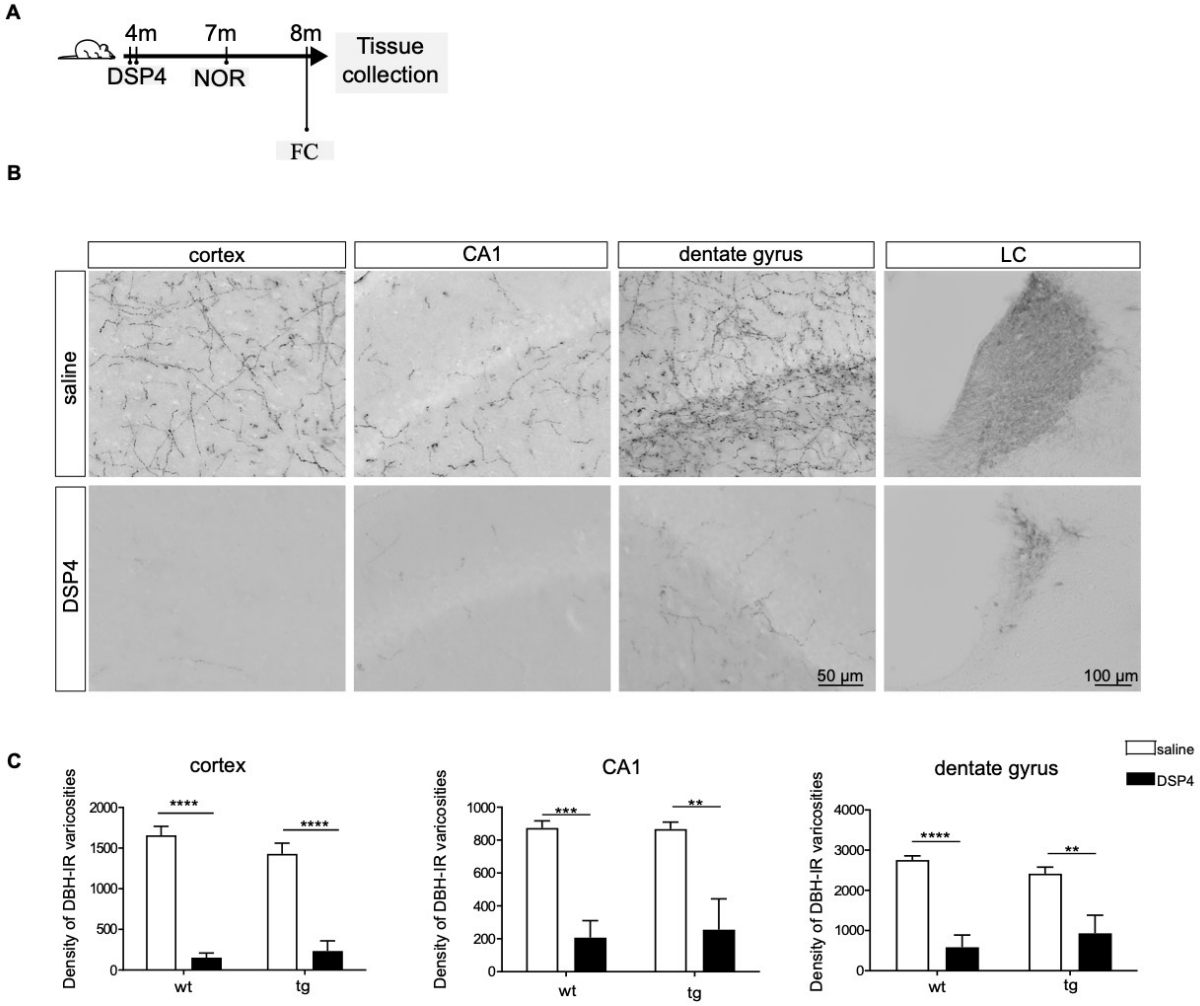


Figure 4- 1. Decrease on noradrenergic innervation following LC lesion.

(A) Experimental timeline: wt and tg rats received two intraperitoneal injections of DSP4 (50mg/kg) or saline at 4 months of age. At 7 months-old, rats were tested in the NOR and FC tests. Following cognitive testing, rats were euthanized, and the brain was harvested and processed for biochemical and histological analyses. (B) Representative images displaying DBH-IR in the cortex, hippocampus, and LC from wt rats treated either with saline or DSP4. (C) The graphs illustrate noradrenergic varicosity quantification in the cortex and hippocampus (CA1 and DG). Note that DSP4 reduced noradrenergic innervation in the cortex and hippocampus. Data were analyzed with a two-way ANOVA followed by Tukey's *post-hoc* test. Data are represented as mean ± SEM. wt rats = 14, tg rats = 10-11. Abbreviations: NOR = novel object recognition, ANOVA = analysis of variance, MCT = multiple comparisons test, LC = locus coeruleus, DBH-IR = dopamine beta hydroxylase-immunoreactivity, DG = dentate gyrus, wt = wild type, tg = transgenic, SEM = standard error of the mean. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

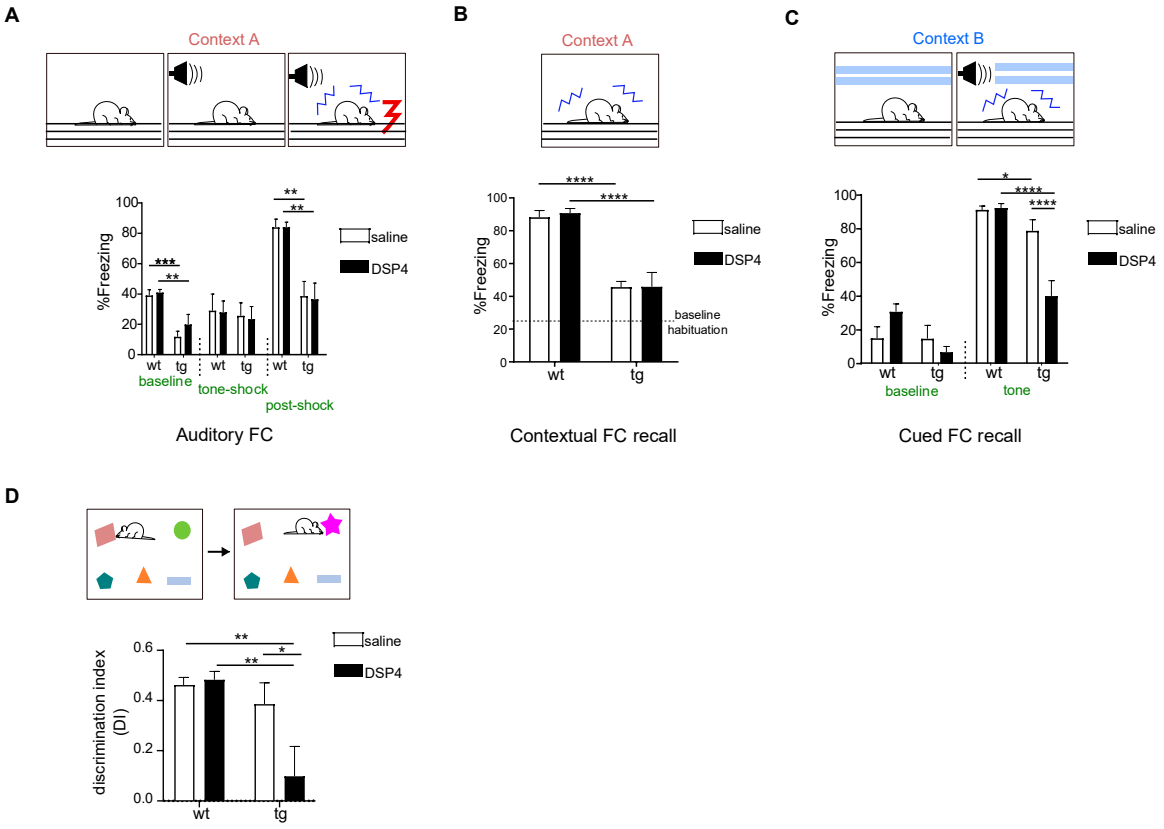


Figure 4- 2. LC demise accelerated and worsened cognitive deficits at the pre-plaque stage of the amyloid-like pathology.

Rats were tested in the FC and NOR tests after DSP4 or saline treatment. (A) Rats were habituated in the auditory FC test. Note that following the foot shock, wt rats displayed an increase in freezing behavior as compared to APP tg rats. (B) 24 hours after, the animals were placed in the same context were the tone-shock was presented and the freezing response was measured. Compared to wt rats, APP tg rats displayed decreased freezing behavior. (C) The cued fear response test revealed cognitive deficits in APP tg rats as compared to wt rats. Following LC demise, APP tg displayed marked cognitive deficits when compared to saline-treated APP tg rats. (D) DSP4 treatment promoted working memory deficits in APP tg rats as revealed by the NOR test. Data were analyzed with a two-way ANOVA followed by Tukey's *post-hoc* test. Data are represented as mean ± SEM. wt rats = 14, tg rats = 10-11. Abbreviations: NOR = novel object recognition, FC = fear conditioning, ANOVA = analysis of variance, MCT = multiple comparisons test, wt = wild type, tg = transgenic, SEM = standard error of the mean. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Noradrenergic impairments and amyloid pathology

Several studies have reported that, once the amyloid plaque pathology is established, lesions in the LC promote an increase in Aβ levels. We investigated whether LC lesion influenced cortical Aβ deposition or soluble Aβ levels. Impairment of the LC did not promote an increase in soluble Aβ38, Aβ40 and Aβ42 levels or amyloid plaque deposition (Figure 4-3). Therefore, at the pre-plaque stage, LC demise does not potentiate the amyloid pathology in the APP tg rat.

LC lesion promoted cholinergic deficits

While cholinergic innervation is not affected at the pre-plaque stage of the amyloid-like pathology in the APP tg rat, a decrease in cholinergic innervation in the hippocampus and cerebral cortex at the amyloid plaque stage has been observed (Iulita *et al.*, 2017). To examine if noradrenergic deficits at the pre-plaque stage have an impact in cholinergic innervation, we assessed the density of hippocampal cholinergic varicosities. Computer-assisted analysis revealed a 30 % decrease in cholinergic varicosities in the CA1 and DG of DSP4-treated APP tg rats when compared to saline-treated wt rats ($p = 0.0001$ and $p = 0.01$, respectively) and a 20% reduction in CA1 cholinergic varicosities when compared to saline-treated APP tg rats ($p = 0.02$) (Figure 4-4). Thus, the loss of LC noradrenergic input accelerated cholinergic deficits.

Decreased neurotrophin expression following LC lesion

NA can act as a neuroprotective molecule by promoting the release of the neurotrophins BDNF and NGF (Counts *et al.*, 2010). Western-blot analyses revealed that, overall, DSP4 treatment reduced the expression of the NGF precursor, proNGF (Figure 4-5A). Moreover, DSP4-treated tg rats displayed decreased levels of BDNF than saline-treated wt rats as revealed by gene expression analyses (Figure 4-5B).

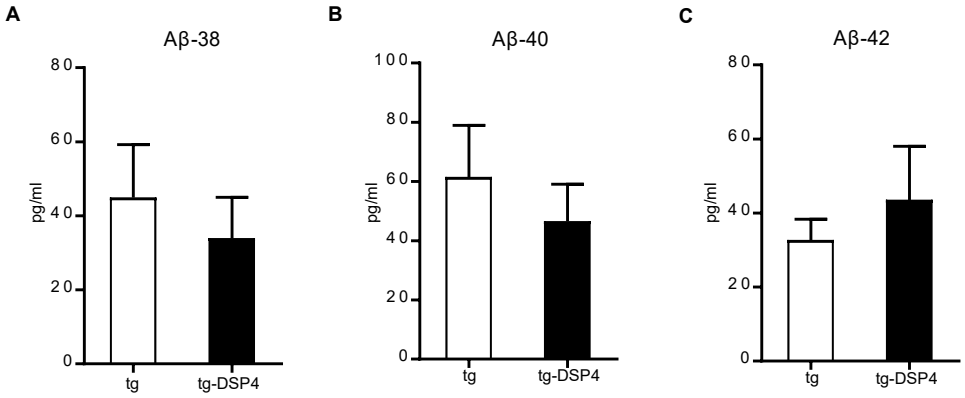


Figure 4- 3. Aβ levels are not affected following LC demise at early stages of the amyloid-like pathology.

(A-C) Aβ38, Aβ40, and Aβ42 were measured with an electrochemiluminescent assay in cortical homogenates of saline and DSP4-treated rats. No differences were found in expression levels between the groups. Student's t-test. Data were analyzed using a two-tailed Student t-test. Data are represented as mean ± SEM. tg rats = 10-11. Abbreviations: Aβ = amyloid beta, tg = transgenic, SEM = standard error of the mean.

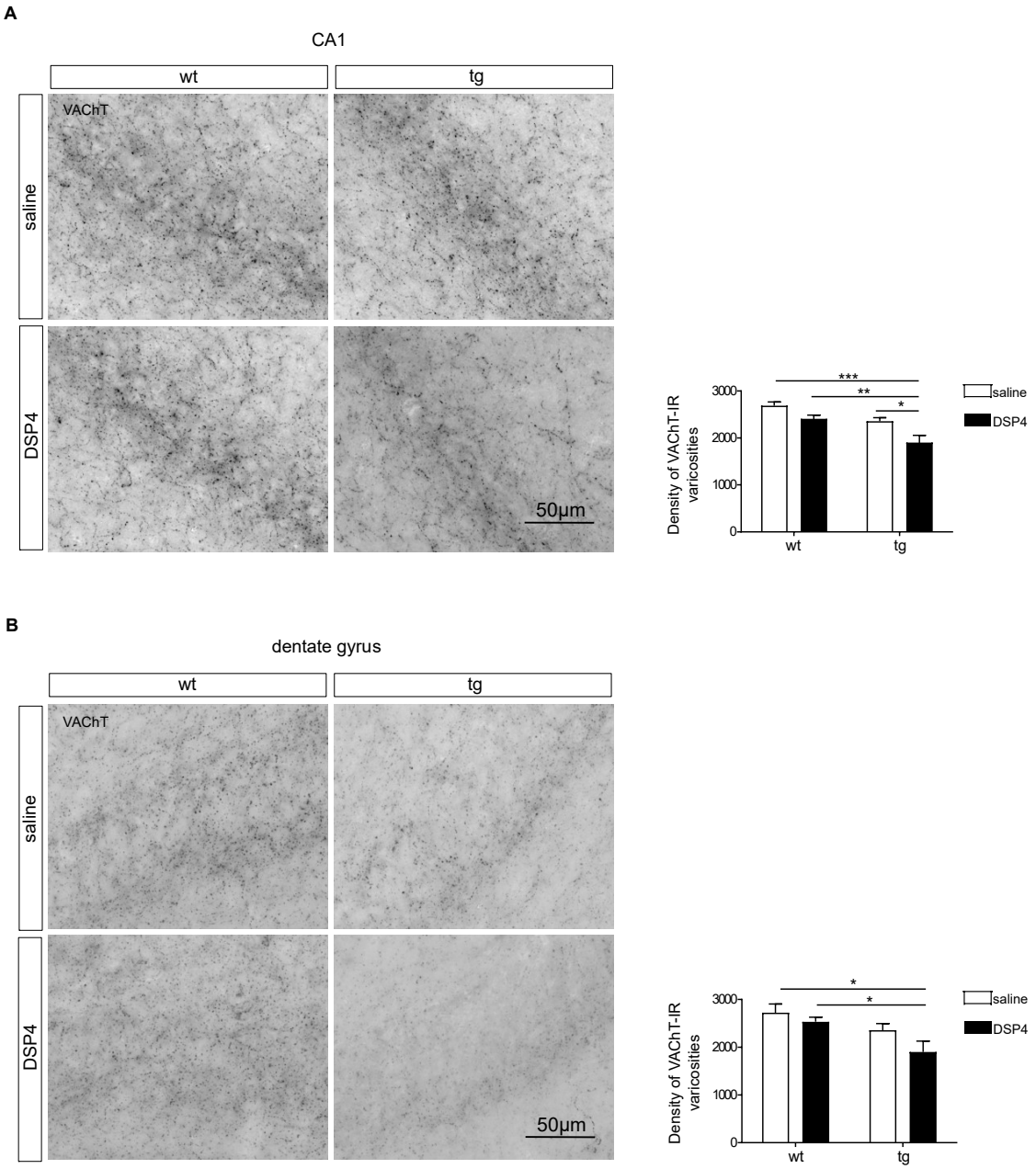


Figure 4- 4. Hippocampal cholinergic varicosities are downregulated after LC lesion in APP transgenic rats.

(A-B) Representative micrographs of VAChT presynaptic boutons in the CA1 and dentate gyrus. Note that DSP4 treatment promoted a downregulation of cholinergic varicosities at the pre-plaque stage of the AD-like amyloid pathology. Data were analyzed with a two-way ANOVA followed by Tukey's *post-hoc* test. Data are represented as mean ± SEM. wt rats = 14, tg rats = 10-11. Abbreviations: ANOVA = analysis of variance, MCT = multiple comparisons test, VAChT-IR = Vesicular acetylcholine transporter-immunoreactivity, wt = wild type, tg = transgenic, SEM = standard error of the mean. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

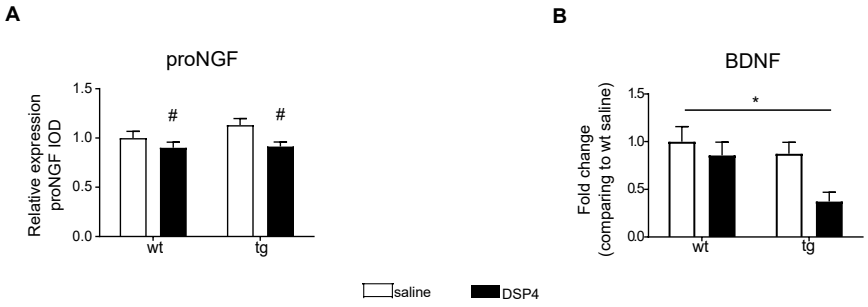


Figure 4- 5. Early locus coeruleus demise promoted a downregulation in pro-NGF and BDNF.

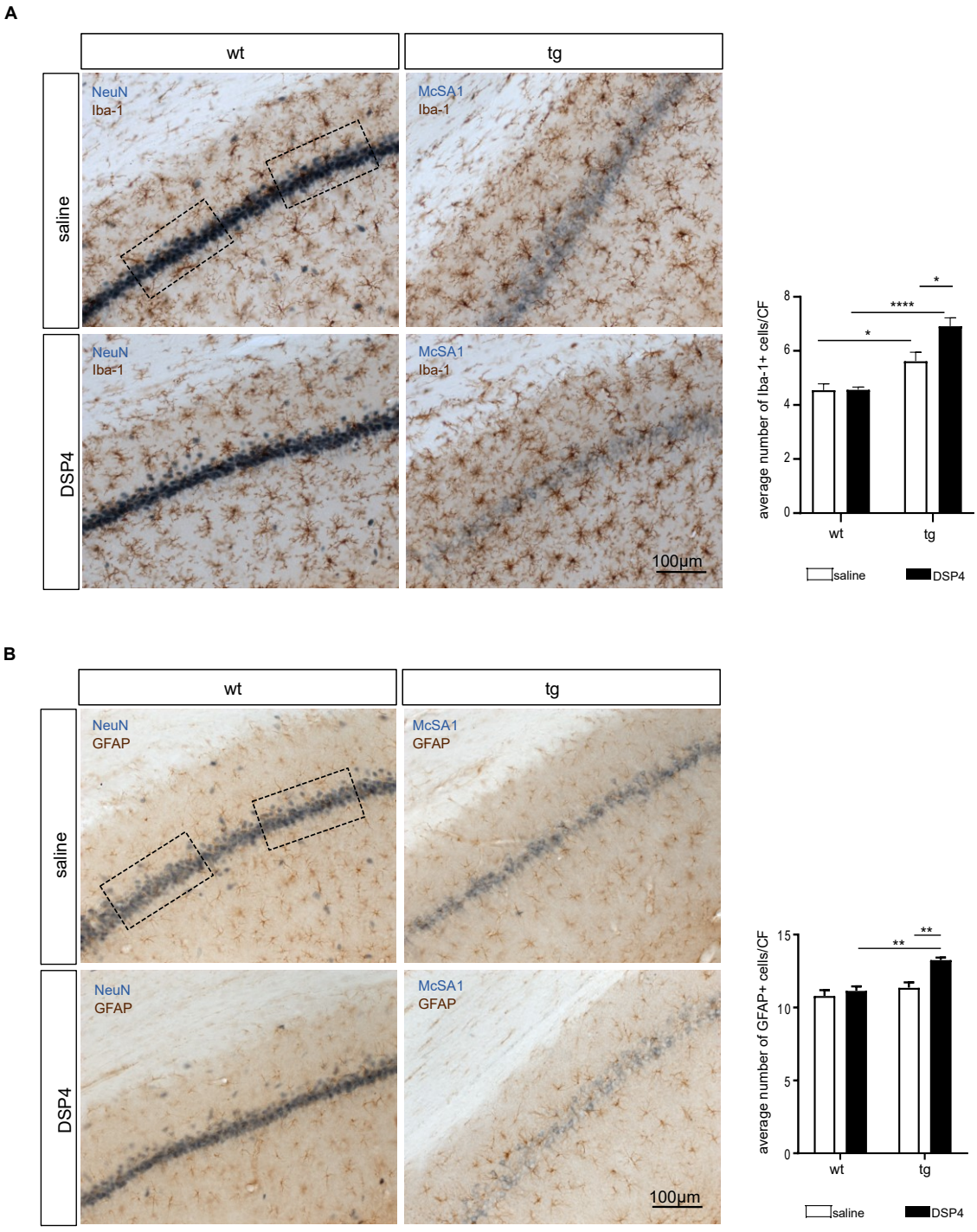
Relative proNGF and BDNF expression levels were analyzed in cortical homogenates. (A) Western blots revealed that DSP4 treatment affected proNGF levels in APP tg and wt rats, (effect of treatment, $F_{(1, 19)} = 6.789$, $\# p = .01$) (B) BDNF levels were markedly downregulated in the tg-DSP4 treated group as compared to wt control levels. Data were analyzed with a two-way ANOVA followed by Tukey's *post-hoc* test. Data are represented as mean ± SEM. wt rats = 14, tg rats = 10-11. *p<.05. Abbreviations: BDNF = brain-derived neurotrophic factor, ANOVA = analysis of variance, MCT = multiple comparisons test, wt = wild type, tg = transgenic, SEM = standard error of the mean. $*p < 0.05$.

LC demise potentiated the early neuroinflammatory process

In the APP tg rats, microglial and astroglial recruitment towards hippocampal Aβ-burdened neurons occurs before amyloid-plaque deposition (Hanzel *et al.*, 2014). To further assess if LC lesion exacerbated this inflammatory response, we quantified microglia and astroglial recruitment towards the CA1 neuronal layer. As illustrated in Figure 4-6A; an increase in microglial recruitment was observed in APP tg-saline rats as compared to wt-saline treated rats ($p = 0.01$). LC demise further potentiated microglial recruitment in DSP4-treated APP tg rats as compared to saline-treated tg rats ($p = 0.01$). Moreover, an increase in astroglial recruitment in the APP tg-DSP4 group was observed when compared to the saline-treated tg ($p = 0.008$) (Figure 4-6B).
To investigate whether inflammatory protein expression was affected by the loss of LC noradrenergic terminals, we examined the expression of inflammatory markers in the frontal cortex of lesioned and control rats. Elevated levels of the proinflammatory chemokine CXCL1 accompanied by a decrease in the anti-inflammatory cytokine IL-13 were found in the DSP4-treated APP tg rats when compared to saline-treated APP tg rats (Figure 4-6C-D). No other inflammatory markers were deregulated at this stage (Figure 4-6E-G).

LC lesion promotes microglial morphological changes

We further investigated if LC lesion had an impact on microglial morphology. To this end, we examined whether there were differences in the total length of microglial cell processes and soma, and in the total area covered by microglia in the CA1 region (Figure 4-7). The total area covered by microglia and the length of their cell body and processes tripled in the APP tg rats as compared to wt rats ($p < 0.0001$) (Figure 4-7B). These morphological changes were more pronounced in the APP tg rats after LC degeneration as compared to APP tg-saline treated rats ($p < 0.0001$) (Figure 4-7B). Furthermore, LC demise in tg rats promoted an increase in the total microglial process and soma length as compared to APP tg-saline treated rats ($p < 0.0001$) (Figure 4-7C).



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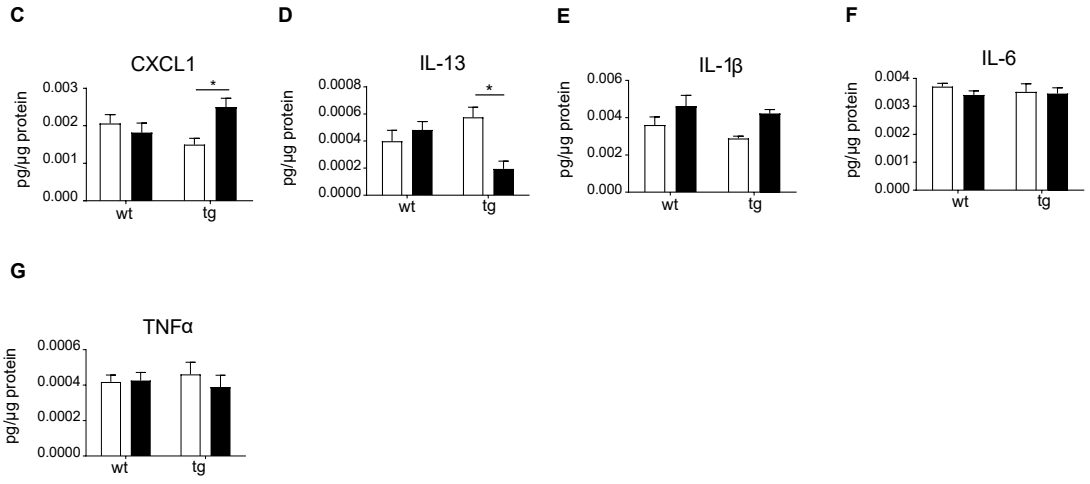

Figure 4- 6. Increased microglial and astroglial recruitment and cytokine expression after LC lesion at the pre-plaque stage of the amyloid-like pathology.

(A) Representative micrographs of brain sections immunostained against Iba-1 (brown), NeuN (dark blue), and McSA1 (light blue) in the CA1 region of the hippocampus. APP tg rats displayed an increase in microglial recruitment as compared to wt rats. Microglial recruitment was further exacerbated following LC demise in App tg rats (B) Micrographs depicting GFAP-1 immunostaining (brown), NeuN (blue, in wt rats), and McSA1 (blue, in APP tg rats). LC lesion promoted an increase in astroglial recruitment in APP tg rats. (C) Inflammatory markers were quantified in cortical homogenates using an electrochemiluminescent assay. CXCL1 levels were increased in APP tg- rats following LC lesion. (D) DSP4-treated APP tg rats displayed lower levels of the anti-inflammatory cytokine IL-13 than APP tg-saline treated rats. (E-G) LC lesion did not affect IL-1β, IL-6 and TNF-α. Data were analyzed with a two-way ANOVA followed by Tukey's *post-hoc* test. Data are represented as mean ± SEM. wt rats = 14, tg rats = 10-11. Abbreviations: Iba-1 = ionized calcium binding adaptor molecule 1, NeuN = neuronal nuclei, GFAP = glial fibrillary acidic protein, Aβ = amyloid beta, ANOVA = analysis of variance, MCT = multiple comparisons test, wt = wild type, tg = transgenic, IL = interleukin, TNF = tumor necrosis factor, SEM = standard error of the mean. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

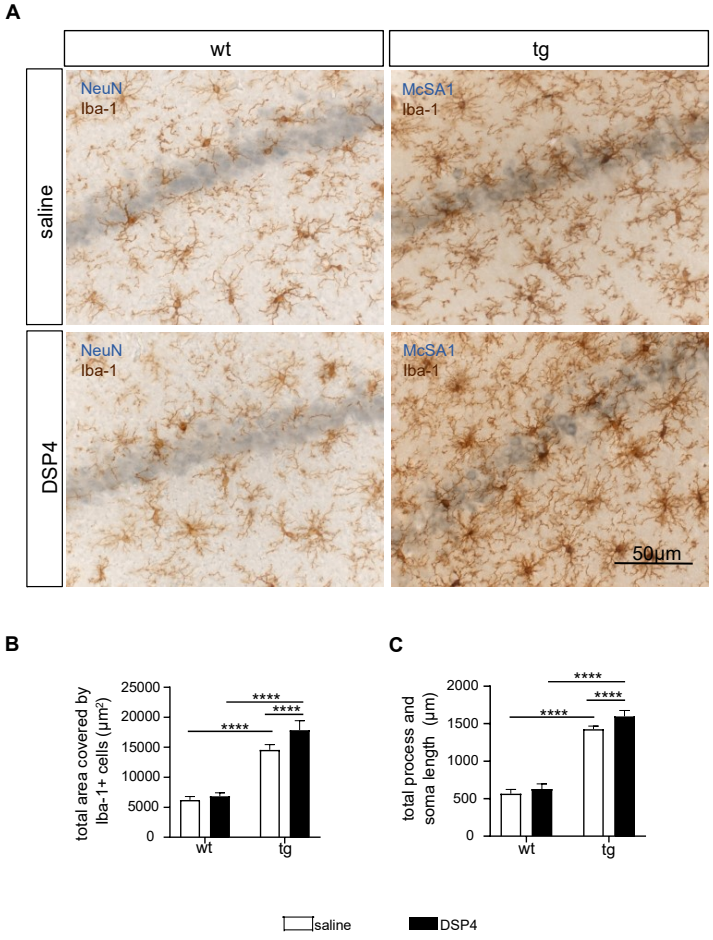

Figure 4- 7. LC degeneration enhances microglia activation at the earliest stages of the amyloid-like pathology.

(A) Representative micrographs of brain sections immunostained against Iba-1 (brown), NeuN (blue, wt rats), and McSA1 (blue, tg rats). (B) An increase in the total area covered by microglia was found in APP tg rats. LC degeneration significantly increased microglial area in APP tg rats. (C) Although APP tg rats displayed an increase in the total length of their somas and processes as compared to wt rats, DSP4 treatment further exacerbated the total length in APP tg rats. Data were analyzed with a two-way ANOVA followed by Tukey's *post-hoc* test. Data are represented as mean ± SEM. wt rats = 14, tg rats = 10-11. Abbreviations: Iba-1 = ionized calcium binding adaptor molecule 1, NeuN = neuronal nuclei, ANOVA = analysis of variance, MCT = multiple comparisons test, wt = wild type, tg = transgenic, SEM = standard error of the mean. ****$p < 0.0001$.

Discussion

In this study, we investigated whether early LC lesion had an impact on AD pathogenesis before the overt deposition of amyloid plaques in the McGill-R-Thy1-APP tg rat model which mimics the earliest stages of the human-like amyloid pathology. The present study indicates that LC degeneration at early stages of the AD-like amyloid pathology accelerates cognitive deficits, promotes cholinergic impairments, aggravates early neuroinflammation, and diminishes the expression of neurotrophic factors (Figure 4-8).

Noradrenergic signaling is involved in the modulation of memory, learning, and long-term potentiation (LTP) (Berridge *et al.*, 2003; Bliss *et al.*, 1983; Neuman *et al.*, 1983; Sara, 2009). Notably, LC neuronal loss and cytopathological changes strongly correlate with ante-mortem cognitive decline (Grudzien *et al.*, 2007; Kelly *et al.*, 2017; Wilson *et al.*, 2013). Heneka and colleagues have reported that LC degeneration in the APP23 and APP/PS1 mouse models accentuated and promoted spatial and working memory deficits (Heneka *et al.*, 2006; Jardanhazi-Kurutz *et al.*, 2010). In this study, ablation of the LC-noradrenergic system at the pre-plaque stage resulted in short-term memory impairments in the APP tg-lesioned rats as revealed by the NOR test. Importantly, LC lesion in wt animals did not promote cognitive deficits, underscoring that intraneuronal Aβ oligomer accumulation could be a contributing factor to the observed cognitive deficits in APP tg rats following LC lesion. Given that the LC is highly involved in the formation and retrieval of emotional memories, we also tested the animals in the Pavlovian fear conditioning test. While the presence of the human mutated APP transgene promoted deficits in the fear conditioning context recall test, the added LC lesion in these animals further aggravated the cued fear memory response. The cued fear response is known to be largely mediated by NA signaling in the amygdala [for a review see (Giustino *et al.*, 2018)]. Notably, the McGill-R-Thy-APP is a tg model which displays intraneuronal Aβ accumulation in the amygdala (Iulita *et al.*, 2014). In this case, intraneuronal Aβ accumulation in the amygdala might further exacerbate the cognitive deficits promoted after DSP4 LC ablation in this animal model.

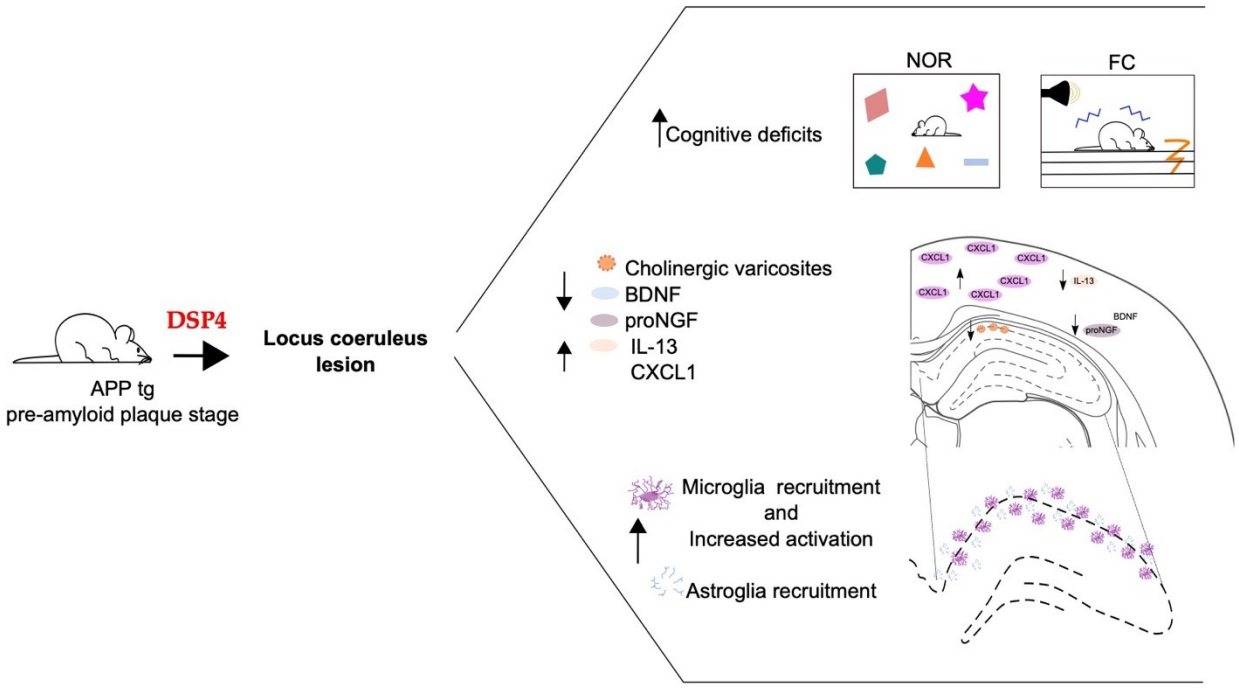

Figure 4- 8. Effects of LC degeneration at the pre-plaque stage of the amyloid-like pathology.

LC lesion in APP tg rats promoted and accelerated cognitive deficits, depleted cholinergic varicosities, NGF and BDNF cortical levels. Moreover, increased microglial and astroglial recruitment towards Aβ burdened neurons, microglial morphological changes indicative of an intermediate activation state and deregulation of inflammatory cytokines were observed following LC lesion.

It is well-established that the basal forebrain cholinergic system is importantly involved in learning and memory mechanisms [for a review see (Hampel *et al.*, 2019)]. The cholinergic compromise in AD is the result of neuronal atrophy in the cholinergic nucleus basalis of Meynert, resulting in decreased cortical and hippocampal acetylcholine levels in AD brains (Bowen *et al.*, 1976; Davies *et al.*, 1976; Etienne *et al.*, 1986; Hampel *et al.*, 2019; Pearson *et al.*, 1983; Perry *et al.*, 1977; Whitehouse *et al.*, 1982). Downregulation of cholinergic varicosities were reported after the appearance of amyloid plaques in the McGill-R-Thy-APP rat and in mouse models of the amyloid-like pathology (Bell *et al.*, 2006; Iulita *et al.*, 2017; Perez *et al.*, 2007). Here, we found that LC demise accelerated the downregulation of cholinergic varicosities, as assessed by VAChT immunoreactivity before the appearance of amyloid plaques, indicating a possible relationship between the steady state number of cholinergic synapses and the status of the noradrenergic synaptic network. As the loss of cholinergic terminals coincides with the onset of cognitive decline in APP tg-lesioned rats, such early cholinergic impairments could be contributing to the observed cognitive deficits following LC lesion.

It has been proposed that NA can exert its neuroprotective functions by modulating neurotrophin expression (Counts *et al.*, 2010). These effects are thought to be mediated by β-adrenoreceptor signaling and activation of the cAMP pathway (Counts *et al.*, 2010). Such activation would lead to increased CREB levels with the ensuing upregulation of NGF and BDNF expression levels (Counts *et al.*, 2010; McCauslin *et al.*, 2006; Tabuchi *et al.*, 2002). Furthermore, *in-vitro* blockage of Trk, the NGF and BDNF receptor, abolishes NA protection against amyloid toxicity (Counts *et al.*, 2010). Finally, antibodies directed against NGF and BDNF can also block NA protective function against amyloid-beta peptides *in-vitro* (Counts *et al.*, 2010). These observations suggest that NA-mediated release of NGF and BDNF might mediate pro-survival and neurotrophic pathways in the healthy CNS. Other studies have shown that loss of BDNF expression results in deficient LTP (Korte *et al.*, 1996a; Korte *et al.*, 1996b) and that NGF signalling mediates the transcription of major cholinergic markers including VAChT (Berse *et al.*, 1999). Therefore, the reductions in cholinergic varicosities discussed above might be reflective of NGF downregulation after LC lesion. The above would be in line with the present observations showing lower levels of proNGF likely representing a decreased expression. Taken together, downregulation of NFG and BDNF after LC ablation might contribute to cognitive deficits, cholinergic system impairments,

and lack of neuroprotection in the APP tg rat at the earliest stages of the amyloid-like pathology; aspects which deserve further investigations.

NA *per se* can also act as an anti-inflammatory agent in the CNS (Feinstein *et al.*, 2016). Previous reports using different APP mouse models at the post-plaque stage have shown that LC ablation promotes an increase in inflammatory cytokine expression, oxidative stress, and microglial and astroglial activation. Our results show that LC lesion further potentiated microglial and astroglial recruitment towards Aβ-bearing neurons in the hippocampal region. Notably, following DSP4 treatment, microglial cells from in APP tg rats adopted a hypertrophic morphology. Such hypertrophic microglia are found in neurodegenerative diseases and commonly reflect an intermediate activation state (Streit *et al.*, 2014). We hypothesize that the loss of noradrenergic innervation primes microglial cells before the overt deposition of plaques. In agreement with this, *in-vitro* studies have shown that NA administration suppresses microglial reactivity (Gyoneva *et al.*, 2013). Therefore, the loss of NA could prime microglia to survey and react promptly to a toxic stimulus. Microglial intermediate activation has been reported in APP tg rodent models at the pre-plaque stage of the amyloid-like pathology along with increased levels of inflammatory mediators (Ferretti *et al.*, 2012a; Ferretti *et al.*, 2012b; Hanzel *et al.*, 2014). Notably, anti-inflammatory treatment rescues the microglial phenotype, ameliorates Aβ pathology and cognitive deficits (Cavanagh *et al.*, 2018; Ferretti *et al.*, 2012a). The above observations emphasize the detrimental nature of the early neuroinflammatory process in AD.

We further examined cortical expression of inflammatory markers. Increased levels of the chemokine CXCL1 and decreased levels of the anti-inflammatory cytokine IL-13 were noted in APP tg rats following DSP4 treatment. CXCL1 is a chemokine that promotes leukocyte extravasation into the CNS (K. Zhang *et al.*, 2013). Moreover, CXCL1 can lead to tau cleavage through the activation of caspase-3 (X. F. Zhang *et al.*, 2015). At the amyloid post-plaque stage, CXCL1 levels are upregulated in the McGill-R-Thy-APP rat (Wilson *et al*., 2019). The early upregulation of CXCL1 provoked by LC demise could promote peripheral monocyte or microglia chemotaxis towards injured neurons, a proposition that awaits further investigation. IL-13 has been regarded to have anti-inflammatory properties mostly in the periphery but also in the CNS (Mori *et al.*, 2016). Intracerebral administration of IL-13/IL-4 in the APP23 mouse model ameliorated cognitive deficits, diminished intraneuronal Aβ and promoted the anti-inflammatory phenotype of

microglia (Kawahara *et al.*, 2012). Thus, decreased IL-13 expression after LC demise might favor neuroinflammation by interfering with the neuroprotective and anti-inflammatory role of IL-13.

Increased Aβ levels and plaque formation have been observed after LC demise at the post-plaque stage of the amyloid pathology in APP mouse models (Heneka *et al.*, 2010; Heneka *et al.*, 2006). Notably, we did not observe any changes in Aβ levels after LC lesion. In agreement with our findings, LC demise before overt amyloid deposition in APP mouse models does not potentiate the amyloid pathology (Jardanhazi-Kurutz *et al.*, 2010; Jardanhazi-Kurutz *et al.*, 2011; Kummer *et al.*, 2014). These studies, and our observations highlight that LC demise might have a primary effect on neuroprotection and neuroinflammation before influencing Aβ levels.

Our studies need to be considered within certain limitations. When studying the effects of LC demise, DSP4 administration continues to be one of the main approaches to promote LC degeneration. However, it has to be noted that acute LC lesions do not resemble the gradual LC degeneration observed in AD. Genetic manipulations of the noradrenergic system in APP tg mouse models have been conducted to impair noradrenergic signalling (Hammerschmidt *et al.*, 2013; Kummer *et al.*, 2014); nevertheless, they pose certain drawbacks as compensatory mechanisms might occur. The generation of better transgenic models resembling the earliest stages of the amyloid pathology and LC tau pathological alterations might help to shed light on the earliest pathological mechanisms associated to LC degeneration. Moreover, addressing the impact of LC co-transmitters such as galanin, neuropeptide Y, and enkephalin on the AD pathology might help to better understand the neuropathological process of LC neurodegeneration in AD.

In sum, in this study, we have shown that LC demise accelerated and worsened cognitive deficits at the pre-plaque stage of the amyloid-like pathology in the McGill-R-Thy1-APP tg rat. Moreover, we also observed depletion of cholinergic varicosities accompanied by a decrease in NGF and BDNF in DSP4-treated APP tg rats. LC demise in APP tg rats was accompanied by a heightened neuroinflammatory response as revealed by microglial intermediate activation and alterations on cytokine expression. Overall, our findings highlight early pathological mechanisms arising from early disruption of NA signaling and LC degeneration. Future studies should elucidate the specific pathways governing these observations.

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CHAPTER 4

Supplemental Figures

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To be submitted

A

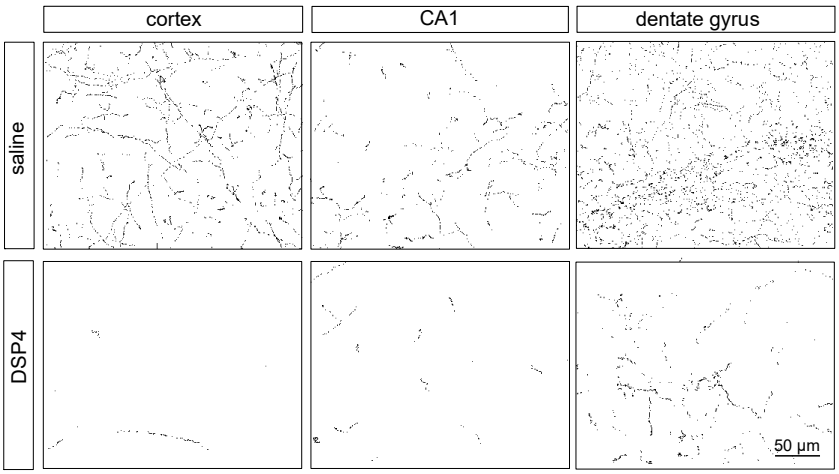

Figure 4-S 1. Noradrenergic varicosities following computer-assisted quantification.

(A) Representative micrographs of DBH presynaptic boutons in the cortex, CA1 and dentate gyrus.

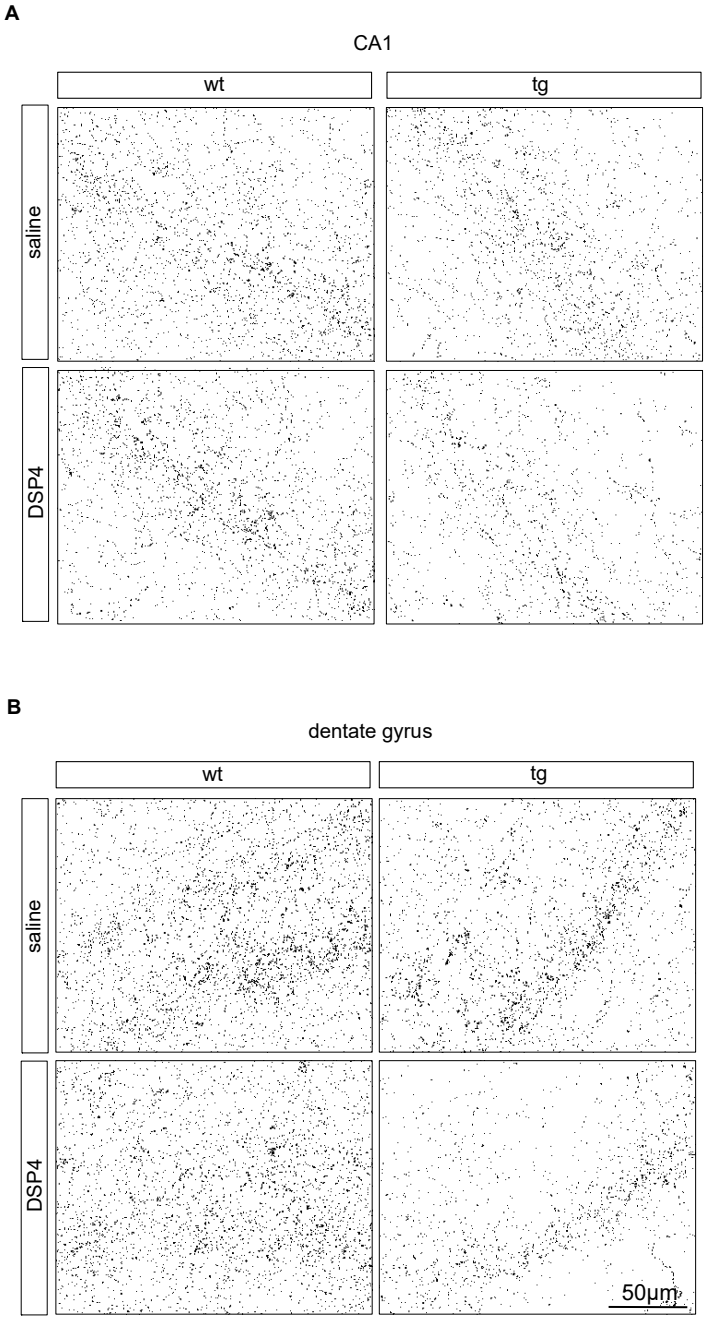

Figure 4-S 2. Cholinergic varicosities following computer-assisted quantification.

(A-B) Representative micrographs of VAChT presynaptic boutons in the CA1 and dentate gyrus.

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CHAPTER 5

Summary of main findings
and
General discussion

Summary of main findings

The main objective of this Thesis has been to understand the initial neuroinflammatory process at the earliest stages of the AD neuropathology. Towards this goal, we have investigated the expression of inflammatory cytokines in the brains of individuals with DS. We have furthered investigated the expression of inflammatory markers and subtle cognitive impairments in older cognitively normal individuals harboring AD pathology. Finally, we have addressed the contributions of noradrenergic system disruption to the neuroinflammatory process, cognitive status and AD-like pathogenesis in a transgenic APP rat model at the earliest stages of amyloidosis, prior to amyloid plaque deposition.

Our investigations provide the most comprehensive analysis of the evolving neuroinflammatory process across the lifespan of individuals with DS (Chapter 2). Our results revealed that prior to the development of an overt AD pathology, individuals with DS displayed a heightened neuroinflammatory profile characterized by the elevation of potent inflammatory cytokines and intermediate activation of microglia. After the development of a full-blown AD pathology, the inflammatory process in DS brains showed a differential inflammatory profile when compared to younger ages as revealed by a decrease in inflammatory mediators, a failure of the tissue resolution response, and the presence of highly dystrophic microglia (Figure 5-1). Furthermore, our investigations indicate that neuroinflammatory marker expression varies between brain regions. Finally, for the first time, we have shown that sex influences the expression of inflammatory mediators in the DS brain, where females with DS have a heightened inflammatory profile than males as revealed by higher IL-6 and IL-8 expression.

To further assess if early neuroinflammation was associated with the incipient accumulation of AD neuropathology, we have analyzed the expression of inflammatory markers in the temporal, parietal, and frontal brain cortices of cognitively normal individuals under the age of 80 with and without cortical Aβ deposition (Chapter 3). Our results indicate that, when compared to individuals without Aβ deposition, individuals harboring Aβ pathology displayed an increase in IL-1β, IL-6, and eotaxin-3 in the temporal cortex; IL-1β, and MCP-1 in the parietal cortex, and a trend towards an increase in IL-1β, IL-6, and MCP-1 in the frontal cortex.

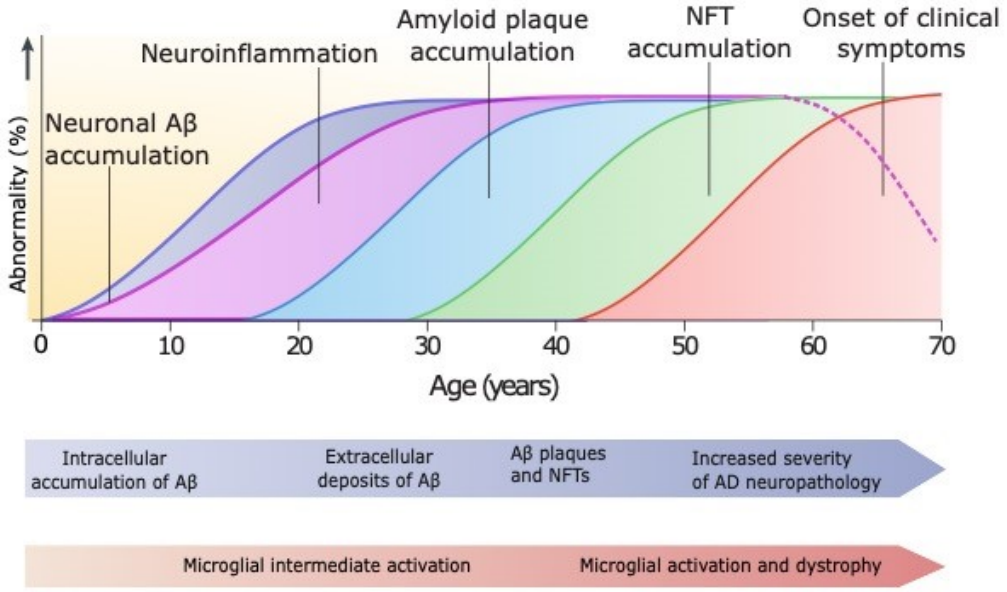

Figure 5- 1. Neuroinflammation is present before the accumulation of overt Alzheimer's pathology in Down syndrome brains and wanes with aging.

Image adapted from (Lott *et al.*, 2019) with permission from *Springer Nature*.

Furthermore, inflammatory marker expression was associated to Aβ deposition. Moreover, individuals with cortical Aβ deposition displayed cognitive deficits in perceptual speed (Figure 5-2).

Finally, we have provided evidence that disruption of the LC-noradrenergic system before amyloid plaque deposition (i.e. pre-plaque stage) promotes and accelerates cognitive deficits. The loss of noradrenergic innervation in the hippocampus and cortex of APP tg rats accelerated the early neuroinflammatory profile as revealed by increased microglial and astroglial recruitment, microglial morphological changes indicative of intermediate activation, and deregulation of inflammatory cytokines. Furthermore, noradrenergic denervation promoted a decrease in neurotrophin expression and cholinergic hippocampal innervation (Chapter 4).

In sum, the results of our investigations indicate that brain inflammation is present before the occurrence of a full-blown AD pathology and onset of cognitive symptoms in AD and in DS. Furthermore, our investigations broaden the current knowledge about the evolution of neuroinflammation along the AD continuum and the contribution of neurotransmitter system dysfunction to the earliest stages of AD.

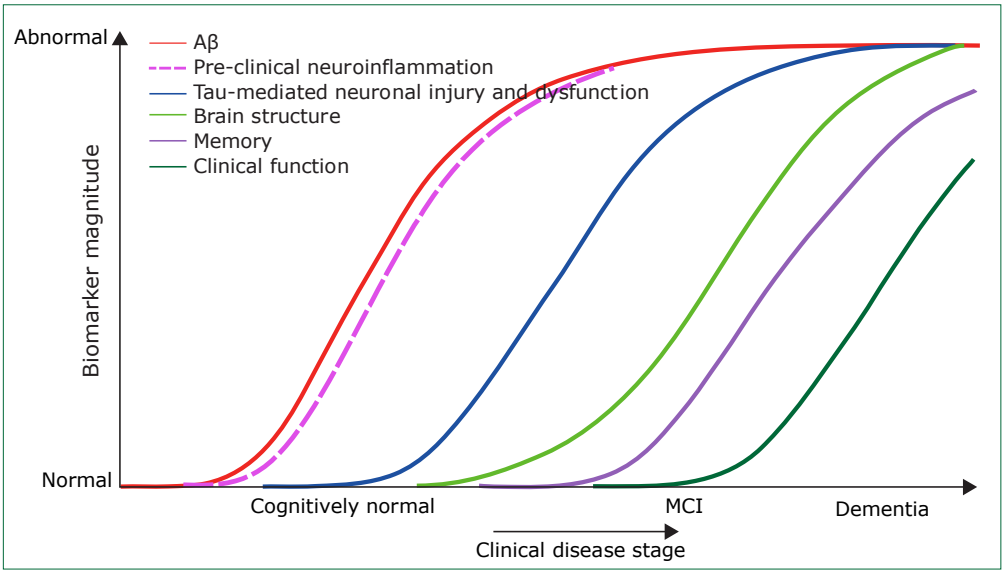

Figure 5- 2. Hypothetical evolution of biomarkers reflecting the Alzheimer's disease pathological cascade.

Aβ is measured by PET or CSFAβ42. Tau-mediated neuronal injury and dysfunction was measured by CSF-tau or fluorodeoxyglucose PET. Brain structure was measured by MRI. Our studies suggest that pre-clinical brain inflammation is parallel to amyloid accumulation. Aβ = amyloid beta, PET = positron emission tomography, MRI = magnetic resonance imaging, MCI = mild cognitive impairment. Reprinted from the Lancet (Jack *et al.*, 2010) and adapted with permission from *Elsevier*.

General discussion

Factors contributing to preclinical inflammation in Alzheimer's disease

Our investigations support the notion that brain inflammation occurs at preclinical stages of AD. While the contribution of Aβ in potentiating the inflammatory response has been discussed in Chapters 2-4, other factors might contribute to the neuroinflammatory process in AD. Aging, peripheral inflammation, genetic risk factors, metabolic disorders, and brain injuries among others can modulate brain inflammation and might have a direct influence in the development of AD. Such events will be discussed in the next sections.

Inflammaging and neuroinflammaging

Inflammation has been classified as one of the mechanisms (or the so-called seven pillars) of aging and age-related diseases (Kennedy *et al.*, 2014). Aging is accompanied by a chronic, sterile low-grade inflammation nowadays referred as "inflammaging" (Franceschi *et al.*, 2018). This increased inflammatory profile is also accompanied by a defective adaptive response (Giunta *et al.*, 2008). Inflammaging can arise from mitochondrial dysfunction, cellular senescence, epigenetic mechanisms, inflammasome activation, and defects in the DNA damage response among other mechanisms (Franceschi *et al.*, 2018). Thus, successful or healthy aging relies on maintaining a low level of inflammation throughout the lifespan while unsuccessful aging might result from maladaptive age-related inflammation; an event possibly leading to the development of age-related diseases. Such a concept is reinforced by the fact that centenarians display an increase in anti-inflammatory mechanisms that might play a role in delaying or preventing age-related diseases in this population (Giunta *et al.*, 2008). As discussed in Chapter 3 of this Thesis, the absence of neuroinflammation in some brains displaying AD pathology might result in sparing of clinical AD in the SuperAger population.

Given that aging is the most prevalent risk factor for AD, potential failure in maintaining low-grade inflammation might contribute to the development of this neuropathology. Such neuroinflammatory events might directly affect the integrity of the CNS. Aged mice displayed

increased TNFα expression in BBB endothelial cells along with a downregulation of tight junction proteins; such events correlate with peripheral inflammation associated to the aging process (Elahy *et al.*, 2015; Holmes, 2013). Further to it, transcriptomic analyses of aged astrocytes have revealed an upregulation of genes related to the astrocytic proinflammatory A1 phenotype (Clarke *et al.*, 2018; Orre *et al.*, 2014). Human studies have shown that hippocampal BBB disruption is associated with aging, occurs before hippocampal atrophy, and worsens after MCI presentation (Montagne *et al.*, 2015); suggesting that BBB disruption precedes the onset of cognitive deficits and neurodegeneration. Taken together, such observations would suggest that age-related BBB breakdown is accompanied and associated to an underlying inflammatory process.

Microglial cells also suffer some relevant changes across aging. Transcriptomic analyses have revealed that aged microglia display a reduced expression in genes involved in actin dynamics, cell adhesion, and axonal guidance when compared to young microglia (Galatro *et al.*, 2017). Such gene deregulation is suggestive of an age-related reduced motility. Further to it, aged-microglia display lower phagocytic activity than young microglia, a factor highly contributing to defective Aβ clearance in AD brains (Hickman *et al.*, 2008; Njie *et al.*, 2012). Histological analyses of human aged microglia show the presence of cytoplasmic morphological changes such as fragmentation, spheroid formation, and deramification suggesting that microglial dystrophy accompanies aging (Streit *et al.*, 2004). Microglial morphological changes are also accompanied by an increased production of inflammatory cytokines and oxidative species (Ritzel *et al.*, 2015). Such inflammatory environment concomitant with repeated inflammatory stimuli that an individual might be subject to throughout the lifespan, is thought to contribute to age-related microglial priming. Indeed, primed microglia are more susceptible to a second stimulus and respond to it with an exacerbated production of inflammatory mediators (V. H. Perry *et al.*, 2014). Therefore, primed microglia lose their ability to perform physiological functions by continuously overexpressing inflammatory mediators, which might contribute to the development of neurodegenerative diseases such as AD.

While most of the research regarding inflammaging has been focused on peripheral cells and inflammatory markers, little is known about brain cytokine expression across the lifespan. The study by Cribbs and colleagues revealed a deregulation on brain inflammatory gene expression between young (20 – 59 years) and old (60 – 99 years) individuals (Cribbs *et al.*, 2012). Such

studies indicate that genes related to inflammasome activation, complement pathway, chemokines, MHC-I and MHC-II type genes are upregulated during aging.

To extend the previous findings, we assessed whether inflammatory protein expression varied across aging in non-demented individuals. Therefore, we investigated cytokine expression in the frontal cortex of infants (1 – 10 years old), young adults (13 – 27 years), and adults (42 – 68 years). As illustrated in Appendix I, a deregulation of inflammatory mediators was found in the frontal cortex of young adults and adults when compared to infants. Remarkably, TNFα was already upregulated in young adults accompanied by a decrease in IL-12p40 and TARC, and increased levels of anti-inflammatory molecules such as IL-4, IL-10 when compared to infants. Such deregulation was maintained in older ages concomitant with increased levels of IP-10, IL-15, MCP-1, MIP-1β, IL-7, and a trend towards an upregulation of IL-1α. Moreover, VEGF-A levels decreased with aging. Therefore, our studies indicate that brain inflammation increases with normal aging; however, such increase is accompanied by high levels of anti-inflammatory molecules. Notably, our results are the first observations of altered brain inflammatory protein expression across the lifespan of normal individuals. Whether such inflammatory process arises in the periphery or in the brain per se awaits further investigations. However, the deregulation of inflammatory markers at a relatively young age (13 – 27 years) would indicate that "neuroinflammaging" might initiate in the brain.

Some contrasting results can be observed when comparing normal brain cytokine expression that accompanies aging and pathological inflammatory expression in neurodegenerative disorders. For example, while IL-10 is upregulated with normal aging (Appendix I), adults with DS (Chapter 2) and AD brains (X. Wang *et al.*, 2015) displayed a downregulation of IL-10 at advanced stages of AD pathology. Moreover, IL-1β and IL-6 did not show an increase with normal aging, however high levels of these cytokines were already present in young DS brains (Chapter 2) and in non-demented individuals with abnormal Aβ accumulation. The above observations highlight the maladaptive nature of neuroinflammation at incipient stages of the AD pathology.

The identification of mechanisms involved in the development of age-related diseases and in factors contributing to healthy ageing and longevity is of outmost importance. Such investigations might reveal some therapeutic targets amenable to therapy aimed to halt or delay the development of age-related diseases. For example, Zhang and colleagues have reported that the hypothalamus

might mediate age-related mechanisms by immune-mediated signaling (Zhang *et al.*, 2013). Aged mice displayed an age-dependent upregulation of NFκB, firstly detectable in hypothalamic microglia and then in neurons (Zhang *et al.*, 2013). Such upregulation was accompanied by an age-related increase in inflammatory mediators such as TNFα (Zhang *et al.*, 2013). Inhibition of NFκB signaling promoted increased lifespan (Zhang *et al.*, 2013). They further demonstrated that NFκB activation led to decreased levels of gonadotropin-releasing hormone (GnRH) (Zhang *et al.*, 2013). Administration of GnRH induced adult neurogenesis and greatly improved lifespan (Zhang *et al.*, 2013). Thus, an immune mediated pathway within the brain might regulate the aging process, highlighting the involvement of the immune system in age-related disorders. Future investigations would provide a deeper understanding of "neuroinflammaging" in the human brain.

Peripheral inflammation and cognitive impairment

Individuals with AD display an underlying peripheral inflammatory process (K. S. P. Lai *et al.*, 2017). It is not yet established whether this inflammatory profile is reflective of brain inflammation or vice-versa. Episodes of immunological challenge can lead to the development of sickness behavior, characterized by anorexia, somnolence, depression, fever, decreased social interaction and concentration (Holmes, 2013); (V. H. Perry *et al.*, 2014). For example, peripheral administration of LPS to humans and non-human primates results in microglial activation, development of sickness behavior and cytokine production, the latter inversely correlated with memory performance (Brydon *et al.*, 2008; Hannestad *et al.*, 2012; Harrison *et al.*, 2009; Krabbe *et al.*, 2005). Some have hypothesized that microglial priming might occur in response to Aβ accumulation, and that systemic inflammation could exacerbate the inflammatory process and accelerate disease progression (Holmes, 2013; V. H. Perry *et al.*, 2014). Limited but compelling evidence indicates that increased peripheral inflammation is associated to the development of AD (Holmes, 2013). For example, periodontal disease caused by periodontal bacteria is a risk factor for the development of AD in the general population and has been suggested to have an impact on the DS population given their susceptibility to periodontal disease (Holmer *et al.*, 2018; Kamer *et al.*, 2009; Kamer *et al.*, 2016; Stein *et al.*, 2007). The presence of systemic infections can also lead to delirium, a condition that increases an individual's risk of developing dementia (Rahkonen *et*

al., 2000). Perhaps one of the most compelling infections associated to the development of AD pathology and dementia is the one cause by the human immunodeficiency virus (HIV). HIV is a retrovirus that depletes CD4+ T cells leading to an impaired immune response. HIV can target the brain, infect microglia, astroglia, macrophages, and neural precursors leading to exacerbated neuroinflammation, neurotoxicity and cognitive impairment in 50 % of infected individuals (Canet *et al.*, 2018). HIV+ individuals over age 50 present neuropathological features similar to those found in AD: decreased CSF $A\beta_{42}$ levels, amyloid plaque deposition, increased BACE-1 activity, increased CSF tau and p-tau, neurodegeneration, BBB disruption, and neuroinflammation (Canet *et al.*, 2018). It remains to be elucidated if HIV infection promotes the development of AD in the HIV+ population; nevertheless, current studies highly suggest that HIV infection may increase the risk of developing AD. Notably, non-infectious age-related disorders such as diabetes type 2, obesity, and hypertension are associated with chronic inflammation. Such metabolic disorders have been found to increase the risk of developing AD (Baumgart *et al.*, 2015; Newcombe *et al.*, 2018). The above evidence would suggest that a cumulative effect of systemic infections or the lifelong presence of a metabolic disorder might have a considerable contribution to the development of AD.

It is important to mention that individuals with DS display an exacerbated systemic inflammatory profile (Maroun, 1980; Sullivan *et al.*, 2017; Sullivan *et al.*, 2016). Given the triplication of genes involved in IFN signaling (i.e. four interferon receptors), individuals with DS display an hyperresponsiveness to IFN. Studies from Sullivan and colleagues indicate that the IFN pathway is consistently activated in individuals with DS since early in life (Sullivan *et al.*, 2017; Sullivan *et al.*, 2016). Such upregulation might lead to the increased systemic inflammation in DS and contribute to autoimmune disorders, leukemia, and developmental abnormalities (Sullivan *et al.*, 2017; Sullivan *et al.*, 2016; Waugh *et al.*, 2019).

It is likely that peripheral inflammation plays an important role in the development of cognitive impairments and AD-dementia in the DS population. Indeed, studies from our laboratory indicate that an inflammatory signature (IL-8 and TNFα) along with Aβ longitudinal changes are good predictors of prospective cognitive decline, highlighting an association between inflammatory marker expression and cognitive impairments in the studied DS population (Iulita *et al.*, 2016).

An important question that remains to be answered is to what extent does the AD-pathology contribute to the neuroinflammatory and systemic inflammatory profile in DS? In Chapter 2, we have presented compelling evidence indicating that inflammatory cytokine production is elevated in DS fetal cortical cells *in-vitro*. To address the above question, we are taking advantage of this unique *in-vitro* system, to study whether inhibition of the amyloidogenic pathway in DS fetal cortical cells has an effect on cytokine expression. The results of these investigations will reveal if Aβ plays a central role in the elevation of inflammatory markers reported in Chapter 2. Furthermore, the investigation of individuals with DS without full triplication of the *APP* gene might reveal whether Aβ pathology exacerbates the inflammatory profile in this population. While these investigations sound straightforward, partial triplications of the *APP* gene in DS are rare, and only few cases have been reported in the literature (Doran *et al.*, 2017). Further research is highly needed to elucidate the contributions of APP overexpression to the inflammatory profile observed in the DS population.

Peripheral immune cells and modulation of neuroinflammation in Alzheimer's disease

Chronic brain inflammation might promote the recruitment of immune cells into the CNS. Indeed, peripheral immune cells such as monocytes and T cells have been observed in the parenchyma of the AD brain (Fiala *et al.*, 2002; Merlini *et al.*, 2018; Town *et al.*, 2005). Induced peripheral monocytic infiltration in amyloidogenic mouse models results in efficient clearing of amyloid plaques, reduced neuroinflammation, and enhanced cognitive performance (Simard *et al.*, 2006; Theriault *et al.*, 2015). Therefore, peripheral monocytes might act as a second line of defense when microglia fail to perform their phagocytic function.

T lymphocytes have also been observed to infiltrate the AD brain (Itagaki *et al.*, 1988; Togo *et al.*, 2002). Notably, T cells of individuals with AD display an increase in the chemokine receptor CXCR2. IL-8 binding to CXCR2 promotes T cell transmigration across an in-vitro endothelial barrier model (Y. J. Liu *et al.*, 2010). Moreover, inhibition of CXCR2 signaling prevents T cell infiltration in the rat brain after parenchymal Aβ injection (Y. J. Liu *et al.*, 2010). Remarkably, IL-8 levels were increased in the frontal cortex of individuals with DS across all the ages analyzed

(Chapter 2). Such elevations might contribute to peripheral immune cell invasion in the DS brain, a proposition that awaits further investigations.

The contribution of specific subsets of T cells such as CD8+ cytotoxic T lymphocytes or CD4+ helper (Th1 and Th2) lymphocytes to the progression of AD has not been widely investigated. Some studies have suggested that inhibition of CD8+ T cells decreases microglial activation and cognitive deficits in a mouse model of tauopathy (Laurent *et al.*, 2017). Migration of Th1 to the brain parenchyma has been observed after immunization with $A\beta_{42}$ in APP models (Monsonego *et al.*, 2006). Such response has been associated to the development of meningoencephalitis, underscoring the role of Th1 responses on increasing the inflammatory response (Orgogozo *et al.*, 2003). Depletion of T and B cells has yielded conflicting results. While some have reported increased Aβ pathology and cognitive deficits (Kipnis *et al.*, 2004; S. E. Marsh *et al.*, 2016) other results indicate the contrary (Spani *et al.*, 2015). Therefore, further investigations are needed in order to understand the role of peripheral immune cells in the progression of AD. Such cells have also been implicated in the resolution of inflammation; a subject that will be furthered addressed in the Discussion of this Thesis.

Genetic risk factors

In chapter 1, we have discussed the contribution of genetic polymorphisms in immune-related genes (i.e. TREM2, and CD33) in increasing the risk of developing AD. Next, we will further discuss the associations of one of the major genetic risk factors, the ApoE4 allele, to the development of inflammation in AD.

While APOE plays a main role in lipid transport, a whole body of evidence indicates that it might modulate inflammatory processes in the brain. APOE knockout mice display increased immune activation, suggesting that APOE has an anti-inflammatory function in the CNS (Y. Liu *et al.*, 2015). Moreover, studies have revealed that the inflammatory response is associated to the APOE genotype and that ApoE4 confers susceptibility to an exacerbated pro-inflammatory profile (Vitek *et al.*, 2009). Transgenic animal models overexpressing ApoE4 allele display an exaggerated upregulation of inflammatory cytokines after immune challenge when compared to those

expressing the ApoE3 allele (Lynch *et al.*, 2003). Likewise, in the human population LPS challenge in ApoE4 -bearing individuals causes a greater increase in IL-6 and TNFα than in individuals with an ApoE3 allele (Gale *et al.*, 2014). Notably, the human ApoE4 isoform increases microglia reactivity in an APP mouse model and promotes cognitive impairments in young mice (Rodriguez *et al.*, 2013; Rodriguez *et al.*, 2014). Therefore, the presence of the E4 allele might contribute to an increased inflammatory profile. In line with this, the frequency of the ApoE4 allele was enriched in non-demented individuals with AD pathology with increased neuroinflammation (Chapter 3). Further investigations in larger cohorts will determine whether APOE alleles are associated to increased cytokine expression levels in such population.

Brain injuries

Traumatic brain injury (TBI) generally results from a primary and a secondary injury. The former is caused by a mechanical force while the latter occurs after BBB disruption, leading to brain swelling, hyperexcitability and increased neuroinflammation. The occurrence of TBI has been posed as a risk factor for AD (Dams-O'Connor *et al.*, 2016). Almost one third of people who suffered fatal TBI display cortical deposition of amyloid plaques and NFT pathology (Gentleman *et al.*, 1997; Johnson *et al.*, 2012). The upregulation of inflammatory cytokines following a secondary TBI usually indicates poor prognosis (Hinson *et al.*, 2015). It is not clear at which moment in the progression of TBI, the inflammatory process converts into maladaptive inflammation. Further to it, the severity of TBI and the risk of AD is increased in individuals bearing the ApoE4 allele (Ponsford *et al.*, 2011). Therefore, the development of AD in individuals with TBI might be favoured by an exaggerated neuroinflammatory profile concomitant with genetic factors such as the presence of the ApoE4 allele. Such proposition highlights a complex interplay between genetic factors, neuroinflammation, and environmental risk factors contributing to the development of AD.

Neuronal-driven inflammation in Alzheimer's disease: the starting point?

The majority of investigations regarding neuroinflammation in AD pose microglial cells as the initiators and main drivers of the neuroinflammatory process. While glia might have a central role in the bulk production of inflammatory mediators at clinical stages of AD, their involvement at the earliest stages of AD is not clear yet. Evidence has come to light that neuronal upregulation of inflammatory markers precedes amyloid plaque deposition and increased glial cytokine expression (Cavanagh *et al.*, 2016; Ferretti *et al.*, 2012a; Ferretti *et al.*, 2012b; Hanzel *et al.*, 2014; McAlpine *et al.*, 2009; Welikovitch *et al.*, 2019, *submitted*). The majority of this evidence comes from transgenic rodent models that mimic the human amyloid pathology. Such transgenic models display intraneuronal accumulation of Aβ oligomers prior to the deposition of amyloid plaques, mimicking the temporal evolution of amyloid accumulation in the human brain. As such, the McGill transgenic rat model displays a pre-plaque stage characterized by the intraneuronal accumulation of Aβ oligomers (Leon *et al.*, 2010). At this stage, neuronal upregulation of IL-1β, COX-2, TNFα, IL-6, MIP-1β, and MCP-1 have been reported (Hanzel *et al.*, 2014; Welikovitch *et al.*, 2019, *submitted*). The upregulation of these cytokines can feed forward the inflammatory cascade and can further increase the processing of Aβ (Shaftel *et al.*, 2008). Supporting the findings in transgenic models of the AD-like pathology, upregulation of neuronal COX-2 has been observed in prodromal AD (Hoozemans *et al.*, 2001), in DS brains, and at postnatal day 30 in a DS mouse model (Mulet *et al.*, 2017). Further to it, upregulation of NF-κB has been observed in neurons adjacent to diffuse amyloid plaques in AD brains (Kaltschmidt *et al.*, 1997). Moreover, an increase in neuronal oxidative stress has been observed in young DS brains before the deposition of amyloid plaques (Nunomura *et al.*, 2000).

In sum, the early upregulation of neuronal inflammatory markers indicates that neurons may be the triggers of neuroinflammation at incipient stages of AD (Cuello, 2017). In our studies, an upregulation of inflammatory markers was found in the conditioned media of DS fetal cortical cells. Oxidative stress and mitochondrial dysfunction have been reported to occur in these cells concomitant with the neuronal accumulation of Aβ (Busciglio *et al.*, 2002; Busciglio *et al.*, 1995; Iulita *et al.*, 2014; Martino Adami *et al.*, 2017). Our studies further demonstrate that a bulk of inflammatory cytokines including IL-1, IL-6, IL-8, and IL-10 are upregulated in the conditioned

media of DS fetal cortical cells. We have furthered examined the frontal cortex of fetuses with DS; however, we did not find an inflammatory deregulation. Such discordance might reflect the incipient stage of inflammation which is not detectable in whole brain homogenates. From these studies, two major questions remain to be answered (i) which is the cellular source of all these inflammatory mediators? (ii) what leads to upregulation of neuronal inflammatory mediators? Based on the above, we hypothesize that neurons should be the main producers of the majority of these cytokines and that amyloid-beta oligomers could be the initial triggers of neuronal inflammation. Future investigations will elucidate such propositions.

Early inflammasome activation in Alzheimer's disease and Down syndrome

The activation of neuronal immune signaling mechanisms elicited by abnormal Aβ accumulation has been barely investigated. Some evidence indicates that neurons might respond to Aβ pathology by mounting an inflammatory response via the inflammasome (Kaushal *et al.*, 2015; Tan *et al.*, 2014; Yu *et al.*, 1998). The inflammasome is a macromolecular complex composed of cytosolic Nod-like receptors (NLRs) and adaptor proteins (Martinon *et al.*, 2002). Inflammasomes assemble after sensing PAMPs or DAMPs; followed by pro-caspase-1 recruitment and conversion to its mature form within the inflammasome (Broz *et al.*, 2016). Mature caspase-1 cleaves the pro-forms of cytokines belonging to the IL-1β family. Additionally, caspase-1 can mediate a form of cell death called pyroptosis (Broz *et al.*, 2016). Compared to glia, neurons and oligodendrocytes express higher levels of the inflammasome receptor NLRP1 (de Rivero Vaccari *et al.*, 2008; Kummer *et al.*, 2007). *In-vitro* studies have shown that Aβ oligomers can promote the assembly and activation of the NLRP1 inflammasome via disruption of the cell membrane and a subsequent disbalance in the K+ current (Yu *et al.*, 1998). Moreover, it has been suggested that inflammasome activation might occur after TLR signalling (Heneka *et al.*, 2018). Remarkably, neuronal NLRP1 expression has been reported to be upregulated in AD brains at advanced stages of the AD pathology (Kaushal *et al.*, 2015).

Activation of the NLPR3 inflammasome has been reported to occur in microglial cells in response to Aβ fibrils and oligomers. Furthermore, upregulation of the NLPR3 inflammasome occurs in APP mouse models, and in MCI and AD brains and promotes worsening of the pathology in APP

mouse models (Heneka *et al.*, 2013). Therefore, inflammasome activation might be an early mechanism leading to neuroinflammation in the AD brain. Several studies have suggested that microglia priming is necessary for the production of NLPR3 inflammasome related proteins. A second signal, such as Aβ oligomers, mitochondrial dysfunction, ATP or other DAMPs promote the assembly and full activation of the NLPR3 inflammasome. Notably, activation of the NLRP3 inflammasome has been reported in mouse models of tauopathy (Ising *et al.*, 2019; Stancu *et al.*, 2019). *Nlrp3* deletion ameliorated tau deposition and cognitive deficits (Ising *et al.*, 2019) in a tau transgenic mouse model. Furthermore, intracerebral injection of Aβ fibrils in NLRP3 deficient tau mice did not aggravate tau pathology, suggesting that inflammasome activation is an important mediator between Aβ and tau pathologies (Ising *et al.*, 2019).

We have conducted a preliminary screening using a qPCR array to investigate whether inflammasome components are deregulated across the lifespan of individuals with DS. As illustrated in Appendix II, preliminary gene expression analyses indicate that NLRP1 and NLRP3 are upregulated early in DS brains. Further to it, caspase-1 gene expression levels are increased across the lifespan of individuals with DS. Moreover, our analyses suggest that other inflammasome receptors such as AIM2, NOD2, NLRP6, and NLRC5 are also upregulated in DS brains. We have also conducted individual qPCR analyses in adult DS brains to confirm the results obtained from the qPCR array at this age-gap. As illustrated in Appendix III, increased levels of caspase-1, NLRP3, NLRP4, CARD18, CARD6, and IL-18 were elevated in adult DS brains. Higher levels of NLRP1 and IL-1β were also detected in DS brains when compared to controls, however it failed to reach statistical significance ($p = 0.07$, $p = 0.05$; respectively). These observations are the first of their kind in DS. Further investigations are needed to characterize protein expression and the cellular source of such inflammatory mediators in the DS brain. Moreover, given that IL-1β levels were elevated in non-demented individuals with AD pathology (Chapter 3), it would be important to investigate whether inflammasome activation is already present at such incipient stages of AD pathology accumulation.

Overall, our investigations would support that inflammasome activation occurs early in AD. Inflammasome inhibition in several neurodegenerative disease animal models has proven to have beneficial outcomes. The development and administration of small molecules that inhibit

inflammasome activation is currently an active field of investigation for the treatment of diseases with an inflammatory component.

Anti-inflammatory mechanisms of neuronal derived noradrenaline and acetylcholine

Besides their role as neurotransmitters, NA and acetylcholine are neuronal-derived anti-inflammatories targeting the CNS as well as the periphery. Such neurotransmitters can exert their immunomodulatory function by binding to noradrenergic and cholinergic receptors located in peripheral immune cells as well as in glial cells.

Noradrenaline as an anti-inflammatory molecule

NA exerts its anti-inflammatory functions via activation of β-adrenergic receptors which is followed by an increase in cAMP levels (Braun *et al.*, 2014). Such increase results in higher nuclear levels of the NFκB inhibitor, IκBα (Gavrilyuk *et al.*, 2001). As a result, NFκB activity is reduced and the production of inflammatory molecules by microglia and astroglia is decreased (Braun *et al.*, 2014; Feinstein *et al.*, 2016). Further studies suggest that NA can also exert its anti-inflammatory function in neurons as revealed by decreased NOS2 levels in primary cortical neurons following NA administration (Madrigal *et al.*, 2005). Pharmacological increase of NA in the CNS also promotes NA anti-inflammatory functions after an inflammatory insult (Braun *et al.*, 2014; Feinstein *et al.*, 2016).

Some studies suggest that NA can also promote an increase in pro-inflammatory mediators. For example, MCP-1 and CX3CL1 astrocytic levels are increased after NA administration (Hinojosa *et al.*, 2013) and pharmacological activation of β-adrenergic receptors increases inflammatory protein levels in the rat brain (Maruta *et al.*, 1997; Tomozawa *et al.*, 1995). Such results suggest that NA might modulate the physiological expression of cytokines under normal conditions. In this line, LPS administration promoted an increase in astrocytic MCP-1 which was downregulated after NA administration, indicating that under inflammatory conditions NA contributes to damp the inflammatory response (Hinojosa *et al.*, 2013).

Will restoring brain NA levels ameliorate AD pathogenesis? Studies in APP transgenic models indicate that raising NA levels via NA precursors, such as the synthetic NA precursor L-threo-3,4,-dihydroxyphenylserine (L-DOPS or droxidopa), or by modulating noradrenergic signaling can rescue cognitive deficits, and ameliorate amyloid pathology and neuroinflammation (Gibbs *et al.*, 2010; Hammerschmidt *et al.*, 2013; Heneka *et al.*, 2010; Kalinin *et al.*, 2012; S. Li *et al.*, 2013; Madrigal *et al.*, 2010; Scullion *et al.*, 2011). Translation of these findings to the human population is challenging. Noradrenergic modulators that increase NA signalling such as the noradrenergic uptake inhibitors, atomoxetine and reboxetine, are currently used for attention-deficit hyperactive disorder (ADHD) and depression, respectively. Notably, atomoxetine administration in adults with ADHD or Parkinson's disease (PD) improves executive function and global cognition (Brown *et al.*, 2011; Ince Tasdelen *et al.*, 2015; L. Marsh *et al.*, 2009; Weintraub *et al.*, 2010) . However, a phase II clinical trial with atomoxetine in patients with mild to moderate AD showed no significant improvement in cognition (Mohs *et al.*, 2009). Nevertheless, this study was underpowered, and individuals were already at clinical stages of AD.

While some evidence indicates that boosting NA levels in neurodegenerative diseases might alleviate disease progression, some observations need to be taken into consideration. Some reports suggest that noradrenergic receptors might be differentially modulated throughout the continuum of AD (Jardanhazi-Kurutz *et al.*, 2011; Szot *et al.*, 2006). As such, Jardanhazi-Kurutz and colleagues have identified distinct expression levels in adrenergic receptors after NA depletion in an APP mouse model (Jardanhazi-Kurutz *et al.*, 2011). While some receptors were downregulated in response to NA deficiency, other receptors such as the α1 adrenergic receptor were increased in this animal model (Jardanhazi-Kurutz *et al.*, 2011). In the human AD brain distinct expression of α and β-adrenergic receptors has been observed; such changes appear to be region-specific (Jardanhazi-Kurutz *et al.*, 2011; Szot *et al.*, 2006). Such differential expression might reflect a compensatory mechanism against the accumulation of AD neuropathology. Deregulation of noradrenergic receptor signaling along with its impact on the inflammatory response in the AD continuum has not been investigated.

Given that LC alterations in AD might start before the onset of cognitive symptoms, pharmacological therapies targeting NA should be directed towards incipient stages of AD. The complexity of noradrenergic signaling and the lack of understanding of the impact of LC

pathological changes pose a great challenge for the development of therapeutics centered in the noradrenergic system at early stages of AD.

Another major question in the field that remains to be answered is why LC neurons are vulnerable at early stages of AD and other neurodegenerative diseases? It has been proposed that the large metabolic demand of LC neurons leads to an increased rate of mitochondrial oxidative phosphorylation and accumulation of oxidative stress (Kelly *et al.*, 2017; Weinshenker, 2018). Furthermore, neuromelanin is known to bind toxic products such as iron and heavy metals, therefore augmenting the concentration of toxic factors in LC neurons (Pamphlett, 2014). Moreover, NA can suffer autooxidation or can be converted to toxic metabolites, contributing to oxidative damage of essential cellular components such as lipids, proteins and nucleotides (Weinshenker, 2018). Lastly, as the LC innervates widely the cerebrovasculature and it is in close contact with the CSF, constant exposure of toxic molecules in such compartments might have a detrimental effect in LC physiology (Weinshenker, 2018). The elucidation of when and why LC neurons degenerate in AD as well as its consequences will contribute to a better understanding of the etiology and progression of AD.

The cholinergic anti-inflammatory pathway

During systemic inflammation, the efferent fibers of the vagus nerve release acetylcholine which signals through nicotinic receptors in immune cells to decrease the production of proinflammatory cytokines via inhibition of the NFκB pathway (Borovikova *et al.*, 2000). Some studies have suggested that lymphocyte-derived acetylcholine activates sympathetic terminals in the spleen leading to NA release which exerts its anti-inflammatory functions in the periphery (Martelli *et al.*, 2014). Further to it, vagus nerve afferents signal to the CNS indicating the occurrence of systemic inflammation (Pavlov *et al.*, 2003). Vagus nerve stimulation leads to activation of CNS structures such as the LC and NBM (Grimonprez *et al.*, 2015; Hulsey *et al.*, 2016; Hulsey *et al.*, 2017; Krahl *et al.*, 1998); thus, central NA and acetylcholine could contribute to modulate brain inflammation. The acetylcholine anti-inflammatory pathway is mediated by α-7 nicotinic receptors (α7 nAChR), as deletion of this receptor abolishes the anti-inflammatory effect (H. Wang *et al.*, 2003). In the CNS α7 nAChR are expressed in astroglial and microglial cells, suggesting

that acetylcholine could exert immunomodulatory functions in these cells (Shen *et al.*, 2012; Shytle *et al.*, 2004). Indeed, activation of α7 nAChR can reduce inflammatory expression in astroglial and microglial cells (Parada *et al.*, 2013; Patel *et al.*, 2017; Revathikumar *et al.*, 2016). Activation of acetylcholine immunomodulatory pathways through the α7 nAChR can decrease neuroinflammation in several neurological diseases such as multiple sclerosis, intracerebral hemorrhage, ischemic stroke, epilepsy, PD, and AD among others [for a review see (Han *et al.*, 2017)]. Downregulation of α7 nAChR has been observed in *post-mortem* brain tissue of individuals with AD (Banerjee *et al.*, 2000; Burghaus *et al.*, 2000; Guan *et al.*, 2000). Notably, in an APP mouse model, an upregulation of α7 nAChR occurs before and after the deposition of amyloid plaques (Bednar *et al.*, 2002). Moreover, it has been reported that Aβ binds with high affinity to the α7 nAChR; such complex is internalized, contributing to the intraneuronal uptake of Aβ (Nagele *et al.*, 2002; H. Y. Wang *et al.*, 2000). Thus, the early upregulation of receptors might reflect a compensatory event against the increased internalization of Aβ. Electrical stimulation of the vagus nerve in the APP/PS1 mouse model at the post-plaque stage of the amyloid-like pathology decreases microglial morphological activation (Kaczmarczyk *et al.*, 2017). Moreover, deletion of the α7 nAChR in the Tg2576 mouse model of the amyloid-like pathology, increased Aβ deposition and worsened pre-existent cognitive deficits suggesting that α7 nAChR confers neuroprotection (Hernandez *et al.*, 2010). In this line, administration of an α7 nAChR agonist also rescues cognitive deficits at advanced stages of amyloid deposition in the 3xTg AD mice (Medeiros *et al.*, 2014). Remarkably, anticholinergic treatment in a mouse model of tauopathy promotes neuronal loss, tau phosphorylation, increased IL-1β expression, and microglial activation (Yoshiyama *et al.*, 2012). The above findings would indicate that acetylcholine plays a role as an anti-inflammatory molecule in the CNS and that disruptions in its immune-mediated signaling might contribute to the progression of several neuropathologies including AD.

Notably, as presented in Chapter 4 of this thesis, we detected a depletion of hippocampal cholinergic varicosities after LC noradrenergic system denervation. Therefore, disruption of the cholinergic system could contribute to the exacerbated inflammatory profile observed after noradrenergic denervation, a proposition that awaits further clarification.

Early microglial activation in Alzheimer's disease

The upregulation of neuronal inflammatory markers at the pre-plaque stage coincides with the recruitment of microglial and astroglial cells towards hippocampal Aβ-burdened neurons (Ferretti *et al.*, 2012b; Hanzel *et al.*, 2014). Such recruitment might be dependent on the neuronal accumulation of Aβ oligomers as microglia are not recruited before the intraneuronal deposition of Aβ, as revealed in an APP mouse model (Ferretti *et al.*, 2012b). Further to it, before the overt deposition of amyloid plaques, microglial cells from transgenic models display morphological changes such as increased soma area and process length; indicative of an intermediate activation state (Ferretti *et al.*, 2012b; Hanzel *et al.*, 2014). Such early inflammatory changes have been observed in other transgenic models of the amyloid-like pathology, further supporting the existence of neuroinflammation before the deposition of amyloid plaques (Cohen *et al.*, 2013; Heneka *et al.*, 2005). Here, we report that such intermediate activation of microglia occurs before the deposition of amyloid plaques in DS brains (Chapter 2). Furthermore, microglia of transgenic rats after noradrenergic depletion displayed a hypertrophic state suggestive of intermediate activation (Chapter 4). In an APP rodent model, intermediately activated microglia upregulate the expression of CD68, a lysosomal marker commonly associated to phagocytosis but phagocytosis of Aβ peptides is absent (Ferretti *et al.*, 2012b). Such an apparent failed attempt of phagocytosis might contribute to microglial priming and constant production of inflammatory mediators, leading to maladaptive neuroinflammation. Possible mechanisms leading to intermediate activation of microglia include the binding of Aβ oligomers, disruption of neuron-glia communication, inflammasome activation by neuronal and glial DAMPS, and loss of neuroprotective molecules such as NA, as exemplified in Chapter 4.

Notably, microglial morphological analyses in individuals with DS revealed a continuum of microglial morphological changes along the progression of AD in DS. Importantly, this continuum reflects early and late activation phases of microglia and suggests that a considerable amount of time and pathology build-up are needed for microglia to adopt a full-activated state. Such changes have been previously observed in transgenic AD models; however, this is the first time that the continuum of microglial morphological changes is reported in a population at risk of AD.

We noted that microglial cells displayed increased soma size and thicker processes in brains from children with DS. As the AD pathology developed, microglia became enlarged and process retraction was evident in most young adult DS cases. Finally, when the AD pathology was fully established in DS brains, microglial cells where highly dystrophic and some of them displayed an amoeboid morphology. We cannot ascertain to what extent Iba-1 positive cells reflected the microglial population. It is likely that monocyte infiltration occurs in DS brains, a possibility that deserves future investigations.

As illustrated in Chapter 2, our analyses also revealed that rod-like microglia were increased in young and adult DS brains. Rod microglia were characterized by Nissl in the early 1900's in the brains of paralytic individuals. Such cells were also described by Alzheimer, Cerletti, Achucarro, Perusini, Ulrich, Bonfiglio, and del Rio Hortega between others in *post-mortem* brain tissue from individuals who suffered distinct neuropathologies with an inflammatory component [for a historical review see (Sierra *et al.*, 2016)]. Although discovered more than 100 years ago, the function of rod microglia remains elusive, partly because of the poor attention this type of cell has received. Some reports suggest that rod-shaped microglia express low levels of proinflammatory cytokines such as IL-1β and TNFα, and high levels of the phagocytic marker CD68 (Tam *et al.*, 2014; Ziebell *et al.*, 2012). Upon LPS stimulation, rod microglia adopt an amoeboid morphology, indicating that these cells can transform their morphology according to the inflammatory milieu (Tam *et al.*, 2014). Moreover, rod microglial cells are commonly found at the initial stages of microglial activation and in brain regions with minimal injury in which amoeboid microglia are scarce (Au *et al.*, 2017). The inflammatory signature of rod-shaped microglia in the DS brain remains to be elucidated.

In line with the above, our observations would suggest that the number of rod microglial cells increases before the appearance of dystrophic and amoeboid microglia. Furthermore, rod microglial cells aligned to neurons harboring tau pathology; suggesting that their appearance and role might be associated to abnormal intraneuronal tau phosphorylation in the DS brain.

Early microglial activation and synaptic dysfunction

Notably, in a 3D AD co-cultures containing neurons, astrocytes and glia, neuronal and astroglial upregulation of MCP-1 led to the activation and recruitment of microglial cells (Park *et al.*, 2018). Park and colleagues further reported that axonal injury and neurite retraction colocalized with microglia (Park *et al.*, 2018). Whether microglial cells promoted synapse engulfment, remains to be elucidated. Synapse pruning by microglia has been reported to occur before the accumulation of amyloid plaques in an APP mouse models (Hong *et al.*, 2016). According to Hong and colleagues, synapses are targeted by complement factors C1q and C3, which are recognized and engulfed by microglial cells. Importantly, microglial engulfment occurs in absence of full microglial morphological activation (Hong *et al.*, 2016). Such immune-mediated mechanisms have been proposed to be responsible for early synaptic deficits in AD (Hong *et al.*, 2016).

Notably, C1q and C3 immunoreactivity is increased in young DS brains (~15 years old) (Stoltzner *et al.*, 2000). Whether synapses are targeted by complement factors in the DS brain has not been addressed as yet. Nevertheless, it has been proposed that given its close alignment to axons and dendrites, rod-like microglia might promote synaptic stripping (Au *et al.*, 2017), a proposition that deserves further investigations in the DS brain.

As presented in Chapter 4, increased microglial recruitment towards Aβ-bearing neurons was exacerbated after noradrenergic depletion. Further to it, we have also reported a decrease in cholinergic boutons in transgenic rats lacking noradrenergic innervation (Chapter 4). Based on the evidence discussed above, it is likely that microglial cells could also contribute to such synaptic deregulation in this rat model, a proposition that awaits further investigations.

While the overall synapse loss might be affected early in the amyloid pathology, it remains to be elucidated how specific synapses are affected along the continuum of AD. As discussed in Chapter 1, Bell and colleagues have demonstrated that synapses are affected in a differential manner by the amyloid pathology (Bell *et al.*, 2006). Cholinergic synapses are downregulated after the appearance of amyloid plaques, followed by glutamatergic and GABAergic synaptic depletion (Bell *et al.*, 2006). Remarkably, an upregulation of cholinergic boutons has been reported in in transgenic APP models preceding the deposition of amyloid plaques (Bell *et al.*, 2006). Concordantly, increased ChAT activity has been reported in the frontal cortex and hippocampus

from individuals with MCI (DeKosky *et al.*, 2002). Shortly after the deposition of plaques, glutamatergic and GABAergic synapses also become upregulated (Bell *et al.*, 2006). We have furthered characterized noradrenergic synaptic changes across the amyloid-like pathology in the McGill transgenic rat. As illustrated in Appendix IV, an upregulation of cortical noradrenergic varicosities was found at the post-plaque stage along with a decrease of noradrenergic synaptic boutons in the hippocampus. At advanced stages of amyloid deposition, the hippocampus displayed a downregulation of noradrenergic synaptic boutons.

Taken together our results support the concept that synaptic dysfunction in AD might evolve in a neurotransmitter-specific manner (Bell *et al.*, 2006). Future investigations should reveal whether immune-directed synapse engulfment displays neurotransmitter specificity. Furthermore, the paradoxical upregulation of synaptic boutons most likely reflects a compensatory mechanism against Aβ-toxicity. Such aberrant sprouting might contribute to microglial activation leading to uncontrolled synapse elimination. These propositions need further investigation in order to define the mechanisms leading to specific synaptic dysfunction along the continuum of AD.

Inflammation in Alzheimer's disease: friend or foe?

The use of transgenic rodent models of the amyloid or tau-like pathologies to study the neuroinflammatory process has yielded conflicting results. While some reports indicate that increased neuroinflammation might be beneficial, others suggest that neuroinflammation exacerbates and contributes to disease progression [reviewed in (Latta *et al.*, 2015; Lee *et al.*, 2013) . Moreover, it appears that modulation of neuroinflammation can exert differential effects on tau and amyloid-like pathologies. Some of these scenarios will be furthered discussed in this section.

The first report indicating that inflammation might be beneficial in an amyloid context was that of DiCarlo and colleagues (DiCarlo *et al.*, 2001). Intrahippocampal LPS injection in the APP/PS1 model promoted clearance of diffuse Aβ plaques (DiCarlo *et al.*, 2001). Moreover, dysfunctional TLR4 signaling in APP mice promoted an increase in Aβ load (Song *et al.*, 2011). On the contrary, LPS administration in a tau mouse model increased tau phosphorylation (D. C. Lee *et al.*, 2010).

Accordingly, systemic LPS administration in the 3xTg mouse model also increased tau pathology and had no effects in amyloid burden (Kitazawa *et al.*, 2005).

The involvement of the complement cascade has also yielded conflicting results. APP mice in a deficient C3 background displayed an increase in amyloid accumulation along with an increase in IL-10 and IL-4 and a decrease in CD68, TNF, and iNOS (Maier *et al.*, 2008). Contrarily, inhibition of the C5a receptor at the initiation of amyloid plaque pathology in the Tg2576 and the 3xTg mice promoted a decrease in amyloid load, glial activation, and rescued cognitive deficits (Fonseca *et al.*, 2009). Deletion of C1q in the Tg2576, APP/PS1, and 3xTg mice did not affect amyloid deposition; however, Tg2576 mice displayed less microglial activation (Fonseca *et al.*, 2004). In sum, complement factors might have opposing roles in the regulation of the amyloid pathology. It is important to note that the use of knockout models might introduce some caveats such as compensatory mechanisms and altered physiological mechanisms. Such mouse models do not necessarily reproduce the human AD neuropathology; therefore, such results and interpretations should be taken with caution.

It has also been argued that the expression of inflammatory cytokines might modulate the expression of the amyloid pathology. Sustained hippocampal expression of IL-1β in the APP/PS1 model ameliorated amyloid deposition and increased microglial activation (Shaftel *et al.*, 2007). On the other hand, although increased hippocampal IL-1β expression in the 3xTg mouse model led to diminished Aβ levels, it also promoted tau pathology and microglial activation (Ghosh *et al.*, 2013). Viral transduction of TNFα into APP mouse promoted a decrease in Aβ pathology (Chakrabarty *et al.*, 2011). In line with this, deletion of TNF receptors resulted in increased Aβ deposition and decreased microglial activation (Montgomery *et al.*, 2011). Similarly, deletion of the CCR2 receptor in APP mice provoked an increase in Aβ deposition (El Khoury *et al.*, 2007). Disruption of fractalkine signaling by deleting the fractalkine receptor (CX3CR1) in APP mice results in diminished Aβ pathology and increased microglial activation (S. Lee *et al.*, 2010; Z. Liu *et al.*, 2010). In turn, CX3CR1 deletion in a tau model resulted in increased tau pathology (Bhaskar *et al.*, 2010). The effects of modulating other inflammatory cytokines have been extensively reviewed in (Lee *et al.*, 2013) and (Latta *et al.*, 2015).

The above studies would indicate that amyloid and tau pathologies are regulated in a differential manner by the immune system. Nevertheless, it appears that activation of inflammatory processes

at the post-plaque stage of the amyloid pathology might be beneficial in some animal models. It has been posed that a failed resolution of inflammation process occurs in AD (X. Wang *et al.*, 2015) in which microglial phagocytosis is likely compromised. Therefore, the beneficial activation of microglia observed in animal studies at late stages of the AD-like pathology might contribute to ameliorate Aβ pathology. It remains to be clarified if such beneficial effect was also caused by the migration of peripheral monocytes, which have greater phagocytic activity than glia (El Khoury *et al.*, 2007).

As outlined above, increased inflammation at late stages of the amyloid pathology promoted tau pathology; highlighting a distinct modulation of amyloid and tau pathologies under inflammatory conditions. Such discrepancy might be explained by the potential capacity of activated microglia to spread pathological tau (Maphis *et al.*, 2015; Perea *et al.*, 2018). Interestingly, we have observed marked microglial activation before and at the time of pathological tau accumulation in the DS brain (Chapter 2 and Appendix IV). Whether microglia contribute to the development of tau pathology in the DS brain remains to be investigated.

Another important factor that needs consideration is that the effect of inflammation in these studies was addressed after amyloid plaque deposition. As discussed in previous chapters, early inflammation might have a deleterious effect in AD pathology. Such proposition is supported by the next body of evidence:

Intraventricular LPS administration before amyloid plaque deposition in APP mice accelerated Aβ deposition (Qiao *et al.*, 2001). In line with this, central administration of LPS to APP mice increased intraneuronal Aβ deposition (Sheng *et al.*, 2003). McAlpine and colleagues have demonstrated that blocking TNFα signaling after LPS administration in pre-plaque 3xTg mice decreased intraneuronal Aβ accumulation (McAlpine *et al.*, 2009). Moreover, administration of a TNFα inhibitor before the deposition of amyloid plaques in the TgCRND8 mouse model prevented synaptic deficits (Cavanagh *et al.*, 2016). Administration of minocycline, a tetracyclic derivative with anti-inflammatory properties, to pre-plaque APP mice resulted in decreased inflammatory protein expression (iNOS, COX-2), microglial activation, reduction of Aβ levels, and decreased BACE-1 activity (Ferretti *et al.*, 2012a). The above observations would suggest that the early inflammatory process is disease-aggravating (Cuello, 2017)(Figure 5-3), a concept also supported by leading investigators (Rogers, 2018).

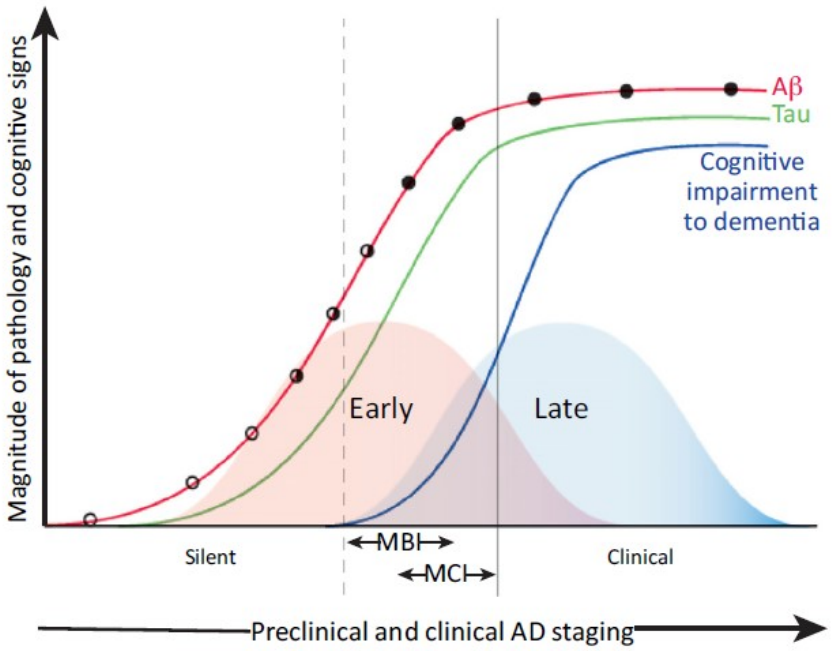

Figure 5- 3. Hypothetical early and late CNS inflammation along the Alzheimer's disease continuum.

It is likely that a certain threshold of intraneuronal Aβ (open circles) unleashes an early pre-plaque neuroinflammatory process. The late inflammatory process might start concomitant with MCI. The early and late inflammatory processes likely overlap at prodromal stages of AD. Aβ = amyloid beta, MBI = mild behavioral impairment, MCI = mild cognitive impairment. Image from (Cuello, 2017) with permission from *Elsevier*.

Differential neuroinflammatory processes along the AD continuum

As outlined above, the early and late inflammatory processes might play a differential role along the progression of AD. As such, some molecules involved in the immune response display a dual role at different timepoints of the AD pathology. In this section, we will discuss some of the most studied examples illustrating such phenomena.

TREM2 variants, associated with a partial loss of function, can triple an individual's risk of developing AD (Carmona *et al.*, 2018; Jonsson *et al.*, 2013) . Investigations regarding the role of TREM2 in APP mouse models have yielded conflicting results (Tanzi, 2015). Although the majority of investigations suggest that TREM2 deficiency leads to decreased accumulation of myeloid cells around amyloid plaques, its effects on the amyloid pathology have differed between different studies (Tanzi, 2015). Such variation might be reflective of the different time points in disease progression in which TREM2 deficiency was analyzed (Jay *et al.*, 2017). Therefore, Jay and colleagues analyzed the effects of TREM2 deficiency along the progression of the amyloid pathology in the APP/PS1 mouse model crossed with a TREM2 deficient mouse (Jay *et al.*, 2017). Remarkably, they found that at 2 months of age, at the early stages of the amyloid pathology, APP/PS1 Trem 2 -/- mice displayed diminished Aβ accumulation, decreased Aβ internalization by myeloid cells, and lower number of CD45+ cells than controls (Jay *et al.*, 2017). On the other hand, transgenic mice at the late stages of amyloid accumulation showed increased Aβ accumulation, an exacerbated decrease in CD45+ cells, diminished myeloid cell proliferation, decreased Aβ internalization by myeloid cells, and lower levels of IL-1β, and TNFα (Jay *et al.*, 2017). Therefore, such observations would indicate that TREM2 exerts distinct roles at different stages of the amyloid pathology, further stressing the occurrence of a differential neuroinflammatory process in AD.

IL-10 has been regarded as an anti-inflammatory molecule as it can suppress inflammatory responses (Strle *et al.*, 2001). Increases in this cytokine are thought to be beneficial to halt inflammation. However, adeno-associated virus (AAV2/1) mediated overexpression of IL-10 in the brains of two APP mouse models (TgCRND8 and Tg2576) increased Aβ deposition, worsened cognitive impairments, decreased synaptic proteins, and impaired Aβ phagocytosis (Chakrabarty *et al.*, 2015). In contrast, APP/PS1 mice deficient in IL-10 displayed decreased Aβ deposition

probably due to increased microglial Aβ phagocytosis (Guillot-Sestier *et al.*, 2015). Furthermore, IL-10 deficiency rescued cognitive deficits and preserved synaptic integrity (Guillot-Sestier *et al.*, 2015). Therefore, elevated levels of the anti-inflammatory cytokine IL-10 disrupt Aβ clearance from the brain and worsen the amyloid-like pathology in transgenic APP mouse models (Chakrabarty *et al.*, 2015; Guillot-Sestier *et al.*, 2015). Guillot-Sestier and colleagues have further reported that the IL-10 receptor (IL-10r) and downstream signaling molecules are also elevated in the AD brain (Guillot-Sestier *et al.*, 2015). Nevertheless, Wang and colleagues have reported decreased IL-10 protein levels in the hippocampus of individuals with AD (X. Wang *et al.*, 2015). Therefore, further research is needed to elucidate how IL-10 levels are affected along the continuum of AD. Our studies in DS brains (Chapter 2) are particularly significant in light of the role that IL-10 might have along the AD pathology. We have found that before the overt accumulation of amyloid plaques, IL-10 brain levels were elevated in children with DS. As previously discussed, such elevation might contribute to Aβ deposition. Remarkably, as the pathology advanced, IL-10 levels decreased. While we cannot ascertain what are the causes and consequences of such decrease, a deregulation in IL-10 levels might have a direct impact on microglial phagocytosis at the late inflammatory stage. However, as reported in Chapter 2, microglia in adult DS brains are highly dystrophic; thus, it is likely that microglial-mediated phagocytosis is compromised even in the presence of low levels of IL-10. Moreover, the chronic neuroinflammation observed across the lifespan of individuals with DS is suggestive of a failed resolution of inflammation. Notably, high levels of IL-10 are associated with successful resolution of inflammation (Schwartz *et al.*, 2014); therefore, the decrease in IL-10 could also reflect a failure in resolving the inflammatory process.

Besides the differential role of inflammatory proteins in the continuum of AD, astroglial and microglial cells also display distinct activation patterns at early and late stages of AD. Imaging studies in a population of autosomal dominant AD mutation carriers have revealed the occurrence of astrocytic activation ~17 years before disease onset concomitant with the first aggregation of amyloid plaques (Rodriguez-Vieitez *et al.*, 2016). Such astroglial activation declines as the AD pathology advances (Rodriguez-Vieitez *et al.*, 2016). Notably, increased cortical thickness, changes in brain structure, and increased hypermetabolism have been found in presymptomatic individuals carrying autosomal dominant AD-related mutations (Benzinger *et al.*, 2013; Fortea *et*

al., 2010; Quiroz *et al.*, 2015; Sala-Llonch *et al.*, 2015). Such changes have been in part attributed to an increased neuroinflammatory state (Fortea *et al.*, 2010).

In this line, increased levels of the pro-inflammatory chemokine IP-10 (CXCL10) have been reported at prodromal stages of AD (Iturria-Medina *et al.*, 2016); (Galimberti *et al.*, 2006). Given the anti-angiogenic role of IP-10 (Angiolillo *et al.*, 1995), it has been proposed that increased IP-10 levels at pre-symptomatic stages of AD could contribute to early vascular impairments (Iturria-Medina *et al.*, 2016). In AD brains and transgenic models of the amyloid-like pathology, IP-10 is mainly expressed in a subset of astrocytes near amyloid plaques (Krauthausen *et al.*, 2015; Xia *et al.*, 2000). Such observations suggest that IP-10 might be also associated to early astrocytic activation. In line with these observations, while IP-10 CSF levels are normal in AD; however, they are upregulated in preclinical AD and MCI individuals (Iturria-Medina *et al.*, 2016) (Galimberti *et al.*, 2006). Such an early increase in IP-10 levels have also been observed in other neuropathologies such as MS and Niemann-Pick disease type C (Galimberti *et al.*, 2006; Shin *et al.*, 2019; Tani *et al.*, 1996). Moreover, byphasic astroglial activation has also been observed in the early inflammatory stage in an experimental model of MS, along with increased levels of IP-10, which are normalized at later stages of the disease (Tani *et al.*, 1996). In Chapter 2 we have shown that IP-10 levels increase early in the lifespan of individuals with DS, this observation goes in line with the early astrocytic activation observed in fetuses and neonates with DS (Griffin *et al.*, 1989). Furthermore, in Down syndrome, IP-10 levels continue to be upregulated during adulthood; such chronic upregulation of IP-10 may exacerbate neuroinflammation, vascular impairments and AD-related neuropathology in DS brains.

Sequential peaks of microglial activation have been observed across the continuum of AD. Longitudinal PET scans to assess microglial activation using the 11C(R)-PK11195 probe were conducted in individuals with MCI and AD (Fan *et al.*, 2017). Increased microglial activation (41%) was observed at baseline in the MCI group, which declined by 18% over 14 months and was associated to an increase in Aβ accumulation (Fan *et al.*, 2017). The AD group displayed a 36% increase on microglia activation which further increased with disease progression (Fan *et al.*, 2017). The authors of the study speculate that the early peak of microglial activation might be protective while the latter could have pro-inflammatory detrimental effects (Fan *et al.*, 2017). However, as previously emphasized, transgenic models and epidemiological evidence support that

the early neuroinflammatory process might have a detrimental effect in the progression of AD (Cuello, 2017; Ferretti *et al.*, 2012a; McGeer *et al.*, 2013). Moreover, it remains to be established if microglia activation occurs at asymptomatic AD stages as it has been shown for astroglia activation, notwithstanding that present PET markers are unable to detect the early intermediately activated and disease-aggravating phenotype.

Resolution of inflammation in Alzheimer's disease and Down syndrome

A successful inflammatory response terminates with the resolution of the inflammatory process. Given the unresolved chronic inflammation present in AD and in DS it is clear that inflammation resolution processes are affected in such neuropathologies (Figure 5-4).

As discussed in (Cuello, 2017; McGeer *et al.*, 2013; Rogers, 2018), anti-inflammatories administered at clinical AD stages can worsen the disease. We hypothesize that anti-inflammatory medications could lead to poor resolution of inflammatory processes. This resolving process has been reported to fail in AD (X. Wang *et al.*, 2015), possibly due to the chronic accumulation of AD pathology and constant overproduction of inflammatory mediators.

There is little knowledge regarding the resolution of inflammation process in AD and DS. However, recent studies suggest that lipid mediators and a regulated invasion of peripheral immune cells are necessary to accomplish a successful resolution response (Bazan, 2018; Schwartz *et al.*, 2014; X. Wang *et al.*, 2015). In the 5xFAD transgenic model displaying amyloid-like and tau-like pathologies the resolution of inflammation is impaired; such failure is attributed to a decrease in the IFNγ-mediated activation of the choroid plexus (Baruch *et al.*, 2015). A decrease in IFNγ-mediated signaling promotes the immunosuppressive actions of regulatory T cells (T_{regs}), impairing the peripheral migration of pro-resolving monocytes and T cells (Baruch *et al.*, 2015). In contrast, T_{regs} depletion was associated to a healthy resolution response along with an increase in IL-10 brain levels and a decrease in the pro-inflammatory mediators IL-12p40 and TNFα (Baruch *et al.*, 2015). Notably, as presented in Chapter 2, the DS adult brain displayed a decrease in IFNγ and IL-10, suggesting that the resolution of neuroinflammation might be impaired in DS. In agreement with our findings, brain IL-10 levels and lipid resolving mediators are also decreased

at late stages of AD (X. Wang *et al.*, 2015). Whether this decrease in cytokines directly affects T_{regs} immunomodulatory functions in the human brain, remains to be elucidated.

Interestingly, blood cells from AD individuals show less responsiveness to an inflammatory stimulus as reflected by reduced release levels of the proinflammatory mediators TNFα, IFNγ and IL-12p40 as compared to blood cells from non-demented individuals (Richartz *et al.*, 2005). Notably, TNFα, IFNγ, and IL-12p40 levels were also reduced in adult DS brains. Such observations in AD and DS, would indicate the presence of defective immunological mechanism or immune senescence at late stages of AD. Supporting such concept, previous studies have shown that the immune response in the AD brain wanes with aging (Hoozemans *et al.*, 2011). While such decrease is due to cell loss or a defective resolution of inflammation process awaits further investigations.

Henceforth, NSAID administration at clinical stages of AD might have a detrimental role in interfering with the poor, but probably present, resolution of inflammation process at this stage. An early and effective intervention aiming to potentiate the resolution of inflammation response could ameliorate the progression of AD neuropathology (Zhu *et al.*, 2018). Further research is needed to understand the mechanisms mediating the resolution of inflammation in AD and DS.

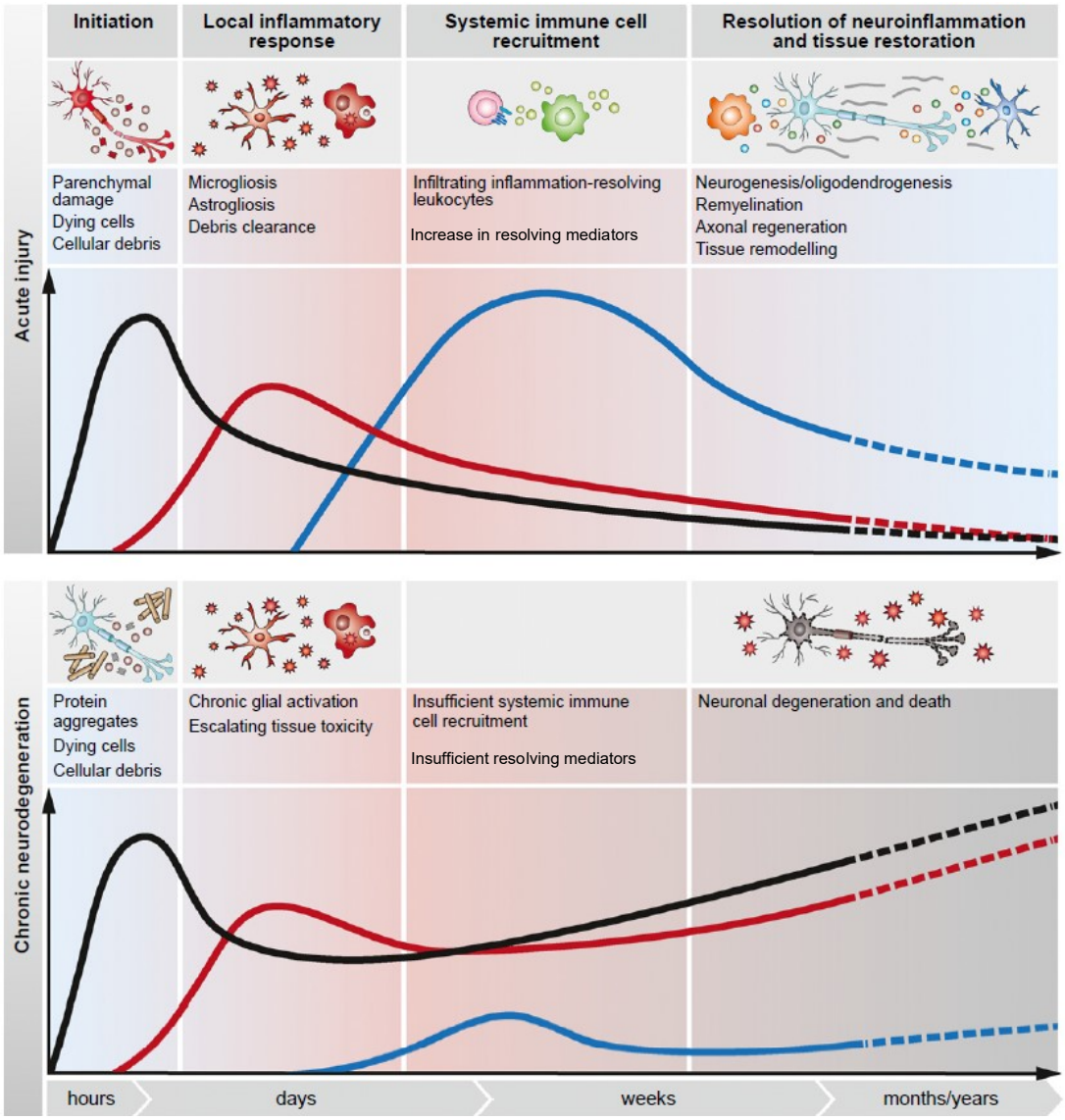

Figure 5- 4. Immune response following an acute CNS injury or chronic neurodegeneration.

Under acute inflammatory conditions, parenchymal damage (black line) leads to glia activation (red line) and to the infiltration of resolving leukocytes (blue line) and inflammation is eventually resolved. In chronic neurodegenerative diseases protein aggregates promote a chronic neuroinflammatory response in which resolving mediators are insufficient to halt the progression of brain inflammation; thus, the resolution of inflammation process fails. Adapted image from (Schwartz *et al.*, 2014) with permission from *John Wiley and Sons*.

Final remarks: fighting inflammation to stop Alzheimer's disease

Throughout this Thesis we have emphasized that anti-inflammatory treatment, if administered early enough, ameliorates AD pathology in APP mouse models and has a sparing effect on AD as revealed by epidemiological studies (Cuello, 2017; McGeer *et al.*, 2013). Nevertheless, although NSAID administration in transgenic models has rendered beneficial effects, its translation into clinical trials in the human population has generally failed. Such failure has been attributed to NSAID administration at late stages of AD, inappropriate targets, or underpowered studies (Figure 5-5). Nevertheless, several clinical trials using compounds with anti-inflammatory properties are currently ongoing, for a list see (Van Eldik *et al.*, 2016). Furthermore, others have suggested that promoting the resolution of inflammatory process might render beneficial effects in AD (Zhu *et al.*, 2018).

Our studies in human *post-mortem* brain tissue from young individuals with DS and non-demented individuals with incipient AD pathology suggest a common upregulation of IL-1β, IL-6, and eotaxin-3. Given that IL-1 is a major cytokine driving the expression of other inflammatory molecules, halting IL-1β inflammatory pathway early in AD progression in DS and in the general population might render beneficial outcomes.

Remarkably, individuals with DS displayed a heightened immune profile before the development of overt AD pathology (Chapter 2) when compared to non-demented individuals with incipient deposition of amyloid plaques (Chapter 3). One possibility is that triplication of inflammatory genes encoded in chromosome 21 might exacerbate the immune brain response in the DS population (D. M. Wilcock *et al.*, 2013). A second possibility stems from the fact that non-demented individuals already displayed amyloid plaques while children and young individuals with DS did not have an established amyloid pathology. Therefore, it might be possible that a distinct neuroinflammatory profile could be present in non-demented individuals solely displaying pathological intraneuronal accumulation of Aβ. Such process might display some similarities to the one we have found in children and young adults with DS (Chapter 2).

The above observations suggest that anti-inflammatory treatment design needs to consider the time of intervention, patient characteristics, optimal therapeutic targets, and treatment duration. Therefore, while a selected anti-inflammatory treatment might fail for some populations it might

show beneficial effects for others, as exemplified by a reduced cognitive decline in ApoE4 carriers with mild AD treated with ibuprofen (Pasqualetti *et al.*, 2009). In line with this, the majority of epidemiological studies reporting a reduced risk of AD were conducted in populations with underlying inflammatory conditions such as rheumatoid arthritis. Thus, such observation led us to hypothesize that anti-inflammatory medication could ameliorate AD progression in individuals with DS as they display a heightened peripheral immune response (Sullivan *et al.*, 2017; Sullivan *et al.*, 2016; Waugh *et al.*, 2019).

Another course of treatment that has recently shown beneficial effects aims to reduce amyloid deposition in the brain. Aducanumab (BIIIB037) is a human monoclonal antibody that binds Aβ fibrils and oligomers leading to clearance of Aβ plaques in the AD brain (Sevigny *et al.*, 2016). Aducanumab reached phase III of a clinical trial conducted by Biogen, but on March 2019, the clinical trial was terminated following a futility analysis. On October 2019, after analyzing a larger data set, Biogen announced that aducanumab reached its primary endpoint by reducing clinical decline along with reduced Aβ deposition and pTau in the CSF (http://investors.biogen.com/news-releases/news-release-details/biogen-plans-regulatory-filing-aducanumab-alzheimers-disease). Biogen aims to pursue regulatory approval by the U.S. Food and Drug Administration (FDA) by 2020. If aducanumab becomes approved, it would be the first treatment to slow cognitive decline in early AD and to directly show that Aβ clearance is associated to better cognitive function, further supporting the amyloid hypothesis. Such results would indicate that the A4 secondary prevention clinical trial currently in Phase III (R. A. Sperling *et al.*, 2014) might hold promising results to prevent AD in cognitively normal individuals displaying Aβ accumulation. Whether anti-inflammatory treatment administered at the right stage would be more beneficial than targeting Aβ, remains to be elucidated.

The exciting news released by Biogen reflect the extensive research and knowledge that we have about amyloid-driven mechanisms. Given that the immune response is tightly linked to the progression of AD related pathology and that certain polymorphisms in immune related genes confer an increased risk of developing AD, the understanding of the longitudinal progression of the neuroinflammatory process is still an unmet need in the field. Addressing such need might reveal possible targets related to the neuroinflammatory process in AD.

The studies presented in this Thesis provide a deeper understanding of the evolution of neuroinflammation at the very early stages of AD. Our findings indicate that (i) neuroinflammation occurs concomitant with incipient AD pathology, (ii) displays a distinct profile across the AD continuum and that (iii) it might be potentiated by the degeneration of neurotransmitter systems. Such processes represent a "disease-aggravating" scenario and therefore a legitimate therapeutic target pending the unequivocal diagnosis of early preclinical stages of the evolving AD pathology.

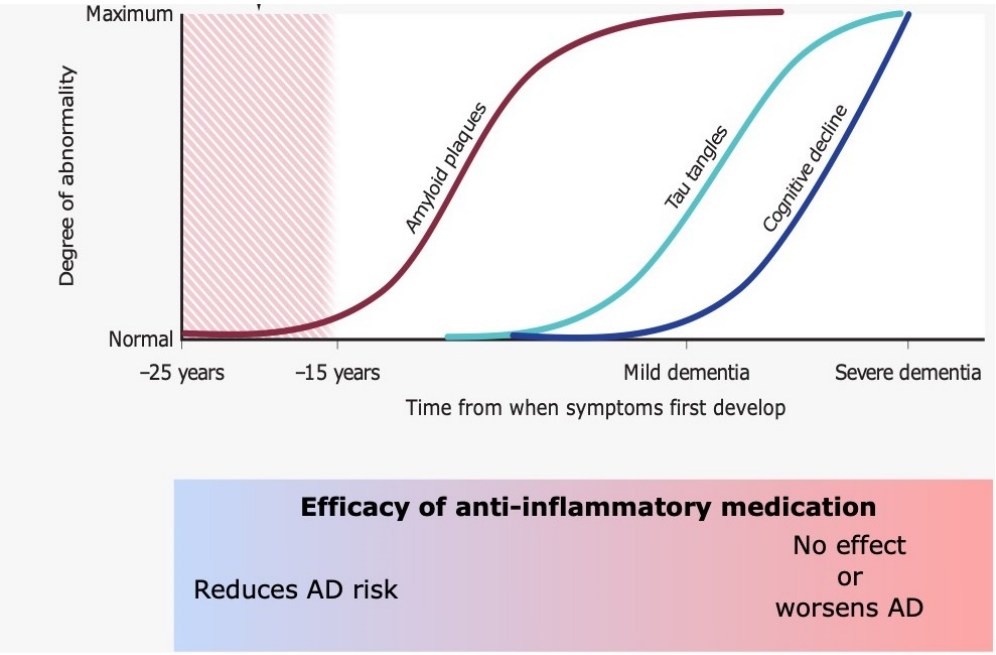

Figure 5- 5. Scheme representing the time of intervention of primary, secondary, and symptomatic trials.

Anti-inflammatory medications have shown to reduce the risk of Alzheimer's disease before the onset of cognitive decline. Image adapted from (McDade *et al.*, 2017) with permission from *Springer Nature*.

Original Contributions

- I demonstrated that neuroinflammation evolves in a differential manner across the lifespan of individuals with Down syndrome. Specifically, I identified that cytokine expression is exacerbated before the development of a full-blown AD pathology in DS brains and that it wanes after AD pathology is well-established.
- I identified a differential cytokine expression between the frontal cortex and hippocampus from adults with DS. Such expression might be dependent on the extent of AD neuropathology.
- I demonstrated that IL-6, IL-8, and eotaxin-3 brain levels were higher in females (euploid and DS) than in males (euploid and DS). Moreover, I identified that females with DS had increased expression of IL-6 and IL-8 than males with DS.
- I identified that microglial cells are intermediately activated in the frontal cortex of children and young adults with DS as reflected by an increase in their dimensional ratio, likely reflecting a "disease-aggravating" neuroinflammatory process.
- I identified the presence and increase of rod microglia, a rare and poorly characterized phenotype, in the frontal cortex of young adults and adults with DS.
- I detected increased expression of inflammasome components in the frontal cortex of adults with DS.
- I established, in *post-mortem* brain tissue, that non-demented individuals with incipient accumulation of amyloid beta display an increase in proinflammatory mediators. Such inflammatory markers positively correlated with the extent of amyloid beta deposition.
- I identified differential inflammatory profiles in *post-mortem* brain tissue from the temporal, parietal, and frontal cortices from non-demented individuals with abnormal amyloid beta deposition.

- I provided evidence that suggests that the temporal cortex is one of the early structures showing a heightened neuroinflammatory profile in *post-mortem* brain samples from non-demented individuals harboring AD pathology.
- I detected that non-demented individuals with higher amyloid beta deposition in *post-mortem brain* sampled displayed faster rates of cognitive decline in perceptual speed and working memory.
- I identified in *post-mortem* brain samples from the cerebral cortex from children, young adults, and adults that normal aging is accompanied by a differential cytokine expression in the brain.
- I identified that noradrenergic denervation at early stages of the human-like amyloid pathology has a detrimental impact on cognition and neuroinflammation in an APP tg rat model of the AD-like amyloid pathology.
- I demonstrated that LC demise provokes microglial morphological changes indicative of an intermediate activation state.
- I identified that cortical noradrenergic synaptic boutons are upregulated at initial stages of amyloid deposition in an APP tg rat model. In turn, I detected a depletion of hippocampal noradrenergic boutons at the post-plaque stage of the AD-like amyloid pathology.

List of Publications and Submitted Manuscripts

1. Evolution of neuroinflammation across the lifespan of individuals with Down syndrome**.** **Flores Aguilar L**, Iulita MF, Kovecses O, Torres MD, Levi S.M., Wisniewski T, Busciglio J, Cuello AC. 2020, *under review.*

2. The Evolving Brain Inflammation in Alzheimer's disease. **Flores Aguilar L**, Welikovitch LA, Foret MK, Do Carmo S, Cuello AC. 2019, *submitted.*

3. Upregulation of cerebral inflammatory markers and cognitive decline in non-demented elderly individuals with Alzheimer's neuropathology. **Flores Aguilar L**, Iulita MF, Tanna N, Bennett D, Cuello AC. 2019, *to be submitted*

4. Locus Coeruleus degeneration aggravates and accelerates early neuroinflammation in a rat model of the human-like amyloid pathology. **Flores Aguilar L**, Hall H, Orciani C, Kovecses O, Foret MK, Ducatenzeiler A, Cuello AC. 2019, *to be submitted*

5. Human Tau overexpressed in the locus coeruleus of rats undergoes progressive pathological changes as seen in human Alzheimer's brains and propagates along efferent projections. Hall H*, **Flores Aguilar L***, Breuillaud L, Cuello AC. 2020, *in preparation for submission. *co-first authors*

6. Neuroinflammaging: differential expression of cytokine expression in the brains of infants, young adults, and adults. **Flores Aguilar L**, AC Cuello, 2020, *in preparation for submission*

7. Inflammasome activation in the brains of individuals with Down syndrome: from infancy to adulthood. **Flores Aguilar L***, Orciani C*, Cuello AC. 2020, *in preparation for submission. *co-first authors*

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APPENDICES

Appendix I

Investigation of cytokine expression in infants, young adults, and adults

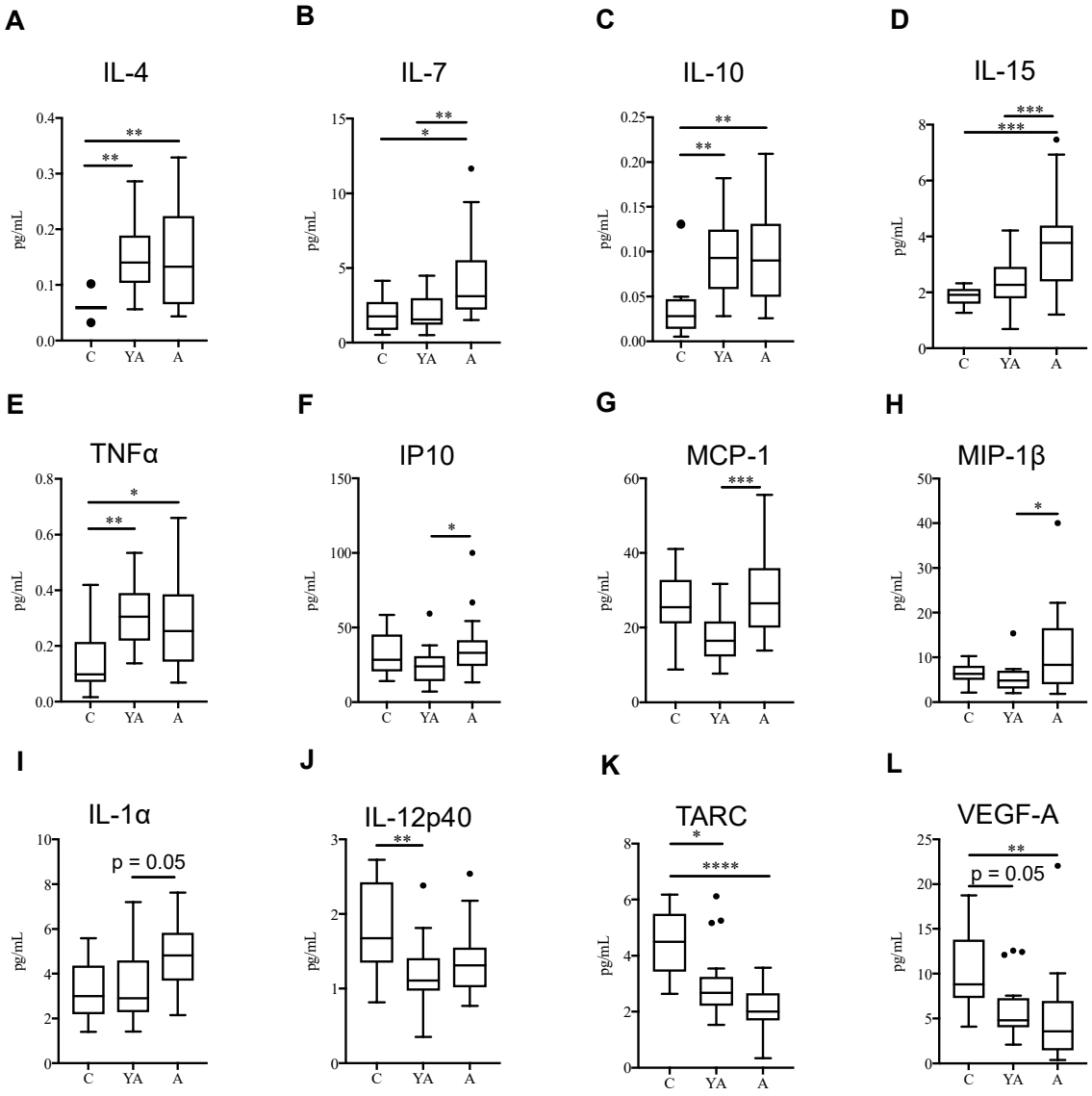

Figure A- 1. Inflammatory markers are deregulated with aging

(A-H) IL-4, IL-7, IL-10, IL-15, TNFα, IP-10, MCP-1, and MIP-1β increased aging while (J-L) IL-1α, IL-12p40, TARC, and VEGF-A levels decrease with aging. A-C, F, H, I, K, L Kruskal-Wallis test followed by Dunn's multiple comparison test; D, E, G, J One-way ANOVA followed by Tukey's multiple comparison test. Error bars represent ± SEM. * *p<0.05, ** p<0.01*, *** p<0.001.* C= Children, YA = young adults, A = adults. *Children n =10, young adults n = 24, adults n = 22.*

Appendix II

Expression of inflammasome components across the lifespan of individuals with Down syndrome

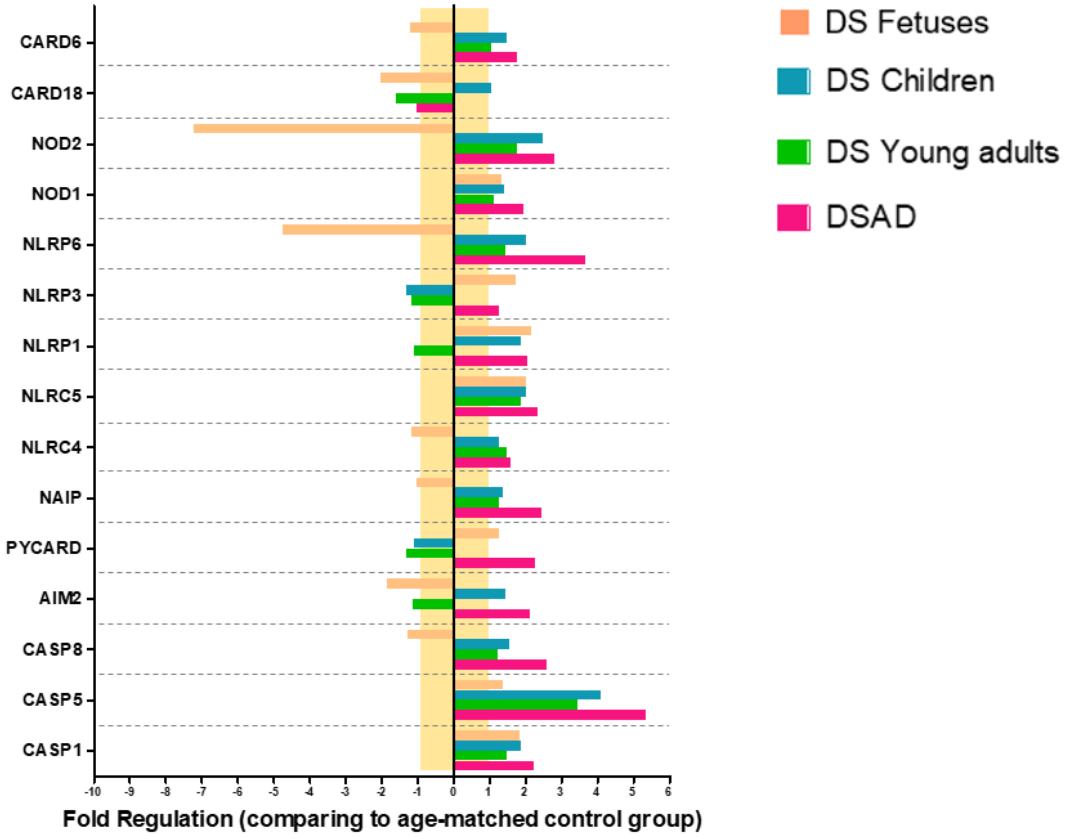

Figure A- 2. Inflammasome components are deregulated across the lifespan of individuals with Down syndrome.

Gene expression analyses was conducted in the frontal cortex of DS and control individuals. The sampled for each age-group were pooled (thus, no statistical analyses are presented), and a qPCR array for inflammasome components was conducted. Each age-group was normalized to their own age matched controls and the fold change was calculated. For simplicity, only the DS groups are presented. The yellow background represents control levels. Note the upregulation of classical inflammasome components such as caspase-1, NLRP1, NLRC5, and NLRP6.

Appendix III

Increased expression of inflammasome components in the frontal cortex of adults with Down syndrome

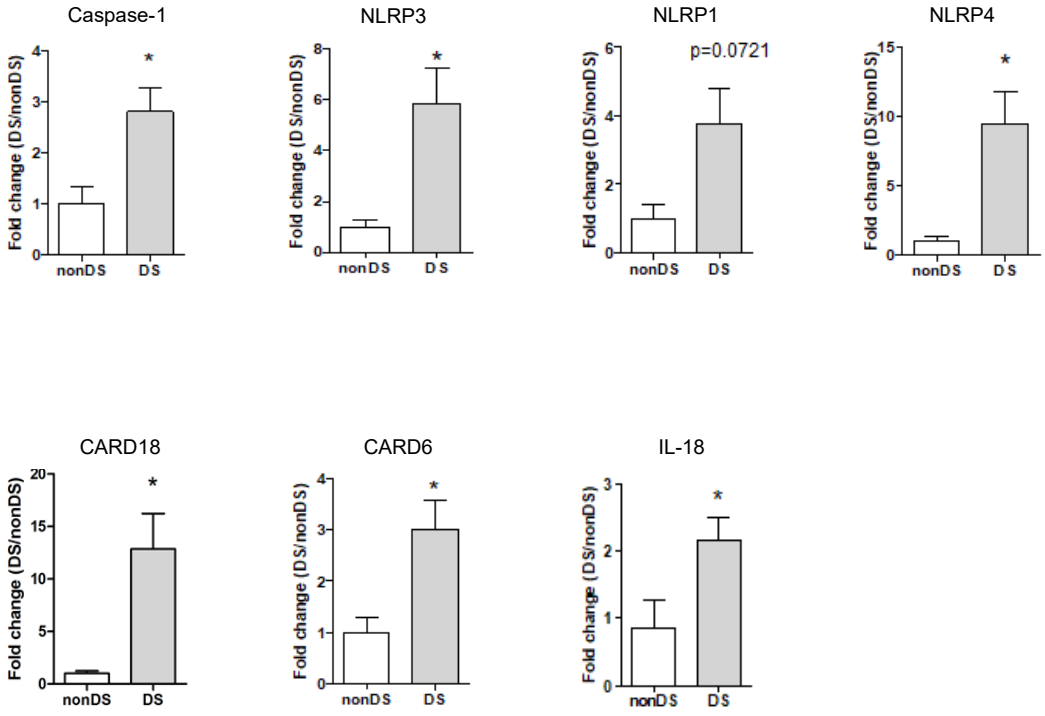

Figure A- 3. Inflammasome components are upregulated in adult Down syndrome brains.

Fold change inflammasome-associated genes in frontal cortex of adults with Down syndrome as analyzed by qPCR. Controls, *n = 6;* DS, *n = 11*. **p<0.05*, t-test.

Appendix V

Noradrenergic boutons at the pre plaque and post-plaque stages of the amyloid-like pathology in the McGill-R-Thy1-APP rat

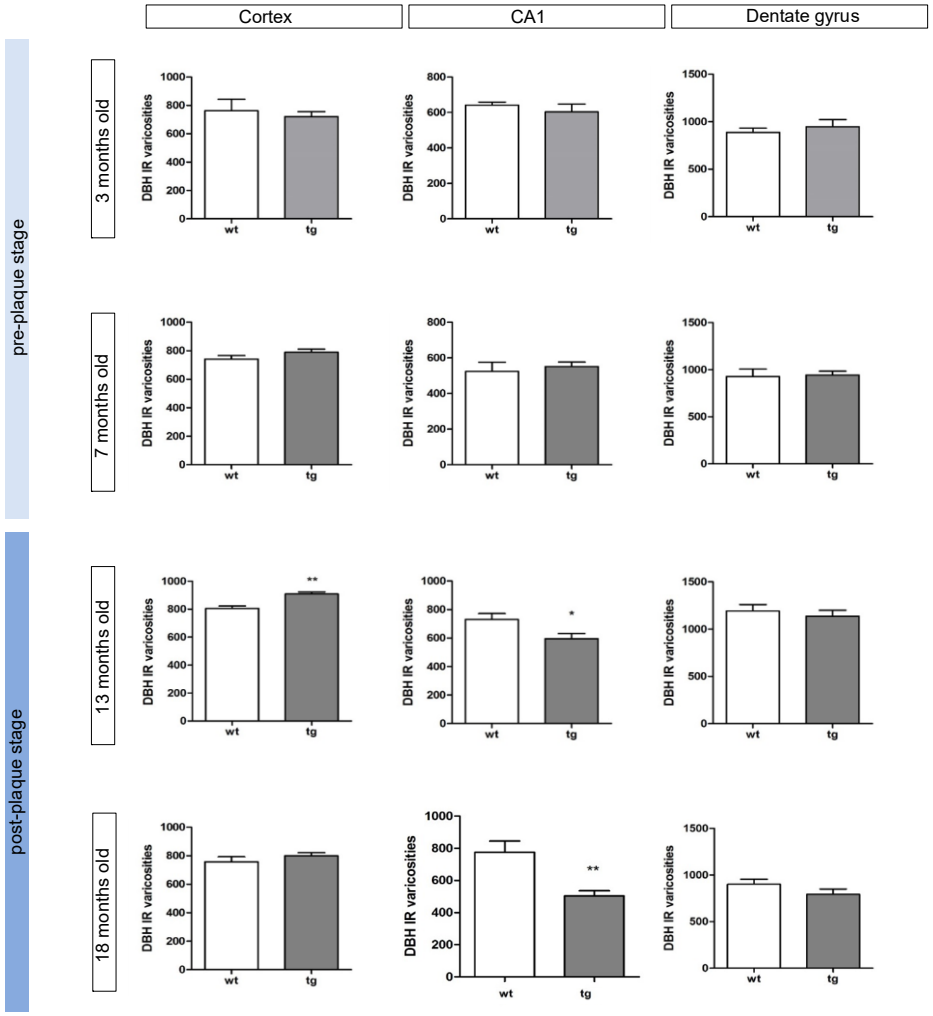

Figure A- 4. Noradrenergic varicosities are deregulated at late stages of the amyloid pathology in the McGill rat

Noradrenergic boutons were detected by immunohistochemistry against DBH (dopamine beta hydroxylase) in the frontal cortex, CA1, and dentate gyrus at the pre-plaque (3 months and 7 months) and post -plaque (13 and 18 months) stages of the amyloid-like pathology in the McGill rat. Computer assisted quantification revealed an increase in DBH-IR in the cortex of 13 months old tg rats accompanied by a decrease in CA1 noradrenergic varicosities. Such decrease was accentuated at 18 months in the tg rats. No significant changes in noradrenergic varicosities were noted at the pre-plaque stage. *$p<0.05$, ** $p<0.01$, t-test. DBH = Dopamine beta-hydroxylase, tg = transgenic.

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