Studying Alzheimer's Disease Pre-clinical Stages: Insights from Down's Syndrome and Transgenic Animal Models

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April, 2014

A Thesis submitted to McGill University

in partial fulfillment of the requirement of the degree of

Doctor of Philosophy

This Thesis is dedicated to my grandparents,

Carlos R. Iulita and *Aida E. Savioli*

Abstract

The recent failure of major Alzheimer's disease (AD) clinical trials would indicate that, at the moment of diagnosis, the damage to the brain is likely irreversible. There is also a growing recognition that the pathological changes that lead to AD develop many years before cognitive decline. This pre-clinical stage deserves further attention, as it should offer a critical opportunity for successful treatment.

Unfortunately, the investigation of this stage is complicated by the fact that current diagnostic methods cannot *unequivocally* identify asymptomatic individuals bound to develop dementia. In that sense, transgenic animal models offer an attractive alternative. Even better, Down's syndrome (DS) brains offer the unique opportunity to investigate the temporal development of AD pathology in humans. Due to triplication of the amyloid-β (Aβ) precursor protein (APP) in chromosome 21, all DS subjects gradually develop amyloid plaques, neurofibrillary tangles and gliosis. Therefore, taking advantage of these models, the main objective of this Thesis was to better understand the early stages of AD pathology.

The progressive degeneration of basal forebrain cholinergic neurons (BFCN) is central to the manifestation of cognitive deficits in AD and DS. BFCN have a life-long dependence on nerve growth factor (NGF) for their phenotypic maintenance. Previous studies from the Cuello lab have shown deficits in NGF's extracellular metabolism in AD brains, which compromise proNGF maturation and enhance NGF degradation. The possibility that this occurs early during AD progression has not been investigated and it is relevant given the need for new disease biomarkers.

Consequently, we first examined whether an NGF-metabolic deficit occurred in DS. Our investigations revealed for the first time a compromised extracellular NGF metabolism in adult DS brains and in trisomic DS mice. Importantly, alterations in NGF metabolism were detected early, in primary cultures from DS fetal cortex.

The NGF metabolic cascade was further examined in the cerebro-spinal fluid (CSF) of subjects with AD and mild cognitive impairment (MCI). Matrix metallo-protease 3 (MMP-3) and tissue plasminogen activator (tPA) were identified as novel biomarker candidates in AD CSF, with significant correlations between the levels of these analytes and clinico-pathological parameters. Although these markers were normal in individuals with MCI, this group exhibited increased neuroserpin, plasminogen and TIMP-1 in CSF, compared to control subjects.

Finally, taking advantage of our APP transgenic rat, we have shown that prior to amyloid plaque deposition, Aβ peptides are present in pyramidal neurons of the cortex and hippocampus as well as in the rat CSF. The early accumulation of intracellular pathology (which includes Aβ, APP and its C-terminal fragments) was accompanied by cognitive deficits, which paralleled the evolution of Aβ pathology.

Together, these results demonstrate that the early accumulation of Aβ is sufficient to induce CNS damage (e.g. NGF deregulation, learning and memory deficits), before the development of full-blown AD pathology. These studies may also provide clues for the development of novel neuroprotective therapies and hint at potential new biomarker candidates for the staging of individuals in the AD trajectory.

Résumé

L'échec récent de plusieurs essais cliniques d'importance suggère qu'au moment du diagnostic de la maladie d'Alzheimer (MA), les dommages au cerveau sont irréversibles. Il y a aussi une reconnaissance croissante du fait que les altérations neuropathologiques qui conduisent à la MA se développent plusieurs années avant le déclin cognitif. Cette longue phase préclinique mérite attention, car elle serait le moment plus indiqué pour diminuer la progression de la maladie. Malheureusement, l'étude de cette phase est compliquée par le fait que les méthodes diagnostiques actuelles ne permettent pas d'identifier avec certitude les personnes asymptomatiques destinées à développer la démence. En ce sens, les modèles animaux transgéniques représentent une alternative intéressante. De surcroît, les cerveaux provenant de personnes atteintes du syndrome de Down (SD) offrent la possibilité d'étudier l'évolution temporelle de la MA chez l'humain. À cause de la triplication du gène de la protéine précurseur d'amyloïde (APP) situé sur le chromosome 21, tous les sujets atteints de SD développent progressivement des plaques amyloïdes séniles, de la dégénérescence neurofibrillaire et de la gliose. En profitant de ces modèles, l'objectif principal de cette thèse était de mieux comprendre les étapes initiales du développement de la MA.

La MA et le SD se caractérisent par la dégénérescence progressive des neurones cholinergiques du cerveau antérieur basal, qui contribue à la manifestation de déficits cognitifs. Ces neurones dépendent de la disponibilité du facteur de croissance nerveuse (NGF) pour le maintien de leur phénotype. Notre laboratoire a démontré des déficits dans le métabolisme extracellulaire de NGF dans les

cerveaux de personnes présentant la MA. Ces altérations compromettent la maturation de proNGF et aggravent la dégradation de NGF. La possibilité que ces déficits se produisent à un stade précoce de la MA n'a pas été étudiée. Cela est pertinent à cause de la nécessité d'identifier de nouveaux biomarqueurs de la MA et de la neurodégénérescence cholinergique.

Ainsi, nous avons d'abord cherché à déterminer si le métabolisme extracellulaire de NGF est affecté dans le SD. Notre recherche a révélé pour la première fois des déficits métaboliques reliés à la maturation et la dégradation de NGF dans les cerveaux provenant de sujets adultes (avec SD) et de souris trisomiques. Ces déficits ont été détectés très tôt, avant l'apparition de plaques amyloïdes, dans des cultures primaires de cortex fétal trisomique.

La cascade de maturation du NGF a aussi été examinée dans le liquide céphalo-rachidien (LCR) de sujets atteints de la MA et de déficience cognitive légère (MCI). Nous avons ainsi identifié la métalloprotéinase-3 (MMP-3) et l'activateur tissulaire du plasminogène (tPA) en tant que nouveaux candidats biomarqueurs, dans le LCR de patients atteints de MA. Les sujets avec MCI, quant à eux, avaient une augmentation de la neuroserpine, du plasminogène et du TIMP-1 dans le LCR.

Finalement, en tirant profit de notre rat transgénique, nous avons démontré que, avant le dépôt de plaques amyloïdes, les peptides Aß sont présents dans les neurones pyramidaux du cortex et de l'hippocampe, ainsi que dans le LCR. L'accumulation intracellulaire précoce de la pathologie (qui comprend le peptide

Aß, APP et ses fragments CTFs) était accompagnée par des déficits cognitifs, qui augmentaient parallèlement à l'évolution de la pathologie Aß.

Ensemble, nos résultats montrent que l'accumulation de Aß est suffisante pour induire des lésions du système nerveux central (par exemple, des déficits métaboliques de NGF, des altérations dans l'apprentissage et dans la mémoire), avant le développement avancé de la pathologie d'Alzheimer. Ensuite, ils offrent de nouvelles pistes pour le développement de thérapies neuroprotectrices cholinergiques et pour l'exploration de nouveaux candidats biomarqueurs pour identifier les sujets asymptomatiques engagés dans la trajectoire de la MA.

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Acknowledgements

There are many so people that I would like to thank, who made this "PhD journey" a most enjoyable experience and one I will never forget.

My biggest "thank you" goes to my supervisor **Dr. Claudio Cuello** for all your patience and guidance, for your trust and for believing in me. Thank you for sharing with us your motivation and your passion for science and discovery. Working with you has taught me how to enjoy science and to never be afraid of pursuing my research dreams. Thank you for all the support you have always provided me and for all your life advices, which I deeply appreciate. You have helped me build the grounds of my scientific career. I am also grateful for all your help in the completion of this Thesis.

I would also like to thank my collaborators **Dr. Jorge Busciglio**, **Dr. Thomas Wisniewski**, **Dr**. **Lotta Granholm** and **Dr. Maria Eriksdötter** for the opportunity of working together and for being supportive of our research. I am especially grateful to **Dr. Gerhard Multhaup**, **Dr. Lisa Münter, Dr. Luise Richter and Dr. Christoph Weise** for many insightful and rich science discussions. I hope that our paths will cross again in the future.

To my Thesis Committee, **Dr. Anne McKinney**, **Dr. Paul Clarke**, **Dr. Guillermina Almazan** and **Dr. Alfredo Ribeiro-Da-Silvia**, I am deeply grateful for your questions, your advice and your guidance throughout these years as a graduate student.

To **Tina Tremblay** and **Hélène Duplessis**, thank you for all help and friendliness, especially for your emotional support when I most needed it.

I would also like to extend my gratitude to the **Alzheimer Society of Canada** for supporting my research during the past three years and to **Dr. Alan Frosst** for his immense interest and continuous support to the work of the Cuello Lab.

To all the members of the Cuello lab: **Adriana, Ted, Lisi, Hélène, Sonia, Molly, Lionel, Lindsay and Ludmilla** thank you for being "my lab family".

A very special thanks to **Sonia Do Carmo**, **Simon Allard** and **Adriana Ducatenzeiler** for all the help and training you have provided me and for always being there with a smile to listen to me. It has been a pleasure and an honour to work with you.

To past members **Martin Bruno**, **Wanda Leon** and **Cecilia Hanzel,** I would like to thank you for all the things you have taught me and for your guidance during my first years as a student.

To **Alison Ower** and **Walter Boiten**, mentoring you has been one of the best experiences I will remember from my PhD.

Finally, I would like to extend an immense "thank you" to my friends and family, because I couldn't have done this without you:

To my friends from University of Quilmes: **Ludmila Frate, Maria Jose Lapponi**, **Albertina Romaniuk**, and **Diego Ferrero**; to my friends from Montreal: **Nouk Tanke**, **Sofia Jacob**, **Diego Pino**, **Emeline Dinparast**, **Maxime Dehaye, Lisi Flores Aguilar, Sonia Do Carmo, Simon Allard, Ilaria Mengoni, Maria Teresa Ferretti** and **Tina Scardochio;** to my lifelong friend: **Ana Davis,** my cousins **Sebastian**, **Camila** and **Tomas**; my aunt **Marta** and uncle **Armando,** my godparents **Maru** and **Yaco**; to **Jorge**, **Alberto**, **Cris** and **Vicky,** thank you everyone (I had to name you all !) for your constant support and encouragement and for many wonderful moments together that I will never forget. Although we are scattered all over the globe, you are always present in me and I feel lucky to have you in my life.

To my mom **Alicia Iulita**, I don't have enough words to thank you for all the things you have done for me. Thank you for always pushing me to do better, for transmitting me the value of education and for raising me to be a "citizen of the world". Your strength will always be a source of encouragement and inspiration to me. To **Pablo Fernandez Busch**, a very special "thank you" for being my pillar and support, for your love and for always believing I could accomplish this. Thank you for helping me with the format of this Thesis, for keeping me company during long nights of writing and figure assembly, for coming with me to the lab during some weekends and for being the wonderful person you are. Thank you for your company to this Canadian adventure. No matter where we are, "home" is being with you. Thank you mami and Pablo for supporting my dreams.

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Appendix I

List of Abbreviations

3xTg	Triple transgenic
AchE	Acetylcholinesterase
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease domain
ADRDA	Alzheimer's Disease and Related Disorders Association
AICD	APP intracellular domain
aMCI	Amnesic Mild Cognitive Impairment
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid-β
BACE1	Beta-site APP cleaving enzyme
BFCN	Basal forebrain cholinergic neurons
CAA	Cerebral amyloid angiopathy
CCNA	Canadian Consortium on Neurodegeneration in Aging
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
ChAT	Choline acetyl transferase

ChEIs Cholinesterase inhibitors

CM Conditioned media

CNS Central nervous system

CR1 Receptor fro complement factor 3b

CRE cAMP response element

CSF Cerebro-spinal fluid

CTF C-terminal fragment (CTF)

Ctrl Control

Cxdar Coxsackie virus and adenovirus receptor

DS Down's syndrome

DSM-IV Diagnostic & Statistical Manual of Mental Disorders, 4th Ed.

ELISA Enzyme-linked immunosorbent assay

ER Endoplasmic reticulum

FA Formic acid

FAD Familial Alzheimer's disease

FDA Food and Drug Administration

FDG Fluorodeoxyglucose

FPRL1 FMLP-receptor-like protein 1

GABA Gamma-amino butyric acid

GCS Global Cognitive Score

HPRT Hypoxanthine-guanine phosphoribosyltransferase

HRP Horseradish peroxidase

i-NOS Inducible nitric oxide synthase

IBM Inclusion body myositis

ICD-10 International Classification of Diseases, 10th revision

IHC Immunohistochemistry

IL- Interleukin-

IOD Integrated optical density

IQ Intelligence quotient

IR Immunoreactivity

LDH Lactate dehydrogenase

LRP LDL receptor related protein

LTP Long-term potentiation

M1 Muscarinic receptor subtype 1

M3 Muscarinic receptor subtype 3

MALDI Matrix assisted laser desorption ionization

MCI Mild Cognitive Impairment

MMP-2 Matrix metallo-protease 2

MMP-3 Matrix metallo-protease 3

MMP-9 Matrix metallo-protease 9

MMPs Metallo-proteases

MMSE Mini Mental State Examination Exam

mNGF Mature nerve growth factor

MRI Magnetic resonance imaging

MS Mass spectrometry

MVB Multivesicular body

nAChR Nicotinic acetylcholine receptor

NAPA National Alzheimer's Project Act

NGF Nerve growth factor

NGS Normal goat serum

NINCD National Institute of Neurological & Communicative Disorders

NMDA-R N-methyl-D-aspartate receptor

NO Nitric oxide

NOL Novel object location

NOR Novel object recognition

NTFs Neurofibrillary tangles

NYU New York University

P-tau	Phosphorylated tau
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline + Triton X-100
PET	Positron emission tomography
PHFs	Paired helical filaments
PiB	Pittsburg compound B
PMI	Post-mortem interval
ProNGF	Pro nerve growth factor (precursor molecule)
PSEN	Presinilin
Pyroglu-Aβ	Pyroglutamate-modified Aβ
qRT-PCR	Quantitative real-time polymerase chain reaction
RAGE	Receptor for advanced glycation end products.
ROS	Religious Orders Study
sAPP	Soluble APP
SCI	Subjective Cognitive Impairment
SEM	Standard error of the mean
SNAP	Suspected Non-Alzheimer Pathology
SSRIs	Selective serotonin reuptake inhibitors
T-tau	Total tau

TACE Tumor necrosis factor-α converting enzyme

TBS Tris-buffered saline

TGN Trans-Golgi network

Tiam1 T-cell lymphoma invasion and metastasis 1

TIMP-1 Tissue inhibitor of metallo-proteases

TNF- α Tumor necrosis factor-α

tPA Tissue plasminogen activator

TREM2 Triggering receptor expressed on myeloid cells 2

TrkA Tyrosine kinase receptor A

UCI University of California, Irvine

α7nACh Alpha7 nicotinic acetylcholine receptor

Contribution of Authors

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

M. Florencia Iulita (MFI): *MFI* was the leading investigator for all described projects (Chapters 2-4), including that resulting in the 2014 Journal of Alzheimer's Disease (JAD) publication, in which the project was led equally by *MFI* and Cecilia E. Hanzel (*CEH*). *MFI* designed all the experiments, in consultation with other collaborators. *MFI* ran the vast majority of the experiments presented, including data quantification and analysis and the generation of figures for publication. *MFI* independently wrote the first draft of all manuscripts, which were then edited by all other co-authors. For the 2014 JAD publication, the first manuscript draft was written by *MFI* (introduction, results, discussion) and *CEH* (methods, results, discussion), and then edited by all other co-authors.

A. Claudio Cuello (ACC): *ACC* was the principal investigator and coordinator of all projects. As my doctoral thesis supervisor he has been the main intellectual influence behind the described work. *ACC* provided me with training and contributed significantly to all original ideas addressed. For all publications, he offered intellectual guidance and edited the writing of all manuscripts and the preparation of figures.

CHAPTER 2

Nerve growth factor metabolic dysfunction in Down's syndrome brains
Iulita MF, Do Carmo S, Ower AK, Fortress AM, Aguilar LF, Hanna M, Wisniewski T, Granholm AC, Buhusi M, Busciglio J, Cuello AC. *Brain*. 2014 Mar; 137 (Pt 3):860-72. Epub 2014 Feb 11.

Sonia Do Carmo: *SDC* performed the qRT-PCR experiments and provided significant intellectual contribution to the generation of the final manuscript.

Alison K Ower: *AKO* performed Western blotting, ELISA and zymography analysis in primary cultures of Down's syndrome fetal cortex.

Ashley M Fortress: *AMF* performed Western blotting in Ts65Dn mice and quantified the resultant data depicted in figure 6.

Lisi Flores Aguilar: *LFA* performed Western blotting in human cortex homogenates.

Michael Hanna: *MH* generated primary cultures from fetal cortex tissue and collected conditioned media for analysis.

Thomas Wisniewski: *TW* provided frontal cortex tissue and related clinical and pathological information of the cases used in this study. He contributed significantly to the generation of the final manuscript.

Ann-Charlotte Granholm: *ACG* provided frozen brains from Ts65Dn mice for analysis by *MFI*. *ACG* provided intellectual contribution and assistance in the generation of the final manuscript.

Mona Buhusi: *MB* provided technical assistance to *AMF*.

Jorge Busciglio: *JB* has been the principal collaborator who provided human brain tissue and human fetal cortex cultures, as well as the clinical and pathological information associated with each case studied. He provided intellectual contribution for the initiation and completion of the project and assisted in the generation of the final manuscript.

CHAPTER 3

Analysis of Matrix Metallo-Proteases and the Plasminogen System in Mild Cognitive Impairment and Alzheimer's Disease Cerebro-Spinal Fluid
Hanzel CE*, **Iulita MF***, Eyjolfsdottir H, Hjorth E, Schutzberg M, Eriksdotter M and Cuello AC. *The Journal of Alzheimer's Disease*. 2014 Feb 14. [Epub ahead of print]
*Denotes co-first authorship.

Cecilia E. Hanzel: *CEH* led the project together with *MFI*. *CEH* performed Western blotting, ELISA and zymography analysis of metallo-proteases. In collaboration with *MFI* she produced the first draft of the manuscript and generated the figures.

Helga Eyjolfsdottir: *HE* carried out the cerebro-spinal fluid extractions and was the leading physician monitoring the participants involved in the study. *HE* contributed significantly to the generation of the final manuscript.

Erik Hjorth: *EH* assisted with the statistical correlation analysis and helped with manuscript editing and completion.

Marianne Schultzberg: *MS* provided significant intellectual contribution for the execution of the project. She also assisted in the generation of the final manuscript version.

Maria Eriksdötter: *ME* was our leading collaborator who gave us access to cerebro-spinal fluid samples and provided significant intellectual contribution for the execution and completions of the project. *ME* also assisted in the generation of the final manuscript.

CHAPTER 4

Intracellular Aβ Pathology and Early Cognitive Impairments in a Transgenic Rat Model Overexpressing Human Amyloid Precursor Protein: a Multidimensional Study

Iulita MF*, Allard S*, Richter L, Munter LM, Ducatenzeiler A, Weise C, Do Carmo S, Klein WL, Multhaup G and Cuello AC. *Acta Neuropathol Commun. 2014 Jun 5;2(1):61*

*Denotes co-first authorship.

Simon Allard: *SA* contributed to the design and execution of the study, together with *MFI*. He conducted the super-resolution microscopy experiments and tested the animals with the Novel Object Recognition and Location paradigm and the Von Frey test. He provided significant intellectual contribution to the generation of the manuscript and also created the microscopy figures.

Luise Richter: *LR* performed the $A\beta_{40}$ and $A\beta_{42}$ ELISAs, analyzed the data and generated the figure. She also helped in editing the manuscript.

Lisa M. Munter: *LMM* contributed to the design of the study and assisted in the Aβ immunoprecipitations from rat cerebro-spinal fluid. She significantly contributed to the generation and completion of the manuscript.

Adriana Ducatenzeiler: *AD* assisted with immunohistochemistry and animal genotyping.

Christoph Weise: *CW* performed the MALDI-MS analysis from rat cerebro-spinal fluid. He also assisted in the manuscript editing and preparation.

Sonia Do Carmo: *SDC* performed the genotyping of the animals. She provided significant intellectual contribution to the generation of the final manuscript version.

William L. Klein: *WLK* contributed to the immunological characterization of the Nu1 antibody and assisted in manuscript editing.

Gerhard Multhaup: *GM* was a leading collaborator who helped in the design of the study. He provided the polyclonal pab27576 antibody. He provided significant intellectual contribution to the generation and completion of the final manuscript

CHAPTER 1

General Introduction

1.1. Preamble

Alzheimer's disease is the most common form of age-related neurodegenerative dementia, affecting 35 million people worldwide (Prince *et al*, 2013b). Unless a cure is found, the number of Alzheimer's disease sufferers is only expected to rise, given the increased life expectancy of the currently aging population.

Present treatment options are only symptomatic and aim at correcting CNS cholinergic deficits. Given the progressive nature of the Alzheimer's disease pathology and the extensive brain damage at the time of clinical diagnosis, these drugs only offer limited therapeutic benefit. Therefore, earlier diagnosis will be critical for the success of novel, disease-modifying therapeutic approaches.

In recent years, it has become evident that Alzheimer's disease has a decades-long, asymptomatic or "incubation" phase, where pathological changes progressively build up in the brain, without apparent disturbances in cognition (Bateman *et al*, 2012; Jack *et al*, 2013). Notably, little is known about this early, "silent" phase, as most studies have examined the Alzheimer's disease pathology at moderate-to-late stages. This "pre-clinical" stage deserves further attention, as it should provide a critical opportunity for successful treatment.

For these studies to be possible, reliable prognostic biomarkers must be available. Although many indicators of Alzheimer's disease pathology are being examined in cerebro-spinal fluid or with brain imaging technology, the challenge is that such biomarker abnormalities are not always firm predictors of future cognitive decline.

In this regard, studies focusing on the convergence between Alzheimer's disease and Down's syndrome are gaining increasing attention. Individuals with Down's syndrome are at increased risk of developing Alzheimer's disease compared to the normal population (Lott, 2012). Down's syndrome subjects progressively develop Alzheimer's pathology such that by middle age, a Down's syndrome brain would not look too different from a brain with Alzheimer's disease (Wisniewski *et al*, 1985). Likewise, individuals with Down's syndrome exhibit cholinergic deficits and other transmitter abnormalities. Therefore, Down's syndrome brains offer the unique opportunity to explore molecular changes that might accompany the development of Alzheimer's disease neuropathology, before the onset of dementia.

Similarly, transgenic animal models of the amyloid pathology continue to be valuable tools to identify early AD pathological events. Despite their limitations in recapitulating the full complexity of human Alzheimer's disease, many mouse and rat models faithfully reflect key aspects of the disease, including the development of extracellular amyloid plaques, the presence of neuroinflammation, synaptic dysfunction as well as cognitive impairments. Because all of them will invariably develop Alzheimer's-like amyloid pathology and progressive cognitive decline, they remain an important asset for Alzheimer's disease research.

In sum, there is an urgent need for better treatments for Alzheimer's disease patients and for earlier diagnosis, to avoid irreversible brain damage and to maximize a successful therapeutic outcome. We propose that a better understanding of the mechanisms of Alzheimer's disease progression will be vital to address these issues.

1.2. The discovery of Alzheimer's disease

1.2.1. 1906: Alois Alzheimer discovers a "peculiar" disease

On November 25th, 1901, Auguste Deter (51 years old) was admitted to the Frankfurt State Asylum (for a historical review see (Berchtold & Cotman, 1998; Hodges, 2006). She was examined and followed by Alois Alzheimer, a young doctor beginning his career in clinical psychiatry and neuropathology. Auguste Deter suffered from memory deterioration and disorientation. She had difficulties naming familiar objects, writing complete sentences and choosing words. She suffered from hallucinations; had strong jealousy toward her husband and felt that someone wanted to kill her. "I have lost myself", she expressed. Auguste Deter died in 1906, 5 years after hospitalization.

In 1903 Alzheimer moved to the Medical School in Munich to work with Professor Emil Kraepelin, an eminent psychiatrist. Encouraged by Kraepelin and by his own interest in Auguste's case, he requested her brain be sent to Munich, where he could perform a pathological analysis, with new silver staining techniques available in his laboratory. Alzheimer revealed significant cortical atrophy and arteriosclerotic changes in Auguste Deter's brain. He noticed the co-occurrence of microscopic extracellular "foci" and "fibrillar bundles" of a chemically modified substance inside degenerating neurons, referred by him as intracellular "tangles of fibrils" (Figure 1-1). He presented his clinico-pathological findings at the 37th Conference of South-West German Psychiatrists in Tübingen (Alzheimer, 1907).

The presence of extracellular "foci" or "plaques" was not a novel discovery; they had previously been noted by Blocq, Marinesco (1892) and Redlich (1898) in patients with epilepsy and advanced senile dementia, respectively. In these studies it was also postulated that plaques, composed of some fibrous material, were nodules of proliferating glial cells. However, they did not comment or reflect upon their possible pathophysiological significance. Alzheimer instead was the first to describe the co-occurrence of plaques and tangles and relate them to his patient's symptoms. These pathological structures are still recognized today as hallmarks of the disease process.

The peculiarity of Alzheimer's patient was the occurrence of these pathological structures in a young individual, as opposed to the much older cases of senile dementia (above 65 years of age), as referred at the time. Moreover, Auguste Deter's symptoms could not be classified into any of the recognized illnesses of the moment. Alzheimer concluded he had encountered a unique clinico-pathological condition, as addressed in his 1907 publication entitled: *"A peculiar serious disease of the cerebral cortex"*; for an English translation of the 1907 paper see (Alzheimer *et al*, 1995). In the same year, Dr. Oskar Fischer reported a comprehensive study of the cortex of 16 cases of senile dementia stained with similar silver staining methods as employed by Alzheimer (Fischer, 1907). He observed the presence of numerous "plaques" of different sizes in the brain. He did not believe in a glial origin for these structures and described them as inclusions of "unknown origin" and compact appearance. The biggest plaques were seen in close proximity to degenerating neurites. He proposed that these neuropathological

lesions could be the substrate of the dementing syndrome. Oskar Fisher correctly provided the first description of a neuritic amyloid plaque; for a historical review see (Goedert, 2009).

Although Alzheimer and Fischer revealed the presence of neuritic plaques in early and late senile dementia, the term "Alzheimer's disease" was coined by Kraepelin in 1910 to describe a *rare* syndrome of pre-senile dementia (under 65 years of age), distinguishable from the more common senile dementia prevalent in people aged 65 and over (Kraepelin, 1910). Alzheimer's disease and senile dementia were considered two separate disorders until the 1970s. A major milestone of this period was a brief but influential article by Dr. Robert Katzman, an American neurologist, concluding that AD and senile dementia were a *single* disorder based on the fact that most patients with senile dementia had AD pathology and similar clinical progression. With this realization he raised awareness that AD was the most prevalent form of dementia (*A Major Killer*) and a serious public health issue. He estimated that AD ranked the 4th leading cause of death in the United States and that ~0.5 million people suffered from dementia in 1970 (Katzman, 1976).

Today, the number of AD suffers in the US alone reaches approximately 6 million (Thies et al, 2013) and 0.5 million in Canada (Alzheimer Society of Canada, 2010). In 2012 the US government announced the creation of a nation-wide initiative called the "National Alzheimer's Project Act", which aims at developing preventative and/or therapeutic approaches for the management of AD by 2025 as well as improving care and quality of life for patients and caregivers (http://aspe.hhs.gov/daltcp/napa/#NAPA).

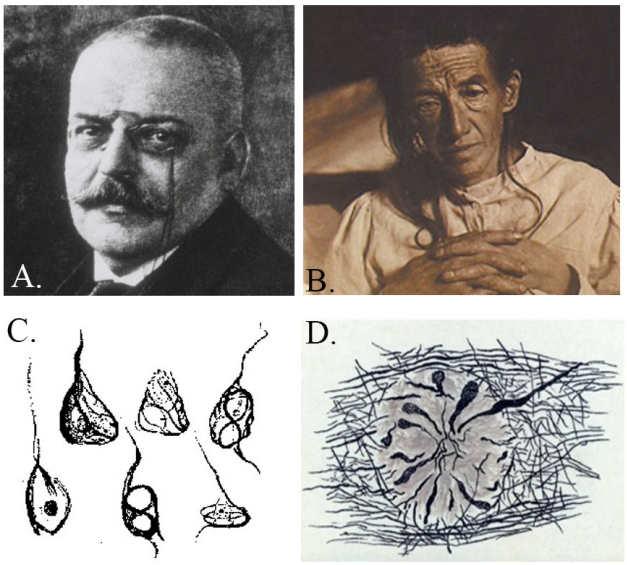


Figure 1-1. Alois Alzheimer and Auguste Deter. A) Alois Alzheimer (14 June 1864 – 19 December 1915) was a German psychiatrist and neuropathologist and the first to describe and identify the clinical and pathological process of pre-senile dementia (known today as Alzheimer's disease). B) Auguste Deter (16 May 1849 - 8 June 1906) was the first patient diagnosed with Alzheimer's disease. C) Drawings of neurofibrillary tangles observed by Alzheimer in Auguste Deter's brain (from Alzheimer A., 1911). D) Oskar Fischer's sketch of a neuritic plaque in the brain parenchyma of patients with senile dementia (from Fischer O., 1907).

A similar Canadian initiative is being launched in 2014, known as the Canadian Consortium on Neurodegeneration in Aging (CCNA). The CCNA aims to create a Canada-wide network to promote research in neurodegenerative diseases that affect cognition, with the goal of preventing or alleviating the impact of dementia for AD sufferers and caregivers (http://www.cihr-irsc.gc.ca/e/46475.html).

1.3. Overview of Alzheimer's disease clinico-pathology

1.3.1. Alzheimer's disease: symptoms, diagnosis and progression

1.3.1.1. Dementia

AD is a progressive neurodegenerative type of dementia. Dementia is a broad term to describe a group of symptoms affecting memory, thinking and social behaviour; ultimately compromising the ability of a person to perform simple activities of everyday life (DSM-IV, 2000). Dementia encompasses a variety of neurodegenerative diseases such as AD, vascular dementia, dementia with Lewy Bodies, fronto-temporal dementia, Parkinson's disease, and Creutzfeld-Jackob disorder. Different types of dementia are associated with a distinct group of symptoms and pathology, as reviewed by (Hou *et al*, 2004; Karantzoulis & Galvin, 2011). AD accounts for approximately 60-80% of dementia diagnoses.

1.3.1.2. Clinical diagnosis

The most common symptoms of AD begin with disturbances in the recall and storage of new information, difficulties in thinking, planning and temporal-spatial disorientation (Cummings & Khachaturian, 2001).

There are three main criteria-based guidelines to identify AD in the clinic: the International Classification of Diseases, 10th revision (ICD-10); the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) and the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDs-ADRDA) criteria. These approaches are geared to identify typical AD symptoms and to exclude other causes of cognitive decline. This is usually assessed with a battery of neuropsychological cognitive tests and structural brain imaging, to identify changes in specific AD-vulnerable areas such as the hippocampus or entorhinal cortex.

Briefly, a diagnosis of AD is defined by the presence of memory impairments and disturbances in at least an additional cognitive domain, which may cause aphasia (i.e. problems in comprehension and formulation of language in speaking or writing), agnosia (i.e. inability to recognize common objects or people) and/or apraxia (i.e. difficulties in executing familiar tasks). These symptoms must be severe enough to disturb the person's independence to carry out normal daily activities (McKhann *et al*, 1984). In addition, other types of neurodegenerative dementias, delirium, or psychiatric disorders which may affect cognition must be excluded. Even between AD-diagnosed subjects, there can be clinical heterogeneity with respect to age of onset, rate of progression and the degree of cognitive compromise; rendering AD diagnosis a challenging task.

Despite great advances in research, there is still no examination capable to determine the presence of AD with certainty. Current guidelines help to define whether AD is the likely cause of a particular group of symptoms, with ~70-80%

diagnostic accuracy. A definite AD diagnosis can only be done post-mortem, by combining the clinical diagnosis with a neuropathological examination.

1.3.1.3. Alzheimer's disease progression

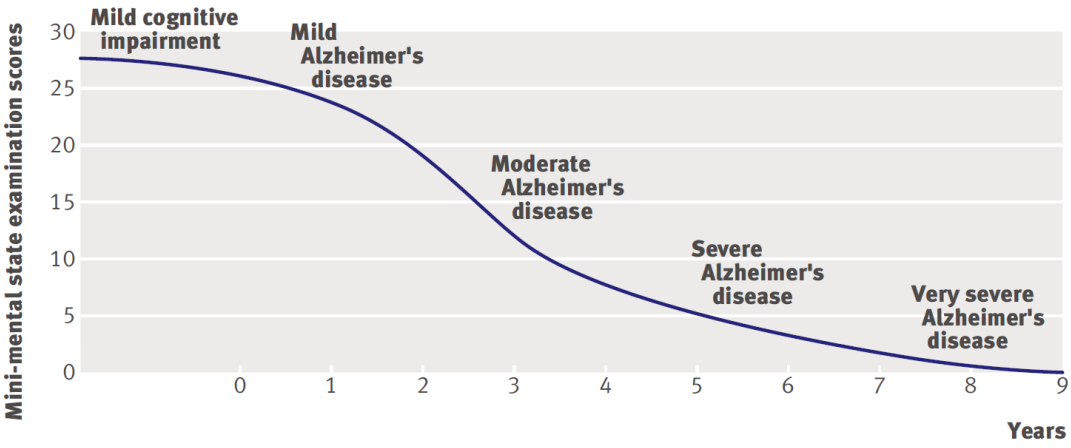
According to the 1984 diagnostic criteria the beginning AD is defined by the presence of progressive cognitive and behavioural impairment, and is differentiated from other forms of dementia by its gradual, slow onset (McKhann *et al*, 1984). Before this phase, patients may exhibit memory complaints and subtle cognitive deficits (i.e. poor judgment or difficulties planning and problem-solving) beyond what is expected for their age and education background; a stage referred as amnesic Mild Cognitive Impairment (aMCI) (Petersen, 2004). Contrary to AD, the neuropsychological deficits detected in MCI are not sufficient to interfere with the person's independence to carry out familiar tasks (Burns & Iliffe, 2009).

People diagnosed with aMCI exhibit a faster decline in cognitive abilities compared to non-impaired subjects (Bennett *et al*, 2002). They develop dementia at a rate of ~10-15% per year; while others may remain cognitively stable for a long period of time. This represents a risk 5-10 times higher than that for the normal aging population (1-2% per year) (Petersen, 2004; Boyle *et al*, 2006). Given the strong correlation between amnestic MCI and AD progression, MCI is considered in effect, a prodromal stage of AD, which may last between 2 to 7 years.

As the disease progresses and the pathology spreads throughout the brain, impairments in memory, thinking and behaviour worsen to such an extent that the person is unable to function independently, needing complete care and supervision

(Cummings & Khachaturian, 2001). At this stage, most memories (even remote and biographical events) are lost. Changes in mood and behaviour may occur, such as aggression, hallucinations, paranoia loss of inhibition and delirium. Motor symptoms manifest in the form of tremors, rigidity and impaired coordination. The patient may also suffer from sleep disturbances, incontinence, severe loss of speech and difficulties in swallowing. Although the chronology of these changes varies between individuals, a patient can be expected to live between 8-10 years after clinical diagnosis (Burns & Iliffe, 2009).

A schematic representation of symptom progression from MCI to AD is illustrated in Figure 1-2.



Mild cognitive impairment: Complaints of memory loss, intact activities of daily living, no evidence of Alzheimer's disease

Mild Alzheimer's disease: Forgetfulness, short term memory loss, repetitive questions, hobbies, interests lost, impaired activities of daily living

Moderate Alzheimer's disease: Progression of cognitive deficits, dysexecutive syndrome, further impaired activities of daily living, transitions in care, emergence of behavioural and psychological symptoms of dementia

Severe Alzheimer's disease: Agitation, altered sleep patterns, assistance required in dressing, feeding, bathing, established behavioural and psychological symptoms of dementia

Very severe Alzheimer's disease: Bedbound, no speech, incontinent, basic psychomotor skills lost

Figure 1-2. Natural history of Alzheimer's disease and symptom progression. The Mini Mental State Examination (MMSE) is a test used to screen for cognitive impairment based on a 30-point questionnaire (30 = maximum score). Image from Burns and Iliffe, *BMJ* 2009; 338:b158.

1.3.2. Alzheimer's disease pathological hallmarks

1.3.2.1. Gross brain alterations

Noticeable gross degenerative changes are evident in the AD brain: a general cortical mass reduction, widened ventricles, shrinkage of the gyri and expanded sulci (Apostolova *et al*, 2012). The narrowing of cortical regions is more prominent in the temporal and frontal cortex but may also severely affect the parietal region. In comparison, the occipital lobe is relatively spared. The hippocampal-entorhinal region is severely (and early) affected. Longitudinal MRI studies have shown that global brain atrophy increases with disease severity, as demonstrated by positive correlations between the rate of global cerebral atrophy and the degree of cognitive decline (measured with the MMSE) (Fox *et al*, 1999). Similarly, the progression of cortical grey matter thinning is already evident in MCI subjects, who exhibit reduced cortical thickness in the medial temporal lobe compared to non-impaired controls (Singh *et al*, 2006). In AD patients, the thinning of cortical layers is more widespread throughout the entire cortex than in MCI. Multiple small infarcts are also noticeable, often in the basal ganglia and thalamus, structures which are otherwise relatively spared of plaque and tangle pathology (Terry *et al*, 1999).

1.3.2.2. Microscopic brain lesions

AD is distinguished by three main pathological hallmarks: 1) amyloid plaques; 2) neurofibrillary tangles and 3) amyloid angiopathy (Wippold *et al*, 2008) (Figure 1-3); as well as by the presence of marked synaptic and neuronal loss (Terry *et al*, 1991; Terry, 1997).

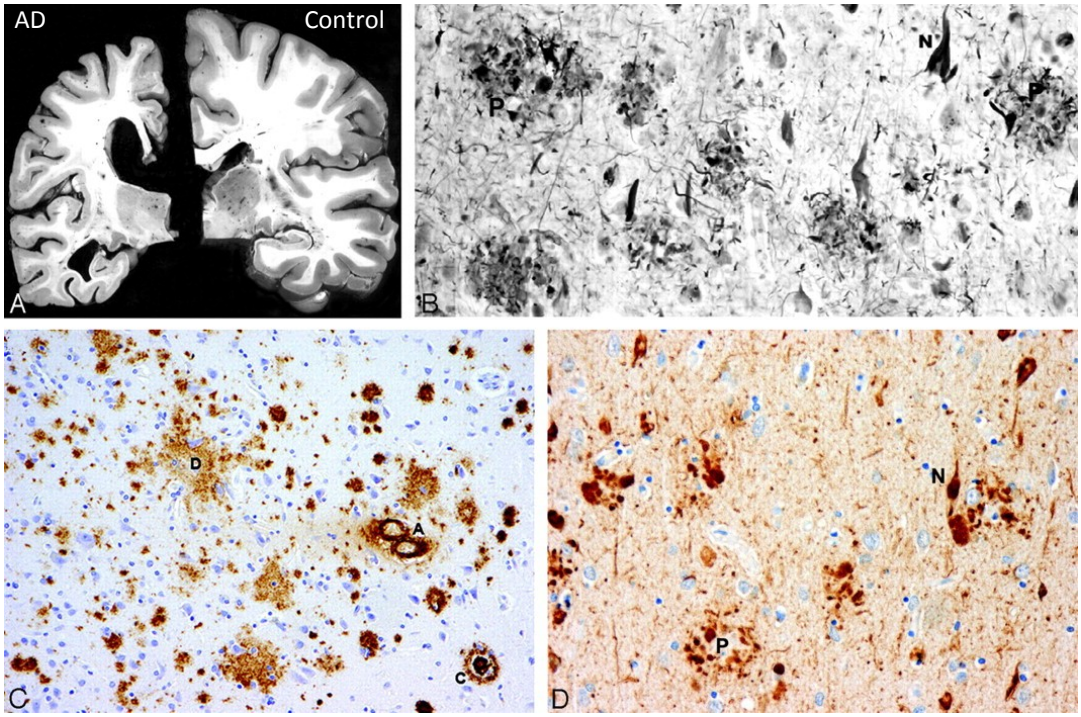


Figure 1-3. Neuropathological hallmarks of Alzheimer's disease. Top left panel (A) depicts a section of a hemibrain from an AD patient (left) and a healthy aged brain (right). Notice the marked cortical atrophy and ventricle enlargement in the AD brain. Top right panel (B): neuritic plaques [P] and neurofibrillary tangles [N] in the hippocampus, as seen with the modified Bielschowsky silver impregnation. Bottom left panel (C): immunostaining of a frontal cortex section with an anti-amyloid-β antibody (10D5) revealing a diffuse amyloid plaque [D], a dense cored plaque [C] and cerebral amyloid angiopathy [A]. (D): immunodetection of neurofibrillary tangles (N) and neuritic plaques [P] in frontal lobe. Modified from Wippold II FJ et al., *Am J Neuroradiol* 2008.

Amyloid plaques

Although Alzheimer identified the occurrence of plaques and tangles in pre-senile dementia over 100 years ago, the molecular composition of these pathological hallmarks was largely unknown until the mid-1980s.

Amyloid plaques are extracellular microscopic deposits, which are found in close proximity to degenerating axons and dendrites, and are generally located within the cerebral cortex and hippocampus. In the mid-1960s Michael Kidd and Robert Terry used the electron microscope to describe the ultrastructural properties of AD brain lesions, demonstrating that plaques are composed of a filamentous material with a structure identical to amyloid, and revealing that this fibrillar substance was different from that of neurofibrillary tangles (Kidd, 1964; Terry *et al*, 1964).

Years later, Glenner and Wong demonstrated that fibres isolated from cerebrovascular amyloid deposits of post-mortem AD and Down's syndrome (DS) brains are composed of a unique 4 kDa β-pleated protein (Glenner & Wong, 1984b; Glenner & Wong, 1984a). This seminal finding was soon confirmed by Masters and collaborators, who instead isolated the amyloid protein from cortical amyloid deposits (Masters *et al*, 1985). The amyloid plaque core protein is known today as "amyloid-β" (Aβ), a short peptide fragment of ~37-43 amino acids, with a high propensity to aggregate (Jarrett *et al*, 1993).

Amyloid plaques exhibit different sizes (10-120 μm) and degrees of compactness, such that two main types can be distinguished: 1) diffuse and 2) neuritic plaques.

Diffuse amyloid plaques are abundant in cortex and hippocampus but can be also found in other brain regions not typically implicated in AD symptomatology (i.e. thalamus, cerebellum, striatum). They are composed of non-fibrillar aggregates of Aβ, mostly ending at amino acid 42, a slightly longer, more hydrophobic form, prone to aggregation. The amorphous Aβ-immunoreactivity in diffuse plaques reflects the absence of a compact, dense core, which is associated with little or no neuritric dystrophy. Given their presence in AD vulnerable regions, it has been hypothesized that these structures may be the precursors of neuritic plaques. Supporting this case, diffuse plaques can be found in the brains of non-demented subjects, in regions vulnerable to AD pathology (Bennett *et al*, 2006). They are also the first types of plaques to develop in young DS brains (Lemere *et al*, 1996).

Neuritic plaques (also referred as mature or senile plaques) are abundant in cortex and hippocampus. They are composed of compact, fibrillar deposits of Aβ ending at amino acid 40 and 42. Activated microglia can be found in close proximity to the central amyloid core, while reactive astrocytes and dystrophic neurites typically surround the outside of the plaque. This classification of plaques is relevant as it is used in pathological examinations to confirm a clinical AD diagnosis. The CERAD criteria utilize a semi-quantitative score based on the density of neuritic plaques to provide a possible, probable or definite AD diagnosis (Mirra *et al*, 1991).

Neurofibrillary tangles

Neurofibrillary tangles (NFTs) are insoluble intracellular protein inclusions found inside neurons, typically occupying the entire cytoplasm. With disease progression

and neuronal demise, NFT "thread-like" material can also be found sparsely in the neuropil as "ghost tangles" (Mena *et al*, 1991). The core of NFTs is composed of bundles of paired helical filaments (PHFs) (Kidd, 1963; Terry, 1963; Terry *et al*, 1964), containing all brain isoforms of the tau protein in a hyperphosphorilated state (Grundke-Iqbal *et al*, 1986; Goedert *et al*, 1988). Tau is a microtubule-associated protein, which is essential for axonal microtubule assembly and stability (Weingarten *et al*, 1975; Lindwall & Cole, 1984). As a result of hyperphosphorilation, tau loses affinity for microtubules, interfering with the neuron's metabolic transport system (Bramblett *et al*, 1993). This chemical modification renders it insoluble, highly prone to aggregation and favours filament formation (Abraha *et al*, 2000; Haase *et al*, 2004). Increased tau phosphorylation may be a consequence of an altered balance between the actions of specific kinases (i.e. GSK-3b, cdk5, MAPK) and phosphatases (PP2A, PP-1), which may exhibit enhanced and reduced activities, respectively; for a review see (Wang *et al*, 2013).

Tau tangles are not an exclusive "signature" of AD brains; they can also be found in other types of dementia referred as tauopathies in the absence of amyloid plaques (Spillantini & Goedert, 2013). Notably, the extent of NFT pathology positively correlates with the severity of dementia (Arriagada *et al*, 1992; Wischik *et al*, 1992; Bierer *et al*, 1995), suggesting that tau tangles play a prominent role in neurodegeneration.

Neurofibrillary tangles develop and "spread" throughout the brain in a highly ordered manner. Braak & Braak have provided the most comprehensive classification of the extent of tau pathology development during AD progression

(Braak & Braak, 1991). The earliest tau deposits are seen in the transentorhinal region (Stage I-II, associated with prodromal AD), followed by the hippocampal formation (Stage III-IV, linked with MCI and mild AD) and finally the rest of the neocortex, including the primary motor and sensory cortex (Stage V-VI, during the final stages of dementia).

Such a view suggests a dissociation between the appearance of Aβ and tau pathology in the brain; with tau aggregation as the initiating event. Supporting this case, recent immunohistochemical studies have revealed the presence of hyperphosphorilated tau in the locus coereulus of young non-demented individuals under 30 years of age (Braak & Del Tredici, 2011); suggesting that tau pathology may initiate in the brainstem from where it may spread to other CNS areas. Conversely, several studies indicate that Aβ facilitates tau aggregation *in vivo* in animal models (Gotz *et al*, 2001; Lewis *et al*, 2001; Oddo *et al*, 2003a). Similarly, in individuals with DS, who invariably develop AD pathology by middle age, Aβ accumulation is evident first, before the presence of tau pathology (Lemere *et al*, 1996; Mori *et al*, 2002). Whether Aβ or tau appears first in sporadic AD remains an issue of great controversy and of active research in the field.

Amyloid angiopathy

Deposition of amyloid in the walls of cerebral vessels is noticeable in >90% of AD-affected brains, although its extent and severity may be highly variable (Skoog *et al*, 1999). In 1927 Paul Divry, a Belgian neurologist, reported on the amyloid nature of plaques in senile dementia, using the Congo red dye under polarized light

(Divry, 1927). Years later the German neuropsychiatrist Willibald Scholz, revealed congophilc amyloid deposits in cerebral blood vessels, concluding that they were similar to those recognized by Divry in cortical plaques (Scholz, 1938). In fact, the first isolation and purification of the Aβ peptide by Glenner and Wong derived from amyloid in the meningeal arteries of post-mortem AD and DS brains (Glenner & Wong, 1984a). In AD, amyloid angiopathy is often more prevalent in the meninges than in vessels from the white matter (Terry *et al*, 1999; Selkoe, 2001) and Aβ ending at residue 40 has been reported as the most prominent peptide detected in cerebral vessels (Suzuki *et al*, 1994). CAA is not uniquely observed in AD; it may be present in the aging brain, even in the absence of cortical senile plaque and neurofibrillary tangle lesions (Vinters & Gilbert, 1983).

Synaptic loss

Another consistent feature of AD brains is progressive synaptic dysfunction; for review see (Selkoe, 2002; Bell & Cuello, 2006). In fact, synaptic alterations better reflect the degree of cognitive impairment than any other AD-related neuropathological feature (DeKosky & Scheff, 1990; Terry *et al*, 1991). Studies with transgenic animal models overexpressing human APP have revealed marked structural synaptic deficits, beginning with a decrease in cholinergic presynaptic bouton numbers, followed by atrophy of glutamatergic terminals and later by GABAergic neurites (Bell *et al*, 2006). Importantly, loss of synapses in the dentate gyrus and CA1 region have been reported in mild AD (~50% reduction) and as early as MCI (~20% reduction) (Scheff *et al*, 2007), indicating that synaptic

deficits occur early during AD progression. Such hippocampal alterations may account for the manifestation of memory deficits early in the course of the disease.

Whether synaptic dysfunction is a consequence of Aβ toxicity or tau pathology is still a controversial issue. Synaptic failure has been attributed to soluble Aβ aggregates called "oligomers"; which have affinity for and localize to synaptic contacts (Deshpande *et al*, 2006). Soluble Aβ assemblies are capable of inhibiting long-term potentiation and of disrupting cognitive function *in vivo* (Walsh *et al*, 2002; Cleary *et al*, 2005; Shankar *et al*, 2008). Moreover, studies employing oligomer-specific conformational antibodies in AD brains have shown that soluble fibrillar Aβ assemblies correlate with decreased cognitive function, as measured with the MMSE (Tomic *et al*, 2009).

At present, these soluble aggregates are considered the most synaptotoxic Aβ species. However due to the heterogeneous conformations and sizes that oligomers may have, attention is being raised to better define the impact of these biochemical differences in biological activity (Benilova *et al*, 2012; Lesne, 2013). Despite the prominent role Aβ oligomers play in AD, this should not imply that amyloid plaques are inert structures. Some studies suggest that plaques may act as a reservoir of oligomers which could diffuse away and cause neuritic dystrophy (Koffie *et al*, 2009). Further to it, tau aggregation may also contribute to synaptic dysfunction by causing axonal transport deficits which hinder the traffic of metabolites from the cell body to the synapse, affecting the growth and maintenance of neuronal processes (Mandelkow *et al*, 2003).

1.3.2.3. Other aspects of the Alzheimer's disease pathology

Although plaques and tangles constitute the signature lesions of AD, this should not give the impression that AD is simply a disease of aggregated proteins. Cholinergic degeneration (discussed in Chapters 1, and 2 of this Thesis), synaptic dysfunction, inflammation and oxidative damage are also prominent components of this complex neurodegenerative disorder. For detailed reviews on these additional aspects of AD pathology see (Perry *et al*, 2002; McGeer *et al*, 2006; Mufson *et al*, 2008; Querfurth & LaFerla, 2010; Ferretti & Cuello, 2011).

The role of inflammation is gaining increasing attention in AD. Besides being potent neurotoxins, Aβ peptides further contribute to CNS damage by activating microglia which respond by producing pro-inflammatory cytokines, complement factors and reactive oxygen species; which are cytotoxic to neurons and promote oxidative damage of lipids and proteins; for review see (Block *et al*, 2007). This response can also lead to astrocyte activation and the production of acute-phase proteins (i.e. α2-macroglobulin and α1-antichymotrypsin) that further stimulate the inflammatory response (Pasternack *et al*, 1989; Bauer *et al*, 1991).

Analysis of microglial cells in AD brains has revealed their presence in close proximity to amyloid plaques as well as the expression of activation markers such as CD68 (a lysosomal protein) and MHC-II (a receptor used for antigen presentation) (McGeer *et al*, 1987; Zotova *et al*, 2011). While these changes would anticipate microglia-induced damage they also suggested that these cells might as well play a beneficial role in the CNS by clearing Aβ via phagocytosis.

It is likely that microglia exhibit different modulatory roles which may vary depending on the stage of the disease (Hickman *et al*, 2008). For instance, epidemiological studies had associated the long-term use of anti-inflammatory drugs with diminished dementia incidence (McGeer *et al*, 1990; McGeer & Rogers, 1992; Stewart *et al*, 1997). Despite their lack of benefit in clinically diagnosed AD (Thal, 2000; Aisen *et al*, 2003), recent results from the AD Anti-inflammatory Prevention Trial have revealed that naproxene can be effective in delaying AD onset in asymptomatic subjects (Breitner *et al*, 2011); suggesting that an early, pro-inflammatory process may feed-forward AD progression. Similarly, administration of minocycline (an anti-inflammatory drug) in APP transgenic mice reduced the AD-like Aβ pathology only at early stages, free of extracellular amyloid deposits (Ferretti and Cuello, unpublished observations).

1.4. On the origin of Aβ peptides

Although Aβ is known as the pathogenic molecule in AD, this peptide is in fact a normal product of cellular metabolism. Aβ can be detected in the conditioned media of APP-expressing cells under normal cell culture conditions (Haass *et al*, 1992); and it is present in the CSF and plasma of healthy subjects throughout life (Seubert *et al*, 1992).

Is there, then, a physiological role for Aβ besides being the toxic peptide fragment that aggregates in AD brains? Recent *in silico* studies have revealed that the gene encoding for the Aβ parent protein (APP) is ancient and highly conserved in vertebrates and invertebrates (Tharp & Sarkar, 2013). Notably, although potential

amyloidogenic sequences are present across vertebrates, certain species-specific factors (which are largely unknown) are critical for Aβ formation.

Soluble $A\beta_{42}$ (in picomolar concentrations) can enhance LTP and hippocampal-dependent short-term memory *in vivo* (Puzzo *et al*, 2008). Similarly, *in vitro* studies from the Cuello lab have indicated that different Aβ concentrations (low/moderate vs. high) can elicit opposite responses in the stimulation or inhibition of CRE-dependent gene expression, an important signalling pathway for synaptic plasticity (Echeverria *et al*, 2005; Arvanitis *et al*, 2007). Taken together, these investigations suggest that physiological, low levels of Aβ may have a positive modulatory role on synapses, whereas the accumulation of higher levels Aβ may favour its aggregation resulting in an opposite, toxic effect.

But where does Aβ come from? The purification of Aβ peptides (Glenner & Wong, 1984a; Masters *et al*, 1985) allowed the identification and cloning of the gene encoding its precursor (Kang *et al*, 1987; Tanzi *et al*, 1987), known as APP; and located in chromosome 21. APP is a transmembrane glycoprotein (~100kDa), which is widely expressed in many tissues (Tanzi *et al*, 1987; Koo *et al*, 1990; Konig *et al*, 1992). Alternative splicing of APP mRNA gives rise to three major isoforms of varying lengths: APP770, APP751 and APP695. The latter is the main isoform expressed in neurons; whereas the other isoforms are mostly expressed in non-neuronal cells. Contrary to the vast knowledge about the functions of APP and Aβ in the CNS, the physiological role of these molecules in other tissues is largely unknown (Joachim *et al*, 1989).

APP contains an N-terminal extracellular domain much larger than its intracellular C-terminal tail and its single transmembrane spanning region (Figure 1-4 A). Although the exact physiological role of APP remains elusive, neuronal APP has been implicated as a cell-surface-like receptor, important for neurite outgrowth, synaptogenesis and neuronal arborisation, for a review see (Reinhard *et al*, 2005).

In the non-amyloidogenic route of APP processing, APP is cleaved by α-secretases, a group of enzymes belonging to the ADAM family (ADAM = a disintegrin and metalloprotease domain) (Lammich *et al*, 1999). ADAM10 and ADAM17 (also known as TACE = tumor necrosis factor-α converting enzyme) can cleave APP within the Aβ region (Figure 1-4 B), generating a soluble ectodomain (sAPP-α) and the retention of a 83-amino-acid-long fragment within the membrane (C83 or α-CTF). This molecule can be further cleaved by another group of enzymes known as the γ secretase complex, generating a short fragment named p3. Importantly, this pathway not only precludes the formation of Aβ but also generates sAPP-α, which has a neurotrophic role for the generation of cortical synapses (Bell *et al*, 2008).

Alternatively, APP can be sequentially cleaved by β and γ secretases (Figure 1-4 C). In the amyloidogenic pathway, a protease known as BACE1 (beta-site APP cleaving enzyme 1) or β-secretase cleaves APP at the N-terminal domain generating an extracellular molecule (sAPP-β) and leaving a 99-amino-acid-long C-terminal fragment (within the membrane) known as C99 (or β-CTF). Further cleavage of this fragment by the γ secretase complex generates free Aβ peptides and the APP intracellular domain (AICD) molecule, for review see (Selkoe, 2001; LaFerla *et al*, 2007). This amyloidogenic processing of APP does not generate *a*

single, uniform Aβ peptide but rather a heterogeneous mixture of peptides with different solubility, toxicity and aggregation properties (Mori *et al*, 1992; Qi-Takahara *et al*, 2005). For instance, γ secretase can give rise to Aβ peptides of varying lengths ($A\beta_{37}$; $A\beta_{38}$; $A\beta_{40}$; $A\beta_{42}$; $A\beta_{43}$). Other enzymes, for instance, glutaminyl cyclases can generate further heterogeneity with N-terminal modifications; such as pyroglutamate-modified Aβ (pyroglu-Aβ), which is also prone to fast aggregation and insoluble plaque formation (Mori *et al*, 1992; Saido *et al*, 1995). The presence of pyroglu-Aβ is not an uncommon event, in fact, pyroglu-Aβ is a major component of amyloid plaques and vascular amyloid in AD and DS brains and is also present in the brains of vervets and canines, animals which naturally accumulate amyloid in an age-dependent manner (Frost *et al*, 2013).

Upon APP cleavage, the most common form of Aβ produced through the non-amyloidogenic pathway is 40 amino acids in length (denoted $A\beta_{40}$). A less common but more toxic variant is $A\beta_{42,}$ which has a higher tendency to form fibrils and is found in abundance within neuritic plaques in human AD and DS brains (Iwatsubo *et al*, 1994; Iwatsubo *et al*, 1995). The molecular mechanisms that favour the formation of shorter or longer forms of Aβ remain largely unknown and the focus of intense research. Notably, recent studies from Münter, Multhaup and colleagues have revealed that the amino acid motif GxxxG on APP's transmembrane sequence has an important impact on the variants of Aβ produced, favouring $A\beta_{42}$ production over shorter, less toxic forms of Aβ (Munter *et al*, 2007; Munter *et al*, 2010).

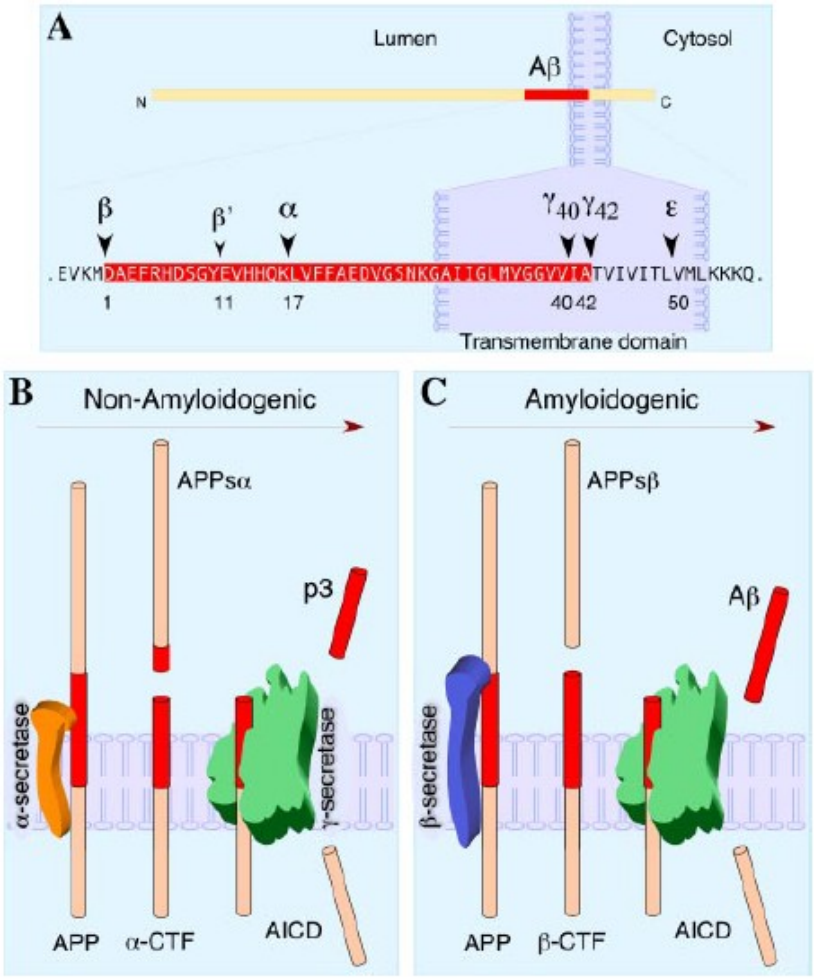


Figure 1-4. Processing of the amyloid precursor protein. A) APP structure, depicting the Aβ domain in red. Arrowheads indicate the main cleavage site of the different secretases. B) In the non-amyloidogenic pathway, APP is sequentially processed by α- and γ-secretase, generating fragments known as p3 and AICD. C) In the amyloidogenic pathway, β- and γ-secretase cleave APP, leading to the formation of Aβ. CTF = C-terminal fragment; sAPP = soluble APP; AICD = APP intracellular domain. Image from Thinakaran and Koo, *J Biol Chem* 2008.

1.4.1. Cellular sites of Aβ production

The generation of Aβ may occur at different subcellular compartments (Figure 1-5); for review see (Gouras *et al*, 2005; LaFerla *et al*, 2007). APP can be found at the plasma membrane, in the secretory pathway (Busciglio *et al*, 1993) as well as in endosomal membranes (Koo & Squazzo, 1994). Only a small fraction of APP localizes to the plasma membrane; and the majority is found in the endoplasmic reticulum (ER) and trans-Golgi network (TGN) (Thinakaran & Koo, 2008). Given the presence of β- and γ-secretases within intracellular vesicles of the ER and TGN, most Aβ is generated intraneuronally. When APP processing occurs at the plasma membrane or secretory pathway Aβ is liberated outside the cell, where it can further aggregate and form extracellular amyloid deposits. Conversely, binding of previously secreted Aβ to receptors on the cell membrane (e.g. α7nAChRs, LRP and RAGE) may also contribute to the intracellular Aβ pool via internalization into early endosomes (Sasaki *et al*, 2001; Nagele *et al*, 2002; Bu *et al*, 2006).

Further evidence for the intracellular generation of Aβ peptides within neurons has been provided by ultrastructural and biochemical analysis of cultured cells overexpressing APP (Wertkin *et al*, 1993; Turner *et al*, 1996; Hartmann *et al*, 1997; Grant *et al*, 2000). These studies have demonstrated a predominant localization of $A\beta_{40}$ peptides to the TGN and the presence of $A\beta_{42}$ within the ER and Golgi compartments in neuronal cells (Cook *et al*, 1997; Hartmann *et al*, 1997). In non-neuronal cells, the production of Aβ occurred mainly at the plasma membrane rather than within intracellular vesicles (Hartmann *et al*, 1997).

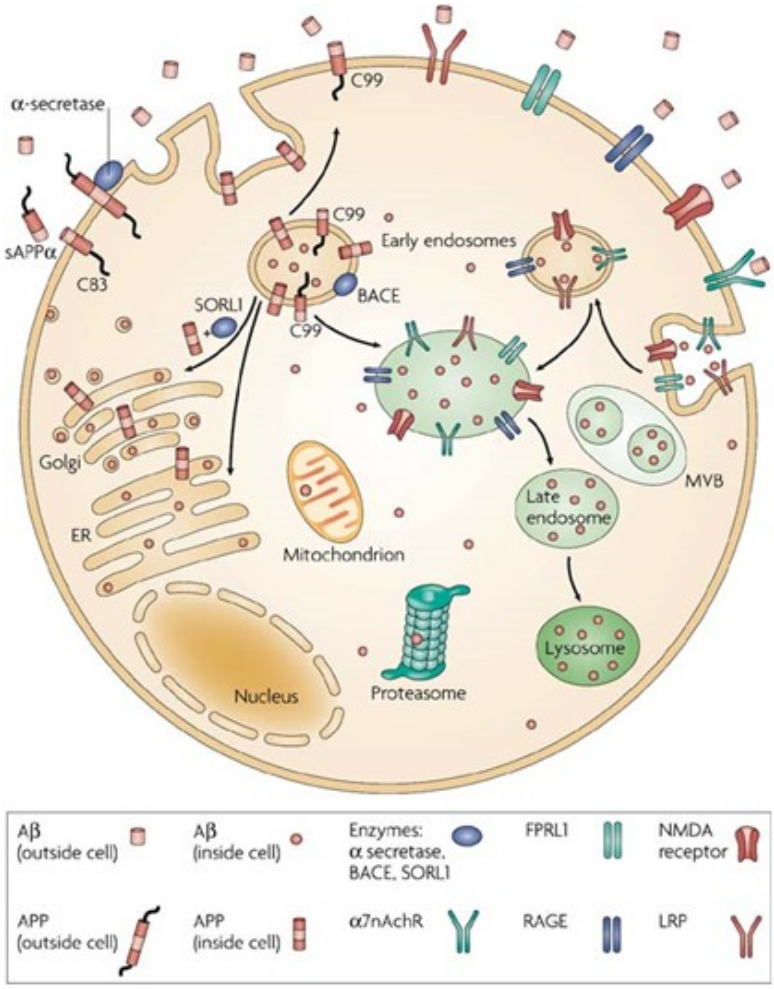


Figure 1-5. Cellular sites of Aβ production. APP can be cleaved at the plasma membrane by α-secretase, producing sAPP-α and C83. APP may be internalized into early endosomes, which contain BACE1 that cleaves APP into C99. Uncleaved APP may be shuttled back to the Golgi by binding to SORL1 (sortilin receptor 1). C99 may remain within vesicles or be trafficked to the endoplasmic reticulum (ER) or plasma membrane. In all these sites γ-secretase may further cleave C99 into Aβ. The generation of Aβ within the ER and Golgi network leads to Aβ secretion through the constitutive secretory pathway. Secreted Aβ can bind to cell surface receptors (i.e. LRP, RAGE, FPRL1, NMDA and α7nACh) and return to the intracellular compartment. Within the cell Aβ can be found in multivesicular bodies, endosomes, lysosomes, mitochondria, the proteasome, the ER and Golgi. Image from LaFerla F et al, *Nat Rev Neurosci* 2007.

Subsequent investigations in post-mortem AD and DS brains, as well as in transgenic animal models, have provided further evidence to support the presence of Aβ within pyramidal neurons; an event that precedes the deposition of extracellular amyloid plaques and that is often accompanied by signs of CNS damage (i.e. cognitive impairments, gliosis) (Lemere *et al*, 1996; Gouras *et al*, 2000; Mori *et al*, 2002; Echeverria *et al*, 2004; Billings *et al*, 2005; Philipson *et al*, 2009; Ferretti *et al*, 2011). Despite this evidence, the topic of intracellular Aβ remains an issue of debate and ongoing investigations; particularly because of the possibility that other molecules besides Aβ co-exist intraneuronally (i.e. APP; CTF-s). Dissecting the relative role of each species to the manifestation of cognitive dysfunction will be fundamental for the development of rationally designed therapies for AD.

Interestingly, the accumulation of intracellular Aβ is not exclusive to the brain. Inclusion body myositis (IBM) is a degenerative human muscle disorder affecting the elderly, characterized by the intracellular accumulation of Aβ in muscle cells (Askanas & Engel, 2006). The fact that IBM results in progressive muscle weakness and that Aβ accumulates only intracellularly highlights the pathogenic relevance of intracellular Aβ peptides. This aspect of Aβ pathophysiology, with particular emphasis to the impact of its intracellular accumulation in the CNS, will be discussed in further detail in Chapter 4 of this Thesis.

1.5. Alzheimer's disease treatment: is dementia too late?

Current therapies available to treat AD cannot prevent disease progression and offer limited symptomatic relief (Gauthier, 2002). None of these medications target the underlying pathological hallmarks of the disease; therefore, the identification of novel targets for drug development is still an area of active research.

1.5.1. Treatment of cognitive symptoms

There are currently five licenced drugs for the treatment of AD (Table 1-1); four are cholinesterase inhibitors (tacrine, donepezil, rivastigmine, galantamine) and one is a partial NMDA receptor antagonist (memantine). These medications act by modulating specific neurotransmitter alterations in AD brains (Moreira *et al*, 2006).

1.5.1.1. The cholinergic deficit in Alzheimer's disease

The rationale for the use of cholinesterase inhibitors is based on studies demonstrating a significant loss of cholinergic markers in several cortical regions of post-mortem AD brains (Bowen *et al*, 1976b; Davies & Maloney, 1976; Perry *et al*, 1977). These investigations have revealed severe deficits in choline acetyl transferase (ChAT), the enzyme that synthesizes acetylcholine from choline and acetyl-CoA, as well as in acetylcholinesterase (AChE), the enzyme that catalyzes the biochemical breakdown of acetylcholine. Importantly, ChAT activity correlated with the ante-mortem clinical severity of dementia, (Perry *et al*, 1981; Etienne *et al*, 1986), as measured with the MMSE. Concomitant with these alterations, nucleus basalis cholinergic neurons, which provide the bulk of cholinergic

innervation to the cerebral cortex, become atrophic (and later die) in AD (Whitehouse *et al*, 1982; Pearson *et al*, 1983; Mufson *et al*, 1989).

The observation that scopolamine (a muscarinic receptor antagonist) induced memory deficits in non-human primates (Bartus & Johnson, 1976) and in young, healthy individuals (Drachman & Leavitt, 1974) and that such deficits could be recovered with phystogtigmine administration (a cholinesterase inhibitor) provided the grounds for the so called *Cholinergic hypothesis of memory dysfunction* formulated by Bartus and colleagues (Bartus *et al*, 1982). In this seminal work Bartus conceptualized that the cholinergic deficits seen in AD patients and in aged individuals were at the root of the cognitive and behavioural deterioration characteristic of both conditions. The cholinergic hypothesis dominated the field in the 1970s and 80s (Coyle *et al*, 1983), in the sense that AD was viewed mainly as a cholinergic disorder, just like Parkinson's was considered a dopaminergic disease. Likewise, it was thought that AD could be treated with cholinergic drugs just like Parkinson's is managed with dopaminergic medications.

Consequently, these studies led to the development of the first-approved cholinesterase inhibitor in 1993 (tacrine) for the treatment of advanced AD-dementia (Summers *et al*, 1986). This drug acts by preventing the breakdown of acetylcholine and thereby enhancing cholinergic neurotransmission. Despite the evidence for clinical improvement following tacrine administration serious side effects were reported, such as hepatotoxicity and gastrointestinal effects (Watkins *et al*, 1994). This led to the development of second-generation ChEIs (donepezil, rivastigmine and galantamine) between 1999 and 2001 (Table 1-1), with better

tolerability profiles (Giacobini, 1998). Importantly, the administration of these drugs resulted in positive therapeutic outcomes compared to placebo; however these benefits were mainly due to reduced symptom worsening rather than cognitive improvement. Moreover, not all patients seemed to respond to treatment (Wilcock, 2008). Regardless of these limitations, ChEIs lead to significant improvement in many people affected by moderate-to-severe AD and they are still first line therapy in Canada, as recommended by the 4th Canadian Consensus Conference on the Diagnosis and Treatment of Dementia (Gauthier *et al*, 2012a).

Although ChEIs are considered symptomatic drugs, there is evidence to suggest that they may also have disease-modifying properties. Stimulation of M1 and M3 muscarinic receptors with carbachol (a cholinomimetic drug) in stably-transfected HEK293 cells favours the cleavage of APP by the non-amyloidogenic pathway (Nitsch *et al*, 1992). Therefore, activation of cholinergic neurotransmission should diminish the generation of Aβ peptides. Similarly, stimulation of muscarinic signalling with small molecule M1 agonists (i.e. AF267B) alleviated Aβ and tau pathology in the triple transgenic (3xTg) mouse model of AD-like pathology (Caccamo *et al*, 2006). Given that M1 receptors are relatively spared in AD (Svensson *et al*, 1992; Overk *et al*, 2010), finding novel ways of activating of these receptors (with minimal peripheral side effects) remains an area of active research.

Pioneered by Dr. Abraham Fisher at the Israel Institute for Biological Research several novel M1 agonists (AF710B, AF267B, AF292) have been developed, reviewed in (Fisher, 2012), and are currently being tested in pre-clinical studies, showing promising therapeutic outcomes (Fisher *et al*, 2012; Fisher *et al*, 2014).

Novel treatment strategies for cholinergic neuroprotection continue to be investigated given the important role of this neuronal network in the modulation of learning, memory and attention; processes which are dysfunctional in AD. A peculiarity of basal forebrain cholinergic neurons is their continuous dependence on the neurotrophin nerve growth factor (NGF) for their phenotypic maintenance and synaptic integrity, even in the fully matured CNS (Sofroniew *et al*, 1990; Debeir *et al*, 1999; Allard *et al*, 2012). The causes of cholinergic atrophy in AD had been a perplexing issue for a long time, given the normal NGF mRNA levels in AD brains (Goedert *et al*, 1986; Fahnestock *et al*, 1996) together with elevated levels of its precursor molecule (proNGF) (Fahnestock *et al*, 2001; Pedraza *et al*, 2005a; Bruno *et al*, 2009a).

Recent studies from the Cuello lab have revealed marked deficits in NGF metabolism in post-mortem AD brains, particularly a failure in proNGF maturation and an enhanced activation of the main NGF-degrading protease (Bruno *et al*, 2009a). This important investigation provided a mechanistic explanation to the atrophy of cholinergic neurons in AD and opened the question whether NGF metabolic deficits may appear at earlier stages of AD pathology accumulation (Cuello & Bruno, 2007; Cuello *et al*, 2010). This question will be further discussed and investigated in Chapter 2 of this Thesis.

1.5.1.2. Glutamatergic defects in Alzheimer's disease

AD is a complex disorder, characterized by alterations in many transmitter networks beyond the cholinergic system. Glutamate is the primary excitatory

neurotransmitter in the CNS and like acetylcholine; glutamatergic neurotransmission is also important for higher cognitive functions such as learning and memory (Fonnum, 1984).

Besides cholinergic deficits, AD brains are also affected by impaired glutamate clearance from the synaptic cleft, which may result in increased excitotoxicity and contribute to neuronal death (Francis, 2003). Importantly, glutamatergic alterations may occur early in the course of AD. Bell and colleagues have revealed an up-regulation of glutamatergic presynpatic boutons in frontal cortex of MCI subjects, which correlated with decreased cognitive function (Bell *et al*, 2007).

The beneficial effects of memantine, a non-competitive NMDA receptor antagonist, can thus be recognized in view of these glutamatergic disturbances. Memantine was approved in 2003 for the treatment of moderate to severe AD dementia. See Table 1-1 for further details on the current approved drugs for AD.

Table 1-1. Licensed treatments for the management of Alzheimer's disease

Drug	Indication	Mechanism of Action	Side effects
ChEIs			
Donepezil	All stages of AD	Non-competitive, reversible inhibition	Bradycardia, gastrointestinal effects
Rivastigmine	Mild-to-moderate AD	Non-competitive, reversible inhibition	Nausea, vomiting, weight loss, bradycardia
Galantamine	Mild-to-moderate AD	Competitive, pseudoirreversible	Nausea, diarrhea, fatigue, bradycardia
NMDA-R antagonist			
Memantine	Moderate-to-severe AD	NMDA receptor channel blocker	Agitation

The table includes only second-generation ChEIs. ChEIs = cholinesterase inhibitors; NMDA-R = N-methyl-D-aspartate receptor; nAChR = nicotinic acetylcholine receptor. Adapted from (Downey, 2008)

1.5.2. Treatment of non-cognitive symptoms

AD patients receive treatment to manage their declining memory and cognition (as listed in Table 1-1) as well as additional medications to control other neuropsychiatric disturbances affecting their quality of life. Examples of non-cognitive symptoms are depression, physical aggression, agitation, hallucinations, sleep alterations; which can be common at advanced disease stages but may occur at any point during disease progression in 50-90% of cases (Parnetti *et al*, 2001). Medications may include antipsychotics such as risperidone for the management of aggression and hallucinations; and anti-depressants in the form of selective serotonin reuptake inhibitors (SSRIs) (Gauthier *et al*, 2012b).

The expression of these symptoms reflects the compromise of other important CNS transmitters, including noradrenaline, serotonin, and somatostatin (Davies *et al*, 1980; Yates *et al*, 1981; Yates *et al*, 1986) and further reinforces the notion that AD is a complex, multifactorial neurodegenerative disorder.

1.5.3. Disease-modifying drugs: does timing of treatment matter?

The last 30 years have witnessed major advances in AD research; we now understand how the disease progresses, how it may start and how we may target it. As a result of this knowledge, hundreds of disease-modifying drug candidates have gone through clinical development. Yet since the development of cholinergic symtopmatic medications in the late 1990s, none of these new putative drugs has made it to the market (Mullane & Williams, 2013).

Some recent disappointing failures include semagacestat (developed by Eli Lilly) and avagacestat (by Bristol-Myers Squibb), two putative disease-modifying drugs that acted by inhibiting γ-secretase, the enzyme which catalyzes the last step in Aβ generation. Clinical development was halted in 2010 and 2012, respectively, due to lack of cognitive benefit and worsening of functional abilities (compared to placebo) and serious adverse effects (gastrointestinal, increased skin cancer episodes and infections) (Doody *et al*, 2013).

Equally disappointing were the results of recent phase III trials, investigating the efficacy of anti-Aβ humanized monoclonal antibodies bapinezumab (by Johnson & Johnson and Pfizer) and solanezumab (by Eli Lilly) in mild-to-moderate AD. This approach, referred as active immunization, aims to engage the immune system in the clearance of aggregated Aβ. Both drugs failed to improve memory and cognition in patients with AD dementia, compared to placebo-treated subjects. However, despite no evidence of cognitive improvement, bapinezumab slowed down Aβ accumulation (as monitored by PET) and reduced phospho-tau levels in CSF (Blennow *et al*, 2012). Moreover, re-analysis of solanezumab's trial results revealed a small -yet significant- slowing of cognitive and functional worsening, in a subset of patients with milder forms of AD (Eli Lilly and Company, 2012).

Following the disappointing outcome of many clinical trials, important conclusions emerged for the future development of treatments:

1) **Rethinking the timing of intervention.** When a clinical diagnosis of AD is made, that is, when dementia is present, the neurological damage is extensive.

There is widespread and abundant deposition of plaques and tangles as well as severe neuronal loss throughout many brain regions. Even MCI subjects exhibit abundant neuropathology and neuronal loss in areas important for memory before dementia onset (Markesbery *et al*, 2006). Therefore, testing drugs at the symptomatic stage of AD does not seem beneficial. In fact, there is increasing consensus that disease-modifying treatment should be most successful if administered before AD becomes symptomatic and even before MCI appears (Selkoe, 2012). A corollary of this hypothesis is that, for secondary prevention to be possible, reliable pre-symptomatic biomarkers must be available.

2) **Dissociation between target engagement and clinical benefit**. Supporting the need of earlier treatment, although bapinezumab failed to improve cognition in patients with clinically diagnosed AD, it slowed down Aβ accumulation and reduced phospho-tau levels in CSF (Blennow *et al*, 2012). This preliminary evidence of "efficacy" suggests a dissociation between AD pathology burden and symptom alleviation at late disease stages. In other words, the clearance of Aβ alone may not be sufficient to alter the clinical course of the disease, once extensive neuronal loss has occurred. It further reinforced the need of including biomarkers in clinical trials to evaluate the effectiveness of disease-modifying drugs, in combination with cognitive assessments. As well, it suggested that targeting Aβ alone may be sufficient to produce downstream changes in phospho-tau, which is a central mediator of neurodegeneration.

1.6. When does Alzheimer's disease begin?

1.6.1. The amyloid cascade hypothesis

The amyloid cascade hypothesis remains the leading paradigm to explain the aetiology of AD (familial and sporadic). It was initially formulated by John Hardy, David Allsop and Dennis Selkoe in the early 1990s (Hardy & Allsop, 1991; Selkoe, 1991) and recently revisited (Hardy & Selkoe, 2002). The amyloid hypothesis theorizes that the abnormal accumulation of Aβ in the brain is a central, primary event in AD pathogenesis that will lead to the appearance of amyloid plaques, followed by neurofibrillary tangles, synaptic dysfunction, neuronal loss and dementia. The amyloid hypothesis is supported by several lines of evidence:

1) **Early onset AD is inherited in an autosomal dominant manner.** All currently known mutations associated with the development of familial AD (before age 65) affect either the production or the aggregation of Aβ. The most common mutated genes are those encoding for PSEN1, PSEN2 and APP, all of them implicated in the generation of Aβ (St George-Hyslop & Petit, 2005).

2) **Down's syndrome subjects are at increased risk of developing AD.** A common complication associated with DS is the development of AD in adulthood (Lott, 2012). As a result of chromosome 21 trisomy, individuals with DS overexpress APP and Aβ peptides from early life. By middle age, all of them will exhibit the neuropathological hallmarks of AD (Wisniewski *et al*, 1985).

3) **Reducing Aβ levels is associated with a reduced risk of developing AD.** A recent report by Johnson and colleagues identified a rare mutation in the APP gene that protects against AD and age-related cognitive decline in a group of Icelanders. This single nucleotide polymorphism is adjacent to the BACE1 cleavage site in APP and *in vitro* studies revealed that it reduces the formation of Aβ peptides by ~40%, compared to wild type APP (Jonsson *et al*, 2012).

4) **One of the major risk factors for sporadic AD favours Aβ accumulation.** Humans possess three alleles for the apolipoprotein E (APOE) gene: ε2, ε3 and ε4 variants; the latter has been identified as an important risk factor for sporadic AD (Poirier *et al*, 1993; Roses, 1994). APOE is a glycoprotein highly expressed in the liver and in brain, where it functions as a ligand in receptor-mediated endocytosis of lipoproteins. The ε4 variant favours Aβ aggregation and impairs its clearance, compared to the other two alleles; for review see (Kim *et al*, 2009).

Despite the above solid arguments to support the amyloid hypothesis, it remains one the most highly debated topics in the field to date. Some of the scepticism in placing Aβ as the central pathogenic agent stems from the fact that all therapeutic approaches targeting Aβ have failed to provide any benefit in patients with clinical AD. Secondly, there is no consistent correlation between the extent of amyloid pathology and the severity of dementia (Terry *et al*, 1991; Bierer *et al*, 1995; Nagy *et al*, 1995). Finally, substantial amyloid deposits may be present in brains from non-demented individuals; sometimes in sufficient amounts to meet neuropathological criteria for AD (Tomlinson *et al*, 1968; Hulette *et al*, 1998;

Bennett *et al*, 2006; Price *et al*, 2009). Unfortunately, these immunohistochemical studies represent a "static snapshot" of the brain; and the antemortem absence of dementia may not necessarily rule out the occurrence of incipient memory deficits.

In view of the fact that AD is not an inevitable consequence of aging (Roth *et al*, 1966), one may hypothesize that the presence of amyloid deposits in non-demented individuals could reflect the initiation of a "silent" pathology, which would have eventually advanced, had these subjects lived longer.

1.6.2. The onset of Alzheimer's disease: before dementia?

According to the 1984 diagnostic criteria the beginning of AD is distinguished by the presence of progressive cognitive and functional impairment, and is differentiated from other forms of dementia by its gradual, slow onset (McKhann *et al*, 1984). Conversely, the absence of such symptoms defines a healthy person, free of AD. However, converging evidence from cognitive, neuropathological and biomarker studies would indicate that this binary definition of AD must be revisited.

Firstly, impaired memory function is not exclusive of aMCI or AD. Some investigations suggest that subtle deficits in memory, particularly in the acquisition and retrieval of new information, may occur before full-blown AD, both in sporadic and genetic at-risk pre-symptomatic cases (Small *et al*, 1999; Wahlund *et al*, 1999). Although this subtle decline, referred as Subjective Cognitive Impairment (SCI), may not be sufficient to meet the threshold for MCI in

psychometric tests (i.e. MMSE), it is in effect an indicator of an early pathological process distinguished from normal aging (Small *et al*, 2002; Reisberg *et al*, 2008).

Secondly, between 20-40% non-demented older subjects may have evidence of CSF biomarker changes consistent with the pathophysiological process of AD (Moonis *et al*, 2005; Peskind *et al*, 2006; Fagan *et al*, 2009; De Meyer *et al*, 2010). Such proportion of putative pre-symptomatic subjects is in agreement with autopsy studies showing significant plaque and tangle burden in cognitively healthy adults, as discussed previously. AD-like changes in CSF include reduced concentrations of $A\beta_{42}$, increased concentrations of total tau and tau phosphorylated at specific residues; alterations which reflect the accumulation of β-amyloid in the brain, axonal degeneration and tau pathology, respectively. Importantly, in individuals with MCI, evidence for CSF biomarker positivity can be a good predictor of future conversion to AD-dementia (Hansson *et al*, 2006; Buchhave *et al*, 2012; Mattsson *et al*, 2012).

A major scientific breakthrough of recent years came with the possibility to examine amyloid plaque pathology in living patients by neuroimaging. Developed by Drs. William Klunk, Chester Mathis and colleagues at the University of Pittsburg, Pittsburg compound B (^{11}C-PiB) is a radioactive analogue of Thioflavin T, which can be used to visualize neuritic amyloid plaques *in vivo* by positron emission tomography (PET) (Klunk *et al*, 2003). Individuals with brain amyloidosis, such as AD and MCI subjects, will exhibit increased retention of the ^{11}C-PiB tracer with high affinity and specificity (Klunk *et al*, 2004). Conversely, in areas typically devoid of AD pathology (i.e. cerebellum, pons) ^{11}C-PiB uptake is

low and comparable to amyloid free subjects. Cerebellar ^{11}C-PiB retention units are often used to normalize cortical uptake values (Jack *et al*, 2008).

The study by Klunk and colleagues was the first to show that it was possible to obtain quantitative information on neuritic amyloid plaque burden in a living patient, and its use was soon validated by other groups (Edison *et al*, 2007; Rowe *et al*, 2007; Ikonomovic *et al*, 2008). The link between the development of brain amyloid-β pathology and the emergence of clinical disease could now be studied for the first time in a prospective, longitudinal manner.

Supporting the hypothesis that AD pathology develops before the onset of clinical disease, cognitively healthy individuals aged 60-90 years may exhibit ^{11}C-PiB positive PET scans, in the absence of MCI or AD symptoms (Rowe *et al*, 2007). Importantly, the extent of brain amyloid accumulation in asymptomatic subjects (as determined by ^{11}C-PiB-PET) has been associated with subtle deficits in episodic memory (Pike *et al*, 2007). This observation was confirmed and extended in a recent study demonstrating that asymptomatic individuals with high ^{11}C-PiB retention presented faster rates of memory decline and grey matter and hippocampal atrophy, compared to ^{11}C-PiB negative asymptomatic subjects, within a follow-up period of 4-6 years (Villemagne *et al*, 2013). Interestingly, the rate of Aβ deposition decelerated at advanced disease stages, suggesting that by the time MCI or AD develops, the disease may become disconnected from Aβ.

Perhaps the most compelling evidence indicating the existence of such a pre-clinical AD stage came from studies on genetic at-risk subjects (i.e. asymptomatic

familial AD mutation carriers), which provided insight into the temporal order of biomarker changes. Briefly, these findings confirm that of all biomarkers, Aβ becomes abnormal first and precedes cerebral atrophy and cognitive decline by decades (~15-20 years before expected symptom onset). This is followed by evidence of tau phosphorylation, hippocampal atrophy, and subtle episodic memory deficits, detectable before MCI / AD symptoms (Acosta-Baena *et al*, 2011; Bateman *et al*, 2012; Ringman *et al*, 2012a). A similar temporal order of biomarker alterations has been recently proposed for sporadic AD (Jack *et al*, 2010; Jack *et al*, 2013).

In sum, these studies are consistent with the hypothesis that AD has a long, asymptomatic phase during which pathologic changes advance gradually over time, in the absence of clinically detectable dementia. Based on the above, diagnostic criteria for AD have been recently revisited to expand the definition of AD into three stages (Figure 1-6) (Sperling *et al*, 2011a). The work by Sperling and colleagues provided a framework for the definition and staging of pre-clinical AD (Table 1-2); and recommended the inclusion of biomarker analysis (e.g. ^{11}C-PiB-PET, CSF Aβ and phospho-tau levels) with neuropsychological evaluations to assist in diagnosis. At the moment, these guidelines can only be implemented in research settings, not in the clinic.

Table 1-2. Staging of pre-clinical Alzheimer's disease

Stage 0	Older individuals with no biomarker evidence of AD pathology
Stage 1	Asymptomatic cerebral amyloidosis (i.e. ^{11}C-PiB+ ; low CSF $A\beta_{42}$)
Stage 2	Amyloidosis + evidence of neurodegeneration or neuronal injury (i.e. high CSF tau or p-tau; grey matter atrophy)
Stage 3	Amyloidosis + neurodegeneration + evidence of subtle cognitive decline
SNAP	Individuals showing biomarkers of neurodegeneration without positive markers of amyloid accumulation

SNAP = Suspected Non-Alzheimer Pathology (SNAP). Adapted from (Sperling *et al*, 2013b)

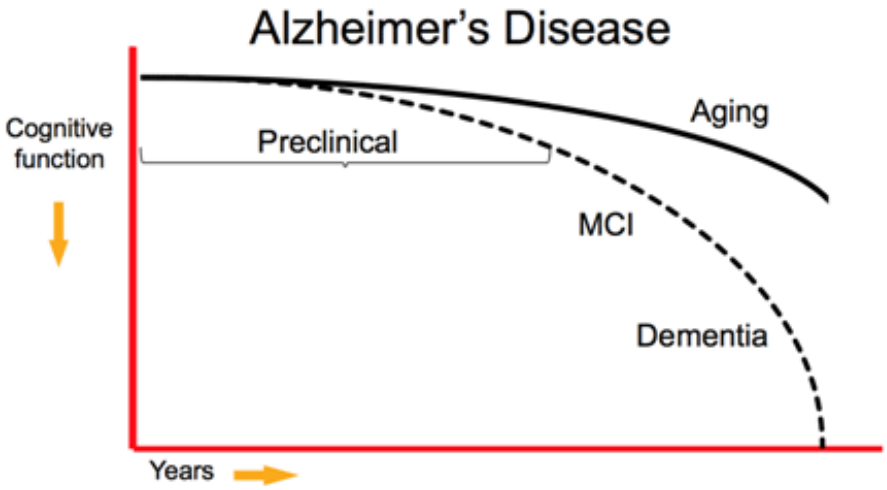


Figure 1-6. The clinical progression of Alzheimer's disease. Individuals with pre-clinical AD (also referred as "asymptomatic" or "pre-symptomatic") exhibit biomarker changes consistent with the pathophysiological process of AD, but are otherwise "cognitively healthy". Some may experience subtle memory complaints. This stage may last between 10-20 years and is followed by MCI, which is characterized by memory impairments and other cognitive disturbances, in the absence of dementia. As the disease progresses and the pathology spreads through the entire brain, the patient has lost complete independence, needing full care and supervision. Image from Sperling R et al., *Alzheimers Dement* 2011.

Following the delineation of these criteria, recent studies have provided evidence that individuals classified as "pre-symptomatic, stage 3" are at increased risk of developing prodromal or symptomatic AD compared to subjects free of biomarker evidence of AD pathology (stage 0); within a follow-up period of 3-5 years (Knopman *et al*, 2012; Vos *et al*, 2013). In this way, the paradigm that AD is a clinical syndrome of progressive cognitive impairment has changed towards defining it as a continuum of neuropathological changes of which the cognitive and functional decline represent the end stage of a long, chronic disease process.

Following the example of other diseases which can be identified before the emergence of symptoms (e.g. high blood cholesterol as a predictor of myocardial infarction); one could contemplate that the long pre-symptomatic phase of AD should provide a critical opportunity for successful treatment to preserve cognition. The major challenge to this would be to establish a firm link between the development of AD biomarker changes and the emergence of clinical disease. Although new studies continue to validate the association between pre-clinical AD and future cognitive decline, some amyloid positive subjects do not progress to AD within their lifetime; and even some individuals with AD may have no evidence of biomarker abnormalities in CSF (Wallin *et al*, 2012). Thus, at the moment, clinicians and researchers lack the resources to predict with precision an eventual "conversion" to dementia. The identification of novel biomarkers to assist in earlier diagnosis or prognosis is therefore one of the most active areas of AD research today. This subject will be further discussed in Chapter 3 of this Thesis.

1.7. The link between Alzheimer's disease & Down's syndrome

Another area of growing interest in the field is focusing on the convergence between Alzheimer's disease and Down's syndrome. Because these individuals are at high risk of developing dementia compared to the normal population, studies on DS can provide valuable insight into the early stages of AD onset and progression. Likewise, novel treatments approaches that benefit AD patients may also help the quality of life of individuals with DS.

1.7.1. A brief historical overview of the discovery of Down's syndrome

The first clinical description of DS in the scientific literature can be attributed to Jean-Étienne Esquirol (1838) who published the first psychiatry handbook of mental diseases, including a section that covered "Idiocy" or "mental deficiency". Years later, Edouard Séguin (1846) extended this report, describing typical features of DS such as the open mouth, the thick morphology of the tongue, their susceptibility to infections and the mental deficiency characteristic of such children. However, the first distinction of DS as a separate entity from other types of mental retardation was made by John Langdom Down, an English physician devoted to the study of children with learning disabilities, who at the time were referred as "idiots" or "imbeciles". As Head Physician of the Earlswood Asylum for Idiots in Surrey, UK, Down noticed that children with learning disabilities displayed a wide variety of clinical phenotypes. To better understand these differences he sought to establish a systematic classification that would distinguish the different degrees of cognitive impairment and other symptoms. He published a

paper in 1866 entitled "Observations on the Ethnic Classification of Idiots" where he described the facial resemblance of many children with mental retardation with individuals of Mongolian descent, including a flattened face and small nose, up-slanted eyes and a large tongue. He coined the term "mongoloids", a term which is no longer used today (Allen *et al*, 1961), to refer to these individuals and to distinguish them from other children with cognitive impairment (Down, 1866). Although he noticed that children with DS exhibited a short life expectancy and that their mental condition was congenital, he failed to provide an accurate cause for their intellectual retardation; attributing its origin to maternal tuberculosis. In fact, it took almost 100 years to have a correct answer to this question.

Following Down's observation, many theories were put forward to explain the origin of DS, including maternal infection, endocrine dysfunction, syphilis, co-sanguinity, etc. Interestingly, as early as the 1930s some physicians, such as the Dutch ophthalmologist Petrus J. Waardenburg had correctly suggested (without much attention from his colleagues) that DS might be due to a chromosomal abnormality (Allen, 1974). A major breakthrough came with the development and refinement of karyotyping techniques in the mid 1950s, which allowed Jerome Lejeune, Raymond Turpin and Marthe Gautier to identify an extra copy of chromosome 21 in karyotypes prepared from DS cells (Lejeune *et al*, 1959). This finding was further confirmed by other independent groups (Jacobs *et al*, 1959; Fraccaro *et al*, 1960) and it was soon accepted that triplication of chromosome 21 is the primary cause of Down's syndrome. For more detailed historical reviews, see (Patterson & Costa, 2005; Megarbane *et al*, 2009).

1.7.2. Genetic spectrum leading to Down's syndrome

The most common genetic abnormality leading to DS is the presence of three full copies of chromosome 21 in all cells, as a result of chromosomal non-disjunction during meiosis (~95% of cases). The incidence of full trisomy is higher with increasing maternal age; therefore, prenatal diagnosis of DS is offered routinely to pregnant women aged 35 or older. A smaller number of DS cases (~5%) may be caused by chromosomal translocations, where a region on chromosome 21 is transferred to another chromosome (i.e. chromosome 14, 15, or 22). Because translocations can be inherited, this type of chromosomal anomaly is often referred as familial DS (Carter *et al*, 1960). A third less frequent cause of DS (~1% cases) is known as mosaic DS, where some cells are trisomic for chromosome 21 and others are not, with varying extents of mosaicism from 1-99% (Clarke *et al*, 1963).

There are two main theories to explain the pathogenesis of the DS phenotype; both are based on the consequences of increased gene number. The first is known as *The Gene-Dosage Hypothesis*, and it states that the entire Down syndrome phenotype is the direct result of triplication of chromosome 21 genes. In other words, this theory posits that the increased dosage of a specific gene on chromosome 21 will result in a specific clinical/pathological phenotype. For instance, the presence of several inflammatory-related genes in chromosome 21 (e.g. S100-β; Tiam1) may promote an exacerbated pro-inflammatory response in DS (Wilcock, 2012). Similarly, the altered dosage of certain transcription factors and kinases important for brain development may contribute to the occurrence of mental retardation (Roizen & Patterson, 2003). The second model is *The Amplified Developmental Instability*

Hypothesis, which postulates that an increase in gene copy number will lead to a global unbalanced genetic homeostasis that will result in developmental abnormalities (Pritchard & Kola, 1999). Both hypotheses acknowledge the important contribution of increased gene number on the manifestation of DS.

1.7.3. Developmental and neurological abnormalities in Down's syndrome

The excess gene dosage in DS results in physical and functional anomalies in the brain and other organs; some of which are congenital and others which are progressive. DS is the most common chromosomal abnormality leading to learning disabilities in children and lifelong mental retardation, occurring in approximately 1 in 800 live births regardless of age, gender or socioeconomic status (Parker *et al*, 2010). Due to improved health and social care and advances in research, the estimated life expectancy of a person with DS has dramatically increased in developed countries, being close to ~50-60 years; 57·8 years for women and 61·1 years for men (Glasson *et al*, 2002). As a comparison, the median age of death of an individual with DS was ~9 years at the beginning of the 20th century, ~25 years in 1983 and ~49 years in 1997 (Penrose, 1949; Yang *et al*, 2002).

DS is a heterogeneous disorder and its symptoms may vary in severity and number depending on the nature of the chromosomal anomaly. However, the most distinctive –and perhaps most invalidating- aspect of this condition is the consistent cognitive deficits present during their lifetime. Most individuals with DS have low intelligence quotients (IQ), ~50 on average, ranging from mild (IQ 50-70) to moderate (IQ 35-70) disability (Chapman & Hesketh, 2000). The broad impairment

in mental function manifests early during childhood as deficiencies in language and speech, impaired verbal short-memory and explicit long-term memory. Such cognitive deficits ultimately further decline in adolescence and adulthood. In contrast, other forms of mental abilities are relatively preserved in DS, such as associative learning and procedural memory (Lott & Dierssen, 2010).

The early cognitive impairments and associated neurological complications in DS have been mainly attributed to brain developmental deficits. Briefly, individuals with DS exhibit a global reduction in brain weight (~20% in magnitude) with particularly smaller frontal and temporal lobes and a prominent reduction in cerebellar volume (Raz *et al*, 1995; Teipel *et al*, 2004). Other areas of the brain are relatively preserved, including the occipital and parietal lobes as well as subcortical nuclei. Paradoxically, and for reasons that are not yet understood, the parahippocampal gyrus appears to be larger in DS. A schematic summary of these changes is depicted in Figure 1-7.

At the cellular level, histological studies in DS have demonstrated a reduced number and size of neurons in cortex and hippocampus, as well as altered distribution of neurons within cortical laminae (Ross *et al*, 1984; Golden & Hyman, 1994). Moreover, inhibitory GABA neurotransmission is enhanced, altering the balance between excitatory and inhibitory networks. These changes may further contribute to the manifestation of cognitive dysfunction in DS (Rissman & Mobley, 2011). The DS brain is also characterized by decreased neurogenesis, abnormal synaptic density and reduced dendritic arborisation of pyramidal neurons (Becker *et al*, 1986). Taken together, these changes lead to a

global dysfunction in cortical network connectivity, which results in a limited capacity for plasticity and for the acquisition, storage and retrieval of information.

Alterations in the cerebellum may further contribute to compromise higher CNS functions in DS. The development of the cerebellum is delayed and incomplete and is characterized by a reduced number of granule cells (Baxter *et al*, 2000). Besides its important role in motor control, several studies suggest that altered cerebellar function may further contribute to impair cognitive abilities, particularly attention, language learning and visuo-spatial skills; as reviewed in (Baillieux *et al*, 2008).

Although deficits in cognition are a consistent hallmark of DS, other aspects of the DS phenotype include distinctive facial and physical features, delayed growth, congenital heart defects, immunological, haematological and endocrinal problems (i.e. recurrent infections, childhood onset leukemia and hypothyroidism) as well as muscle and articulatory complications; reviewed in (Roizen & Patterson, 2003).

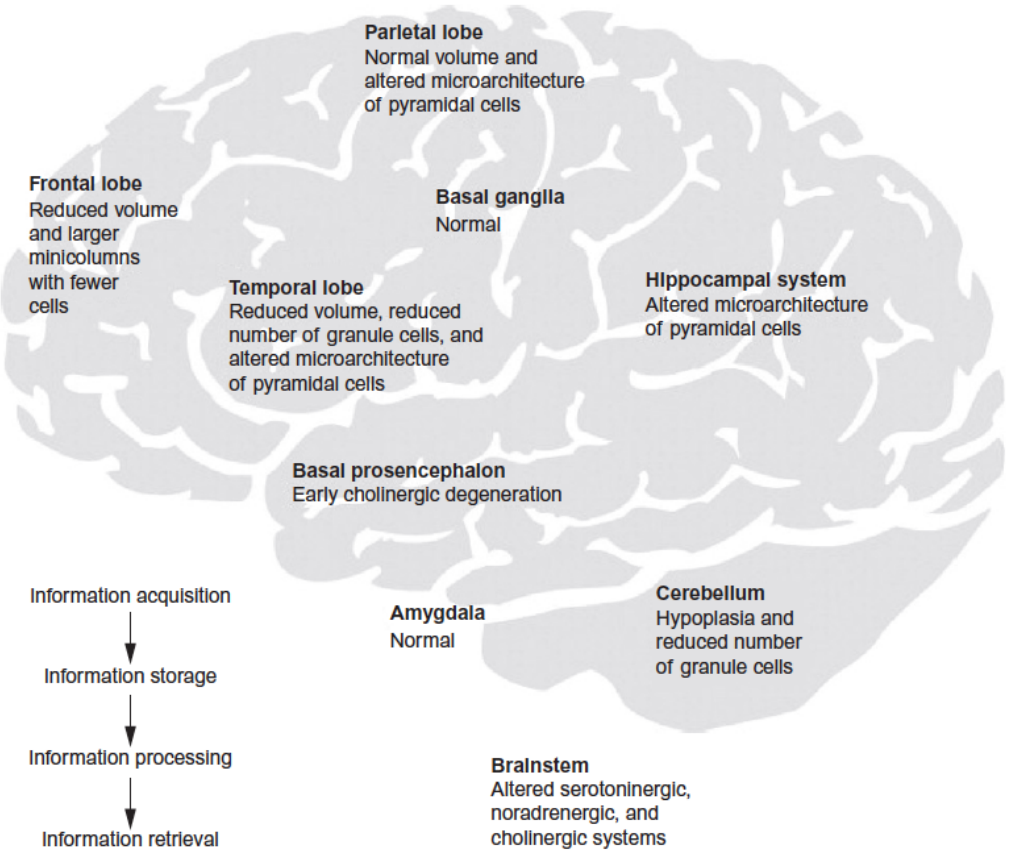


Figure 1-7. Pathological basis for cognitive dysfunction in individuals with Down's syndrome. The arrows represent the altered stages of information flow during the processes of learning and memory, which are affected in DS. Image from Lott and Dierssen, *Lancet* 2010.

The onset of this wide variety of symptoms in DS is generally age-specific; some problems may arise in childhood, while other may appear in adult life. During the first 10 years of life, the main causes of mortality are associated to heart defects (which may be corrected with early surgery) and the high risk of developing leukemia. Later in life common complications are pneumonia and respiratory infections and in general, other disorders associated with precipitated aging, including renal failure, cerebrovascular accidents, coronary artery disease and the development of dementia due to Alzheimer's disease (Bittles *et al*, 2007). Other signs of premature aging may include early changes in skin tone, hearing loss, increased frequency of cataracts and loss of adaptive abilities (Zigman *et al*, 1996).

1.7.4. Increased risk of Alzheimer's disease in adults with Down's syndrome

The first recognition that DS is associated with premature senility came in the late 1800s from British physicians John Fraser and Arthur Mitchell, who published an extensive report describing the physical and mental condition of 62 individuals with mental retardation of different ages, ranging from 0 – 45 years. Fraser and Mitchell commented on their short life expectancy and accurately identified maternal age as a risk factor for this condition. They further added, *"In not a few instances, however, death was attributed to a general decay - a sort of precipitated senility"* (Fraser & Mitchell, 1876). This important realization was noticed 30 years before Alois Alzheimer described Auguste Deter's case and ~70 years before an association between AD and DS was reported for the first time (Jervis, 1948).

Dr. Jervis, former director of the Institute for Basic Research of the New York State Office of Mental Retardation and Developmental Disabilities, documented the case of three individuals with DS (between 35 and 47 years of age) and who exhibited noticeable personality changes and a progressive deterioration of intellectual function. Importantly he pointed out that the brains of these subjects were in fact characterized by the pathological changes that defined senile dementia (i.e. Alzheimer's disease), namely, a marked loss of cortical neurons and atrophy of the brain, dilated ventricles, the widespread presence of senile plaques throughout the cortex as well as intraneuronal tangles, particularly abundant in hippocampus (Jervis, 1948). The presence of amyloid plaques in DS had been noticed in an earlier, single case study of a 37 year-old individual with mental retardation, but with no documentation of the clinical status of this subject (Struwe, 1929). Therefore, Jervis's observations provided the grounds to establish a link between AD and DS; a subject that paved the way for future important investigations.

1.7.4.1. Development of Alzheimer's disease pathology in Down's syndrome

Today, it is well established that age-related disorders begin earlier in DS than in the general population; and particularly, that a common complication associated with trisomy 21 is the increased risk of developing AD in adulthood. Following the studies of Struwe and Jervis, numerous investigations have provided further evidence to support that, by middle age (from 35 onwards), virtually all subjects with DS will exhibit the hallmark neuropathological changes of AD (Ropper & Williams, 1980; Whalley, 1982; Wisniewski *et al*, 1985; Mann, 1988b; Lemere *et al*, 1996; Mori *et al*, 2002) (Table 1-3).

Table 1-3. Prevalence of Alzheimer's disease pathology in Down's syndrome

Age range (years)	Total no. of patients	No. showing plaques and tangles	Percentage of patients affected
0-9	37	0	0
10-19	80	6	7.5
20-29	58	9	15.5
30-39	35	28	80
40-49	55	54	98.2
50-59	87	85	97.7
60-69	43	43	100
70-79	3	3	100

From Mann D., *Histopathology* 1988; 125-37

These brain lesions are rather uncommon in individuals with other types of intellectual disabilities and, in DS, they are further associated with additional deterioration of their mental function and the clinical development of dementia.

The invariable development of AD pathology in DS is attributed to the triplication of chromosome 21, where the gene coding for APP is located (Rumble *et al*, 1989). As a result of increased APP gene dosage (approximately ~1.5 fold in magnitude), individuals with DS overexpress APP and Aβ peptides from early life (Oyama *et al*, 1994). Soluble Aβ is increased in DS brains as early as the second trimester during development, and increases exponentially with age and with the development of extracellular amyloid deposits (Teller *et al*, 1996). In DS, the deposition of $A\beta_{42}$ precedes that of $A\beta_{40}$, however, while $A\beta_{40}$-immunoreactive amyloid plaques increase with age, the burden of $A\beta_{42}$ plaques remains stable across time (Lemere *et al*, 1996).

Before amyloid plaques appear, Aβ peptides are initially detected intraneuronally in young DS subjects, particularly within large pyramidal neurons of the entorhinal cortex and hippocampus (Lemere *et al*, 1996; Gyure *et al*, 2001; Mori *et al*, 2002). This is followed by the development of the first extracellular diffuse amyloid plaques, which may appear as early as 8-12 years of age, together with intraneuronal Aβ (Lemere *et al*, 1996; Mori *et al*, 2002). Recent imaging studies have demonstrated the presence of PiB+ extracellular deposits in middle-aged DS individuals (~30 years old) (Handen *et al*, 2012). At this age, the first neurofibrillary tangles may appear, which then become more abundant at later

ages. By 40-60 years mature amyloid plaques predominate and are significantly abundant throughout the association cortex and subcortical nuclei. Amyloid angiopathy is also common at advanced disease stages. In sum, the deposition of Aβ in the brain of DS subjects precedes neurofibrillary tangle formation, neuronal loss and the development of clinical signs of dementia by several decades (Figure 1-8).

Further evidence implicating triplication of APP in the development of AD pathology in DS is illustrated by the case study of a 78-old woman with partial trisomy 21, who had a normal number of *APP* copies (2 rather than 3) and did not manifest clinical AD or its main pathological hallmarks (Prasher *et al*, 1998).

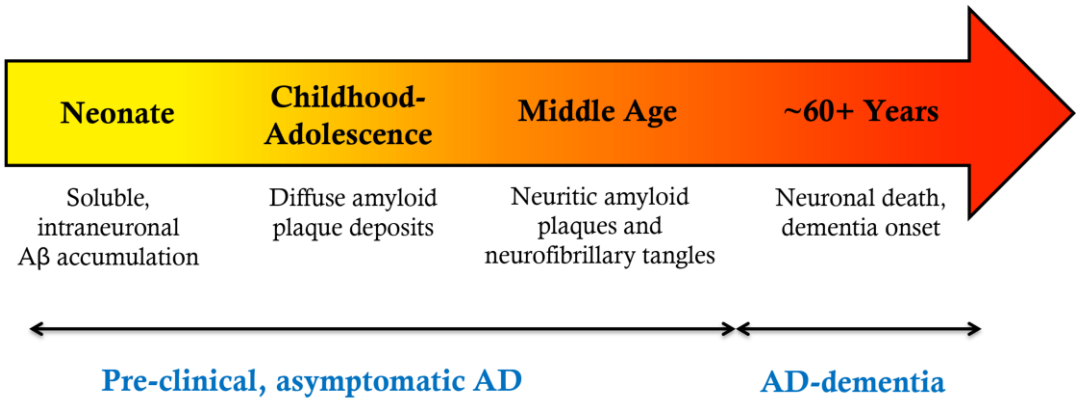


Figure 1-8. Temporal development of Alzheimer's disease pathology in Down's syndrome. The gradual, progressive accumulation of Aβ peptides, amyloid plaques and neurofibrillary tangles occurs over many decades preceding the onset of clinical AD and dementia. From Iulita MF and Cuello AC 2014, unpublished.

1.7.4.2. Do all individuals with Down's syndrome develop Alzheimer's disease?

While the development of AD neuropathology in adults with DS is almost universal; the clinical manifestation of dementia is more heterogeneous. There is some discrepancy in the literature regarding the prevalence of clinical AD in DS.

Most studies will agree that the frequency of dementia increases with age; with a mean onset of AD around ~50-55 years of age, but which may vary widely (Zigman *et al*, 1996). In fact, some investigations have reported prevalence rates ranging from 6 – 75 % (Visser *et al*, 1997); or indicating rates of 2-5% at age 40; 30-65% at age 60 and >70% at age 70 (Lai & Williams, 1989). Such heterogeneity may be explained by a phenomenon known as *diagnostic overshadowing*, which reflects the difficulty in diagnosing the cognitive and functional decline characteristic of dementia in a population with pre-existing cognitive impairments. Added to this is the challenge to distinguish such changes from the expected cognitive decline associated with normal aging. Furthermore, because of this pre-existing mental dysfunction, individuals with DS may never have developed the skills required to perform the common neuropsychological tests used in the general population. Therefore, psychometric tests must be adapted to address these needs.

Additionally, certain risk factors may influence the development of AD symptoms in DS, as reviewed by (Schupf & Sergievsky, 2002). Cognitive reserve refers to the capacity of the brain to withstand acquired pathology (Stern, 2002; Whalley *et al*, 2004). In the normal population factors such as education, higher literacy, and an engaging social life are associated with a reduced risk for AD dementia (Bennett *et*

al, 2014). Similarly, individuals with DS with higher IQ, good education and advanced verbal abilities may be at a reduced risk of developing dementia compared to subjects with a more severe mental handicap. Moreover, as mentioned earlier, certain case reports of adults with atypical DS karyotypes (i.e. partial trisomy or mosaicism) have shown no evidence of dementia, or AD neuropathology, even by age 70 (Prasher *et al*, 1998). Furthermore, the APOE ε4 variant has been associated with a higher incidence of dementia in DS and a faster decline in cognitive and functional abilities (Schupf *et al*, 1996; Deb *et al*, 2000). In sum, the risk of developing AD in adults with DS is influenced by many factors, which may reflect the heterogeneity in dementia onset despite the invariable presence of advanced AD neuropathology.

Another issue to consider in evaluating the prevalence of dementia in DS is the atypical symptomatic manifestation of AD, compared to the normal aging population. Early signs of dementia in DS usually begin with changes in behaviour and the development of seizures in previously unaffected individuals; as opposed to the loss of short-term memory seen in the normal population. Seizures in adults with DS are often a good predictor of future –and rapid- cognitive decline and the development of AD symptoms (Lott & Dierssen, 2010). Typical changes in behaviour that signal the beginning of dementia may include apathy, irritability, social withdrawal, depression and speech deterioration; which are indicators of frontal lobe dysfunction (Lai & Williams, 1989). Dementia is diagnosed when these symptoms are present along with a global deterioration of memory and other cognitive functions, (e.g. temporal disorientation) and a loss of self-care abilities.

Unfortunately, there are no formal guidelines (i.e. DSM-IV, ICD) for diagnosing early signs of dementia in DS subjects. Some studies have highlighted the need for longitudinal cognitive assessments, so that individual baselines can be used to demonstrate an objective decline in cognition (Burt & Aylward, 2000). There are specific screening tests designed for individuals with DS; e.g. the Test for Severe Impairment and the Down Syndrome Mental State Exam. These tests are easy to administer and screen several cognitive domains, taking into account the lower IQ of adults with DS. Besides a direct evaluation of the individual, clinicians rely on informant-based assessments to identify changes in behaviour, memory, speech and practical skills using observer-rated scales (O'Caoimh *et al*, 2013).

Although some studies have suggested that changes in plasma Aβ were associated with future development of dementia in adults with DS (Schupf *et al*, 2010), the identification of biomarkers for pre-symptomatic diagnosis is still in its infancy. The major challenge to address is that with current screening tools, the diagnosis of AD in individuals with DS is made at relatively late stages of the disease. Furthermore, there is currently no effective therapeutic approach capable of preventing the development of AD pathology in DS subjects. This is an issue that needs serious consideration, given the increased life expectancy of adults with DS and as a result, the greater likelihood of dementia manifestation. Therefore, identifying novel molecular signatures of an evolving AD pathology is a major unmet objective, which will benefit both AD and DS sufferers. Likewise, novel treatment paradigms aiming at arresting the progression of AD in the general population may also help individuals with DS.

1.8. Thesis objectives and rationale

Based on the overview provided in this Introduction, it is clear that AD is a major debilitating neurodegenerative disease without an effective treatment. There is a growing recognition that the best strategy to achieve disease modification should be early intervention. Consequently, the main objective of this Thesis was to better understand the molecular changes that occur during the early stages of AD pathology development. Toward this goal we have taken into consideration that:

1. Given the uniformity in which DS individuals acquire AD pathology, they represent an ideal population to investigate early pathological events.

2. NGF-dependent basal forebrain cholinergic neurons have key roles in memory and cognition, and this neuronal network is severely affected in AD and DS. In AD, NGF fails to mature and its degradation is enhanced, compromising the integrity of CNS cholinergic neurons. NGF dysmetabolism has not been studied in other β-amyloid pathologies or at earlier disease stages.

3. Because the cerebro-spinal fluid is in direct equilibrium with the extracellular CNS milieu and is of easy access, it represents an ideal environment to investigate novel biomarkers that reflect the presence of AD pathology.

4. Transgenic animal models reflect important aspects of the disease and continue to be valuable tools to explore early consequences of AD pathology progression.

Based on the above, we have formulated the following three working hypotheses:

Hypothesis I: NGF metabolic dysfunction occurs in DS brains. Alterations in NGF metabolism are an early consequence of Aβ accumulation.

Hypothesis II: Given that AD brains exhibit NGF metabolic deficits, alterations in NGF pathway markers should be reflected in cerebro-spinal fluid from AD subjects.

Hypothesis III: Aβ initially accumulates within neurons of the cortex and hippocampus and the early development of AD-like amyloid pathology is accompanied by cognitive deficits, even prior to amyloid plaque deposition.

The testing and outcome of these specific hypotheses will be covered in Chapters 2, 3 and 4 of this Thesis, respectively.

CHAPTER 2

Nerve Growth Factor Metabolic Dysfunction in Down's Syndrome Brains

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Brain. 2014 Mar; 137 (Pt 3):860-72. doi: 10.1093/brain/awt372. Epub 2014 Feb 11.

2.1. Abstract

Basal forebrain cholinergic neurons play a key role in cognition. This neuronal system is highly dependent on NGF for its synaptic integrity and the phenotypic maintenance of its cell bodies. Basal forebrain cholinergic neurons progressively degenerate in Alzheimer's disease and Down's syndrome, and their atrophy contributes to the manifestation of dementia. Paradoxically, in Alzheimer's disease brains, the synthesis of NGF is not affected and there is abundance of the NGF precursor, proNGF. We have shown that this phenomenon is due to a deficit in NGF's extracellular metabolism which compromises proNGF maturation and exacerbates its subsequent degradation. We hypothesized that a similar imbalance should be present in Down's syndrome.

Using a combination of qRT-PCR, ELISA, Western blotting and zymography, we have investigated signs of NGF metabolic dysfunction in post-mortem brains from the temporal (n = 14), frontal (n = 34) and parietal (n = 20) cortex obtained from Down's syndrome subjects and age-matched controls (age range 31-68 years). We further examined primary cultures of human fetal Down's syndrome cortex (17-21 gestational age weeks) and brains from Ts65Dn mice (12-22 months), a widely used animal model of Down's syndrome.

We report a significant increase in proNGF levels in human and mouse Down's syndrome brains, with a concomitant reduction in the levels of plasminogen and tissue plasminogen activator mRNA as well as an increment in neuroserpin expression; enzymes that partake in proNGF maturation. Human Down's syndrome

brains also exhibited elevated zymogenic activity of MMP-9, the major NGF-degrading protease.

Our results indicate a failure in proNGF maturation in Down's syndrome brains and a likely enhanced proteolytic degradation of NGF, changes which can compromise the trophic support of basal forebrain cholinergic neurons. The alterations in proNGF and MMP-9 were also present in cultures of Down's syndrome fetal cortex; suggesting that this trophic compromise may be amenable to rescue, before frank dementia onset. Our study thus provides a novel paradigm for cholinergic neuroprotection in Alzheimer's disease and Down's syndrome.

2.2. Introduction

It is well established that individuals with Down's syndrome are at increased risk of developing premature aging and dementia due to Alzheimer's disease (Lott, 2012). Amyloid-beta (Aβ) peptides progressively deposit in Down's syndrome brains from early life (Lemere *et al*, 1996; Mori *et al*, 2002) and recent studies have shown accumulation of PiB-positive amyloid plaques in Down's syndrome subjects already in their 30s (Handen *et al*, 2012). By middle-age (40-60 years) virtually all Down's syndrome sufferers have the neuropathological hallmarks characteristic of Alzheimer's disease, including senile amyloid plaques surrounded by dystrophic neurites and neurofibrillary tangles (Wisniewski *et al*, 1985; Mann, 1988a). Thus, the study of Down's syndrome brains represents a unique opportunity to explore the molecular changes accompanying the over-production of APP and of its amyloidogenic peptide products.

A major consequence of Alzheimer's disease and Down's syndrome pathology is basal forebrain cholinergic neuron degeneration (Bowen *et al*, 1976a; Davies & Maloney, 1976; Yates *et al*, 1980; Whitehouse *et al*, 1982). These cells are highly dependent on target-derived NGF for the phenotypic maintenance of their cell bodies and synaptic integrity at post-natal stages (Thoenen, 1995; Debeir *et al*, 1999; Sofroniew *et al*, 2001). In consequence, it has been hypothesized that the atrophy of these neurons in Alzheimer's disease was caused by NGF deficits (Hefti, 1983). However, in post-mortem Alzheimer's disease brains the synthesis of NGF is not affected (Goedert *et al*, 1986; Jette *et al*, 1994; Fahnestock *et al*, 1996) and

the levels of its precursor (proNGF) are increased (Fahnestock *et al*, 2001; Pedraza *et al*, 2005b; Bruno *et al*, 2009a).

Recent data from our lab demonstrated that upon neuronal activity, proNGF is released to the extracellular space, along with the enzymes necessary for its conversion to mature NGF (mNGF) and for its subsequent degradation (Bruno & Cuello, 2006). ProNGF is cleaved and matured by plasmin, which derives from plasminogen by the action of tissue plasminogen activator (tPA). tPA activity is inhibited by neuroserpin in the CNS (Miranda & Lomas, 2006). Matrix metallo-protease 9 (MMP-9) is the main mNGF-degrading enzyme (Bruno & Cuello, 2006). In post-mortem Alzheimer's disease brains, we have shown that there is accumulation of proNGF due to a failure in its maturation, as well as increased MMP-9 activity (Bruno *et al*, 2009a). Notably, these changes are also present in MCI brains, a stage in which the increase in proNGF and MMP-9 activity positively correlates with the degree of pre-mortem cognitive decline (Peng *et al*, 2004; Bruno *et al*, 2009b).

Thus, the above opens the question whether an analogous compromise in NGF metabolism would occur in Down's syndrome. In consequence, we embarked on an extensive study involving post-mortem adult Down's syndrome brains, primary cultures of human fetal Down's syndrome cortex and brains from a widely used Down's syndrome mouse model (Lockrow *et al*, 2012). In line with our findings in Alzheimer's disease and MCI, we report marked deficits in NGF metabolism in Down's syndrome brains, evidenced by increased MMP-9 activity and proNGF accumulation. These alterations were accompanied by increased neuroserpin and

reduced plasminogen levels and tPA synthesis, changes which will impair the maturation of proNGF. In addition, there is a probable enhanced degradation of mNGF, reflected by the up-regulation of MMP-9 activity in human Down's syndrome brains and by the reduction of mNGF levels in the brains of Ts65Dn mice. Our results may offer new vistas to prevent or decelerate cholinergic neurodegeneration in individuals with Down's syndrome-related Alzheimer's disease.

2.3. Materials and methods

2.3.1. Human brain tissue

Frozen grey matter tissue from the temporal (n = 14), frontal (n = 20) and parietal (n = 20) cortices of adult Down's syndrome subjects and age-matched control cases were obtained from the Alzheimer's Disease Research Center, University of California, Irvine. Additional frontal cortex tissue (n = 14) was obtained from New York University School of Medicine. These additional samples did not result in any demographic difference such as post-mortem interval or age between the three regions studied. For further demographic information see Table 2-S1 (Chapter 2: Supplementary Tables and Figures).

Clinico-pathological analysis, performed at the respective research centers in Irvine and New York, revealed the presence of Alzheimer's disease dementia, confirmed by the widespread deposition of senile plaques and neurofibrillary tangles in all Down's syndrome brains included in this study. Diagnosis of Alzheimer's disease in Down's syndrome subjects was done following neurological examination using

DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, American Psychiatric Association) and ABC criteria (Montine *et al*, 2012) as well as considering medical and behavioral history obtained through a knowledgeable informant such as a parent/caregiver. Control cases showed no evidence of chromosomal or neuropathological abnormalities and displayed no signs of cognitive decline. The project has been approved by the McGill University Research Ethic Board.

2.3.2. Generation of mixed primary cultures

Mixed primary cultures were established from the cerebral cortex of normal (n = 5) and Down's syndrome (n = 9) fetuses of 17-21 gestational age weeks, following previously described protocols (Kerkovich *et al*, 1999; Pelsman *et al*, 2003; Helguera *et al*, 2013). The protocols for obtaining post-mortem fetal brain complied with all US federal and institutional guidelines with special respect for donor identity confidentiality and informed consent.

To avoid widespread Down's syndrome neuronal degeneration in culture (Busciglio & Yankner, 1995), we prepared higher density cortical cultures (600 cells per mm^2), maintained in Neurobasal medium plus N2 supplement (Invitrogen, USA). Under these conditions Down's syndrome cortical neurons survived well, developed an extensive network of processes and appeared no different than controls in terms of morphological features. The cells were maintained for 20 days in cultures, after which conditioned media and cellular pellets were collected and frozen at -80 °C.

2.3.3. Ts65Dn mice

Trisomic Ts65Dn mice (Reeves *et al*, 1995) (*n* = 6-9) were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). Age-matched (12-22 month-old), normosomic littermates were used as controls (*n* = 8-11). Only males were used. Animals were housed at the Animal Resource Facilities, Medical University of South Carolina, having access to food and water *ad libitum*. Animals were subjected to isofluorane overdose prior to decapitation. Frontal cortex tissue and hippocampus were dissected and stored at -80˚C. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

2.3.4. Human Aβ ELISA

Human $A\beta_{42}$ and $A\beta_{40}$ levels were measured using sandwich ELISA kits (Invitrogen, USA), following manufacturer's instructions. Briefly, frozen brain samples were homogenized in 5M guanidine hydrochloride and incubated 3 h at room temperature in agitation. Homogenates and conditioned media samples were diluted in standard dilution buffer (supplied in the kit) supplemented with 1 mM PMSF. All samples including the standard curve were run in duplicate. The detection limit for this assay is 15.63 pg/ml of $A\beta_{42}$ and 7.81 pg/ml of $A\beta_{40}$.

2.3.5. Western blotting

Frozen brains and fetal cortical cells were homogenized in cold lysis buffer (50 mM Tris HCl pH 7.4; 150 mM NaCl; 1% NP-40; 0.1% SDS; 0.1% sodium deoxycholate; 2 μg/ml leupeptin; 2 μg/ml aprotinin and 1mM PMSF), followed by

sonication. Homogenates were incubated 15 min on ice and centrifuged at 13.000 rpm, 45 min at 4 °C. Protein concentration was measured from the supernatant fraction with the DC™ Protein Assay kit (BioRad, USA). Proteins from tissue homogenates (15-50 µg) and conditioned media (10-30 µl) were separated on 8-12% polyacrylamide gels under reducing conditions and semi-dry transferred to PVDF membranes (BioRad, USA). Membranes were blocked 1 h in 5% skim milk and incubated with primary antibodies overnight at 4°C (Table 2-S2, Supplementary Tables and Figures). Peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch, USA. Blots were developed with an enhanced chemiluminiscence system (GE Healthcare Life Sciences, USA) on Hyblot CL films (Denville, Canada). Densitometry (IOD) was quantified with the Gel-Pro Analyzer software (Media Cybernetics, Inc., USA). Relative protein levels were expressed in relation to β-actin, or normalized to total protein concentration in each conditioned media sample.

2.3.6. RNA extraction and quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from 20 mg human or mouse brains using the RNeasy Mini Kit (Qiagen, USA), following manufacturer's instructions. RNA was retro-transcribed with the Omniscript RT Kit (Qiagen, USA) using an oligo-dT primer to generate cDNA. Quantification of transcript expression was assessed by qRT-PCR with EvaGreen® (MBI EVOlution EvaGreen qPCR Mix, Montreal Biotech Inc.) using the Illumina Eco Instrument and Software (Illumina Inc., USA). Expression of each gene was normalized to the housekeeping gene hypoxanthine-guanine

phosphoribosyltransferase (*HPRT*). For a list of primers used see Table 2-S3 (Supplementary Tables and Figures).

2.3.7. Gelatin zymography

The proteolytic activity of proMMP-9 (proenzyme, ~105kDa), MMP-9 (Gelatinase B, ~92kDa) and MMP-2 (Gelatinase A, ~72kDa) was determined by gelatin zymography, as previously described (Bruno *et al*, 2009a; Bruno *et al*, 2009b). Briefly, 70 µg of cortical homogenates and 10 µl of conditioned media were separated on 8% polyacrylamide gels containing 0.2% gelatin (Sigma-Aldrich, USA). Zymograms were renatured with 2.5% Triton X-100 (Sigma-Aldrich, USA) for 45 min and incubated in developing buffer (50 mM Tris, 0.2 M NaCl, 5 mM $CaCl_2$, Brij-35 0.02%) for 24-48 h at 37 ˚C. Gels were stained with 0.25% Coomasie R-250 and incubated with destaining solution (methanol: water: acetic acid; 50:40:10) until clear areas of protease activity were seen. Zymograms were quantified with the Gel-Pro Analzyer software (Media Cybernetics, Inc., USA).

2.3.8. tPA-casein zymography

tPA activity was measured as detailed in (Fabbro & Seeds, 2009). Proteins from cortical homogenates (70 µg) and conditioned media (20 µl) were separated on 10% polyacrylamide gels containing casein (Sigma-Aldrich, USA) and plasminogen (Chromogenix, USA). Gels were renatured in 2.5% Triton X-100 for 45 min at room temperature and developed with 0.1 M Tris, 0.5 mM amiloride, pH 8.1 (18-24 h; 37 ˚C). Gels were stained in identical conditions as for gelatin zymography.

2.3.9. Statistical analysis

Two-group comparisons were analyzed with a 2-tailed Student's *t*-test. Spearman rank analysis was used for correlations (Graph Pad Prism 5.01). Significance was set at $P < 0.05$. Error bars represent mean ± SEM. All experiments were run in triplicate.

2.4. Results

2.4.1. Analysis of APP and Aβ peptides in adult Down's syndrome brains

In order to establish whether NGF metabolic deregulation occurs in other Aβ pathologies than Alzheimer's disease and MCI, we first confirmed the presence of APP and Aβ neuropathology in our cohort of adult Down's syndrome brains. Down's syndrome brains exhibited a significant increase in APP levels (~2.5 fold) in temporal (Figure 2-1 A), frontal (Figure 2-1 B) and parietal cortex (Figure 2-1 C) compared to control cases, in agreement with prior reports (Cheon *et al*, 2008). $A\beta_{40}$ and $A\beta_{42}$ peptides were also highly elevated in Down's syndrome brains (~3-20 fold, depending on the brain region). In temporal cortex, Down's syndrome cases exhibited mean concentrations of $A\beta_{40}$ and $A\beta_{42}$ of 7.97 and 7.65 μg/g tissue, respectively (Figure 2-1 D). In frontal cortex, Aβ expression was more robust, with mean concentrations of $A\beta_{40}$ and $A\beta_{42}$ of 19.02 and 9.76 μg/g tissue, respectively (Figure 2-1 E). Aβ expression was lower in parietal cortex, with mean concentrations of $A\beta_{40}$ and $A\beta_{42}$ of 4.47 and 2.11 μg/g tissue (Figure 2-1 F), respectively.

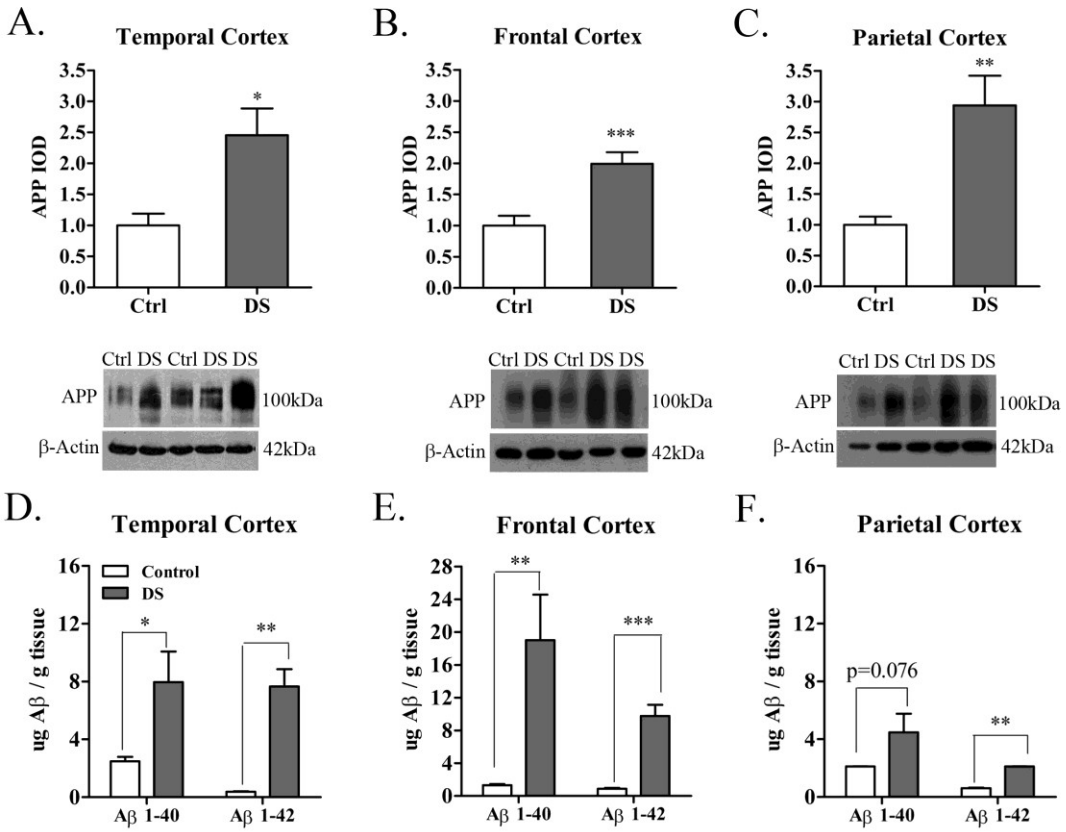

Figure 2-1. Increased APP and Amyloid-β levels in Down's syndrome brains. Western blot analysis from human cortical homogenates revealed increased APP levels in Down's syndrome brains compared to control cases in A) temporal (P = 0.017; n = 14), B) frontal (P = 0.0004; n = 34) and C) parietal (P = 0.004; n = 20) cortex. Representative immunoblots probed with 22C11 and β-actin antibodies are shown. D-F) ELISA analysis of $A\beta_{40}$ and $A\beta_{42}$ peptides from guanidine hydrochloride-homogenized brains. Down's syndrome brains exhibited significantly higher levels of $A\beta_{40}$ and $A\beta_{42}$ peptides in D) temporal (P = 0.003, P = 0.002) E) frontal (P = 0.007, P < 0.0001) and F) parietal cortex (P = 0.076, P = 0.005). Data are expressed as ug of Aβ / g tissue. Error bars represent mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001, Student's t test.

2.4.2. Increased proNGF levels in Down's syndrome brains

We next investigated whether post-mortem Down's syndrome brains exhibited proNGF accumulation similar to MCI and Alzheimer's disease brains. The ~32kDa band quantified here corresponded to that previously identified as proNGF (Fahnestock *et al*, 2001; Bruno *et al*, 2009a). The immunoreaction was abolished by preadsorbing the primary antibody with a proNGF peptide supplied by the manufacturer. ProNGF levels were significantly higher (~2-3 fold) in Down's syndrome temporal (Figure 2-2 A), frontal (Figure 2-2 B), and parietal cortex (Figure 2-2 C). This accumulation was not because of increased transcription, as NGF mRNA levels were not significantly different between control and Down's syndrome cases, in all regions investigated (Supplementary Figure 2-1).

In temporal and frontal cortex, proNGF positively correlated with APP (Figure 2-2 D and F) and with $A\beta_{42}$ levels (Figure 2-2 E and G). In parietal cortex, there was no significant correlation between proNGF and increased APP expression ($r = 0.309$, $P > 0.05$) or $A\beta_{42}$ peptides ($r = 0.342$, $P > 0.05$). These findings suggest a region-specific relationship between the amount of APP and Aβ peptides and reduced proNGF processing.

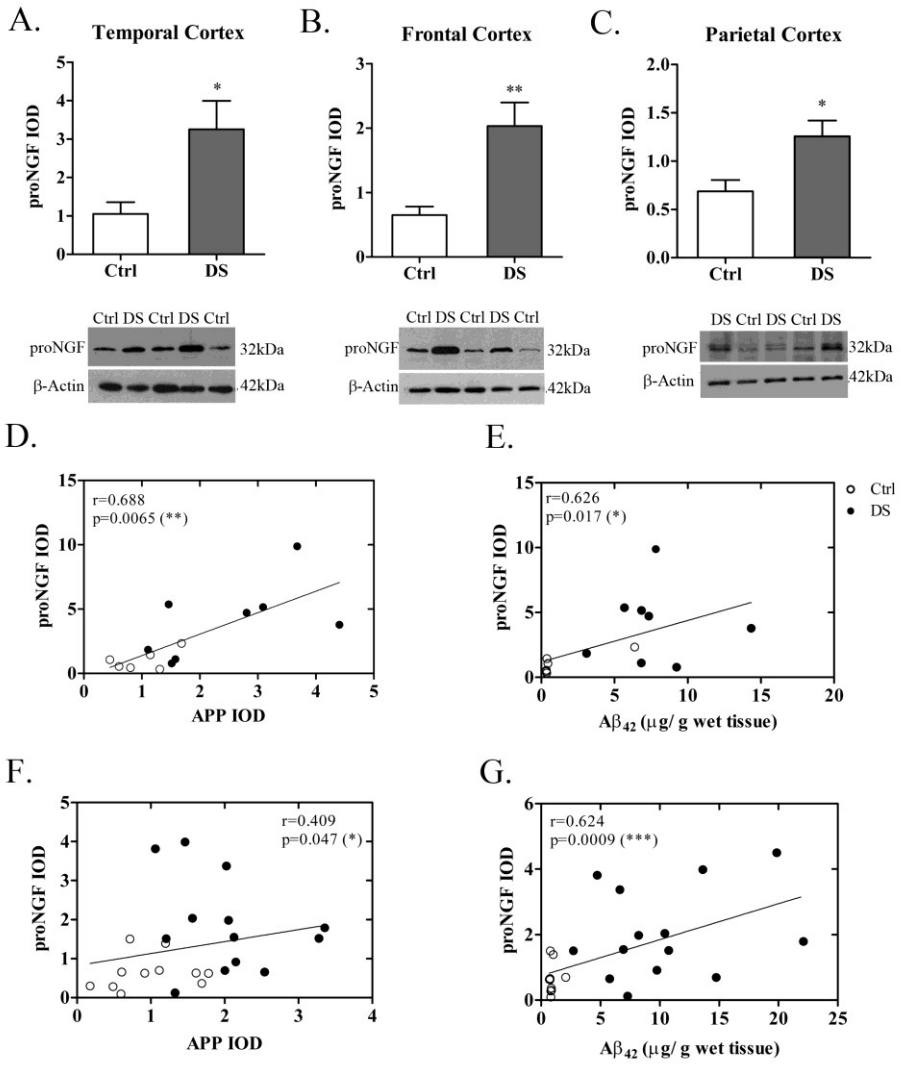

Figure 2-2. Increased proNGF levels in Down's syndrome brains. Western blot analysis of proNGF in cortical brain homogenates. Down's syndrome subjects exhibited significantly higher proNGF levels compared to age-matched control cases in A) temporal ($P = 0.026$), B) frontal ($P = 0.004$) and C) parietal ($P = 0.018$) cortex. Representative immunoblots probed with proNGF and β-actin antibodies are shown. D-G) Scattergrams showing positive correlation between proNGF and APP in D) temporal ($r = 0.688$, $P = 0.007$) and F) frontal cortex ($r = 0.409$, $P = 0.047$) and between proNGF and $A\beta_{42}$ in E) temporal ($r = 0.626$, $P = 0.017$) and in G) frontal cortex ($r = 0.629$, $P = 0.001$). Error bars represent mean ± SEM. *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$, Student's t test; Spearman Rank analysis for correlations.

2.4.3. Increased MMP-9 activity and TIMP-1 mRNA in Down's syndrome brains

Despite no apparent changes in MMP-9 mRNA expression between control and Down's syndrome cases (Supplementary Figure 2-1), we observed an increased proMMP-9 and MMP-9 activity in Down's syndrome temporal (Figure 2-3 A), frontal (Figure 2-3 B) and parietal cortex (Figure 2-3 C). No significant changes in MMP-2 zymogenic activity were observed in adult Down's syndrome brains in all areas investigated (Figure 2-3 A-C).

We found strong associations between MMP-9 activity, Aβ pathology and proNGF. In temporal cortex, analysis revealed a significant correlation between MMP-9 activity and $A\beta_{42}$ (Figure 2-3 D), as well as a strong association between proNGF levels and MMP-9 (Figure 2-3 E). This pattern followed in frontal cortex. MMP-9 activity correlated with APP (Figure 2-3 F), $A\beta_{42}$ (Figure 2-3 G) and proNGF expression (Figure 2-3 H). We further observed a significant increase (~3 fold) in TIMP-1 mRNA levels, the endogenous MMP-9 inhibitor, in Down's syndrome frontal (Figure 2-3 J) and parietal cortex (Figure 2-3 K). TIMP-1 mRNA positively correlated with MMP-9 activity in frontal cortex (Figure 2-3 I) but not in parietal cortex ($r = 0.093$, $P > 0.05$). A trend reflecting higher TIMP-1 expression was observed in Down's syndrome temporal cortex (Supplementary Figure 2-2 A). There was also a strong association between MMP-9 activity and TIMP-1 in this region (Supplementary Figure 2-2 B), excluding one Down's syndrome case with very high MMP-9 activity (dark square) and a significant medical history of asthmatic bronchitis, an inflammatory disease in which strong elevations in MMP-9 activity have been documented (Vignola *et al*, 1998).

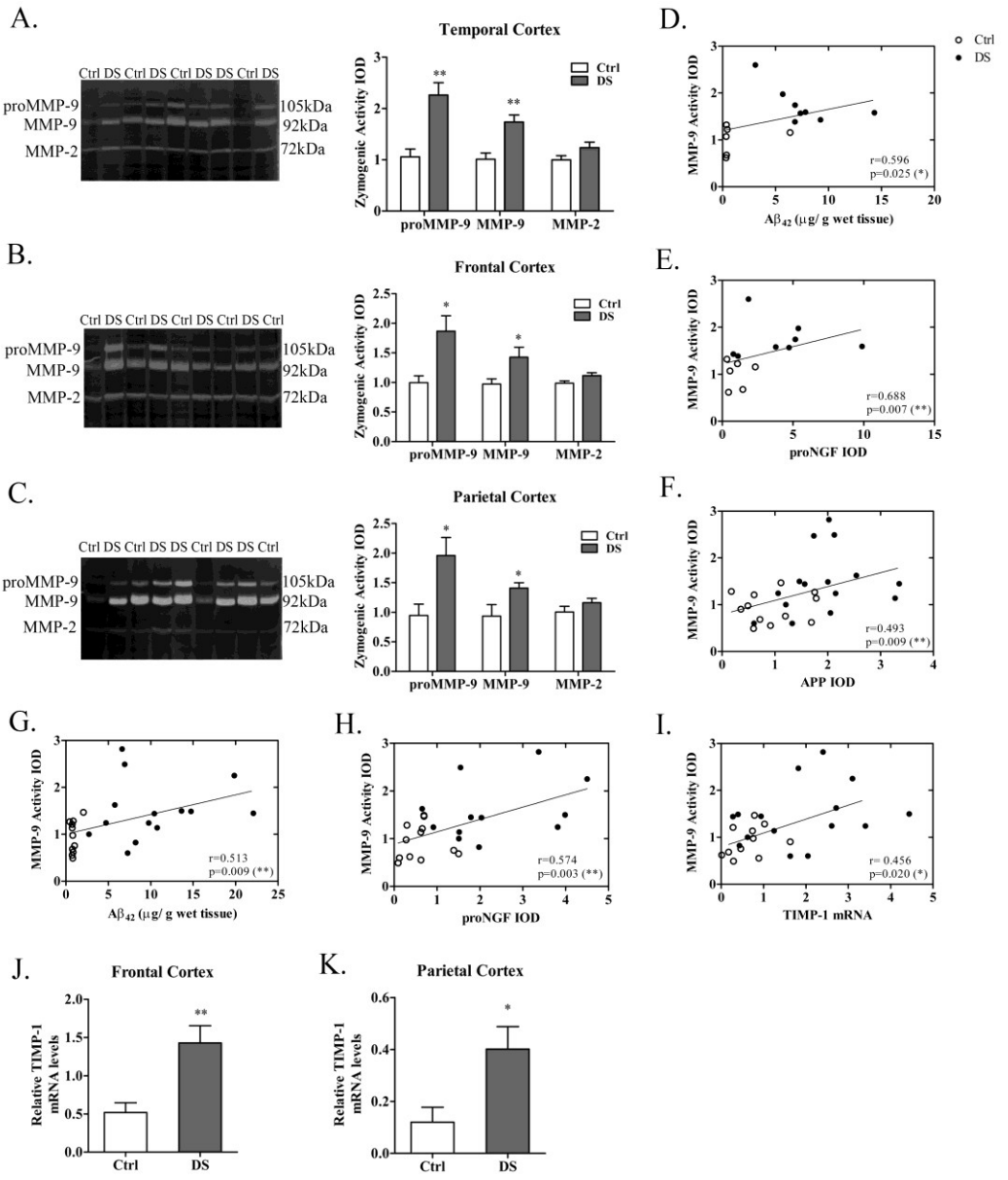

Figure 2-3. Increased MMP-9 activity and TIMP-1 expression in Down's syndrome brains. A-C) Representative gelatin zymographs depicting proMMP-9, MMP-9 and MMP-2 proteolytic activity. Analysis revealed significantly elevated proMMP-9 and MMP-9 zymogenic activity in Down's syndrome cortical homogenates compared to control cases in A) temporal (P = 0.002 and P = 0.003, respectively) B) frontal (P = 0.011 and P = 0.043) and C) parietal cortex (P = 0.018, P = 0.039). MMP-2 activity did not differ between Down's syndrome and control subjects in none of the areas investigated. Values are expressed as fold increase versus control. Independent statistical analysis was done for each metallo-protease, comparing its levels between control and Down's syndrome cases. In

temporal cortex there was a positive correlation between D) MMP-9 activity and $A\beta_{42}$ levels (r = 0.596, P = 0.025) as well as a strong link between E) MMP-9 activation and proNGF accumulation (r = 0.688, P = 0.007). Correlation analysis in frontal cortex also revealed positive associations between F) MMP-9 zymogenic activity and APP levels (r = 0.493, P = 0.009), G) MMP-9 and $A\beta_{42}$ (r = 0.513, P = 0.009) and H) MMP-9 and proNGF (r = 0.577, P = 0.003). qRT-PCR analysis revealed significantly higher TIMP-1 mRNA levels in J) frontal (P = 0.0003) and K) parietal cortex (P = 0.015). TIMP-1 mRNA expression was normalized to the housekeeping gene *HPRT*. I) Scattergram showing positive correlation between MMP-9 activity and TIMP-1 mRNA levels in frontal cortex (r = 0.457, P = 0.020). Error bars represent mean ± SEM. *P < 0.05; **P < 0.01; Student's t test; Spearman Rank analysis for correlations.

2.4.4. Alterations in neuroserpin, plasminogen and tPA expression in brains from Down's syndrome subjects

Given that we observed proNGF accumulation in Down's syndrome brains we sought to establish whether Alzheimer's disease-like neurochemical changes in the plasminogen / tPA / neuroserpin proNGF-conversion pathway are also present in this condition. PCR analysis revealed higher neuroserpin mRNA levels (~1.5 fold) in Down's syndrome temporal (Figure 2-4 A), frontal (Figure 2-4 B) and parietal cortex (Figure 2-4 C). Neuroserpin protein levels were accordingly elevated (Figure 2-4 D-F). In frontal cortex $A\beta_{42}$ positively correlated with neuroserpin mRNA (Figure 2-4 G) and protein levels (Figure 2-4 H).

The neuroserpin upregulation in Down's syndrome was accompanied by marked reductions in tPA mRNA levels (~70% decrease) in frontal (Figure 2-4 I) and parietal cortex (Figure 2-4 J). No significant changes in tPA mRNA levels were detected in temporal cortex (Supplementary Figure 2-1 G). Plasminogen levels were also compromised in Down's syndrome brains, as evidenced by lower protein expression (~40% reduction) in frontal (Figure 2-4 K), parietal (Figure 2-4 L) and a strong trend in temporal cortex (Supplementary Figure 2-2 C). We did not observe any significant reduction in plasminogen mRNA synthesis in Down's syndrome temporal (Supplementary Figure 2-2 D), frontal (Supplementary Figure 2-2 E) or parietal cortex (Supplementary Figure 2-2 F).

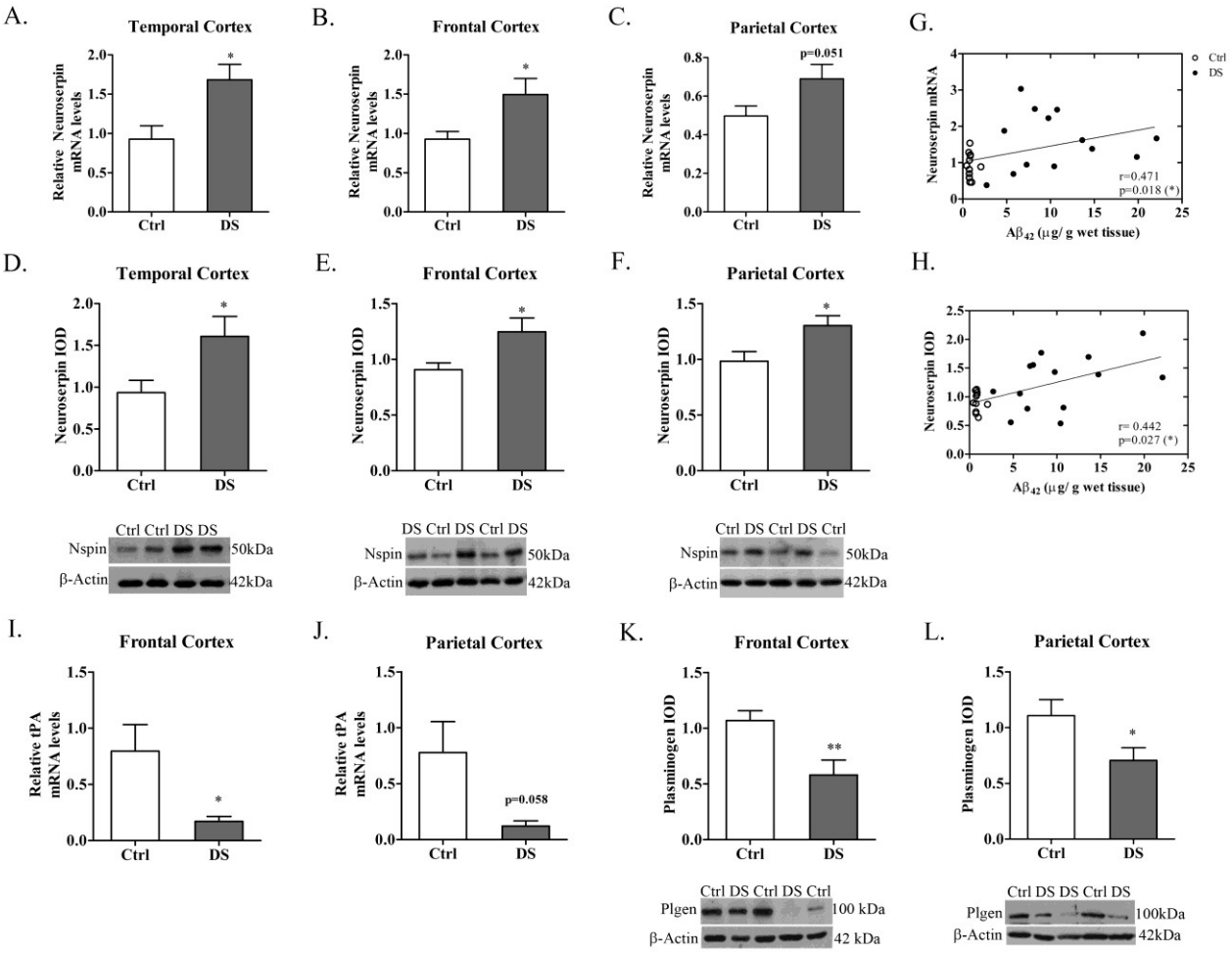

Figure 2-4. Alterations in neuroserpin, tPA and plasminogen in Down's syndrome brains. Down's syndrome brains exhibited significantly higher neuroserpin mRNA levels in A) temporal ($P = 0.017$), B) frontal ($P = 0.029$) and C) parietal cortex ($P = 0.051$), compared to age-matched control cases. D-F) Western blot analysis revealed a significant increase in neurosepin protein levels in D) temporal ($P = 0.047$), E) frontal ($P = 0.045$) and F) parietal cortex ($P = 0.031$). G-H) Scattergrams showing positive correlation between G) neuroserpin mRNA and $A\beta_{42}$ ($r = 0.471$, $P < 0.05$) and H) neuroserpin protein levels and $A\beta_{42}$ ($r = 0.442$, $P < 0.05$) in frontal cortex. PCR analysis revealed marked reductions in tPA mRNA levels in I) frontal ($P = 0.016$) and J) parietal ($P = 0.058$) cortex. Down's syndrome brains also exhibited reduced plasminogen protein levels in Down's syndrome K) frontal ($P = 0.0079$) and L) parietal cortex ($P = 0.045$). D-F, K-L) Representative immunoblots probed with neuroserpin, plasminogen and β-actin antibodies are shown. A-C, I-J) PCR data are expressed as the normalized ratio between each protein of interest and *HPRT*. Error bars represent mean ± SEM. *$P < 0.05$; **$P < 0.01$; Student's *t* test; Spearman Rank analysis for correlations.

Importantly, we verified that alterations in NGF metabolism were not influenced by differences in post-mortem interval (PMI). We found no significant correlation between protein, mRNA or enzymatic activity of NGF pathway markers and PMI, in all of the cortical regions investigated.

2.4.5. Analysis of soluble APP and Aβ peptides in primary cultures of Down's syndrome fetal cortex

We observed a significant increase in soluble APP (Supplementary Figure 2-3 A) and soluble APP-β levels (~2.5 fold) in Down's syndrome conditioned media compared to control cultures (Figure 2-5 A). This was accompanied by higher levels of secreted $A\beta_{40}$ (Figure 2-5 B) and $A\beta_{42}$ (Figure 2-5 C) peptides.

2.4.6. Alterations in NGF pathway markers in primary cultures of Down's syndrome fetal cortex

In line with our results in adult brains, proNGF levels were significantly increased in Down's syndrome conditioned media (Figure 2-5 D). The specificity of the ~40 kDa band corresponding to secreted proNGF (Bruno & Cuello, 2006) was confirmed by its disappearance after incubation of the proNGF primary antibody with a proNGF peptide, provided by the manufacturer. Down's syndrome conditioned media also exhibited marked deficits in tPA proteolytic activity (Figure 2-5 E), indicating a likely compromise of proNGF maturation, at this early stage.

We next examined MMP-9 activity by gelatin zymography and observed a significant increase in MMP-9 and MMP-2 activity (Figure 2-5 F) in Down's syndrome conditioned media (extracellular milieu). The changes in MMP-9

activity were also accompanied with increases in protein expression (Supplementary Figure 2-3 B). In accordance with our analysis in adult brains, MMP-9 activation positively correlated with $A\beta_{42}$ levels (Figure 2-5 H) and with increased proNGF expression (Figure 2-5 I). Likewise, Western blot analysis revealed significantly elevated TIMP-1 protein levels (~3 fold) in Down's syndrome conditioned media compared to control cultures (Figure 2-5 G). There was a strong correlation between MMP-9 activity and TIMP-1 levels (Figure 2-5 J). Importantly, the changes we observed in Down's syndrome cells were not due to increased cell death and subsequent release of cellular contents to the media, as revealed by low, comparable lactate dehydrogenase levels in control and Down's syndrome cultures (Supplementary Figure 2-3 C).

In accordance with our results in conditioned media, Down's syndrome cortical cell homogenates exhibited higher APP (Figure 2-5 K) and MMP-9 protein levels (Figure 2-5 L), (by ~2 fold) compared to control lysates. We also found a significant reduction in tPA proteolytic activity in Down's syndrome primary cortical culture homogenates (Figure 2-5 M). However, we found no significant differences in the intracellular levels of proNGF between control and Down's syndrome cell culture lysates (Supplementary Figure 2-3 D).

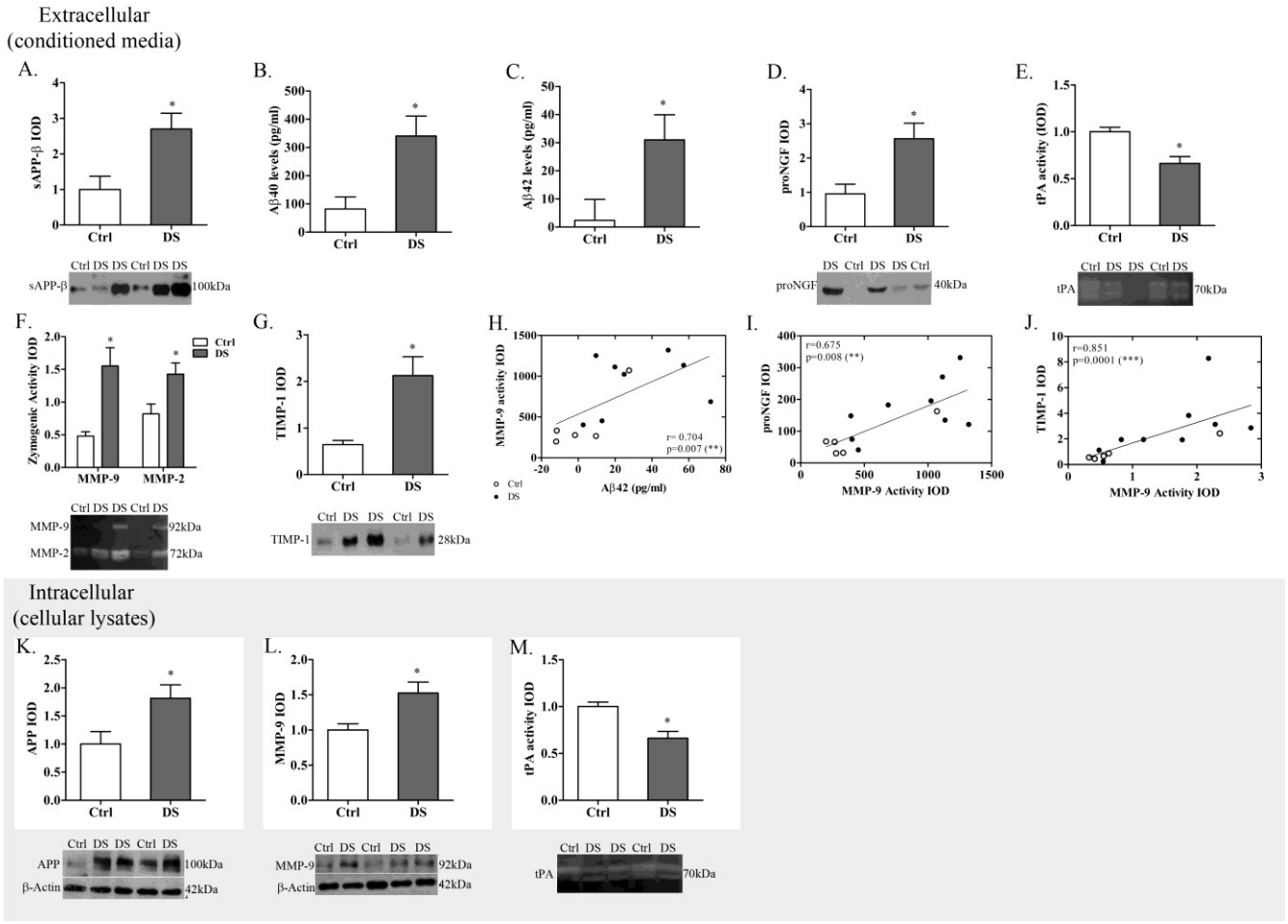

Figure 2-5. Early signs of NGF dysmetabolism in primary cultures from fetal Down's syndrome cortex. A) Western blot analysis revealed significantly higher soluble APP-β levels (P = 0.015) in conditioned media from Down's syndrome cultures. Down's syndrome cultures exhibited significantly higher levels of secreted B) $A\beta_{40}$ (P = 0.036) and C) $A\beta_{42}$ (P = 0.047), compared to controls. The concentration of Aβ peptides is expressed as pg/ml. D) Western blot analysis revealed a significant increase in proNGF levels in conditioned media from Down's syndrome cultures (P = 0.029). E) Reduced tPA zymogenic activity in Down's syndrome conditioned media (P = 0.046). F) The activity of MMP-9 and MMP-2 was significantly increased in Down's syndrome conditioned media (P = 0.011 and P = 0.026, respectively) H) Scattegram showing positive correlation between MMP-9 activity and $A\beta_{42}$ (r = 0.718, P = 0.009). I) Positive correlation between proNGF and MMP-9 activity (r = 0.676, P < 0.011). G) Western blot analysis revealed significantly higher TIMP-1 levels in Down's syndrome conditioned media (P = 0.029). J) Positive correlation between TIMP-1 and MMP-9 activity (r = 0.851, P = 0.0001). For Western blot and zymography analysis, IOD

values were normalized by total protein concentration in each conditioned media sample. K-M) Western blot analysis from cortical cell homogenates revealed K) increased APP ($P = 0.042$) and L) MMP-9 protein levels ($P = 0.030$) in Down's syndrome. M) There was a marked reduction in tPA activity in Down's syndrome cells, as determined by casein zymography ($P = 0.017$). Representative tPA zymogram and immunoblots probed with 22C11, MMP-9 and β-actin antibodies are shown. Error bars represent mean ± SEM, ($n = 5$ for control and $n = 9$ for Down's syndrome cultures). *$P < 0.05$; **$P < 0.01$; Student's t test, Spearman Rank analysis for correlations.

2.4.7. NGF dysmetabolism in a genetic mouse model of Down's syndrome

Ts65Dn mice, trisomic for a segment of chromosome 16 (orthologue to chromosome 21), possess three copies of the APP gene (Reeves *et al*, 1995) and exhibit increased APP expression (mRNA and protein levels) in cortex and hippocampus by 8-12 months of age (Granholm *et al*, 2003; Hunter *et al*, 2003; Hunter *et al*, 2004; Seo & Isacson, 2005; Salehi *et al*, 2006; Choi *et al*, 2009; Lockrow *et al*, 2009).

In this study we examined whether similar NGF-metabolic alterations were evident in Ts65Dn Down's syndrome mice. Trisomic mice (12-22 months of age) exhibited increased hippocampal proNGF (Figure 2-6 A) and a significant reduction in mNGF levels (Figure 2-6 B). We also found marked deficits in plasminogen (Figure 2-6 C) and tPA (Figure 2-6 D) suggesting a compromise in proNGF maturation in the basal forebrain target tissue. Such a decrease in plasminogen protein occurred without a reduction in its mRNA levels (Supplementary Figure 2-2 G), consistent with the results obtained in human Down's syndrome brains. Representative immunoblots are shown in Figure 2-6 E. Trisomic mice also exhibited significantly increased neuroserpin (Figure 2-6 H), MMP-9 (Figure 2-6 F) and TIMP-1 (Figure 2-6 G) mRNA expression in frontal cortex at this time-point, in accordance with our results in human Down's syndrome brains.

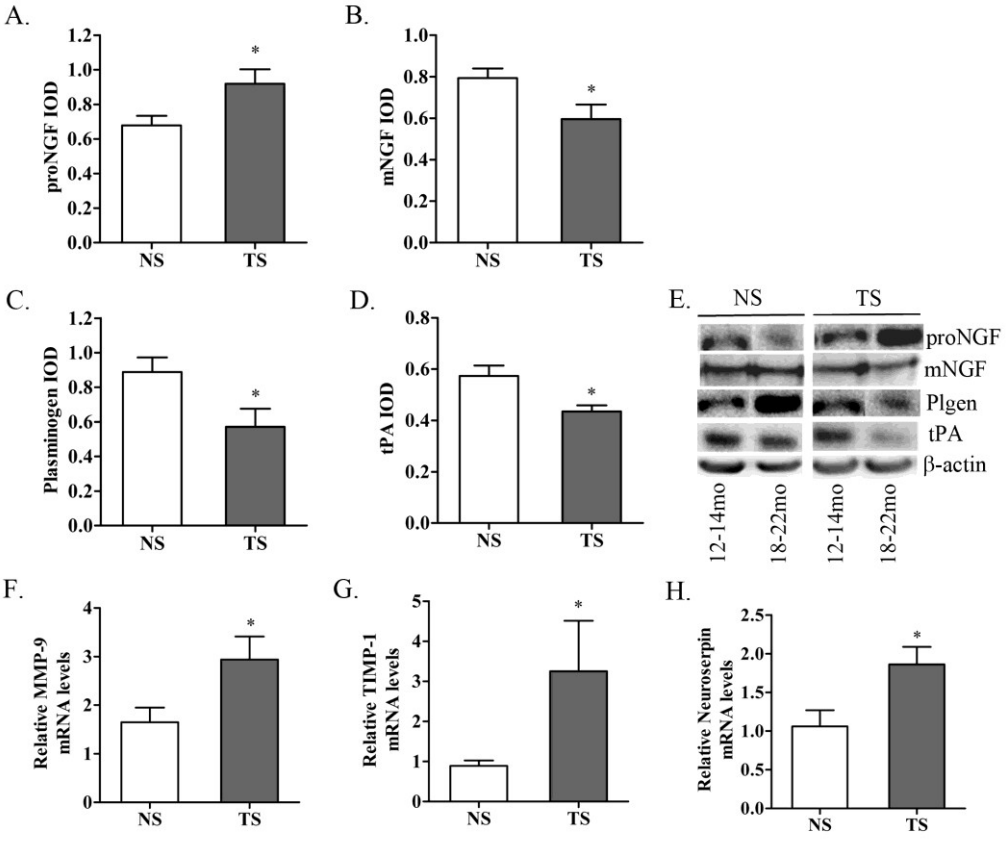

Figure 2-6. Deficits in proNGF cleavage in Ts65Dn mice, a genetic mouse model of Down's syndrome. Western blot analysis of NGF pathway markers in basal forebrain target tissue (hippocampus) of 12-14 and 18-22 month-old mice. trisomic mice exhibited significantly higher A) proNGF levels (P = 0.035) and lower B) mNGF levels (P = 0.010), compared to normosomic littermates. Analysis also revealed a significant reduction in C) plasminogen (P = 0.031) and D) tPA (P = 0.026) protein levels. Graphs depict data combined from the two time points. E) Representative immunoblots probed with NGF, plasminogen, tPA and β-actin antibodies are shown. F-H) qRT-PCR analysis of NGF pathway markers in frontal cortex from trisomic mice and normosomic littermates. Trisomic mice exhibited higher F) MMP-9 (P = 0.044) and G) TIMP-1 mRNA levels (P = 0.049). H) Increased neuroserpin mRNA levels in trisomic mice (P = 0.039). Data are expressed as the normalized ratio between each protein of interest and the housekeeping gene *Hprt*. Error bars represent mean ± SEM. *P < 0.05; Student's t test. NS = normosomic; TS = trisomic.

2.5. Discussion

This comprehensive study is, to the best of our knowledge, the first demonstration that human post-mortem Down's syndrome brains exhibit robust deficits in NGF metabolism which are replicated in cultured Down's syndrome fetal cortical cells and in Ts65Dn trisomic mice. Figure 2-7 illustrates schematically the normal NGF metabolic pathway and its deregulation in Down's syndrome brains.

2.5.1. A link between NGF metabolic deregulation and CNS cholinergic neuron dysfunction

In this study we report accumulation of proNGF (in human Down's syndrome brains) in basal forebrain target tissue (cortex), with concomitant alterations in the plasminogen / tPA / neuroserpin metabolic loop responsible for proNGF maturation. These changes suggest an impaired conversion of proNGF into its mature, biologically active molecule, resulting in an accumulation of proNGF. We also demonstrate an enhanced activity of the metallo-protease responsible for mNGF degradation (MMP-9), which should further diminish the brain availability of mNGF, aggravating the imbalance between proNGF and mNGF. Despite the scarcity of human Down's syndrome post-mortem brain material, we were able to investigate tissue samples from three different brain regions from each individual (i.e. temporal, frontal and parietal cortex) from two highly reputable brain banks. We have further validated the neurochemical alterations reported in human brains in two additional Down's syndrome experimental paradigms (i.e. in the cell culture system and in the genetic mouse model), confirming our observations.

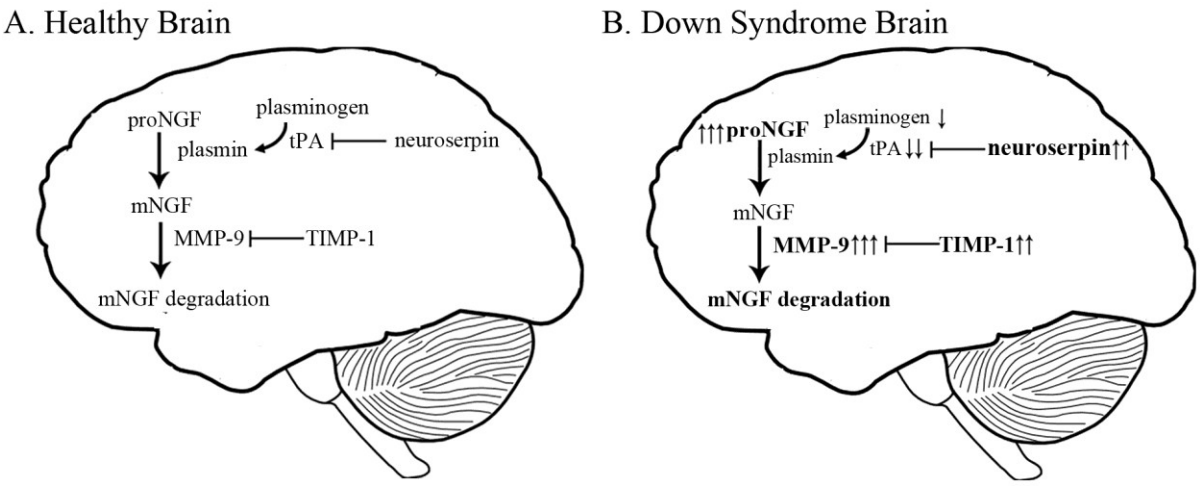

Figure 2-7. Schematic representation of the NGF metabolic pathway in healthy brains and its deregulation in Down's syndrome. A) The NGF precursor is released to the extracellular space along with the convertases and zymogens necessary for its maturation and degradation. ProNGF is cleaved extracellulary and converted to mNGF by plasmin. Plasmin derives from plasminogen by the action of tPA. Neuroserpin is the endogenous tPA inhibitor in the CNS. mNGF is degraded by MMP-9, which is also released from neurons along with its endogenous inhibitor TIMP-1. B) In Down's syndrome brains there is a failure in proNGF maturation due to reduced plasminogen and tPA as well as enhanced neuroserpin levels. Down's syndrome brains also exhibit increased MMP-9 activity and higher TIMP-1 levels, likely contributing to enhanced mNGF degradation.

The observation that proNGF maturation is affected is highly relevant in the context of Alzheimer's disease pathology in Down's syndrome. NGF is a key neurotrophin for basal forebrain cholinergic neurons. This neuronal system is highly dependent on endogenous NGF supply to maintain synapse numbers and TrkA mRNA expression (Venero *et al*, 1994; Figueiredo *et al*, 1995; Debeir *et al*, 1999). The basal forebrain cholinergic system progressively degenerates in Alzheimer's disease and Down's syndrome, deficits which contribute to the manifestation of cognitive impairments (Yates *et al*, 1980; Bartus *et al*, 1982; Pepeu & Giovannini, 2004). Therefore, the present findings provide a mechanistic explanation for the atrophy of basal forebrain cholinergic neurons in Down's syndrome, related to an impaired NGF metabolism and a concomitant trophic disconnection.

In line with the above, we have shown that pharmacological inhibition of proNGF maturation with α_2-antiplasmin resulted in atrophy and reduction of pre-existing cholinergic synapses, leading to cognitive impairments in young rats (Allard *et al*, 2012). Likewise, transgenic mice expressing a furin cleavage-resistant form of proNGF exhibit learning and memory deficits and cholinergic cell loss in the medial septum (Tiveron *et al*, 2013).

Importantly, a likely aggravating factor to proNGF accumulation and BFCN degeneration in Down's syndrome is impaired NGF retrograde transport due to endosomal dysfunction (Cooper *et al*, 2001; Salehi *et al*, 2006). Such deficits in NGF trophic support and subsequent cognitive impairment can be rescued with exogenous NGF application, in Ts65Dn mice (Cooper *et al*, 2001), emphasizing the

importance of NGF availability to the maintenance of the basal forebrain cholinergic phenotype.

2.5.2. NGF metabolic dysfunction in Down's syndrome

The increase in proNGF in Down's syndrome is consistent with the finding of proNGF accumulation in MCI and Alzheimer's disease brains (Fahnestock *et al*, 2001; Peng *et al*, 2004; Bruno *et al*, 2009a). Importantly, the deficits in tPA and plasminogen levels illustrated in this study reproduce the changes that we have reported in post-mortem Alzheimer's disease brains (Bruno *et al*, 2009a). Besides affecting proNGF maturation, a reduction in tPA synthesis can further contribute to neurodegeneration given that endogenous tPA has been shown to be neuroprotective in the ischemic brain and in hypoxic conditions (Wu *et al*, 2013).

Higher neuroserpin protein levels have also been reported in post-mortem Alzheimer's disease brains (Fabbro & Seeds, 2009) and CSF (Nielsen *et al*, 2007). The fact that increased neuroserpin might have deleterious effects in the CNS is suggested by knock-out studies in J20 hAPP transgenic mice, resulting in a reduction of $A\beta_{40}$ and $A\beta_{42}$ peptides, a decline in amyloid plaque burden and rescue of cognitive deficits (Fabbro *et al*, 2011). It is likely that increased neuroserpin levels in Down's syndrome are also related to the pathological accumulation of Aβ peptides. The positive correlation between neuroserpin and $A\beta_{42}$ reported in this study reinforces such observations.

We have gathered significant biochemical evidence supporting a very early proNGF accumulation and MMP-9 activation in cortical fetal Down's syndrome

primary cultures. These changes were accompanied by increased sAPP-β and Aβ peptides, under culture conditions that favor survival, avoid oxidative stress and degeneration. We have found strong correlations between MMP-9 activity, proNGF and $A\beta_{42}$ pathology, both in adult and in fetal tissue. Notably, Cho and colleagues have recently reported an elevation of MMP-2 levels in amniotic fluid from Down's syndrome pregnancies (Cho *et al*, 2011). Thus, MMP-9 and MMP-2 could be early molecular signals responding to Alzheimer's disease pathology accumulation in Down's syndrome.

We did not observe an upregulation of proNGF levels in Down's syndrome cellular lysates. The fact that a build-up of proNGF was only detected extracellularly (i.e. in the conditioned media, the secreted fraction) supports the hypothesis that this is a result of diminished proNGF conversion, rather than due to an increased NGF expression. In this regard, the culture system has the added advantage that the cellular and extracellular fractions can be physically separated. These findings reinforce the concept that proNGF is released to the extracellular space where it is cleaved and converted to its mature form (Bruno & Cuello, 2006).

Down's syndrome-related NGF metabolic dysfunction is further supported by the fact that trisomic Ts65Dn mice also exhibited NGF-metabolic deficits. In agreement with previous studies (Cooper *et al*, 2001; Salehi *et al*, 2006), proNGF levels were higher in basal forebrain target tissue (hippocampus). We further report a significant reduction in mNGF, plasminogen and tPA levels in trisomic mice, together with increased MMP-9 and TIMP-1 mRNA synthesis. These changes suggest that proNGF cleavage is compromised in the brains of trisomic mice,

reinforcing the observations in adult and fetal human Down's syndrome tissue. Importantly, at the time point examined, trisomic mice exhibit marked cholinergic deficits and neurodegeneration (Chen *et al*, 2009; Lockrow *et al*, 2012).

2.5.3. Possible causes of NGF metabolic dysfunction in Down's syndrome, MCI and Alzheimer's disease

We have previously shown that Aβ oligomers injected in the hippocampus of naïve rats are sufficient to induce NGF metabolic alterations, including an increase in proNGF levels, up-regulation of MMP-9 activity and increased expression of classical inflammatory markers (Bruno *et al*, 2009a). Moreover, pro-inflammatory mediators such as interleukin-1β, TNF-α and nitric oxide are known to be potent stimulators of TIMP-1 production and MMP-9 activation (Pagenstecher *et al*, 1998; Gu *et al*, 2002). Notably, several genes in chromosome 21 (i.e. S-100β, Cxdar, Tiam1; for review see (Wilcock, 2012)) may contribute to an over-activated pro-inflammatory response in Down's syndrome brains and as such, may be implicated in deregulating MMP-9 and TIMP-1 expression at fetal stages.

Supporting a role for Aβ in the occurrence of NGF metabolic deficits is the fact that in human Down's syndrome brains this compromise was most evident in frontal cortex, the region with the greatest extent of Aβ pathology. Notably, amyloid deposits in Down's syndrome appear first in the frontal and entorhinal cortex and further progress to other areas with advancing age (Azizeh *et al*, 2000). As such, regional differences in NGF metabolism could be a consequence of differential Alzheimer's disease pathology severity. However, we do not exclude

the possibility that other non-Aβ pathways are involved in compromising NGF's extracellular metabolism. Mitochondrial dysfunction and oxidative stress are well-established features of Down's syndrome brains which can induce alterations in APP metabolic processing (Busciglio *et al*, 2002) and may similarly impair NGF metabolism in Down's syndrome.

2.6. Concluding remarks

This study revealed novel and robust CNS alterations in NGF metabolism in Down's syndrome brains. Overall, these results strengthen the concept that NGF dysfunction is a relevant component of the β-amyloid pathology. This knowledge may provide new strategies to prevent –or arrest- the compromised NGF metabolism and hence protect CNS cholinergic neurons. The fact that NGF metabolic deficits were evident in human Down's syndrome fetal tissue indicates that alterations in proNGF cleavage and in MMP-9 activation occur prior to the full-blown Alzheimer's disease neuropathology. This may suggest that successful neuropreventive therapy could be initiated decades prior to frank memory loss in adults with Down's syndrome. Finally, a further relevant observation is the strong association between proNGF, MMP-9 and $A\beta_{42}$ in Down's syndrome brains, also evident in fetal tissue. These findings are relevant to consider the investigation of novel biomarkers signaling a progressive CNS trophic factor deregulation.

2.7. Acknowledgements

We would like to thank Dr. Wayne Poon for his assistance in the selection of human brain tissue and Dr. Daniel Lawrence for the generous gift of the

neuroserpin antibody. The Cuello Lab wishes to thank Dr A. Frosst, the Frosst family and Merck Canada for their unrestricted support.

2.8. Funding

This research was supported by a Canadian Institute for Health and Research grant (MOP-97776) to AC Cuello, a National Institutes of Health (NIH) grant (HD38466) and a UCI's Alzheimer's Disease Research Center grant (AG16573) to J Busciglio, a National Institutes on Aging grant (AG012122) to AC Granholm and NIH grants (AG08051 and NS073502) to T Wisniewski. AC Cuello is the holder of the McGill University Charles E. Frosst/Merck Chair in Pharmacology. MF Iulita is the recipient of a Biomedical Doctoral Award from the Alzheimer Society of Canada. S Do Carmo is the holder of the Charles E. Frosst-Merck Post-Doctoral Fellowship. L. Flores Aguilar is the recipient of a scholarship granted by the National Council for Science and Technology (CONACyT) of Mexico and by the Secretariat of Public Education and the Mexican government.

CHAPTER 2

Supplementary Tables and Figures

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Brain. 2014 Mar; 137 (Pt 3):860-72. doi: 10.1093/brain/awt372. Epub 2014 Feb 11.

Table 2-S1. Demographic information of the cases used in this study

UCI Brain Brank

		Ctrl (*n* = 7)	**DS (*n* = 13)**	***P* value**
Age (years)	Age range	41-67	40-63	-
	Mean ± SEM	58.7 ± 4.2	53.8 ± 2.0	$P > 0.05$
Gender (F/M)		3/4	9/4	-
Post-mortem delay (hours)	Range	2-10.4	0-10.5	-
	Mean ± SEM	6.7 ± 1.1	4.6 ± 0.8	$P > 0.05$
Braak Stage (0-VI)		0	III-VI	-
Neuropathological Diagnosis		Control[#]	DS+AD*	-

NYU Brain Bank

		Ctrl (*n* = 6)	**DS (*n* = 8)**	***P* value**
Age (years)	Age range	31-68	43-62	-
	Mean ± SEM	53.0 ± 6.9	53.0 ± 2.9	$P > 0.05$
Gender (F/M)		3/3	6/2	-
Post-mortem delay (hours)	Range	3-14	3-72	-
	Mean ± SEM	6.2 ± 1.7	25.2 ± 12.5	$P > 0.05$
Braak Stage (0-VI)		0-III	V-VI	-
Neuropathological Diagnosis		Control[#]	DS+AD*	-

*DS brains exhibited widespread deposition of senile plaques and neurofibrillary tangles. #Control cases showed no evidence of chromosomal or neuropathological abnormalities and displayed no signs of cognitive decline

Table 2-S2. List of primary antibodies used

Antibody Target	Antibody and Source	Epitope	Source / Company
APP total (22C11)	Mouse monoclonal Anti-APP	N- terminus of APP (66-81 aa)	Millipore, USA
sAPP-ß	Rabbit polyclonal to human soluble APP-ß	C-terminus of human sAPPβ-Wild Type	IBL, Germany
proNGF	Rabbit polyclonal to proNGF	Pro-domain of rat NGF (84-104 aa)	Alomone Labs, Israel
NGF (H-20)	Rabbit polyclonal to NGF	N-terminus of human NGF	Santa Cruz Biotechnology, USA
MMP-9	Rabbit polyclonal to MMP-9	Full-length MMP-9 native protein	Abcam, USA
TIMP-1 (7-6C1)	Mouse monoclonal to TIMP1	Bovine TIMP-1	Millipore, USA
Plasminogen (H-90)	Rabbit polyclonal to plasminogen	N-terminus of human plasminogen	Santa Cruz Biotechnology, USA
Neuroserpin	Rabbit polyclonal to neuroserpin	Human neuroserpin	Dr D. Lawrence, University of Michigan
tPA	Rabbit polyclonal to tPA	Human tPA	American Diagnostica
ß-Actin	Rabbit polyclonal to ß-actin	Residues 1-100 of human ß-actin	Abcam, USA

Table 2-S3. List of primer sequences used

mRNA analysis in human cortex

gene	Forward primer (5'-3')	Reverse primer (5'-3')
TIMP-1	TGACATCCGGTTCGTCTACA	TGCAGTTTTCCAGCAATGAG
NEUROSERPIN	GTAGCCGTGGCCAACTACAT	CCCTTGGGGATACCAAATCT
PLASMINOGEN	GCCCCATAGACACAGCATTT	TAGCACCAGGGACCACCTAC
TPA	GACGTGGGAGTACTGTGATGTG	CCCTCCTTTGATGCGAAACTGA
HPRT	TTGCTTTCCTTGGTCAGGCA	ATCCAACACTTCGTGGGGTC

mRNA analysis in mouse cortex

gene	Forward primer (5'-3')	Reverse primer (5'-3')
Mmp-9	GTCCAGACCAAGGGTACAGC	ATACAGCGGGTACATGAGGC
Timp-1	TCCTAGAGACACACCAGAGCA	AGCAACAAGAGGATGCCAGA
Neuroserpin	GATCCGGAGCAGTCTCAGC	GCCAGCAAAGCAAGCAGTTC
Plasminogen	ATAGCCCCAGAGTGGGTTCT	GAACATCCGACCCACGGATA
Hprt	CTGGTGAAAAGGACCTCTCG	TGAAGTACTCATTATAGTCAAGGGCA

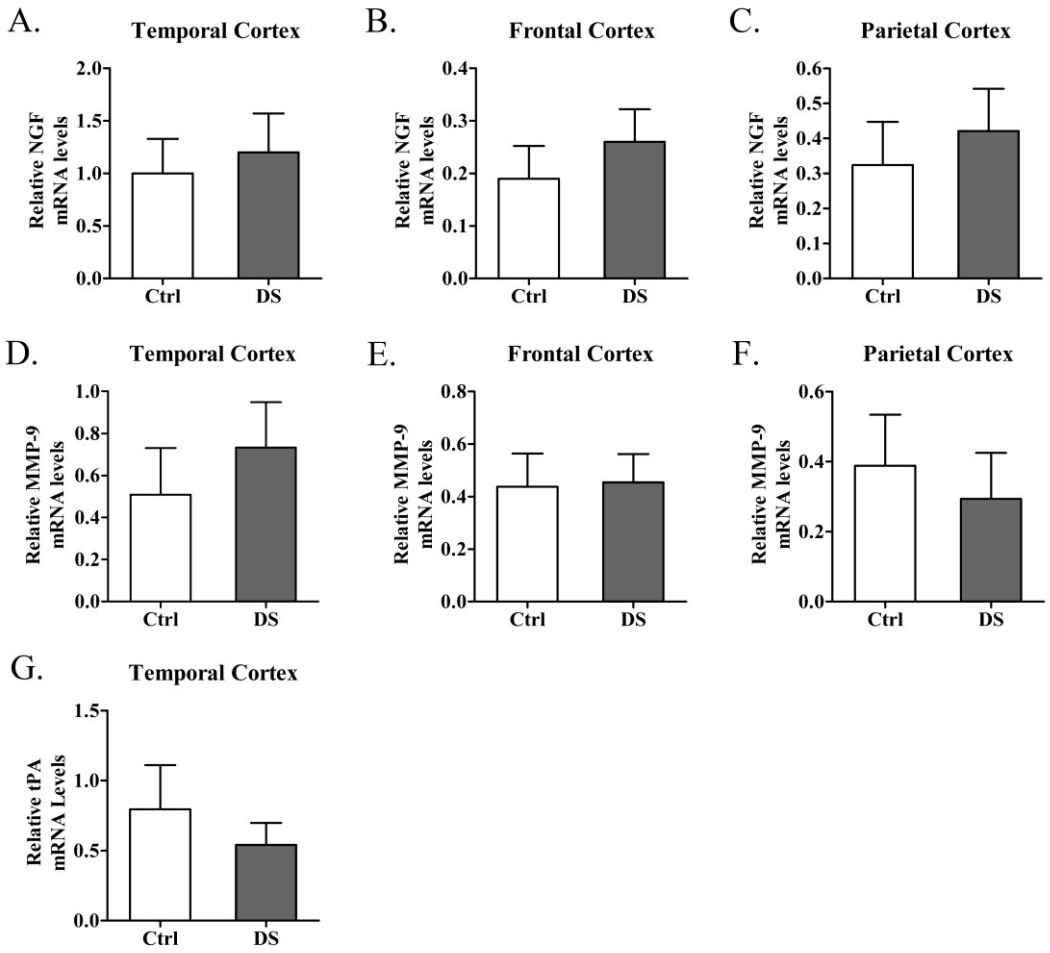

Figure 2-S1. Analysis of NGF, MMP-9 and tPA mRNA expression. qRT-PCR analysis of NGF mRNA levels (A-C), revealed no significant differences in Down's syndrome brains compared to control cases in A) temporal (n = 14), B) frontal (n = 34) and C) parietal (n = 20) cortex. D-F) MMP-9 expression levels were also similar between Down's syndrome and control cases in D) temporal, E) frontal and F) parietal cortex. G) tPA mRNA levels in temporal cortex were similar in control and Down's syndrome cases. Error bars represent mean ± SEM. $P >$ 0.05, Student's t test.

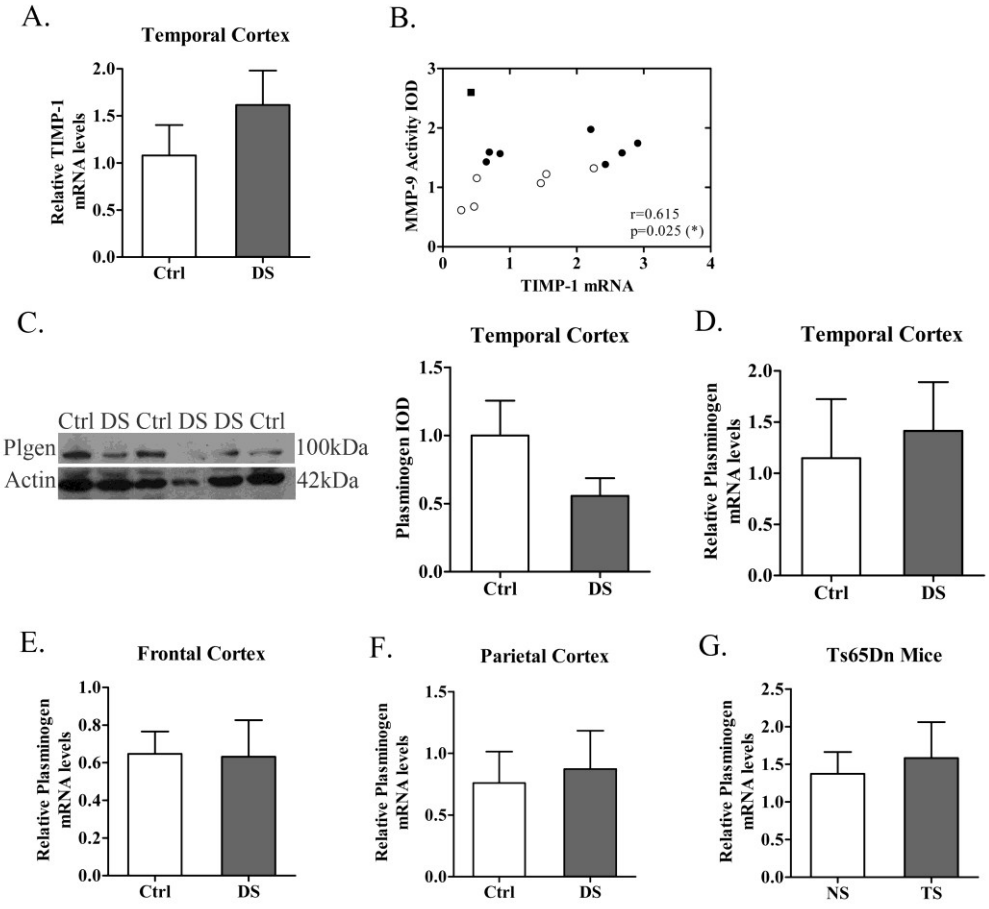

Figure 2-S2. Analysis of TIMP-1 and plasminogen expression. qRT-PCR analysis of TIMP-1 and plasminogen mRNA expression in cortical brain homogenates. A) TIMP-1 mRNA expression was not significantly different in Down's syndrome temporal cortex ($P > 0.05$). B) Scattergram depicting positive correlation between MMP-9 activity and TIMP-1 mRNA expression in temporal cortex ($r = 0.615$, $P = 0.025$), excluding one Down's syndrome case (dark square) with very high MMP-9 activity and a medical history of asthmatic bronchitis. C) Western blot analysis revealed a strong trend toward a reduction in plasminogen protein levels in Down's syndrome temporal cortex ($P = 0.138$). D-G) Plasminogen mRNA levels remained unchanged in D) temporal ($P > 0.05$), E) frontal ($P > 0.05$) and F) parietal cortex ($P > 0.05$) as well as in G) brains from Ts65Dn mice ($P > 0.05$). Error bars represent mean ± SEM.

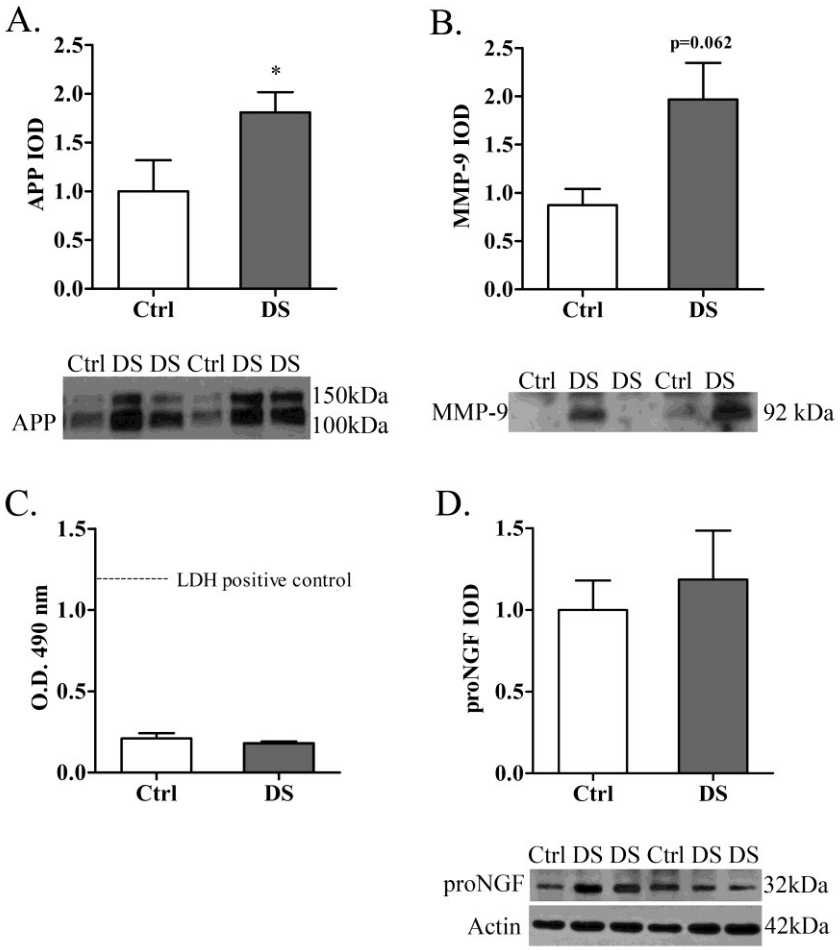

Figure 2-S3. APP, MMP-9 and proNGF levels in Down's syndrome fetal primary cortical cultures. Western blot analysis revealed higher A) soluble APP (P = 0.045) and B) MMP-9 (P = 0.062) protein levels in conditioned media from Down's syndrome cortical cultures. C) Comparable, low lactate dehydrogenase levels in conditioned media from Down's syndrome and control cultures (P > 0.05), as measured with a colorimetric enzymatic assay (CytoTox 96® Assay, Promega) and comparing it with an LDH positive control (supplied by manufacturer). D) No differences in proNGF protein levels in the intracellular fraction between control and Down's syndrome cell lysates (P > 0.05). Error bars represent mean ± SEM.

Connecting Text: Chapter 2 to 3

In Chapter 2 we have investigated whether individuals with DS exhibited deficits in NGF metabolism, similar those observed in AD brains. These studies have demonstrated that adult subjects with DS and dementia, manifested elevations in proNGF and in MMP-9 activity, together with reduced levels of plasminogen, tPA and increased neuroserpin. An important observation from this Chapter was the fact that the increase in proNGF, MMP-9 and the reduction in tPA were evident in primary cultures derived from DS fetal cortex. Since DS cultures also secreted higher levels of sAPP-β, $A\beta_{40}$ and $A\beta_{42}$ peptides compared to normal cultures, these observations suggested that NGF-metabolic deficits may arise early in the course of AD, before the development of advanced neuropathology.

If NGF dysfunction were an early consequence of AD progression, it would be desirable to monitor such changes in biological fluids, to assess signs of trophic dysfunction and prevent (or delay) cholinergic neurodegeneration. Therefore, as a continuation of the studies presented in Chapter 2 we next sought to establish whether the NGF metabolic pathway could be monitored in CSF, during the progression of clinical AD. At the time we started this research there were other reports on metallo-proteases and the plasminogen system in CSF; however, most of these studies had focused on AD patients and very few had examined these markers at previous stages, such as MCI. Therefore, our analysis included CSF derived from healthy, non-cognitively impaired controls, as well as subjects with amnestic MCI and individuals with AD dementia. The outcome of this investigation will be described in the subsequent chapter.

CHAPTER 3

Analysis of Matrix Metallo-Proteases and the Plasminogen System in Mild Cognitive Impairment and Alzheimer's Disease Cerebro-Spinal Fluid

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J Alzheimers Dis. 2014 Feb 14. [Epub ahead of print]

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3.1. Abstract

The expression of matrix metallo-proteases (MMP-2, MMP-3, MMP-7 and MMP-9), plasminogen and their regulators (TIMP-1, tissue plasminogen activator and neuroserpin) was investigated in cerebro-spinal fluid (CSF) from subjective cognitive impairment (SCI) subjects, mild cognitive impairment (MCI) and Alzheimer`s disease (AD) cases. ELISA analysis revealed a significant increase in MMP-3 protein levels in CSF from AD subjects, compared to age-matched SCI and MCI cases. No significant differences in MMP-2 and MMP-9 protein levels were detected between the three groups. MMP-7 was undetectable in all three groups. MCI individuals exhibited increased levels of the metallo-protease inhibitor TIMP-1 in CSF as well as higher plasminogen and neuroserpin expression, compared to SCI subjects. Levels of tPA were significantly reduced in AD CSF. Correlation analysis revealed a significant positive association between MMP-3, p-tau and total-tau levels. Conversely, there was a significant negative correlation between this protease and MMSE scores. tPA positively correlated with Aβ levels in CSF and with MMSE scores. Our results suggest that MMP-3 and tPA, in combination with current Aβ and tau biomarkers, may have potential as surrogate indicators of an ongoing AD pathology.

3.2. Introduction

Numerous clinico-pathological studies suggest that the neurochemical changes that lead to Alzheimer`s disease (AD) begin many years (or decades) before the first symptoms of cognitive decline (Jack *et al*, 2010; Sperling *et al*, 2011a; Bateman *et al*, 2012). Therefore, reliable biomarkers are essential for an early therapeutic intervention and to monitor disease progression.

Compared to blood, the CSF reflects changes in the brain more precisely, as it is in direct equilibrium with the extracellular CNS milieu. CSF amyloid-β (Aβ) and tau are useful markers to segregate healthy aged subjects from mild cognitive impairment (MCI) or AD cases (Hansson *et al*, 2006; De Meyer *et al*, 2010). Despite being accurate indicators of AD neuropathological hallmarks, there is often overlap between the three clinical groups. Therefore, the identification of novel CSF biomarker candidates is central for the development of more robust, early diagnostic platforms of AD.

Matrix metallo-proteases (MMPs) have been implicated in the pathogenesis of neurodegenerative diseases, including AD, multiple sclerosis and ischemia (Yong *et al*, 2001). Interestingly, recent studies showed that certain MMPs, in combination with the AD biomarkers Aβ and tau, are relevant for discrimination between vascular dementia and AD given their role in blood brain barrier permeability (Bjerke *et al*, 2011). MMPs are a family of zinc-dependent proteases which are involved in a variety of processes such as tissue remodelling, synaptic plasticity and protein degradation (Yong *et al*, 2001). Recent evidence has implicated MMPs in the regulation of other functions, including survival,

angiogenesis, inflammation and Aβ catabolism (Yong *et al*, 2001; Hernandez-Guillamon *et al*, 2010).

MMPs can be classified according to the substrates they digest; i.e. collagenases (MMP-1, MMP-8 and MMP-13), stromelysins (MMP-3 and MMP-10), matrilysin (MMP-7), and gelatinases (MMP-2 and MMP-9) (Snoek-van Beurden & Von den Hoff, 2005). Their physiological activity is regulated at the level of transcription (e.g. inflammatory stimuli can induce MMP expression) and also by endogenous MMP inhibitors referred as tissue inhibitors of metallo-proteases (TIMPs) (Rosenberg, 2009).

MMP-3 is an attractive candidate biomarker in the context of AD. It is significantly increased in AD brains and in plasma, compared to control subjects, localizing in close proximity to amyloid plaques (Yoshiyama *et al*, 2000; Horstmann *et al*, 2010). MMP-3 up-regulation by neurons occurs in response to apoptotic signals (Kim *et al*, 2005) and neuron-derived MMP-3 is a signal for microglia recruitment and activation *in vitro* and *in vivo*, leading to the production of classical pro-inflammatory stimuli (Kim *et al*, 2007). This is of great relevance as inflammation accompanies the early accumulation of AD pathological hallmarks in MCI and AD (Akiyama *et al*, 2000; Okello *et al*, 2009). Equally significant is the observation that MMP-3 has been shown to activate certain MMPs, including MMP-9 (Ogata *et al*, 1992). Increased MMP-9 activation occurs in AD and MCI brains (Bruno *et al*, 2009a; Bruno *et al*, 2009b) and its expression is primarily localized around amyloid plaques (Asahina *et al*, 2001).

The tPA/plasminogen system is widely known for its roles in thrombolytic initiation; however it also has important functions in the CNS; for review see (Melchor & Strickland, 2005). tPA is highly expressed in regions of the brain important for learning and memory such as the hippocampus and amygdala. It is released from neurons in an activity-dependent manner, resulting in a rapid, localized expression at the synapse (Bruno & Cuello, 2006). Its activity is tightly regulated by the endogenous inhibitors PAI-1 and neuroserpin (Miranda & Lomas, 2006).

Plasmin is a serine-protease produced from the zymogen plasminogen by the action of tPA. Plasmin has important roles in the CNS. It participates in Aβ catabolism and in pro-neurotrophin (proNGF and proBDNF) cleavage to its mature forms (Pang *et al*, 2004; Bruno & Cuello, 2006). Recent studies have demonstrated that in AD brains there is proNGF accumulation and a reduction in plasminogen, plasmin and tPA, suggesting an impaired proNGF maturation (Bruno *et al*, 2009a). Increased levels of neuroserpin, have also been reported in post-mortem AD brains (Fabbro & Seeds, 2009) and in Down's syndrome (Iulita *et al*, 2014).

Therefore, examining the CSF levels of metallo-proteases and the tPA / plasminogen system is relevant given their crucial role in neuroinflammation, blood brain barrier permeability, synaptic plasticity, and neurotrophin metabolism.

3.3. Materials and methods

3.3.1. Study population

The base population for the study consisted of referrals (due to memory complaints) to the Memory Clinic at the Karolinska University Hospital in

Huddinge, Sweden, between 2007 and 2009. Selection criteria for the study included lumbar puncture (LP) performed as a part of the clinical work-up, diagnosis of Alzheimer's disease according to the ICD-10 criteria, diagnosis of mild cognitive impairment according to the Winblad criteria (Winblad *et al*, 2004), or no objective cognitive impairment (subjective cognitive impairment, SCI). Patients with SCI were cognitively healthy as their cognition was normal based on neuropsychological examination. All individuals underwent a work-up assessment at the memory clinic, including patient history and history from proxy, medical examination including neurological and psychiatric assessments, MRI imaging, cognitive testing and lumbar puncture for CSF biomarkers. Cognitive screening was performed with the Mini-Mental State Examination (MMSE) (Folstein *et al*, 1975). Subjects were excluded if they presented any physical disease (i.e. congestive heart failure, diabetes mellitus, etc.) or severe depression significantly affecting cognitive performance or dementia due to diseases other than AD. A total of three subjects were taking NSAIDs during the work-up: two subjects in the SCI group and one in the MCI group (none in the AD group). A total of 15 subjects were taking acetylsalicylic acid: six in the SCI group, six in the MCI group and three in the AD group. Two subjects in the AD group were taking corticosteroids perorally. Two subjects had insufficient registration of current medication.

The demographic characteristics of the population studied are shown in Table 3-1. All subjects signed an informed consent prior to lumbar puncture. The study was approved by the regional Ethical review board of Stockholm (2011/680-31) and by the McGill University Review Board following the Canadian Tri-Council Policy on Ethical Conduct for Research Involving Humans.

Table 3-1. Clinical characteristics of SCI, MCI and AD groups

	n	Age (years)	P-Tau (ng/L)	Total tau (ng/L)	$A\beta_{42}$ (ng/L)	MMSE Score	Gender
SCI	31	59±5	53±20	242±96	1140±153	29±2	20 F / 11 M
MCI	23	69±11	56±22	293±118	635±246	27±2	18 F / 5 M
AD	32	69±9	102±40	705±368	491±123	22±3	21 F / 11 M

The data is presented as mean ± SD. SCI=subjective cognitive impairment; MCI= mild cognitive impairment; AD= Alzheimer's disease. MMSE= Mini-Mental State Examination.

3.3.2. CSF analysis

LP was performed in a standardized manner with the patient in a sitting position, between 8 and 12 am to avoid bias by possible circadian fluctuation of CSF biomarkers. The tap was performed with a non-traumatic cannula placed in the intervertebral space L3 / L4 or L4 / L5. A small amount of CSF was used for routine analysis, including total cells (leucocytes and erythrocytes), total protein, and glucose. CSF was aliquoted in polypropylene tubes of 0.5 or 1 ml and stored at –70 °C until further analysis. CSF $A\beta_{42}$, total tau (t-tau), and phosphorylated tau (p-tau) were measured with commercially available sandwich ELISAs (INNOTEST®, Innogenetics, Ghent, Belgium). No significant differences in total protein levels were found between the three clinical groups (mean ± SEM; 375 ± 23 ng/µl SCI, 365 ± 26 ng/µl MCI, 338 ± 16 ng/µl AD), as determined by the Bradford assay (BioRad, USA) and analyzed by One-way ANOVA followed by Bonferroni post-hoc test.

3.3.3. Western blot analysis

20 µl of CSF were loaded on 10-12% sodium dodecyl sulfate (SDS)-polyacrylamyde gels and wet-transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for one hour before incubation with the primary antibody overnight at 4°C. The following primary antibodies were used: rabbit anti-plasminogen (H-90), 1:1000; rabbit anti-tissue inhibitor of metalloproteinase 1 (TIMP-1) (H-150), 1:500 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit anti-matrix-metalloproteinase-9 (MMP-9) 1:1000 (Abcam, Ontario, Canada), rabbit anti-

neuroserpin, 1:5000 (provided by Dr. D Lawrence, University of Michigan, USA) and rabbit anti-MMP-7 1:2000 (Novus Biologicals, USA). Membranes were subsequently washed in TBS-T and incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies 1:10000 (Jackson ImmunoResearch, West Grove, PA, USA) in 5% non-fat milk in TBS-T for 90 minutes. Immunoreactive bands were visualized with ECL prime (GE Healthcare Life Sciences, Baie d'Urfe, Qc, Canada) using either Kodak Biomax XAR imaging film kit with exposure times varying from 5 to 20 minutes or a Storm Phosphoimager 840 (Molecular Dynamics, Sunnyvale, CA, USA). All experiments were performed at least in triplicate. The software Gel-ProPlus (Media Cybernetics Inc., Rockville, USA) was used to quantify the optical density of each band.

3.3.4. Gelatin zymography

20 µl of CSF samples were resolved on 8% (SDS)-polyacrylamyde gels containing 0.1% gelatin. After electrophoresis on ice, the gel was incubated in zymogram renaturing buffer (Triton X-100, 2.5% vol/vol in water) with gentle agitation during 45 minutes at room temperature. Gels were further incubated 45 h at 37°C in developing buffer (50 mmol/L Tris-HCl, 0.2 mol/L NaCl, 5 mmol/L $CaCl_2$, 0.02% Brij 35, pH 7.6). The gels were finally stained with 2.5% Coomassie blue R-250 (in methanol: acetic acid: water (50:10:40)) for 30 minutes. Areas of metallo-protease activity appeared as clear bands against a dark blue background after incubation of the gel with the destaining solution (methanol: water: acetic acid; 50:40:10). All experiments were performed at least in triplicate. The software Gel-

ProPlus (Media Cybernetics Inc., Rockville, MD, USA) was used to quantify the optical density of each band.

3.3.5. MMPs ELISA

Levels of MMPs -2, -3 and -7 were assessed with commercially available sandwich ELISAs (R&D Systems, Minneapolis, MN, U.S.A), according to manufacturer's instructions. Briefly, 50 - 100 µl of CSF were diluted 1:1 in assay diluent and further incubated in the coated wells for 2 hours before adding an HRP-conjugated polyclonal antibody mixture. Four washes were done in between each step. Subsequently, a peroxidase-conjugated substrate was added followed by a stop solution before reading the plates at 450 nm. A standard curve was run in parallel and results are expressed as ng/ml. Minimum detectable levels ranged from 0.016-0.289 ng/ml for MMP-2; 0.002-0.045 ng/ml for MMP-3 and 0.005-0.094 ng/ml for MMP-7.

3.3.6. tPA ELISA

Quantitative measurement of tPA levels in 100 ul CSF was done with a human tPA total antigen ELISA kit, following manufacturer's instructions (Molecular Innovations, USA). Briefly, tPA in CSF was captured by a primary antibody coated on the plate and following appropriate washing, a second anti-human tPA primary antibody was added to react with the captured tPA protein. After this incubation, a secondary antibody conjugated to HRP was applied. Following an additional washing step, the TMB substrate was used for color development at 450 nm. A calibration curve with human tPA standard protein was included. Absorbance was read at 450 nm. This assay measures tPA protein levels in the 0.02-10 ng/ml range.

3.3.7. Data analysis

Data was analyzed using the Graph-Pad Prism 5 software (LaJolla, CA, USA). Unpaired Student's *t* test was performed for two group comparisons. One-way ANOVA followed by Bonferroni's post-hoc tests were performed for multiple group comparison. Spearman rank correlation coefficients (*r*) between the markers are reported. Results are expressed as mean ± SEM. A probability (*P*) value <0.05 was considered statistically significant.

3.4. Results

3.4.1. MMPs and TIMP-1 determination in CSF of SCI, MCI and AD groups

As shown in Figure 3-1 A, MMP-3 protein levels were significantly increased in AD CSF, compared to SCI and MCI cases (One-way ANOVA, Bonferroni's Multiple Comparison Test; $P < 0.05$). The AD group had a 4.6 fold increase in MMP-3 protein compared to SCI and MCI groups, which exhibited almost undetectable MMP-3 levels (Figure 3-1 A). MMP-7 protein levels were undetectable by Western blotting and ELISA in CSF samples from all three groups examined (data not shown).

No differences in MMP-9 (Figure 3-1 B) and MMP-2 (Figure 3-1 C) levels were found between the three groups. In order to determine whether these MMPs, 9 and 2, had changes in their proteolytic activity, a gelatin zymography was performed. While MMP-9 zymogenic activity was undetectable across the groups, MMP-2 activity was clearly visualized as a white band (~70 kDa) against the dark background of the zymogram (Figure 3-1 D). There were no statistically significant

changes for MMP-2 activity along the groups, as analyzed by One-way ANOVA (Figure 3-1 D).

TIMP-1 is the main inhibitor of MMP-9 but it also binds and inactivates MMP-3 (Ogata *et al*, 1995). To further characterize whether the increase in MMP-3 protein was accompanied by changes in the levels of its inhibitor, the content of TIMP-1 was investigated by Western blotting. As shown in Figure 3-2, a significant increase in TIMP-1 was observed in MCI CSF, compared to CSF from SCI subjects. No significant changes in the levels of TIMP-1 were seen in AD (Figure 3-2 A). We next determined the ratio between MMP-3 and TIMP-1 protein levels and observed a higher MMP-3 / TIMP-1 ratio in the AD group compared to both MCI and SCI cases, but no apparent significant difference between MCI and SCI (Figure 3-2 B).

To further study if MMP-3 levels were associated with classical AD biomarkers, we performed correlation analysis between MMP-3 protein levels and age, MMSE scores, p-tau, t-tau and $A\beta_{42}$ levels (Table 3-2). MMP-3 protein content showed a significant positive correlation with p-tau ($r = 0.55$, $P < 0.0001$; Figure 3-3 A) and t-tau ($r = 0.58$, $P < 0.0001$; Figure 3-3 B) levels. Notably, we also found a significant negative correlation between MMP-3 and MMSE scores ($r = -0.36$, $P < 0.05$; Figure 3-3 C). No significant correlation was found with $A\beta_{42}$ (Table 3-2). A summary of the correlations between MMP-3 and the clinico-pathologic data is shown in Table 3-2.

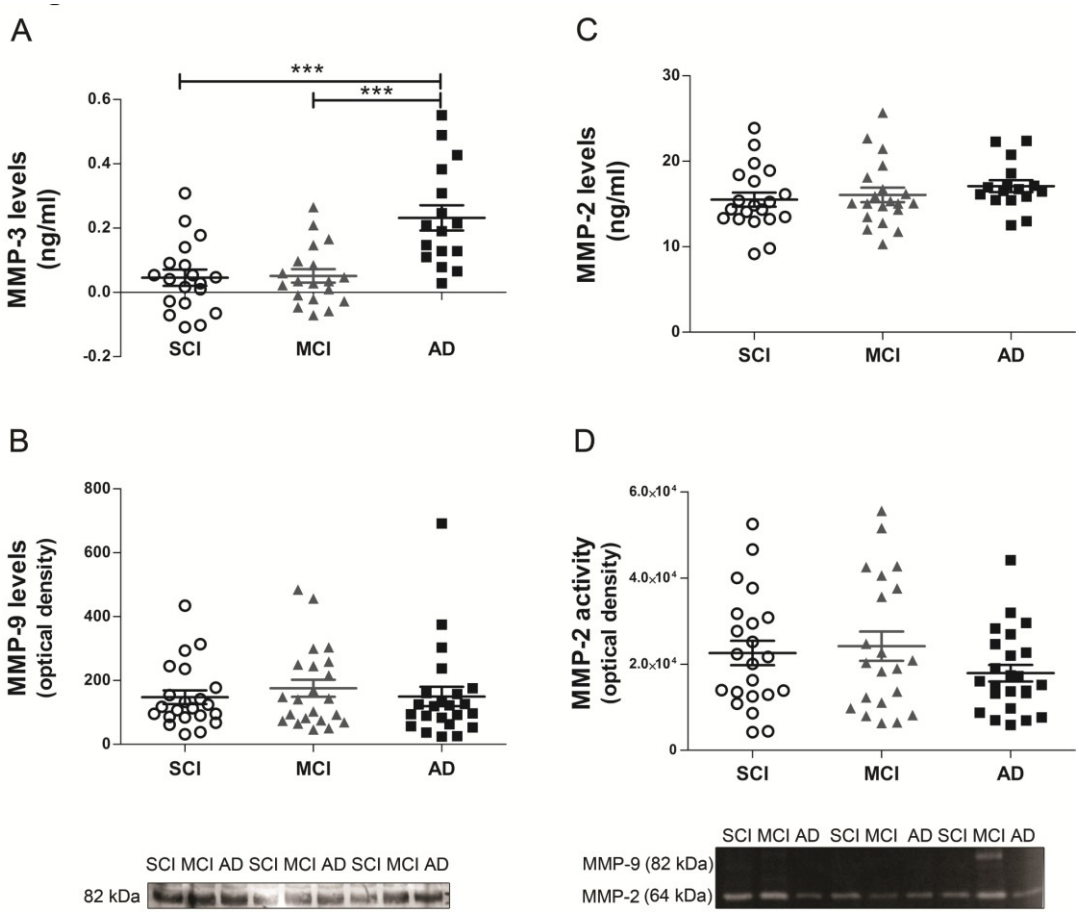

Figure 3-1. Analysis of matrix metallo-protease levels in CSF. A) Increased MMP-3 protein levels (~5 fold) in CSF from AD cases, compared to SCI ($P < 0.001$) and MCI ($P < 0.01$) subjects, determined by ELISA. B) Western blot analysis revealed no significant differences in MMP-9 protein levels between the three clinical groups ($P > 0.05$). C) Comparable MMP-2 protein content between SCI, MCI and AD CSF, assessed by ELISA ($P > 0.05$). D) Gelatin zymography analysis revealed no difference in MMP-2 zymogenic activity across the groups ($P > 0.05$). MMP-9 activity was not detectable and could not be quantified. *** $P < 0.001$; One-way ANOVA, Bonferroni's Multiple Comparison Test. Error bars represent mean ± SEM, n = 16-24/group.

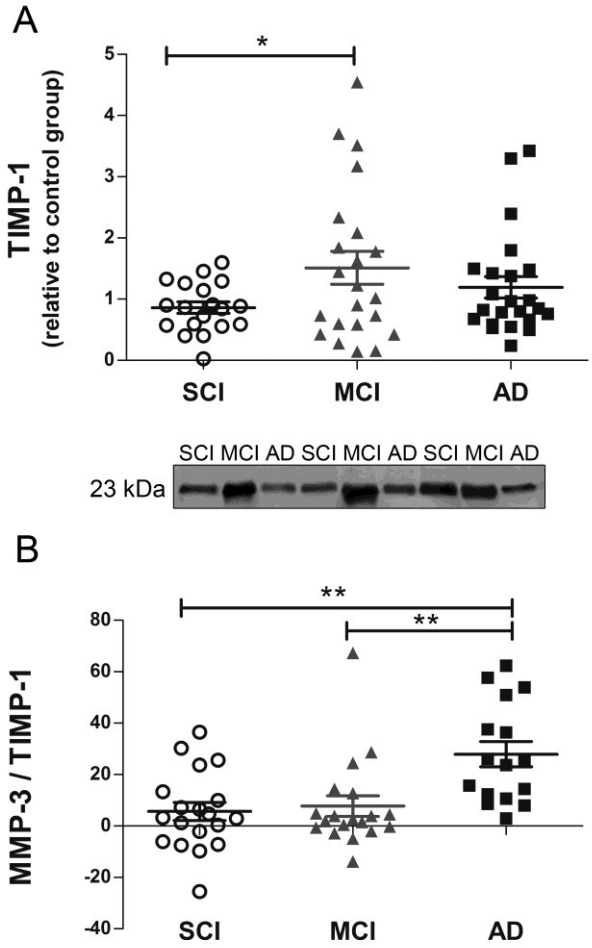

Figure 3-2. Assessment of TIMP-1 levels in CSF. A) Western blot analysis revealed significantly higher (~1.8 fold) TIMP-1 protein levels in CSF from MCI cases ($P < 0.05$), compared to SCI and AD subjects. A trend reflecting higher (~1.4 fold) TIMP-1 content in AD CSF was also evident. B) AD cases exhibited significantly higher MMP-3/TIMP-1 ratios compared to CSF from SCI (~5 fold; $P < 0.01$) and MCI (~3.6 fold; $P < 0.01$) subjects. * $P < 0.05$; ** $P < 0.01$; One-way ANOVA, Bonferroni's Multiple Comparison Test. Error bars represent mean ± SEM, n = 16-23/group.

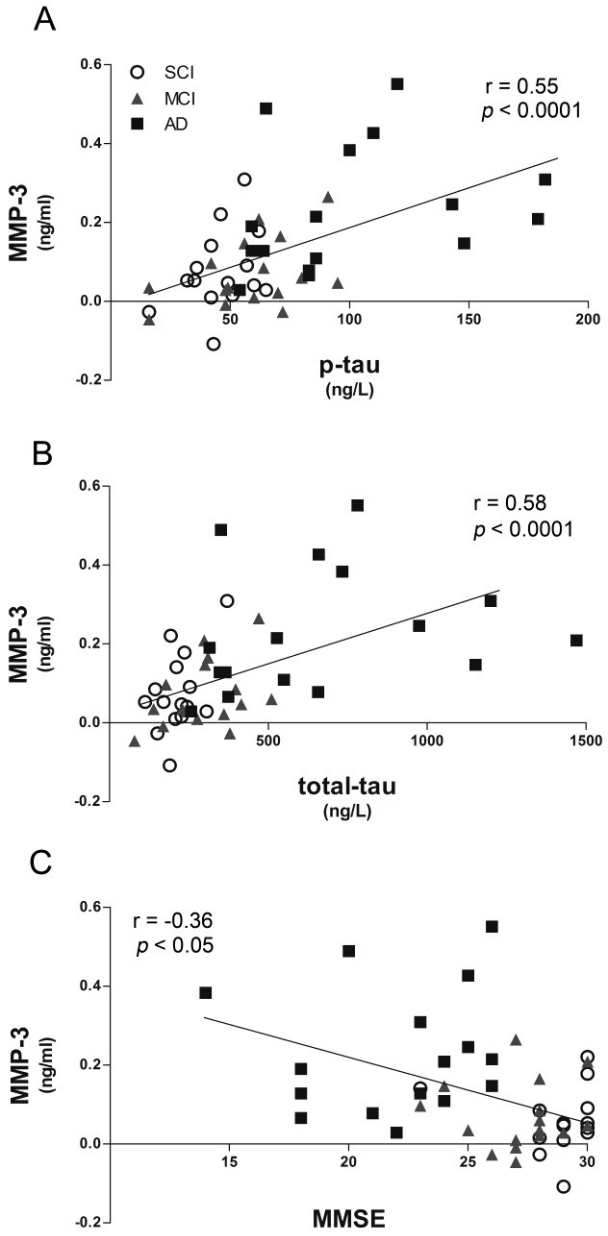

Figure 3-3. Correlation between CSF MMP-3 levels and clinico-pathological biomarkers. A-C) Scattergrams depicting significant positive correlations between MMP-3 and A) p-tau levels (r = 0.55; $P < 0.0001$) and with B) total tau (r = 0.58; $P < 0.0001$). C) Negative correlation between CSF MMP-3 levels and MMSE scores (r = -0.36; $P < 0.05$).

3.4.2. Analysis of plasminogen, tPA and neuroserpin levels in CSF

We then examined plasminogen levels in CSF by Western blotting. MCI cases exhibited significantly elevated (~2.5 fold) plasminogen protein levels compared to SCI subjects (One-way ANOVA, Bonferroni's Multiple Comparison Test $P < 0.01$) (Figure 3-4 A). AD cases exhibited a trend but no apparent significant increase in plasminogen CSF levels (Figure 3-4 A). No significant correlation was found between plasminogen and Aβ, p-tau or MMSE scores. There was a mild but significant negative association with t-tau levels ($r = -0.24$, $P < 0.05$) (Table 3-2).

In the CNS, the activity of tPA is regulated by neuroserpin, which is up-regulated in AD brains (Fabbro & Seeds, 2009). We therefore investigated whether MCI or AD cases exhibited detectable changes in neuroserpin levels in CSF. Western blot analysis revealed a significant up-regulation of neuroserpin in MCI CSF (One-way ANOVA, Bonferroni's Multiple Comparison Test; $P < 0.05$) (Figure 3-4 B). Conversely, in AD, neuroserpin levels were not significantly different from SCI cases. Neuroserpin did not correlate with clinical or pathological AD biomarkers.

ELISA analysis revealed a significant reduction in total tPA protein levels in CSF from AD cases (One-way ANOVA, Bonferroni's Multiple Comparison Test $P < 0.01$) and a clear trend in the same direction for subjects with MCI (Figure 3-5 A). tPA levels positively correlated with $A\beta_{42}$ levels in CSF ($r = 0.43$, $P < 0.01$) and MMSE scores ($r = 0.49$, $P < 0.01$) (Figures 3-5 B and 3-5 C, respectively). There was no evident association between tau biomarkers (t-tau or p-tau) and CSF tPA levels (Table 3-2), as well as no correlation between tPA and MMP-3 levels ($r = -0.05$, $P = 0.76$).

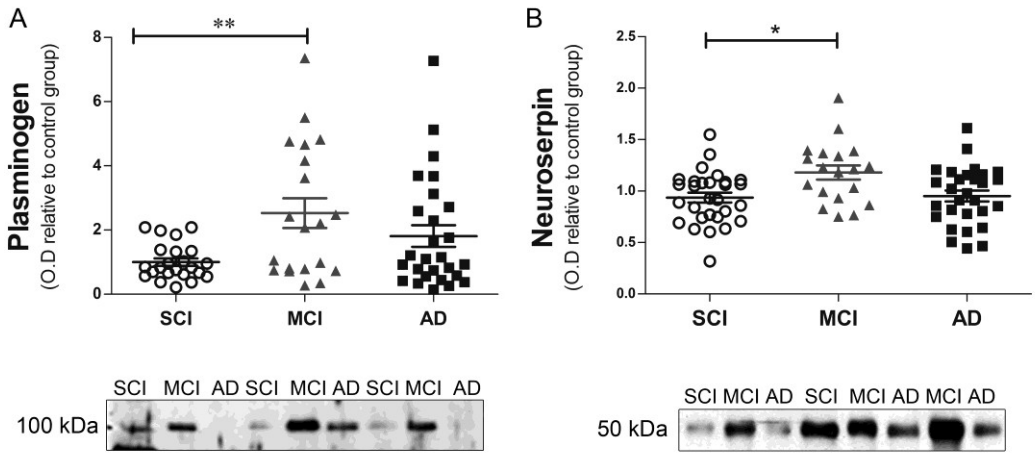

Figure 3-4. Analysis of plasminogen and neuroserpin levels in CSF. A) MCI cases exhibited significantly higher plasminogen levels in CSF (~2.5 fold), compared to SCI and AD subjects ($P < 0.01$), determined by Western blotting. A trend reflecting higher plasminogen protein content (~1.8 fold) was also evident in the AD group ($P > 0.05$). B) Western blot analysis revealed higher (~1.3 fold) neuroserpin protein levels ($P < 0.05$) in CSF from MCI cases, compared to SCI and AD subjects. No significant changes in neuroserpin expression were apparent in AD CSF. ** $P < 0.01$, One-way ANOVA, Bonferroni's Multiple Comparison Test. Error bars represent mean ± SEM, n = 20-29/group.

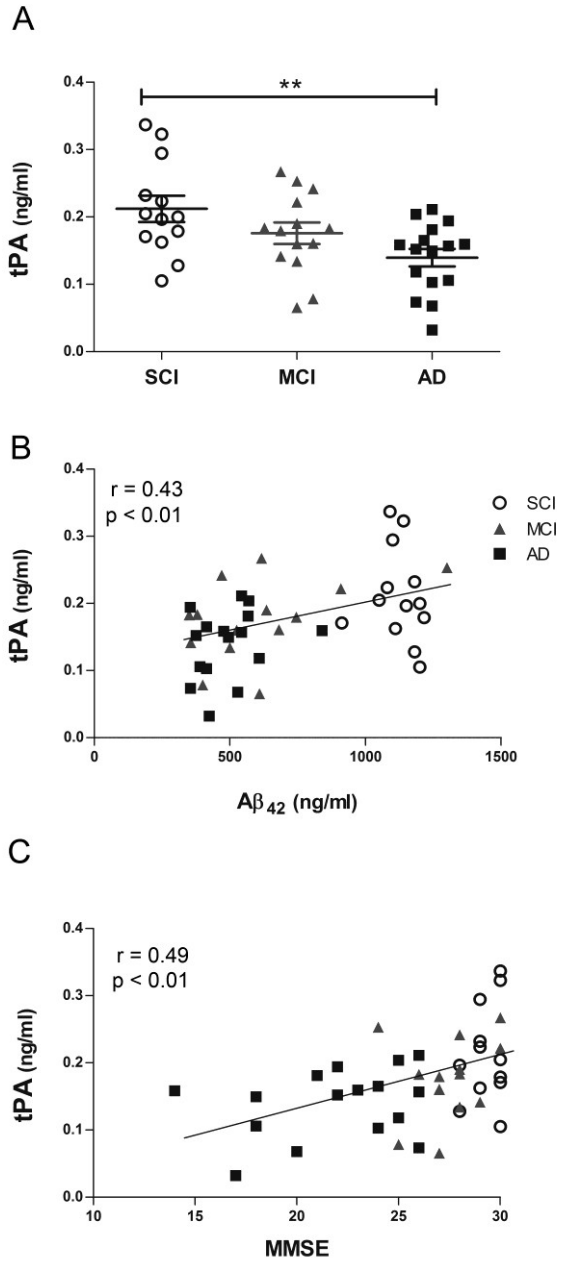

Figure 3-5. Determination of tPA levels in CSF and correlation with clinico-pathological biomarkers. A) ELISA analysis revealed a significant reduction (~30%) in tPA protein levels in CSF from AD subjects ($P < 0.01$), compared to SCI cases. A modest, not significant reduction was evident in MCI CSF (~17% decrease; $P > 0.05$). B-C) Scattergrams depicting significant positive correlations between tPA levels and B) $A\beta_{42}$ in CSF ($r = 0.43$; $P < 0.01$) as well as C) MMSE scores ($r = 0.49$; $P < 0.01$). ** $P < 0.01$, One-way ANOVA, Bonferroni's Multiple Comparison Test. Error bars represent mean ± SEM, n =13-16/group.

Table 3-2. Correlation between biochemical and clinical data for SCI, MCI, AD groups

		P-Tau (ng/L)	Total tau (ng/L)	MMSE	$A\beta_{42}$ (ng/L)	TIMP-1
MMP-3	Correlation*	0.55	0.58	-0.36	-0.06	-0.18
	Significance	< 0.0001	< 0.0001	< 0.05	0.69=ns	0.23=ns
Plasminogen	Correlation*	-0.19	-0.24	0.06	-0.17	0.35
	Significance	0.11=ns	<0.05	0.63= ns	0.14=ns	< 0.01
tPA	Correlation*	-0.18	-0.27	0.49	0.43	-0.23
	Significance	0.25=ns	0.08=ns	<0.01	<0.01	0.24=ns

*Spearman r correlation coefficients; ns= not significant

3.5. Discussion

In the present study we examined the levels of several MMPs (MMP-2, -3, -7 and -9) in CSF samples from AD, MCI and SCI subjects. We have found a significant up-regulation of MMP-3 protein levels in AD CSF, compared to the other clinical groups. Correlation analysis revealed strong associations between MMP-3 and markers of neurodegeneration (tau) (Blennow *et al*, 1995) and cognitive decline (MMSE scores), suggesting a role for MMP-3 as a marker of an ongoing neurodegenerative process.

Our findings confirm and extend previous studies reporting an elevation of MMP-3 levels in CSF and serum of AD patients (Horstmann *et al*, 2010). This up-regulation is likely a reflection of the elevation of MMP-3 protein seen in AD brains; particularly around senile amyloid plaques (Yoshiyama *et al*, 2000). In addition, MMP-3 was found elevated in CSF from cognitively healthy individuals with AD risk markers (defined by the presence of an ApoE ε4 allele and CSF biomarkers within the MCI-AD range), compared to healthy subjects without risk markers (Stomrud *et al*, 2010).

Several studies on CNS diseases like ischemia, multiple sclerosis and AD suggest a role for MMP-3 in neurodegeneration. MMP-3 has been shown to be produced and released mainly from stressed neurons (e.g. under ischemic conditions, apoptosis, oxidative stress) (Sole *et al*, 2004; Kim *et al*, 2005; Kim *et al*, 2007). Furthermore, treatment of microglial cells with MMP-3 induced the expression and release of classical inflammatory markers such as TNF-α, IL-6, IL-1β and IL-1 receptor antagonist (Kim *et al*, 2005). Similarly, the intra-cerebral administration of TNF-α

resulted in increased expression and activation of MMP-3 in naïve rats (Candelario-Jalil *et al*, 2007). These findings support a role for MMP-3 in inflammation; not only as a neuronal stress marker; but also as a signal for microglia activation.

Neuroinflammation is a well-known, pathological component of AD brains (Akiyama *et al*, 2000; Block *et al*, 2007). Transgenic animal models also illustrate that inflammation is one of the earliest events in the progression of the AD-like amyloid pathology, occurring both in transgenic mouse (Tehranian *et al*, 2001; Ferretti & Cuello, 2011; Ferretti *et al*, 2012a; Ferretti *et al*, 2012b) and rat models (Hanzel *et al*, 2014b). Such an early pro-inflammatory process probably starts many years before the diagnosis of AD or MCI, as it occurs at pre-plaque stages in transgenic models. Support for an early inflammatory component in AD is also provided by analysis of CSF samples, revealing increased levels of the soluble IL-1 receptor type II in cases with mild to moderate AD (Garlind *et al*, 1999). Such increase may reflect a negative feedback response to counteract an early up-regulation of the pro-inflammatory cytokine IL-1. In addition *in situ* studies on human subjects using PET-ligands indicate activation of astrocytes (Carter *et al*, 2012) and microglia (Yasuno *et al*, 2012) during prodromal AD. We therefore propose that the increase in MMP-3 is likely due to a combination of the neurodegenerative and inflammatory processes characteristic of AD brains. Our correlation analysis reflecting positive associations between MMP-3, tau and MMSE would support such a proposition.

In contrast to the striking MMP-3 elevation, levels of MMP-7 in CSF were undetectable by Western blotting and ELISA, consistent with other reports (Leppert *et al*, 1998; Shukla *et al*, 2013). Moreover, no significant changes in the levels of MMP-9 and MMP-2 could be detected, a finding that is in agreement with other CSF studies (Lorenzl *et al*, 2003; Adair *et al*, 2004; Mlekusch & Humpel, 2009; Horstmann *et al*, 2010; Stomrud *et al*, 2010). MMP-9 enzymatic activity is highly susceptible to deactivation over time and during thaw-freezing cycles, therefore we cannot rule out a possible increased MMP-9 activation in AD CSF. The study of MMP-9 activity warrants further investigation as its activity is highly elevated in AD and MCI brains (Bruno *et al*, 2009b).

In AD brains, there is also a well-established accumulation of proNGF which is likely due to deficits in its extracellular metabolism (Bruno *et al*, 2009a). ProNGF is converted to mature NGF by plasmin, which derives from plasminogen by the action of tPA. The levels of plasminogen and activity of plasmin are markedly reduced in AD (Ledesma *et al*, 2000; Bruno *et al*, 2009a). Interestingly, plasminogen levels were significantly increased in CSF from MCI cases. Similar observations have been reported in CSF from asymptomatic familial Alzheimer's disease (FAD) mutation carriers (~1.5 fold up-regulation) compared to non-carriers (Ringman *et al*, 2012b). The increase in plasminogen seems to flatten with disease progression, as we have seen a less prominent accumulation in AD CSF. Therefore, these observations may suggest that plasminogen might be an early marker with promise for the detection of an early AD pathology accumulation.

In line with the increase in plasminogen in MCI, we also found a small but significant increase in neuroserpin levels in the same group, but no difference between AD and SCI cases. Neuroserpin is a serine protease which is increased in AD brains (Fabbro & Seeds, 2009). Its role in the CNS is still not completely understood; some studies suggest that neuroserpin is an amyloid plaque-associated protein with neuroprotective properties, which binds $A\beta_{42}$ peptides and prevents the formation of amyloid fibrils (Kinghorn *et al*, 2006). Conversely, neuroserpin knock-out studies in the J20-APP AD transgenic mouse model have demonstrated a reduction in Aβ peptides and a concomitant improvement of AD-related cognitive deficits (Fabbro *et al*, 2011). In line with these observations, Nielsen et al. have reported a mild increase in neuroserpin levels (~25% up-regulation) in CSF from AD patients (but not in Lewy Body dementia), compared to controls. As well, neuroserpin levels positively correlated with tau CSF biomarkers, suggesting a potential role for this inhibitor as a marker of neurodegeneration in AD (Nielsen *et al*, 2007).

Furthermore, our analysis revealed a significant reduction in tPA levels in CSF from AD cases, with a clear trend in the same direction in MCI. Besides its role in plasminogen cleavage and plasmin generation, tPA has been shown to be important for synaptic plasticity and long term potentiation in the CNS (Pang *et al*, 2004; Melchor & Strickland, 2005). Recent evidence indicates that endogenous tPA is neuroprotective for stressed neurons under hypoxic conditions or in the ischemic brain (Haile *et al*, 2012; Wu *et al*, 2013). tPA activity is reduced in brains from transgenic mice over-expressing APP, likely due to a concomitant up-regulation of

PAI-1 expression, a serine protease inhibitor similar to neuroserpin (Melchor *et al*, 2003; Cacquevel *et al*, 2007). The expression of this inhibitor could be induced by Aβ peptides injected in the hippocampus (Melchor *et al*, 2003). Notably, in our study, CSF tPA levels positively correlated with CSF $A\beta_{42}$ and MMSE scores, supporting a role for tPA reduction as a contributor to the neurodegenerative process characteristic of AD.

A further interesting finding of our study constitutes the increased TIMP-1 protein content observed in MCI CSF, with a similar trend observed in AD. In agreement with this observation previous studies have found higher TIMP-1 protein content in CSF from AD patients (Lorenzl *et al*, 2003). The increase in TIMP-1 levels in MCI and AD could be attributed to a counter-reactive, compensatory response to the elevation of MMP-3 levels. TIMP-1 binds non-covalently to the active form of MMP-3 at a molar ratio of 1:1 (Okada *et al*, 1988; Hornebeck *et al*, 2005) and can attenuate MMP-3 activity (Kim *et al*, 2010). It is possible that the state of increased inflammation in AD increases TIMP-1 levels, since raised levels of this protein are associated with inflammatory activation *in vitro* (Roeb *et al*, 1994) and with disorders with a pronounced chronic inflammatory component such as rheumatoid arthritis (Yoshihara *et al*, 1995).

Importantly, none of the markers investigated in this study showed a correlation with the age of subjects; suggesting that the changes reported were not confounded by the mild difference in age between SCI and MCI / AD subjects.

3.6. Concluding remarks

In the present study we have investigated potential early biomarkers of AD, which are related to inflammatory and neurodegenerative processes, as well as proNGF metabolism. Interestingly, significant changes in most of these biomarkers: TIMP-1, plasminogen and neuroserpin were found in MCI CSF, suggesting a possible role for these molecules as early indicators of AD pathology. However, MMP-3 and tPA were found altered in AD CSF. Such change would hint at a more advanced stage, related to an ongoing neurodegenerative process. Future studies are warranted, as these biomarkers should be further validated in other clinical groups (e.g. FAD cases) for the detection and prediction of AD progression, in conjunction with well-established markers of disease such as Aβ, p-tau, and t-tau. In addition, it would be of interest to assess the specificity of these putative biomarkers in other types of neurodegenerative conditions such as vascular dementia, tauopathies or Parkinson dementia. The influence of early inflammation on the plasminogen system and on metallo-proteases provides a framework for future studies on NGF maturation and degradation, which may lead to a better understanding of the pathogenesis of AD.

3.7. Acknowledgements

C.E.H is the holder of a CIHR Neuroinflammation Training Program postdoctoral fellowship. M.F.I is the recipient of a Biomedical Doctoral Award from the Alzheimer Society of Canada. A.C.C is the holder of the Charles E. Frosst/Merck-endowed Chair in Pharmacology and a member of the Canadian Consortium of Neurodegeneration in Aging. This work was funded by CIHR grants (MOP- 97776 and MOP-102752). We also acknowledge the support from the Swedish Research Council (072194 (MS)), the Regional agreement on medical training and clinical research (ALF) between Stockholm County Council and the Karolinska Institutet, the Swedish Brain Power consortium, Gustaf V and Queen Victorias Freemason foundation and the Swedish Alzheimer Foundation. The authors wish to thank Dr. Daniel Lawrence (University of Michigan, USA) for the generous provision of anti-neuroserpin antibodies. The ACC laboratory is grateful for the unrestricted support received from Dr A. Frosst, the Frosst family and Merck Canada.

Connecting Text: Chapter 3 to 4

The investigations described in previous chapters revealed that the study of DS brains is highly valuable for the examination of molecular changes associated with the development of AD pathology. When considering the sequence of events that lead to AD, the intracellular accumulation of Aβ peptides is well supported as one of the earliest changes, before the development of amyloid plaques. However, the existence and pathological relevance of intracellular Aβ remain an enigmatic topic and a matter of controversy in the field. We therefore considered that in order to have a good understanding of the early Alzheimer's pathology this issue deserved a new, multidisciplinary investigation.

CHAPTER 4

Intracellular Aβ Pathology and Early Cognitive Impairments in a Transgenic Rat Model Overexpressing Human Amyloid Precursor Protein: a Multidimensional Study

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Acta Neuropathol Commun. 2014 Jun 5;2(1):61

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4.1. Abstract

Numerous studies have implicated the abnormal accumulation of intraneuronal Aβ as an important contributor to AD pathology, capable of triggering neuroinflammation, tau hyperphosphorylation and cognitive deficits. However, the occurrence and pathological relevance of intracellular Aβ remain a matter of controversial debate. In this study, we have used a multidimensional approach including high-magnification and super-resolution microscopy, CSF mass spectrometry analysis and ELISA to investigate the Aβ pathology and its associated cognitive impairments, in a novel transgenic rat model overexpressing human APP. Our microscopy studies with quantitative co-localization analysis revealed the presence of intraneuronal Aβ in transgenic rats, with an immunological signal that was clearly distinguished from that of APP and its C-terminal fragments (CTFs). The early intraneuronal pathology was accompanied by a significant elevation of soluble $A\beta_{42}$ peptides that paralleled the presence and progression of early cognitive deficits, several months prior to amyloid plaque deposition. $A\beta_{38}$, $A\beta_{39}$, $A\beta_{40}$ and $A\beta_{42}$ peptides were detected in the rat CSF by MALDI-MS analysis even at the plaque-free stages; suggesting that a combination of intracellular and soluble extracellular Aβ may be responsible for impairing cognition at early time points. Taken together, our results demonstrate that the intraneuronal development of AD-like amyloid pathology includes a mixture of molecular species (Aβ, APP and CTFs) of which a considerable component is Aβ; and that the early presence of these species within neurons has deleterious effects in the CNS, even before the development of full-blown AD-like pathology.

4.2. Introduction

Alzheimer's disease (AD) remains the most common form of age-related dementia, a disorder which affects approximately 36 million sufferers worldwide (Prince *et al*, 2013a). Recent studies have revealed the existence of a long asymptomatic phase, where the pathological changes leading to AD begin –at least- decades before the first symptoms of cognitive decline appear (Bateman *et al*, 2012; Jack *et al*, 2013; Villemagne *et al*, 2013).

Although these studies have addressed the temporal order in which AD pathological hallmarks appear, the exact sequence of cellular and molecular events that lead to AD is still poorly understood. However, it is widely accepted that before the overt deposition of amyloid plaques and neurofibrillary tangles, the accumulation of amyloid-β peptides is one of the first steps in the series of pathogenic changes that lead to neurodegeneration and dementia (Hardy & Higgins, 1992; Karran *et al*, 2011).

The concept of a pre-clinical, asymptomatic phase of AD is gaining increasing support. This is best evidenced by the recent revision of research diagnostic criteria which now include a new detailed framework for the diagnosis and pre-clinical staging of individuals at-risk (Sperling *et al*, 2011a). Notably, little is known about this early phase, as most studies of the AD pathology center on moderate-to-late stages. This pre-clinical period deserves further attention, as it should offer a critical window for successful treatment.

Given the lack of definitive AD biomarkers in humans, transgenic animal models of the amyloid pathology continue to be valuable tools to examine molecular

changes preceding the deposition of amyloid plaques and associated pathology (i.e. late inflammation, neuritic dystrophy, etc.). For instance, many studies in transgenic models have demonstrated that Aβ accumulates first intraneuronally, before amyloid plaques appear (Wirths *et al*, 2002; Oddo *et al*, 2003b; Casas *et al*, 2004; Billings *et al*, 2005; Leon *et al*, 2010; Ferretti *et al*, 2011); a finding that has been validated in post-mortem AD and Down's syndrome (DS) brains (Lemere *et al*, 1996; Gouras *et al*, 2000; D'Andrea *et al*, 2001; Gyure *et al*, 2001; Mori *et al*, 2002; Takahashi *et al*, 2002; Fernandez-Vizarra *et al*, 2004).

A deeper understanding of the pre-plaque stage is of great relevance in view of the fact that the intraneuronal compartment is also a place where Aβ can oligomerize (Walsh *et al*, 2000; Takahashi *et al*, 2004; Ferretti *et al*, 2011) forming toxic aggregates which can impair synaptic plasticity (Lambert *et al*, 1998; Walsh *et al*, 2002; Shankar *et al*, 2008; Jin *et al*, 2011), induce cognitive impairments (Cleary *et al*, 2005; Lesne *et al*, 2006) and unleash a pro-inflammatory reaction (Bruno *et al*, 2009a; Ferretti *et al*, 2011). In fact, the nature and pathological relevance of the intracellularly accumulated material have been questioned in a recent study (Winton *et al*, 2011), raising the intriguing possibility that such material is APP, rather than Aβ. The arguments regarding this controversy have been recently reviewed (Cuello *et al*, 2012). The idea that the intraneuronal material is only APP is a challenging proposition, and we considered that this issue deserved a thorough multidisciplinary analysis.

For such study, we have used the McGill-R-Thy1-APP transgenic rat, which is unique compared to other rodent models in that the AD-like phenotype has been

achieved with a single genomic insertion of a mutated human APP transgene; minimizing off-target genetic corruption and therefore being closer to the human disease (Do Carmo & Cuello, 2013). Our microscopy studies at high magnification and high resolution have revealed a clear segregation between Aβ-immunoreactivity and that of APP and its C-terminal fragments (CTFs). Soluble $A\beta_{40}$ and $A\beta_{42}$ peptides were observed elevated early, in cortex and hippocampus from transgenic rats. The increase in Aβ peptides paralleled the manifestation of cognitive deficits, several months prior to amyloid plaque deposition. Notably, $A\beta_{38}$, $A\beta_{39}$, $A\beta_{40}$ and $A\beta_{42}$ peptides could be detected in the rat CSF by MALDI-MS analysis, even at plaque-free stages. Taken together, these results indicate that the early, intracellular accumulation of soluble Aβ in McGill transgenic rats is already accompanied by a CNS compromise (i.e. learning and memory impairments). Further to it, we have demonstrated that the early CNS amyloid pathology is reflected and can be monitored in the rat CSF.

4.3. Materials and methods

4.3.1. Animals

Transgenic rats belonged to the McGill-R-Thy1-APP line, harboring the human APP751 transgene with the Swedish and Indiana mutations under the control of the murine Thy1.2 promoter (Leon *et al*, 2010). The 3-month old group consisted of: transgenic heterozygote (n = 7) and homozygote (n = 7) rats and non-transgenic (n = 10) littermates. The 7-month old group consisted of: transgenic homozygote (n = 4) and non-transgenic (n = 4) littermates. The 13-month old group consisted of: transgenic heterozygote (n = 10) and homozygote (n = 6) rats and non-transgenic

(*n* = 10) Wistar rats (Charles River, Wilmington, MA), housed in identical conditions. Each group had equal gender representation. Animals were maintained on a 12-hour light/12-hour dark cycle and had *ad libitum* access to water and a standard rodent diet. All procedures were approved beforehand by the Animal Care Committee of McGill University, following the guidelines of the Canadian Council on Animal Care.

4.3.2. Tissue and CSF collection

Rats were deeply anesthetized with equithesin, and CSF (~80-150 µl) was collected from the cisterna magna using a glass capillary and subsequently frozen at -80˚C, following an established protocol (Pegg *et al*, 2010). Only clear, blood-free CSF samples were used for analysis.

For tissue collection, animals were perfused transcardially with cold saline for 1 min. The brain was removed, one hemisphere was kept for immunohistochemistry (IHC) and the other was dissected, flash-frozen and kept at -80˚C for further neurochemical analysis. The hemisphere for IHC was immersion-fixed in cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 24 h and transferred to 30% sucrose in 0.1 M PB. After the brains equilibrated in sucrose, they were cut into 40 µm coronal sections with a freezing microtome (Leica SM 2000R, Germany). Brain sections were stored in cryoprotectant solution (37.5% v/v ethylene glycol, 37.5% w/w sucrose, in PBS pH 7.4) at -20˚C until processed for IHC.

4.3.3. Antibodies

A detailed description of the primary antibodies used in this study can be found in Table 4-1.

4.3.4. Bright-field immunohistochemistry

Free-floating immunostaining was done following well-established protocols (Côté *et al*, 1994; Hu *et al*, 2003; Leon *et al*, 2010; Ferretti *et al*, 2011). Sections were incubated in 0.3% hydrogen peroxide in PBS for 20 min, washed in PBS-T (0.01 M phosphate-buffered saline, 0.2% Triton X-100) and blocked 1 h with 10% normal goat serum (NGS) in PBS-T. To examine the evolution of the AD-like amyloid pathology we incubated sections with the monoclonal antibody McSA1 (Grant *et al*, 2000) (MediMabs, Montreal, Canada) at 1:4000 in PBS-T with 5% NGS overnight at 4ºC. The following day, the sections were washed in PBS-T and incubated with a goat anti-mouse secondary antibody (MP Biochemicals, Canada) 1:100 in PBS with 5% NGS for 1 h. The sections were washed in PBS and incubated for 1 h with a mouse anti-peroxidase monoclonal antibody (Semenenko *et al*, 1985) (1:30) pre-incubated with horseradish peroxidase (5 µg/ml) in PBS (MAP kit, Medimabs, Canada). Stainings were developed with 0.06% 3,3'-diaminobenzidine (Sigma-Aldrich, USA) and 0.01% hydrogen peroxide (Sigma-Aldrich, USA) in PBS and then mounted on subbed slides. Sections were dehydrated in increasing ethanol concentrations (70-100%) and xylene, prior to coverslipping with Entellan (EM Science, USA). Images were acquired on an Axioplan Imaging microscope equipped with an AxioCam HRc digital camera (Carl Zeiss, Toronto, Canada); using the Axiovision 4.8 Software.

Table 4-1. List of antibodies used in this study

Antibody	Antibody target	Source	Epitope recognized	Obtained from
McSA1	Human Aβ	Mouse monoclonal	N-terminus of human Aβ (residues 1-12)	Medimabs, Canada
MOAB-2	Human Aβ	Mouse monoclonal	N-terminus of human Aβ (residues 1-4)	Biosensis, Australia
Nu1	Human Aβ oligomers	Mouse monoclonal	Conformational, Aβ oligomers	Dr. William Klein, Northwestern University
pab27576	APP	Rabbit polyclonal	Last 43 C-terminal residues of APP	Dr. Gerhard Multhaup, McGill University
W0-2	Human Aβ	Mouse monoclonal	N-terminus of human Aβ (residues 4-10)	EMI Millipore, USA
G2-10	Human $A\beta_{40}$	Mouse monoclonal	C-terminus of $A\beta_{40}$	EMI Millipore, USA
G2-13	Human $A\beta_{42}$	Mouse monoclonal	C-terminus of $A\beta_{42}$	EMI Millipore, USA

4.3.5. Immunofluorescence and confocal microscopy

Immunofluorescence was done following established protocols (Ferretti *et al*, 2011; Allard *et al*, 2012). Sections were incubated in 50% ethanol for 20 min followed by three 10-min washes in PBS. Blocking was done in 10% NGS in PBS-T during 1 h. Primary antibodies were incubated overnight in 5% NGS at 4˚C. Double labeling was performed between three anti-Aβ antibodies (McSA1 (Grant *et al*, 2000), Medimabs, Canada; MOAB-2 (Youmans *et al*, 2012), Biosensis, Australia and Nu1 (Lambert *et al*, 2007), provided by Dr. William Klein, Northwestern University, USA; at 1:500) and a polyclonal antibody (pab27576, provided by Dr. Gerhard Multhaup, McGill University; at 1:500) directed against the last 43 amino acids of the C-terminal domain of APP. This antibody recognizes full-length APP (~100 kDa) and CTFs (~12 kDa) without crossreactivity for Aβ (Wirths *et al*, 2002; Ferretti *et al*, 2012a; Kaden *et al*, 2012). Alexa 488-conjugated goat anti-mouse (Jackson Immunoresearch, USA) and Alexa 594-conjugated goat anti-rabbit (Molecular Probes, USA) secondary antibodies were applied overnight at 1:400-1:800 in PBS with 5% NGS. Sections were washed three times in PBS and subsequently mounted on gelatin subbed slides, dried overnight at 4°C, and coverslipped with Aqua Polymount (Polysciences, USA).

Images were taken on a Zeiss LSM 510 confocal microscope (Carl Zeiss, Canada) equipped with Argon and Helium-Neon lasers. Three sections per animal were immunolabeled and five pictures per animal (per region) were acquired from area CA1 of the hippocampus and lamina V and III of the parietal cortex. To maximize the sensitivity of co-localization analysis and fully exploit the resolution of the

microscope objective (Zeiss 63X oil immersion objective NA = 1.4), we used a 3.0 scan zoom so that each pixel covered 0.05 μm. Each signal was acquired using separate tracts with the appropriate laser-filter settings to avoid bleed-through of signals, and the pinhole was set so that optical sections were less than 0.7 μm thick. With the given objective settings, the theoretical resolution was of 250 nm in *x*, *y* and 700 nm in the *z* plane.

Confocal images were analyzed and quantified with the JACoP plugin (Bolte & Cordelieres, 2006) of the Image J software, to obtain Pearson and Manders' correlation coefficients. The Pearson coefficient defines the quality of the linear relationship between two signals and varies between 1 and -1. Manders' coefficients (M1 and M2) indicate the proportion of signal overlap; varying from 0 (no co-localization) to 1 (perfect co-localization). In this study, M1 indicated the proportion of McSA1 (or MOAB-2 or Nu1) (green signal) overlapping pab27576 immunoreactivity (red signal) over its total intensity. Conversely, M2 is defined as the proportion of pab27576 signal (red) overlapping McSA1 / MOAB-2 / Nu1 immunolabels (green) over its total intensity. Omission of primary antibodies resulted in no detectable fluorescent staining (data not shown).

4.3.6. Structured illumination microscopy (SIM)

Given that both McSA1 and pab27576 gave robust fluorescent signals, we were able to image them by SIM a super-resolution microscopy technique permitting a resolution of ~100 nm in *x*, *y* and of ~300 nm in *z* (Gustafsson *et al*, 2008). The IHC was done following the same protocol as for the confocal experiments, except that the McSA1 and pab27576 were used at a concentration of 1:1000. Images

were taken on a DeltaVision OMX V4 Blaze super-resolution microscope (Applied Precision, GE Healthcare). Reconstructed images were visualized using the Volocity 3D Image Analysis Software (PerkinElmer, USA). Following image reconstruction and registration, serial *z* planes were assembled to form a 3D model using the Volocity Software.

4.3.7. Human $A\beta_{40}$ and $A\beta_{42}$ ELISA

Brains were homogenized in 8% vol (w/v) of cold TBS buffer (150 mM NaCl, 50 mM Tris / HCl, 5 mM EDTA, pH 7.6) containing a protease inhibitor cocktail (Roche, Germany) using a teflon-glass homogenizer. Homogenates were cleared by centrifugation at 100.000 g for 1 h at 4°C, and supernatants were collected for further analysis (TBS-soluble fraction). The remaining pellets were dissolved in 70% formic acid (FA) with 50% of homogenization volume and sonicated for 30 s at 30% power. Centrifugation at 100.000 g was repeated and supernatants were collected (FA-soluble fraction). Samples from FA-soluble fractions were neutralized with 1 M Tris, 0.5 M Na_2HPO_4 prior to ELISA analysis.

Levels of $A\beta_{40}$ or $A\beta_{42}$ in TBS-soluble and FA-soluble fractions were determined by sandwich ELISA. The C-terminus-specific anti-Aβ monoclonal antibodies G2-10 and G2-13 (EMI Millipore, USA) were used to capture Aβ species terminating at 40 or 42 amino acids, respectively. The monoclonal biotinylated W0-2 (EMI Millipore, USA) recognizing the Aβ N-terminus was used as detection antibody, and the reaction was developed by streptavidin-horseradish peroxidase conjugate and the chromogenic substrate 1-Step Ultra-TMB (Pierce, USA). After stopping the reaction with 1 M H_2SO_4, the enzymatic products were measured at 450 nm in a

microplate reader (Anthos HT2, Germany). Synthetic $A\beta_{40}$ or $A\beta_{42}$ peptides (EMI Millipore, USA) served as standards.

4.3.8. MALDI-MS analysis of Aβ peptides in rat CSF

Aβ was immunoprecipitated from 50 µl CSF with 5 µg of the anti-Aβ antibody W0-2 (EMI Millipore, USA) coupled to protein-G sepharose beads (GE Healthcare, USA) and incubated overnight at 4ºC. The beads were washed three times with PBS and twice with 50 mM ammonium acetate, pH 7.0. Aβ was eluted twice with 50% acetic acid and vacuum-dried overnight. Immunoprecipitated samples were resuspended in 10 µl of TA (33% acetonitrile, 0.1% trifluoroacetic acid) and sonicated in a water bath for 10 min. Samples were spotted by the dried droplet technique mixing 1 µl of sample with 1 µl of a TA solution saturated with sinapinic acid. MALDI-MS analysis was carried out on a Bruker Ultraflex II instrument (Bruker Daltonik, Bremen, Germany).

4.3.9. Behavioral studies

Tests were conducted during the light phase of the circadian cycle. Rats were handled prior to testing for 2 - 3 days. Experimenters were blinded to the genotype during all behavioral studies.

4.3.9.1. Fear conditioning

The fear conditioning protocol was designed adapting a previously reported procedure (Nader *et al*, 2000). Animals were tested in a single chamber connected to a weight transducer system to allow the tracking of movement (Panlab, Spain). The chamber was scented with coconut extract and cleaned with ethanol 70%

between animals. To create a different environment for the fear memory retention test, the walls of the chamber were decorated with visual cues; the chamber was scented with mint extract and cleaned with acetic acid 1% between animals.

On day 1 (habituation) rats were allowed to explore the chamber for 5 min and returned to their home cages. On day 2 (conditioning), after an initial 90 sec phase of exploration (baseline), a 30 sec tone (75 dB, 5 kHz) was presented which co-terminated with a 2 sec foot shock (0.75 mA). Animals were allowed to recover (post-shock; 120 sec) and then returned to their home cages. On day 3, contextual fear conditioning was evaluated by placing the animals in the chamber during 8 min. On day 4, animals were allowed to explore the "new" arena for 120 sec (baseline). This was followed by three consecutive tone presentations (30 sec, 75 dB, 5 kHz), each separated by a 30 sec pause. The Freezing software (Freezing v1.3.01, Panlab) recorded all freezing episodes in each of the test phases, considering freezing as immobility for at least 2 sec.

4.3.9.2. Novel object recognition and location

Before testing, the animals were habituated to the testing environment by allowing them to explore the experimental arena. Following habituation, the rats were subjected to three testing phases: exploration, novel object location (NOL) and novel object recognition (NOR). During exploration, five objects similar in size but different in color, shape and texture were put in the arena. These objects had been tested in pilot studies to ensure that none of them elicited spontaneous preference or avoidance. The animals were allowed to explore the objects for three sessions of 2 min, with a 10 min inter-session interval. An animal was considered as exploring

when the muzzle was touching or in close proximity to the objects. The NOL test consisted of a single 2 min session where one of the objects was moved to a new location. For NOR, one of the undisplaced objects was substituted by a novel one. For NOL and NOR, the time exploring familiar and displaced / novel objects was recorded separately to calculate the discrimination ratio. We defined this ratio as the difference in exploration time for the displaced or novel object divided by total exploration time. Increased time spent exploring the object in the novel location was interpreted as successful spatial memory. Increased time spent exploring the novel object was interpreted as successful recognition memory for the familiar objects versus the novel one.

4.3.9.3. Calculation of cognitive index

For correlation analysis between behavior and Aβ pathology, a global learning and memory score, referred as cognitive index, was calculated based on the animals' performance during each different phase of behavior tests. For auditory and cue fear conditioning, a score was computed by calculating the fold increase in freezing behavior during the post-shock phase and tone presentation (respectively), compared to baseline freezing. Context conditioning freezing percentages were converted to a 10-point scale. For NOL and NOR, a score was obtained by calculating the fold increase between the animal's discrimination ratio and that of chance levels. All behavior scores were added and expressed as a *z* score, referred here as "cognitive index".

4.3.9.4. Von Frey test

Animals were habituated to the testing environment by placing them in individual boxes on a metal mesh floor during 10 min. The following day mechanical sensitivity was assessed by measuring withdrawal responses to a series of calibrated Von Frey filaments with incrementing force (15 g, 26 g, 60 g and 100 g). These were applied perpendicularly to the plantar surface of both hind paws, following the "up and down" method (Chaplan *et al*, 1994). Each hind paw was poked twice, calculating an average of all four pokes for between-animal comparisons.

4.3.10. Statistical analysis

The software Graph Pad Prism 5.01 (La Jolla, CA, USA) was used for statistical analysis. One-way ANOVA was used for three-group comparisons, followed by post-hoc multiple comparison tests (as specified in figure legend). Two-way ANOVA was used to analyze the fear conditioning data. Graphs illustrate mean ± SEM.

4.4. Results

4.4.1. Progression of the amyloid pathology in McGill-R-Thy1-APP rats

This study provides a quantitative biochemical and morphological investigation of the evolution of the Aβ pathology and its associated cognitive impairments in McGill-R-Thy1-APP rats. In this rat model, the expression of Aβ-immunoreactive material is detectable as early as 1 week of age and the first isolated amyloid

plaques may appear between 6 – 8 months starting in the subiculum (Leon *et al*, 2010).

We defined the pre-plaque stage of the amyloid pathology as a stage where Aβ immunoreactivity is limited to the intraneuronal compartment in the absence of extracellular amyloid plaques, as detected with the anti-Aβ McSA1 monoclonal antibody (Figure 4-1). In this cohort, 3 and 7 month-old McGill transgenic rats displayed robust intracellular McSA1 immunoreactivity and no evidence of amyloid plaque deposition (Figure 4-1). By 13 months, homozygote (+/+) animals displayed widespread and abundant amyloid plaques throughout the cortex (with greatest expression in lamina V) (Figure 4-1 C, I), hippocampus (Figure 4-1 C, F) and amygdala (Figure 4-1 L). Heterozygote transgenic rats of comparable age (13 months) did not exhibit amyloid plaques (Figure 4-1 N, O). In this rat model, the amyloid pathology at advanced stages (18-20 months) is further accompanied by strong microglial activation and dystrophic neuries surrounding amyloid plaques (Leon *et al*, 2010). Absence of McSA1-immunoreactivity was observed in non-transgenic animals (Figure 4-1 M), at all time points examined.

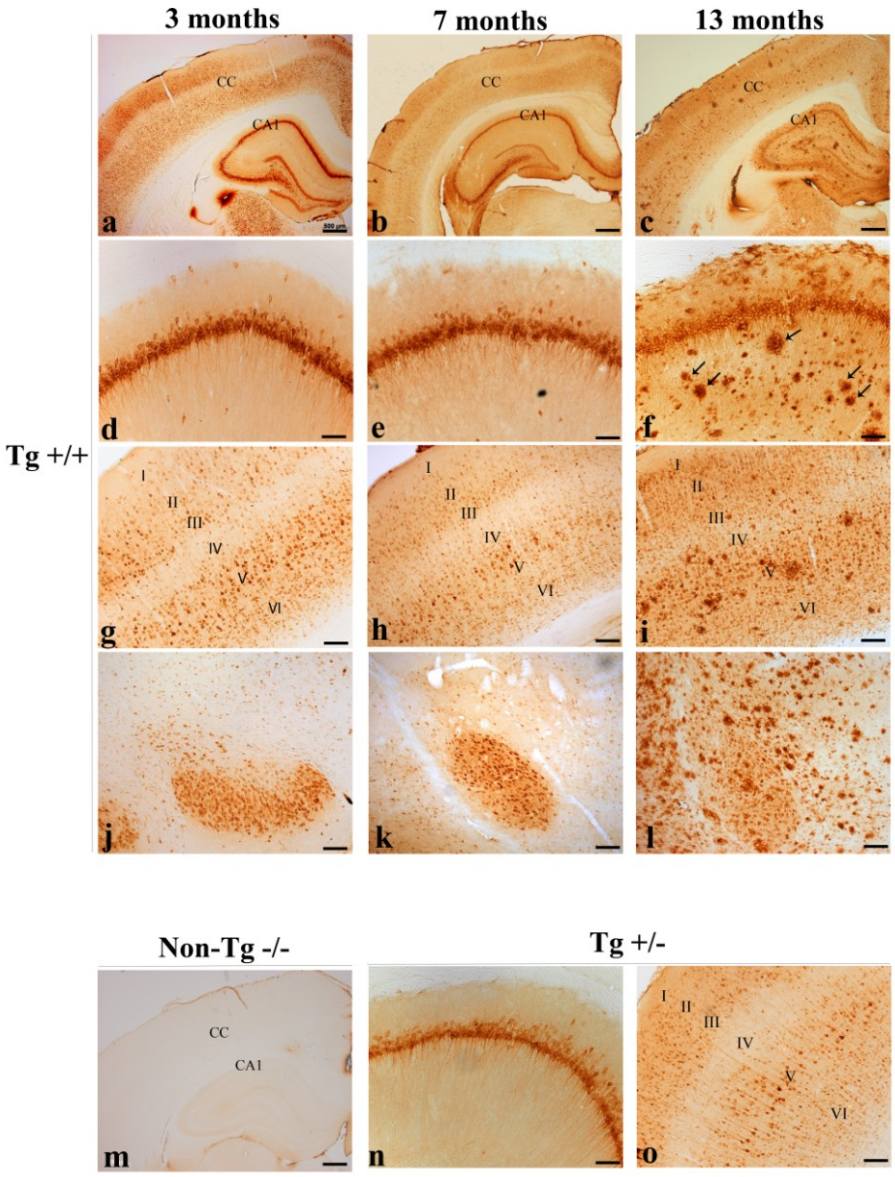

Figure 4-1. Temporal progression of the AD-like amyloid pathology in McGill-R-Thy1-APP transgenic rats. Intense intraneuronal Aβ immunoreactivity is observed in McGill transgenic rats throughout the neocortex (a-c, g-i, o), hippocampus (a-f, n) and amygdala (j-l), as detected with the anti-Aβ McSA1 monoclonal antibody. Note the absence of amyloid plaques at 3 months and 7 months of age. At 13 months, transgenic rats exhibited strong intracellular and extracellular McSA1 immunoreactivity. Abundant amyloid plaque deposition is evident throughout all cortical layers, hippocampal formation and amygdala in homozygote transgenic rats. Arrows indicate the presence of amyloid plaques. McSA1 immunoreactivity remained limited to the intracellular compartment in heterozygote animals of similar age. Note the absence of McSA1-immunoreactivity in non-transgenic animals (m). Scale bar: a-c, m = 500 μm; d-l, n-o = 100 μm.

4.4.2. Co-localization analysis between Aβ and APP/CTF-specific signals

To detect intracellular Aβ we utilized a monoclonal antibody (McSA1), which recognizes the N-terminal region of the human Aβ peptide (residues 1 - 12) (Grant *et al*, 2000). This epitope could theoretically also be found in β-CTF and in APP. However, competition studies have demonstrated that McSA1 is highly specific for Aβ as opposed to APP or sAPP-α (Grant *et al*, 2000; Leon *et al*, 2010).

We performed quantitative co-localization analysis between McSA1 and pab27576 signals at pre- and post-plaque stages of the amyloid pathology. At 3 months of age we observed only a partial co-localization between McSA1 and pab27576 in lamina V (Figure 4-2 B), as reflected by an average Pearson coefficient of 0.44 ± 0.03 (Table 4-2). Quantitative analysis demonstrated only ~30% overlap (M1 = 0.30 ± 0.03) between McSA1 and pab27576 signals in lamina V (Table 4-2), indicating that ~70% of the cellular epitope recognized by McSA1 is not coming from APP or β-CTFs. Similar observations were made in CA1 neurons and lamina III cortical pyramidal neurons (Figure 4-S1), revealing Pearson coefficients of 0.57 ± 0.02 and 0.32 ± 0.03 (Table 4-2) and a degree of overlap of only ~ 20 – 50 % between the two signals (M1 = 0.48 ± 0.02 and M1 = 0.23 ± 0.03; respectively). The clear distinction between intracellular McSA1 and pab27576 immunoreactivity was also evident at later stages of the amyloid pathology (Figure 4-S1). Pearson coefficients ranged between 0.35 and 0.5 and there was approximately ~ 20 - 40% overlap between the two signals in the brain regions investigated (Table 4-2). At this time point, we also observed the presence of extracellular Aβ deposits that were McSA1 positive but pab27576 negative (Figure 4-S2 C).

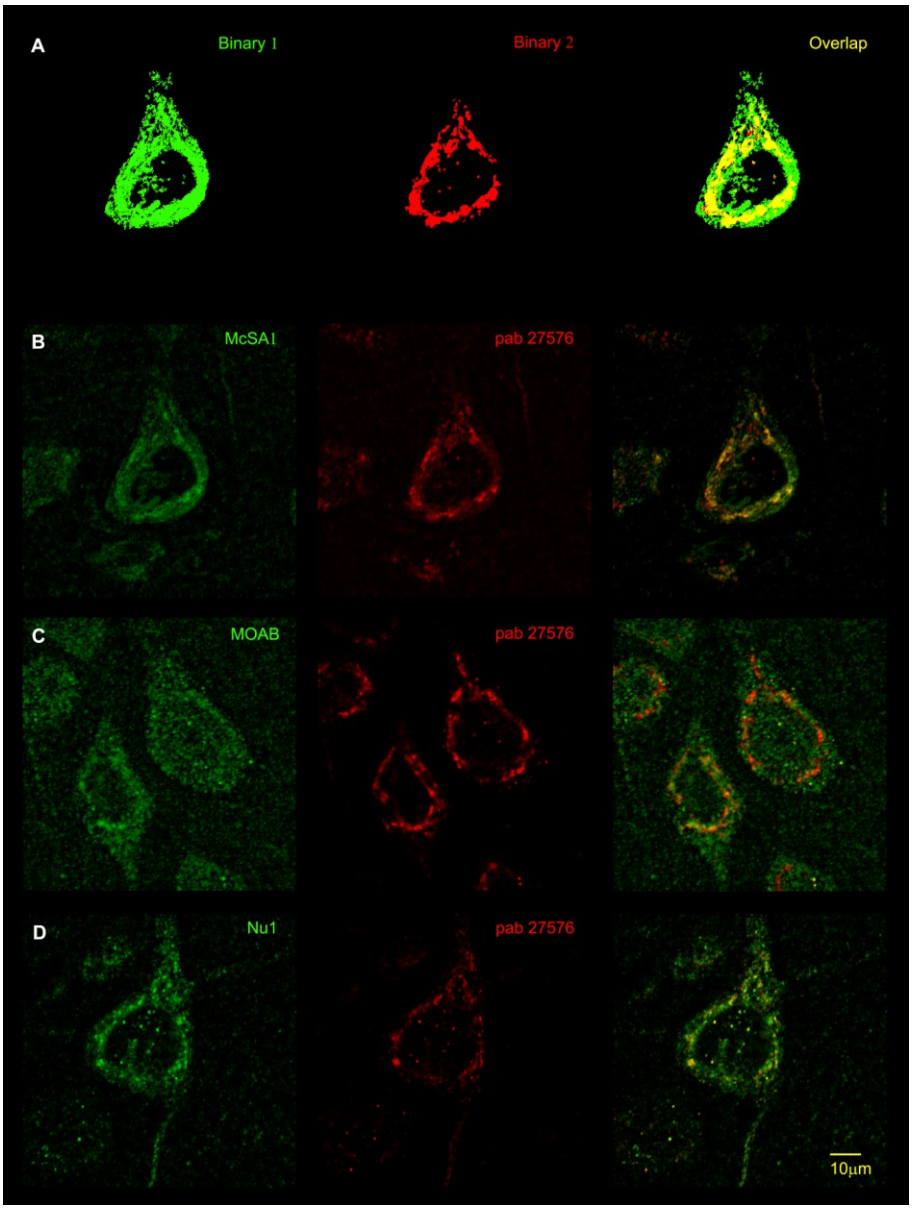

Figure 4-2. Detection of intraneuronal Aβ and APP/CTFs. A) To measure the overlap coefficient of different antibody binding sites, the signals were separated from background using a brightness criterion and made binary. The two images were then superimposed; the M1 coefficient represents the overlap (yellow) divided by the total green signal (yellow + green). The M2 coefficient represents the overlap divided by the total red signal (yellow + red). B-D) Representative high-magnification confocal micrographs depicting co-localization between pab27576 (red) and B) McSA1 (green) binding sites, C) MOAB-2 (green) and D) Nu1 (green) in lamina V neurons of the parietal cortex, at the pre-plaque stage (3 month-old transgenic rats). Note the lack of complete overlap between Aβ- and APP/CTF-specific signals. Scale bar = 10 µm

Table 4-2. Co-localization analysis between McSA1 and pab27576

		Pearson	Manders M1	Manders M2
3 months	**CA1**	0.57 ± 0.02	0.48 ± 0.02	0.83 ± 0.02
	Lamina V	0.44 ± 0.03	0.30 ± 0.03	0.65 ± 0.04
	Lamina III	0.32 ± 0.03	0.23 ± 0.03	0.56 ± 0.04
13 months	**CA1**	0.51 ± 0.04	0.37 ± 0.02	0.79 ± 0.03
	Lamina V	0.39 ± 0.03	0.22 ± 0.02	0.74 ± 0.03
	Lamina III	0.35 ± 0.03	0.22 ± 0.02	0.64 ± 0.04

M1: proportion of McSA1-IR (green) overlapping pab27576 (red) over its total intensity
M2: proportion of pab27576 (red) overlapping McSA1-IR (green) over its total intensity

Strikingly, the intracellular McSA1 immunolabeling appeared diffusely distributed throughout the neuronal cytoplasm, whereas the pab27576 signal was mostly associated to vesicular-like structures within the perinuclear region (Figure 4-2 and S1). To obtain a higher, optimal 3D resolution of McSA1 and pab27576 immunoreactivities at the pre-plaque stage, we applied super-resolution structured illumination microscopy. With this technique, we finally demonstrate that the overlap between McSA1 and pab27576 immunoreactive sites was minimal, where the former diffuse McSA1 signal now became localized to small intracellular vesicles, clearly dissociated from pab27576 immunoreactive structures (Figure 4-3).

To further validate our inclusion/exclusion co-localization analysis we then stained rat brain sections with MAOB-2, a monoclonal antibody directed to residues 1 - 4 of Aβ, and which has been reported as absent of cross-reactivity with APP or CTFs (Youmans *et al*, 2012). In general, the MOAB-2 immunoreaction ranged from low (in some neurons) to hardly detectable in most neurons from the different areas and time points examined. Therefore, we subjected confocal images to quantitative co-localization analysis only when the MOAB-2 signal was strong enough to be clearly distinguished from background, non-specific staining. Corroborating our previous results with McSA1, we observed a differential pattern of immunoreactivity between MOAB-2 and pab27576 in cells with detectable and reliable MOAB-2 staining.

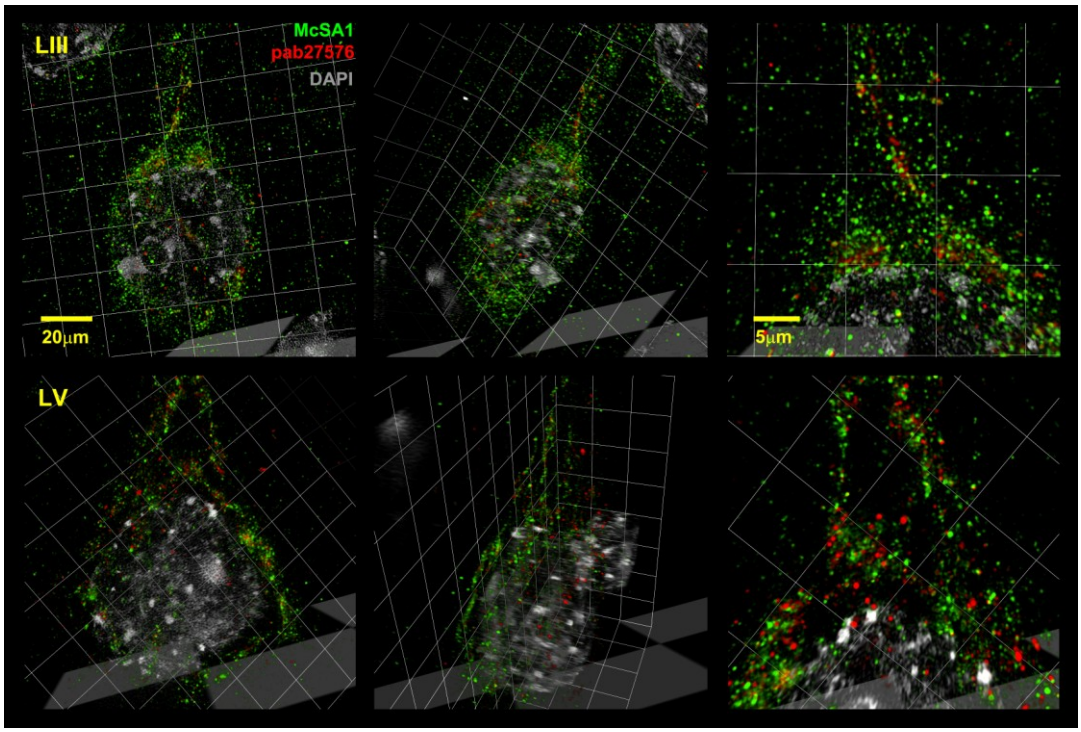

Figure 4-3. Characterization of McSA1 and pab27576 immunoreactivities by super-resolution microscopy. To further characterize the distribution of McSA1 and pab27576 signals, cells from lamina III and lamina V of the parietal cortex were imaged by SIM in 3-month old, amyloid-plaque free animals. Note the distinct intracellular localization of McSA1 immunoreactivity (green) with that of pab27576 (red), denoting Aβ-amyloid peptides and APP/CTFs, respectively. DAPI (gray) indicates the boundaries of the neuronal nucleus. From left to right are frontal and oblique views, followed by higher magnification images of 3D-reconstructions.

At 3 months of age high-magnification quantitative co-localization analysis revealed partial overlap between the two signals in lamina V neurons (Figure 4-2 C) as reflected by an average Pearson coefficient of 0.41 ± 0.01 and M1 coefficient of 0.44 ± 0.02. At 13 months of age, the intracellular MOAB-2 immunolabel was hardly detectable in pyramidal neurons adjacent to amyloid plaques, the latter which were strongly labeled by this antibody and not by pab27576 (Figure 4-S2).

Additional confirmation of the segregation between Aβ-specific and APP/CTF-specific signals was obtained by co-labeling rat brain sections with the Nu1 monoclonal antibody (Aβ oligomer-specific) (Lambert *et al*, 2007) and pab27576. With this approach, a similar clear distinction between the two signals was observed. At the pre-plaque stage we revealed only a partial co-localization between Nu1 and pab27576 immunoreactivity in lamina V (Figure 4-2 D), indicated by an average Pearson coefficient of 0.53 ± 0.02 (Table 4-3). Quantitative analysis also demonstrated only ~40% overlap (M1 = 0.37 ± 0.02) between Nu1 and pab27576 signals in lamina V (Table 4-3). Similar observations were made in CA1 and lamina III (Figure 4-S3), revealing Pearson coefficients of 0.64 ± 0.01 and 0.35 ± 0.02 and a degree of overlap of ~ 25 – 50% between the two signals (M1 = 0.50 ± 0.02 and M1 = 0.24 ± 0.02; respectively) (Table 4-3). At 13 months of age co-localization analysis also revealed a clear distinction between intracellular Nu1 and pab27576 immunoreactivity (Figure 4-S3). Pearson coefficients ranged between 0.4 - 0.7 and there was approximately 40 - 50% overlap between the signals (Table 4-3). At both stages of the amyloid pathology, the intracellular Nu1 immunolabel appeared associated to vesicle-like structures.

Table 4-3. Co-localization analysis between Nu1 and pab27576

		Pearson	Manders M1	Manders M2
3 months	**CA1**	0.64 ± 0.01	0.50 ± 0.02	0.70 ± 0.02
	Lamina V	0.53 ± 0.03	0.37 ± 0.02	0.61 ± 0.04
	Lamina III	0.35 ± 0.02	0.24 ± 0.02	0.43 ± 0.03
13 months	**CA1**	0.63 ± 0.05	0.51 ± 0.03	0.84 ± 0.02
	Lamina V	0.68 ± 0.04	0.49 ± 0.03	0.79 ± 0.03
	Lamina III	0.47 ± 0.02	0.37 ± 0.02	0.56 ± 0.03

M1: proportion of Nu1-IR (green) overlapping pab27576 (red) over its total intensity
M2: proportion of pab27576 (red) overlapping Nu1-IR (green) over its total intensity

4.4.3. Analysis of Aβ peptides in brain

We further quantified the age-dependent accumulation of Aβ peptides in McGill transgenic rat brain homogenates by ELISA following established protocols (Munter *et al*, 2007; Munter *et al*, 2010). We observed significantly higher levels of soluble $A\beta_{40}$ peptides (~3 fold) in cortex and hippocampus from transgenic rats compared to age-matched non-transgenic animals, at 3 months ($P < 0.0001$), 7 months ($P < 0.001$) and 13−15 months of age ($P < 0.01$) (Figure 4-4 A). The levels of soluble $A\beta_{40}$ peptides, however, did not differ significantly between transgenic rats at different stages of the amyloid pathology, with an average of 50.2 ± 4.7 pg/mg total protein. Furthermore, cortical and hippocampal levels of TBS-soluble $A\beta_{42}$ peptides were slightly elevated in young McGill transgenic rats (3 and 7 months) compared to non-transgenic animals (Figure 4-4 B), whereas substantially more $A\beta_{42}$ was found in 13−15 month-old transgenics (171 ± 37.8 pg/mg protein, $P < 0.0001$). This data strongly suggests an age-dependent accumulation of soluble $A\beta_{42}$ peptides in McGill transgenic rats ($P < 0.001$). Notably, levels of TBS-soluble $A\beta_{40}$ and $A\beta_{42}$ were slightly increased in heterozygote (+/-) rats at 13−15 months of age compared to non-transgenic littermates, though not significantly (Figure 4-4).

When analyzing the presence and levels of Aβ in FA fractions we detected highest concentrations of $A\beta_{40}$ (938.3 ± 313.9 pg/mg protein, $P < 0.001$) and $A\beta_{42}$ (3246.1 ± 963.7 pg/mg protein, $P < 0.0001$) in transgenic rats at 13−15 months of age compared to plaque-free transgenic rats (Figure 4-4 C, D), with an average $A\beta_{42}/A\beta_{40}$ ratio of 6 ± 2. Thus, this data revealed that ~95% of the total $A\beta_{40}$ and $A\beta_{42}$ detected in the cortex and hippocampus of transgenic rat at the post-plaque

stage required FA for solubilization. There was no difference or age-dependent increase in the level of TBS-soluble or FA-treated Aβ peptides in cerebellum between non-transgenic and transgenic animals (Figure 4-S4); a finding that is in agreement with the human pathology (Mann *et al*, 1990).

4.4.4. Analysis of Aβ peptides in CSF

In order to detect Aβ peptides in the CSF of transgenic rats, we immunoprecipitated human Aβ with the W0-2 antibody (Table 4-1). MALDI-MS analysis revealed the presence of $A\beta_{42}$ (4513 Da), $A\beta_{40}$ (4329 Da), $A\beta_{39}$ (4230 Da) and $A\beta_{38}$ (4131 Da) peptides in CSF from McGill transgenic rats, both at pre- and post-plaque stages of the amyloid pathology (Figure 4-5). Notably, the pattern of CSF peptides was similar at both time points and comparable to that observed in human CSF (Portelius *et al*, 2006); except for two observations: no $A\beta_{37}$ was detected in the CSF of McGill transgenic rats at any time point and the $A\beta_{38}$ signal was as strong as the $A\beta_{40}$ signal, under these conditions. MALDI-MS analysis revealed an absence of Aβ signal in non-transgenic animals (Figure 4-5) at both time points, indicating that detected Aβ peptides predominantly derive from the human APP transgene expression or reflecting the low level of endogenous rodent Aβ (De Strooper *et al*, 1995). The masses identified as Aβ peptides were absent when immunoprecipitation was performed from PBS instead of CSF.

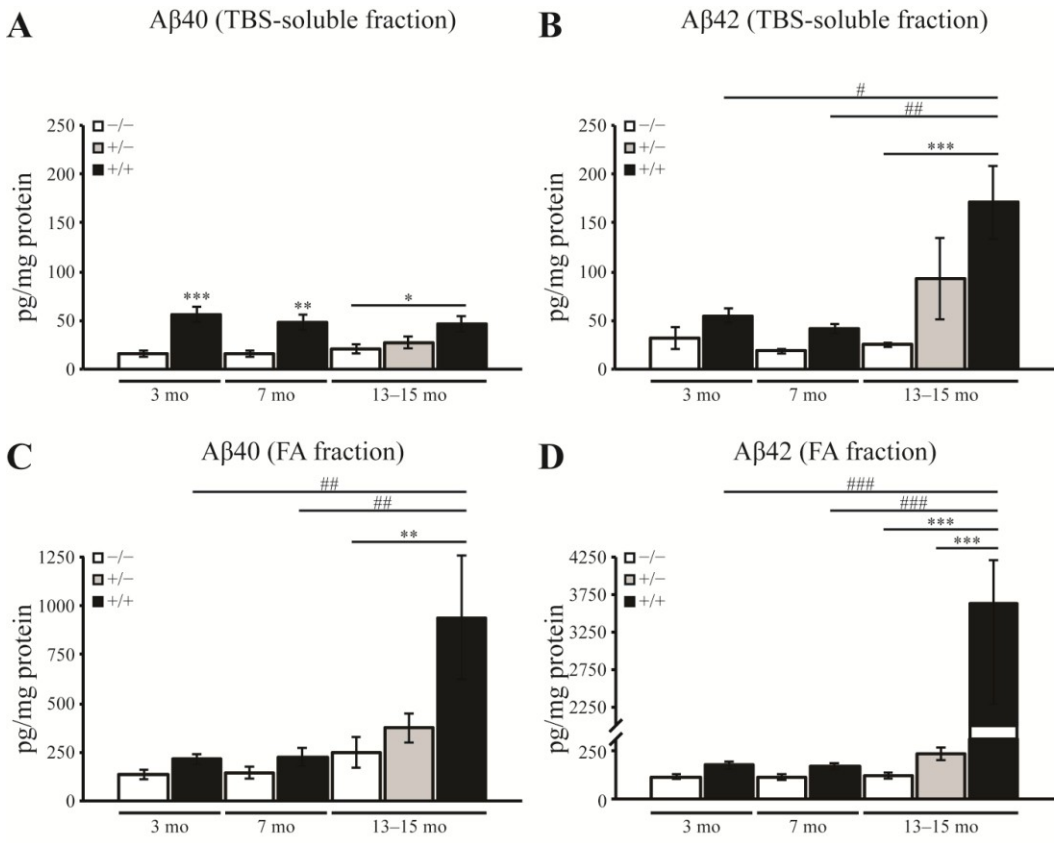

Figure 4-4. Quantification of $A\beta_{40}$ and $A\beta_{42}$ levels in cortex and hippocampus by sandwich ELISA. TBS-soluble A) $A\beta_{40}$ and B) $A\beta_{42}$ levels in cortex + hippocampus homogenates from non-transgenic (−/−), heterozygous (+/−) and homozygous transgenic (+/+) rats at different ages (3 months, 7 months and 13-15 months) were quantified with specific G2-10/W0-2 and G2-13/W0-2 sandwich ELISAs, respectively. TBS-insoluble pellets were extracted with 70% formic acid (FA) and after neutralization, C) $A\beta_{40}$ and D) $A\beta_{42}$ levels in FA fractions were assayed with G2-10/W0-2 and G2-13/W0-2 ELISAs, respectively. Values were normalized to total protein concentration and expressed as means ± SEM. One-way ANOVA, followed by Dunnett's post-hoc test. $^{*}P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.0001$ (across the same time point), $^{\#}P < 0.01$, $^{\#\#}P < 0.001$, $^{\#\#\#}P < 0.0001$ (across ages).

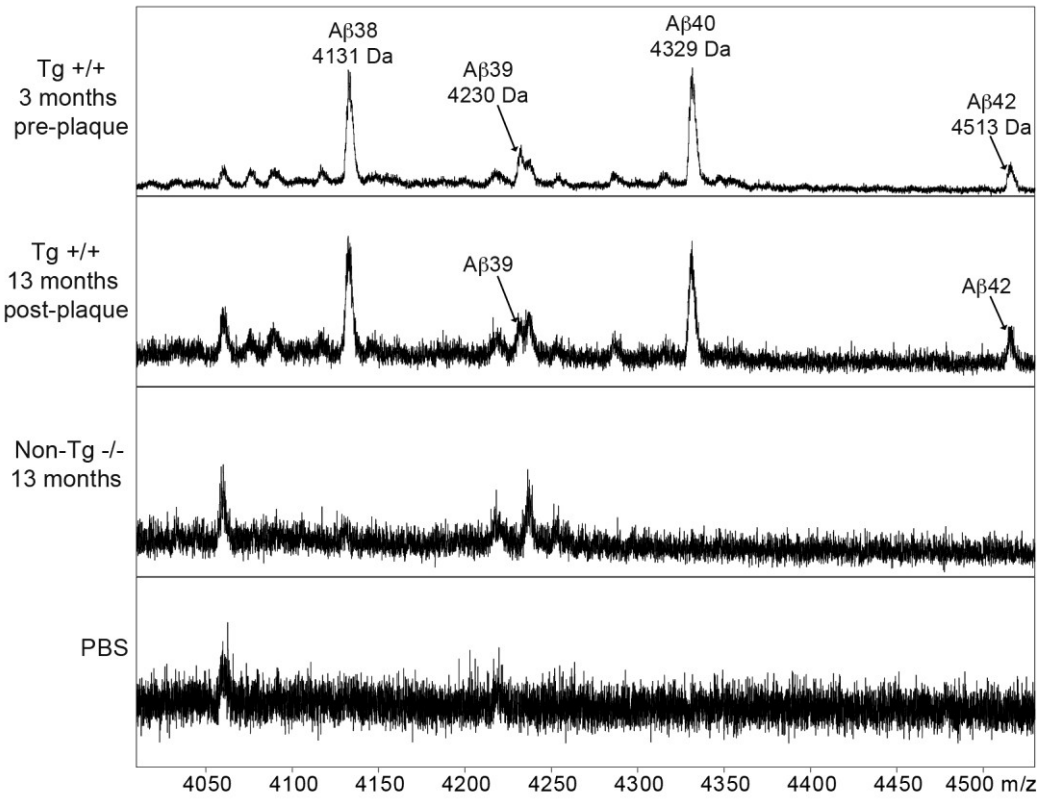

Figure 4-5. MALDI-MS spectra of immunoprecipitated Aβ peptides from rat CSF. Aβ peptides were immunoprecipitated from rat CSF with the W0-2 antibody and further subjected to MALDI-MS analysis. Peaks correspond to signals for $A\beta_{38}$, $A\beta_{39}$, $A\beta_{40}$ and $A\beta_{42}$ with theoretical monoisotopic masses of 4130.0 Da, 4229.0 Da, 4328.2 Da and 4512.3 Da respectively. Note that the differences in measured versus theoretical masses are predominantly due to the linear detection method used. Signals observed in the non-transgenic animals and the PBS control result from either protein-G sepharose, the antibody or other non-specific binding components of the CSF.

4.4.5. Early and progressive cognitive deficits in McGill-R-Thy1-APP rats

4.4.5.1. Impaired fear conditioning

During auditory conditioning training (Figure 4-6 A), 3 month-old transgenic and non-transgenic animals exhibited low, comparable baseline freezing behavior. The initial presentation of the tone, before the foot-shock, did not result in increased freezing behavior in any of the groups. Conversely, we observed marked differences in fear response acquisition between transgenic animals and non-transgenic littermates during the post-shock phase ($F_{2,21}$ =17.03; $P < 0.001$).

In the fear conditioning paradigm, animals can also learn to associate the environment with the aversive stimulus; therefore, we tested contextual fear conditioning behavior 24 h later in the same arena, and compared it to the average freezing response during the pre-training habituation session (which averaged ~10% freezing). No differences in freezing responses between non-transgenic and transgenic rats were detected during contextual fear conditioning testing at this early stage ($F_{2,21}$= 1.043; $P > 0.05$) (Figure 4-6 B). Following contextual fear conditioning, we then tested fear memory retrieval in response to an auditory cue, within a different environment. All rats exhibited low, comparable freezing responses at baseline. However, upon tone presentation, McGill transgenic rats manifested significant deficits in amygdala-dependent auditory fear memory, compared to non-transgenic littermates ($F_{2,21}$= 47.12; $P < 0.001$) (Figure 4-6 C). Therefore, fear conditioning deficits were detectable at early stages of the amyloid pathology, in the absence of amyloid plaques. At this early time-point, transgenic homozygotes and heterozygote rats had comparable fear conditioning responses

throughout all test phases. However, with increasing age and advancing amyloid pathology, fear conditioning deficits severely progressed in homozygote animals (Figure 4-6 D-F).

We observed marked differences in fear response acquisition between transgenic and non-transgenic rats during the post-shock phase at 13 months (Figure 4-6 D). Both heterozygote and homozygote rats exhibited lower freezing responses compared to non-transgenic animals ($F_{2,19}$ =13.59; $P < 0.05$ and $P < 0.001$). Non-transgenic animals presented a significant increase in freezing behavior, with respect to their baseline ($F_{2,14}$=25.6; $P < 0.001$). There was a mild learning response in heterozygote animals ($F_{2,14}$=4.95; $P < 0.05$) with respect to their baseline freezing behavior. Conversely, transgenic homozygote rats exhibited significant fear conditioning deficits as evidenced by comparable freezing levels throughout all phases ($F_{2,10}$ =1.01; $P > 0.05$). All animals exhibited low, comparable freezing behavior at baseline and during the initial tone presentation.

In 13-month old transgenic rats with advanced amyloid pathology we revealed marked contextual fear conditioning deficits in homozygote animals. Non-transgenic and transgenic heterozygote rats exhibited high freezing responses (~70% and ~60% freezing, respectively) (Figure 4-6 E). Conversely, freezing behavior was significantly lower in McGill homozygote transgenic rats ($F_{2,19}$= 9.528; $P < 0.01$). During fear memory retrieval in a different context (Figure 4-6 F), all groups exhibited low, comparable baseline freezing. Upon tone presentation, homozygote rats presented significantly lower freezing responses compared to non-transgenic animals ($F_{2,19}$ = 3.370; $P < 0.05$).

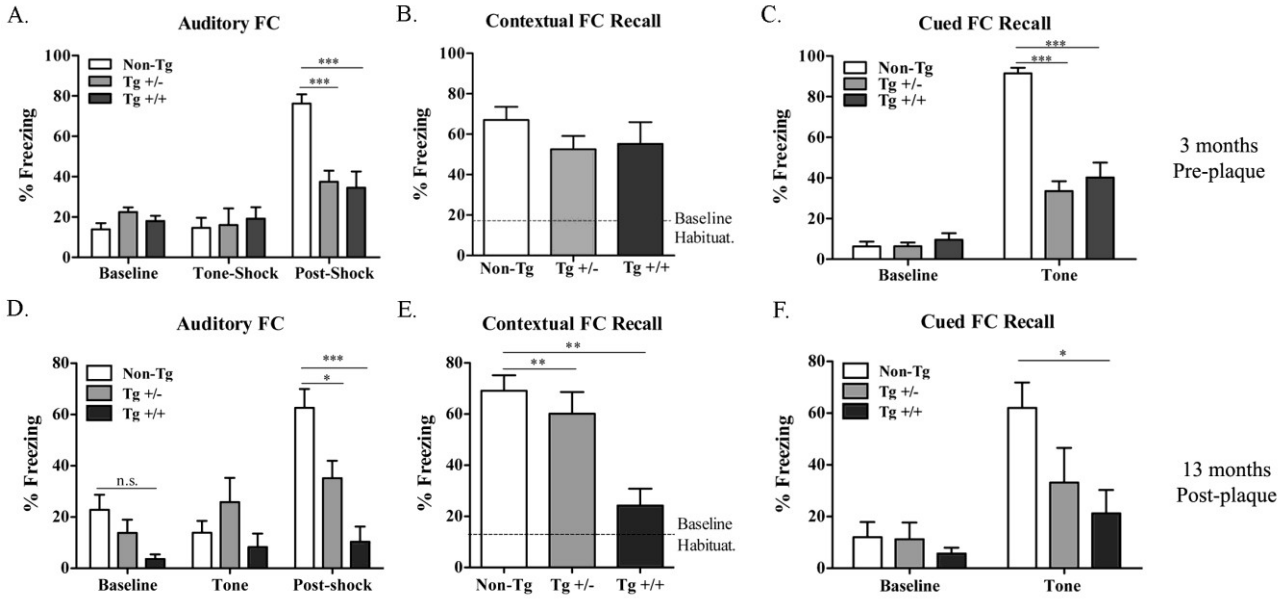

Figure 4-6. Impaired auditory fear conditioning in McGill-R-Thy1-APP transgenic rats. Freezing responses in A-C) 3 month-old and D-F) 13 month-old transgenic rats. Following two days of habituation and animal handling, auditory fear conditioning was tested (A, D) followed by assessment of contextual fear conditioning 24 h later (B, E). The following day (48 hs after auditory conditioning), cued fear responses were examined in a different arena (C, F). Note the progression of auditory and contextual fear conditioning deficits as the amyloid pathology advanced from pre- to post-plaque stages. A, C, D, F) Two-Way ANOVA, followed by Bonferroni post-hoc test. B, E) One-Way ANOVA, followed by Bonferroni post-hoc test. Error bars represent mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Importantly, at both time points examined, there were no differences in locomotor activity and/or pain sensitivity between rat groups (Figure 4-S5), suggesting that the reduced fear conditioning responses (freezing behavior) in transgenic rats were not influenced by any differences in sensitivity to pain or by increased locomotion.

4.4.5.2. Impaired novel object recognition and location

In the novel object recognition test, McGill-R-Thy1-APP rats were able to discriminate the novel object better than chance levels (object recognition index = 0.2), however they exhibited significantly lower object recognition indices compared to wild type animals (Figure 4-7). An impairment in object recognition memory was detectable at early stages of the amyloid pathology, months prior to extracellular Aβ deposition (Figure 4-7 A) in McGill transgenic rats ($F_{2,19}$=10.61; $P < 0.001$ and $P < 0.05$, respectively). Novel object location (Figure 4-7 B) was also significantly impaired in heterozygote animals at this early stage but, despite the trend, did not appear significantly affected in homozygote rats ($F_{2,19}$=3.03; $P < 0.05$ and $P > 0.05$, respectively). At 13 months of age, McGill transgenic heterozygote and homozygote rats showed significant impairments in object recognition (Figure 4-7 C, $F_{2,21}$=13.87; $P < 0.001$), with recognition indices close to chance levels, as well as deficits in location memory (Figure 4-7 D, $F_{2,21}$= 16.41; $P < 0.001$), compared to non-transgenic animals.

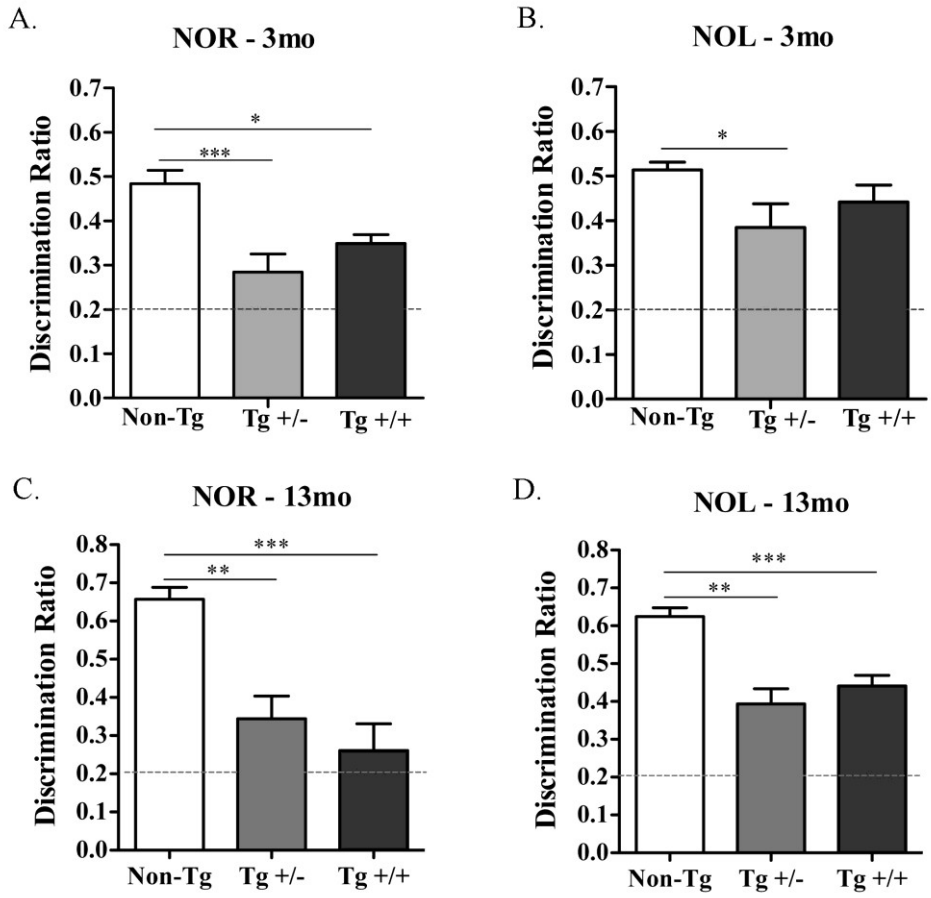

Figure 4-7. Deficits in Novel Object Recognition and location task. In the novel object recognition test (NOR) the object discrimination ratio was defined as the difference in exploration time for the novel object (in sec) divided by the total exploration time (in sec). A, C) McGill APP transgenic rats exhibited lower recognition memory indices than wild type littermates at A) pre-plaque (3 months) and C) post-plaque (13 months) stages. In the novel object location test (NOL) (B, D) the object discrimination ratio was defined as the difference in exploration time for the displaced object (sec) divided by the total exploration time (in sec). Data is expressed as mean ± SEM and analyzed with One-Way ANOVA, followed by Tukey's post hoc test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

4.5. Discussion

The concept that Aβ accumulates within neurons remains a matter of controversial debate. However, it is widely accepted that a central event in AD pathogenesis is the abnormal accumulation of Aβ and its related toxins, either due to increased production or to deficits in clearance mechanisms (Zlokovic *et al*, 2000; Hardy & Selkoe, 2002). Aβ peptides, initially discovered extracellularly, are the core component of amyloid plaques in AD and DS brains (Glenner & Wong, 1984b; Masters *et al*, 1985). Several studies have reported the existence of intracellular Aβ within cultured neurons (Wertkin *et al*, 1993; Turner *et al*, 1996; Hartmann *et al*, 1997; Grant *et al*, 1999; Grant *et al*, 2000) as well as in post-mortem brains from AD (Gouras *et al*, 2000; Mochizuki *et al*, 2000; D'Andrea *et al*, 2001), DS subjects (Lemere *et al*, 1996; Gyure *et al*, 2001; Mori *et al*, 2002) and in transgenic rodent models (Wirths *et al*, 2002; Oddo *et al*, 2003b; Echeverria *et al*, 2004; Philipson *et al*, 2009; Ferretti *et al*, 2011).

Despite the above-mentioned studies, a valid concern has been raised regarding some of the antibodies applied for intracellular Aβ immunodetection, which could theoretically also recognize the Aβ sequence as part of APP and in CTFs. This argument was recently put forward in a study by Winton and colleagues who further concluded that the intraneuronal material in the well-established 3xTg-AD mouse model is not Aβ but in fact, solely APP (Winton *et al*, 2011). The idea that only APP accumulates intraneuronally is thought-provoking and additionally, whether the intracellular Aβ pathology cause some of the symptoms in human AD remains unknown.

In this report, we have investigated the molecular species that accumulate intraneuronally in McGill-R-Thy1-APP rats and examined in parallel the presence of cognitive impairments, in a model which is closer to the human pathology than transgenic mice. To investigate the intracellular Aβ pathology, we have performed high-magnification quantitative co-localization/exclusion analysis, using three different monoclonal antibodies specific for Aβ: 1) McSA1, against Aβ residues 1-12; 2) MOAB-2, against Aβ residues 1-4 and 3) Nu1, a conformation-specific antibody which recognizes Aβ oligomers (Lambert *et al*, 2007). These antibodies were co-incubated with a polyclonal antibody (pab27576) against the C-terminal domain of full-length APP. The present study revealed a consistent subcellular segregation of immunoreactive sites detected by Aβ-specific antibodies and pab27576 within neurons of different brain areas, at pre- and post-plaque stages of the amyloid pathology. Importantly, our co-localization/exclusion analysis also revealed that the intracellularly accumulated material is likely a heterogeneous mixture of Aβ, APP and CTFs.

The examination of the amyloid pathology with super-resolution microscopy permitted us to further reveal a clear separation of the subcellular location of Aβ-immunoreactivity from that of APP/CTFs. Similar compelling evidence supporting the existence of *bonafide* intraneuronal Aβ peptides has been provided by ELISA analysis on laser microdissected pyramidal neurons from sporadic AD cases (Hashimoto *et al*, 2010). Future studies of this nature are warranted in the McGill-R-Thy1-APP model.

We have observed a certain degree of overlap between Aβ antibodies and pab27576 by confocal microscopy. This likely reflects the presence of Aβ and APP/CTFs within the same intracellular location but does not necessarily imply that the antibodies are identifying the same molecules. In the experimental conditions employed for the confocal imaging, the resolution was of 250 nm in the *x*, *y* planes, and 700 nm in *z*; an area sufficient to accommodate thousands of molecules and even neighboring organelles. Notably, when the McSA1 and pab27576 signals were resolved by SIM, McSA1 immunoreactivity became almost completely dissociated from pab27576 and was found in distinct compartments. It should be kept in mind, however, that this super-resolution technique also has its limitations. Because the mathematical algorithms reconstruct images based on Moiré patterns caused by the structured illumination, the technique is less effective at reassigning signal that is diffuse or weak. It is then possible that we have failed to detect low levels of Aβ or APP at plasma membrane locations.

Previous work on the McGill-R-Thy1-APP transgenic rat has provided additional evidence to support that a considerable component of the intracellular material detected by McSA1 is Aβ. Pre-adsorption of this antibody with synthetic $A\beta_{42}$ completely abolished McSA1 immunoreactivity. Conversely, incubation with the same molar concentration of a cleaved fragment of APP did not result in diminished signal intensity (Leon *et al*, 2010), reinforcing the specificity of the McSA1 antibody for Aβ compared to APP-derived peptides (Grant *et al*, 2000).

In this study, we further observed that the intraneuronal Aβ-specific immunolabel was hardly detectable in close proximity to amyloid deposits. One interpretation

could be that the intracellular pool of Aβ may act as a source for the formation of extracellular amyloid deposits, an argument which was put forward for the human pathology (D'Andrea *et al*, 2001). Notably, in DS brains intracellular Aβ immunoreactivity diminishes with increasing age and advanced AD pathology (Lemere *et al*, 1996; Mori *et al*, 2002). Furthermore, in brains from APP transgenic mice conformation-specific antibodies have revealed the early appearance of intraneuronal fibrillar and oligomeric Aβ immunoreactivity, which declined as amyloid plaques appeared, and further became evident in the extracellular space (Ferretti *et al*, 2011). Alternatively, the apparent decline in intracellular Aβ signal may be the result of the intense reactivity of extracellular Aβ, potentially sequestering antibodies and creating steric hindrance, thus lowering the availability of intracellular epitopes.

The biochemical data presented in this report indicates that soluble $A\beta_{40}$ and $A\beta_{42}$ peptides are already abundant during amyloid plaque-free stages. We assert that this material reflects true Aβ (cleaved from APP), as the ELISA approach applied in this study consisted of C-terminus specific anti-Aβ capture antibodies, which do not recognize CTFs or APP molecules. In the McGill transgenic rat, ELISA analysis revealed robust accumulation of soluble Aβ species at 3 and 7 months of age (in cortex and hippocampus), with a preferential progressive and incremental accumulation of $A\beta_{42}$ compared to $A\beta_{40}$ peptides at advanced ages. Such a situation is similar to that observed in human AD and DS brains (Iwatsubo *et al*, 1995; Lemere *et al*, 1996; Gouras *et al*, 2000). This finding is of great significance considering that cognitive deficits paralleled the early accumulation of soluble Aβ

peptides and further progressed across age and with the advancement of the soluble $A\beta_{42}$ pathology.

To further address this issue, we analyzed whether there was an association between the average concentrations of Aβ peptides and the magnitude of behavior deficits, across the temporal progression of the amyloid pathology in transgenic animals. We calculated a cognitive index, adding the scores obtained throughout the different phases of the two behavioral paradigms employed, and expressing them as a *z* score. This analysis revealed a trend reflecting that higher concentrations of soluble $A\beta_{42}$ peptides correlated with lower indices of cognitive performance (Figure 4-8 A). No such correlation was evident for soluble $A\beta_{40}$ peptides (Figure 4-8 B) or insoluble $A\beta_{42}$ (Figure 4-8 C). However, there was a significant association between the levels of insoluble $A\beta_{40}$ peptides and cognitive decline (Figure 4-8 D). Although this correlation analysis cannot establish causality it is consistent with the concept that soluble forms of $A\beta_{42}$ are highly toxic species.

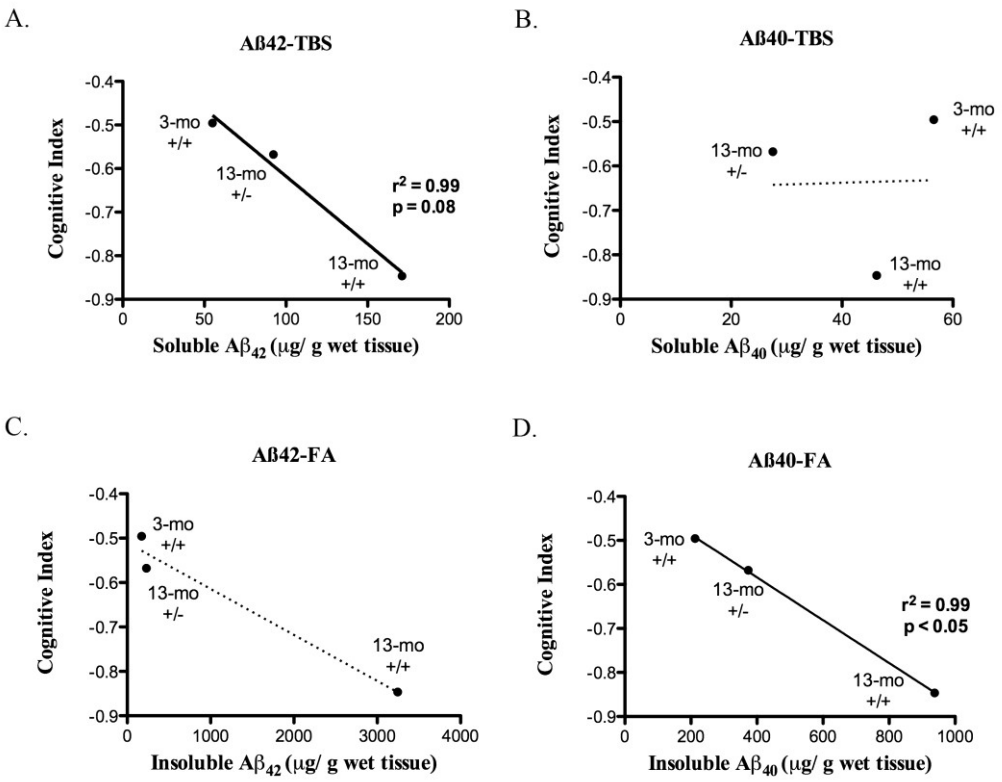

Figure 4-8. Link between soluble Aβ pathology and cognitive impairment. Correlation between average levels of soluble (A, B) and insoluble (C, D) $A\beta_{42}$ and $A\beta_{40}$ and the magnitude of cognitive impairments in transgenic animals, during the temporal progression of the AD-like amyloid pathology. A cognitive index was calculated based on the animals' performance throughout all phases of the two behavioral paradigms employed, and expressed as a z score. Average values were analyzed with linear regression. Note the negative correlation between cognition and the levels of soluble $A\beta_{42}$ peptides but not with soluble $A\beta_{40}$.

To determine the occurrence of Aβ peptides in the rat CSF we resorted to Aβ immunoprecipiation followed by MALDI-MS analysis, before and after the formation of amyloid deposits. This technique allowed the detection of C-terminally truncated peptides, including $A\beta_{38}$, $A\beta_{39}$, $A\beta_{40}$ and $A\beta_{42}$ species, as early as 3 months of age; a time point which precedes amyloid plaque deposition by several months (4-6 months). The distinct pattern of Aβ peptides observed in rat CSF compared to human CSF (which includes more species, ranging from 1-12 to 1-42) may reflect differences in the γ-secretase complex or differential Aβ clearance patterns between human and rodent species. Importantly, this is one of the first reports applying Aβ immunoprecipitation followed by MALDI-MS analysis in CSF from a transgenic AD model, providing a robust, novel platform for the assessment of Aβ expression profiles in biological fluids of transgenic models. Furthermore, the CSF detection of Aβ peptides suggest a dynamic state of these molecules, where some accumulate inside neurons, and some are exported to the extracellular space, from where they can reach the CSF even in the absence of amyloid plaques in the brain parenchyma.

A good number of previous studies support the tenet that the occurrence of intraneuronal Aβ accumulation can have early, deleterious effects in CNS functions (Oddo *et al*, 2003b; Echeverria *et al*, 2004; Billings *et al*, 2005; Ferretti *et al*, 2012b). In our study, we observed strong Aβ-immunoreactivity in the amygdala at pre-plaque (intraneuronal) and post-plaque stages; changes which likely account for the fear conditioning deficits reported at both time points. Emotional disturbances are a common early symptom in AD patients and such changes may

be a reflection of early amygdalar damage (Heun *et al*, 1997; Mizuno *et al*, 2000). The fact that cognitive impairments were seen at the pre-plaque stage, where Aβ could be detected intraneuronally (by IHC) and in CSF (by MALDI-MS), suggests that a combination of intraneuronal and soluble extracellular Aβ may be responsible for impairing neuronal function at early time points. A role for Aβ-mediated toxicity is further supported by the fact that cognitive deficits progressed according with the incremental accumulation of the amyloid pathology, in particular, increased levels of soluble $A\beta_{42}$ (Figure 4-8). However, we do not discard the possibility that the presence of other intracellular species, such as CTFs may also contribute to neuronal dysfunction in McGill transgenic rats. β-CTF can be toxic to primary rat hippocampal neurons in culture (Yankner *et al*, 1989), and is capable of inducing impairments spatial learning and working memory *in vivo* (Nalbantoglu *et al*, 1997; Jinno *et al*, 2009; Mitani *et al*, 2012).

Finally, we propose that an analogous, intraneuronal Aβ pathology during the pre-diagnostic stages of human AD should also have a negative impact on cognitive function. However, due to population-based tests and the well-known aspect of neural reserve (Scarmeas & Stern, 2003) such early deficits may be difficult to reveal in the human species. We therefore suggest that self-to-self assessments that reflect a cognitive decline from an individual-adjusted baseline may be required to detect early (and yet subtle) memory deficits in pre-symptomatic human AD.

4.6. Concluding remarks

This study demonstrates that a considerable component of the intracellular AD-like pathology in McGill-R-Thy1-APP rats consists of Aβ peptides. The intracellular

material reflects a variety of molecular species, including free Aβ, APP, CTFs as well as aggregated Aβ peptides in the form of oligomers. Future investigations on this issue are warranted, particularly to examine which form of Aβ predominates within neurons and in which compartment; as well as dissecting the differential contributions of intraneuronal species to CNS dysfunction. A similar pathological situation is likely to occur at the earliest, silent stages of AD in the human brain.

4.7. Acknowledgements

This research was supported by a CIHR grant (MOP-97776) to ACC, by McGill start-up funds to GM and LMM, and by the Deutsche Forschungsgemeinschaft through MU901, the Sonderforschungsbereich SFB740, the German Federal Ministry of Education and Research through the Kompetenznetz Degenerative Demenzen (Förderkennzeichen 01 GI 0723) to GM. GM is holding a CRC Tier 1 linked to CFI infrastructure support. LMM is also funded by a Young Investigator Award from the Alzheimer Society of Canada (2013-2016). WLK was supported by a Zenith Award from the Alzheimer's Association. MFI is the recipient of a Biomedical Doctoral Award from the Alzheimer Society of Canada. LR is supported by the Richard and Edith Strauss Postdoctoral Fellowship in Medicine. SDC is the holder of the Charles E. Frosst-Merck Post-Doctoral Fellowship. ACC is the holder of the McGill University Charles E. Frosst/Merck Chair in Pharmacology. We would like to thank Dr. Elke Küster-Schöck and the Cell Imaging and Analysis Network Center at the McGill Biology Department for their help with super-resolution microscopy. The Cuello Lab wishes to thank Dr A. Frosst, the Frosst family and Merck Canada for their unrestricted support.

CHAPTER 4

Supplementary Figures

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Acta Neuropathol Commun. 2014 Jun 5;2(1):61

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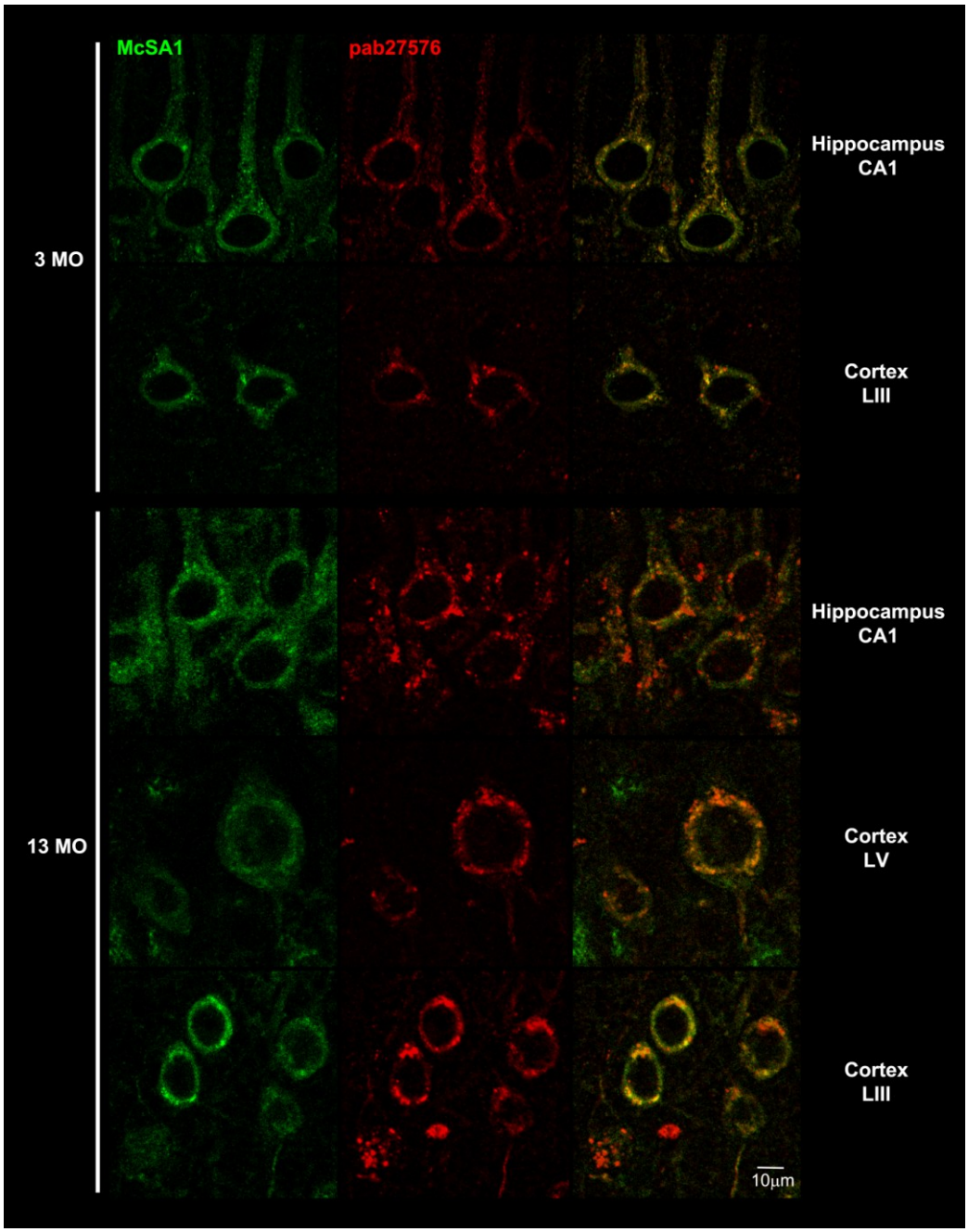

Figure 4-S1. Double immunolabeling with McSA1 and pab27576 antibodies. Representative high-magnification confocal micrographs depicting lack of complete co-localization between pab27576 (red) and McSA1 (green) immunoreactive sites at 3 months and 13 months in CA1 neurons of the hippocampus, and neurons of lamina V and III of the parietal cortex. Note the lack of complete overlap between the intracellular Aβ- and APP/CTF-specific immunoreactive signals at both time points. Scale bar = 10 µm.

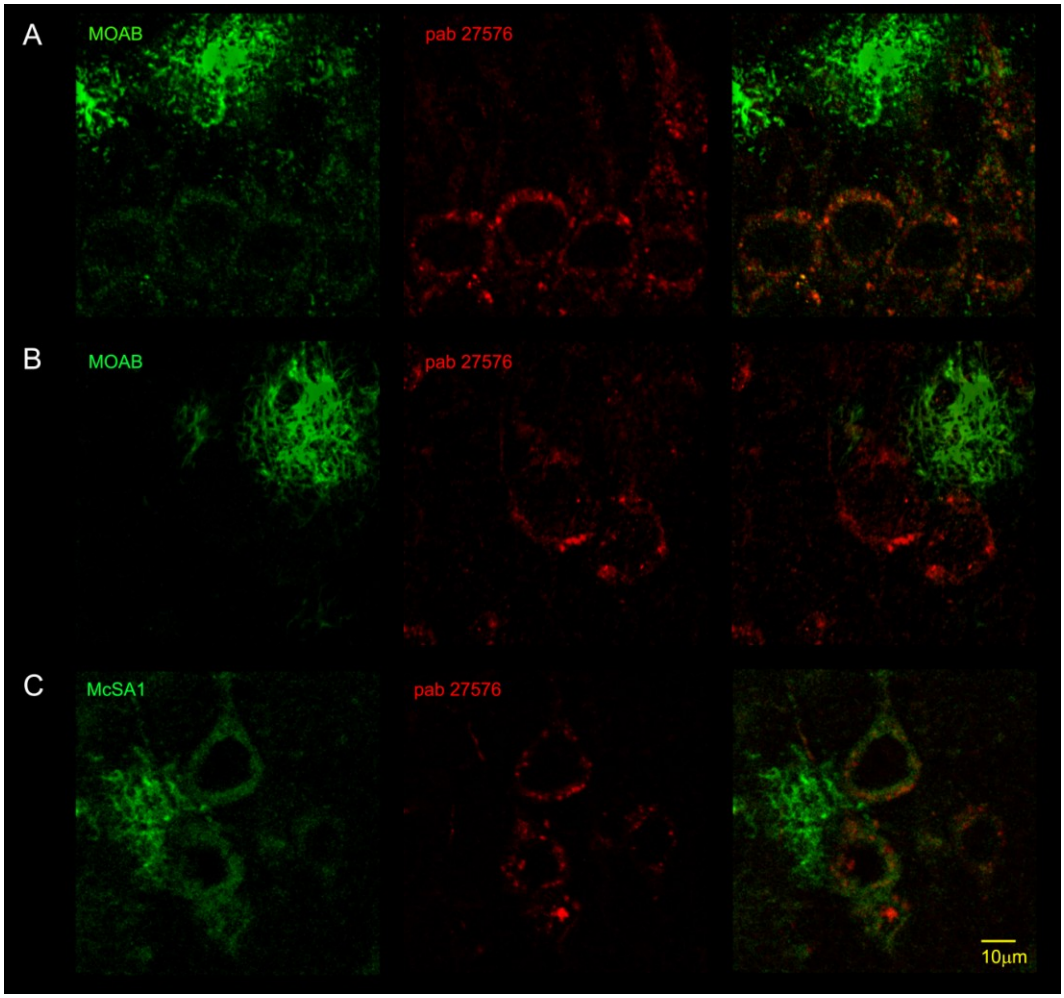

Figure 4-S2. Double immunolabeling with MOAB-2, McSA1 and pab27576. A-B) Representative high-magnification confocal micrographs depicting co-localization between pab27576 (red) and MOAB-2 (green) in CA1 neurons of the hippocampus A) and neurons of lamina V of the parietal cortex B) at post-plaque stages (13 months). C) Co-localization between McSA1 (green) and pab27576 (red) immunoreactive sites in neurons of the cerebral cortex (lamina V) at 13 months. Note the absence of pab27576 immunoreactivity in amyloid plaques (A-C). Scale bar = 10 µm.

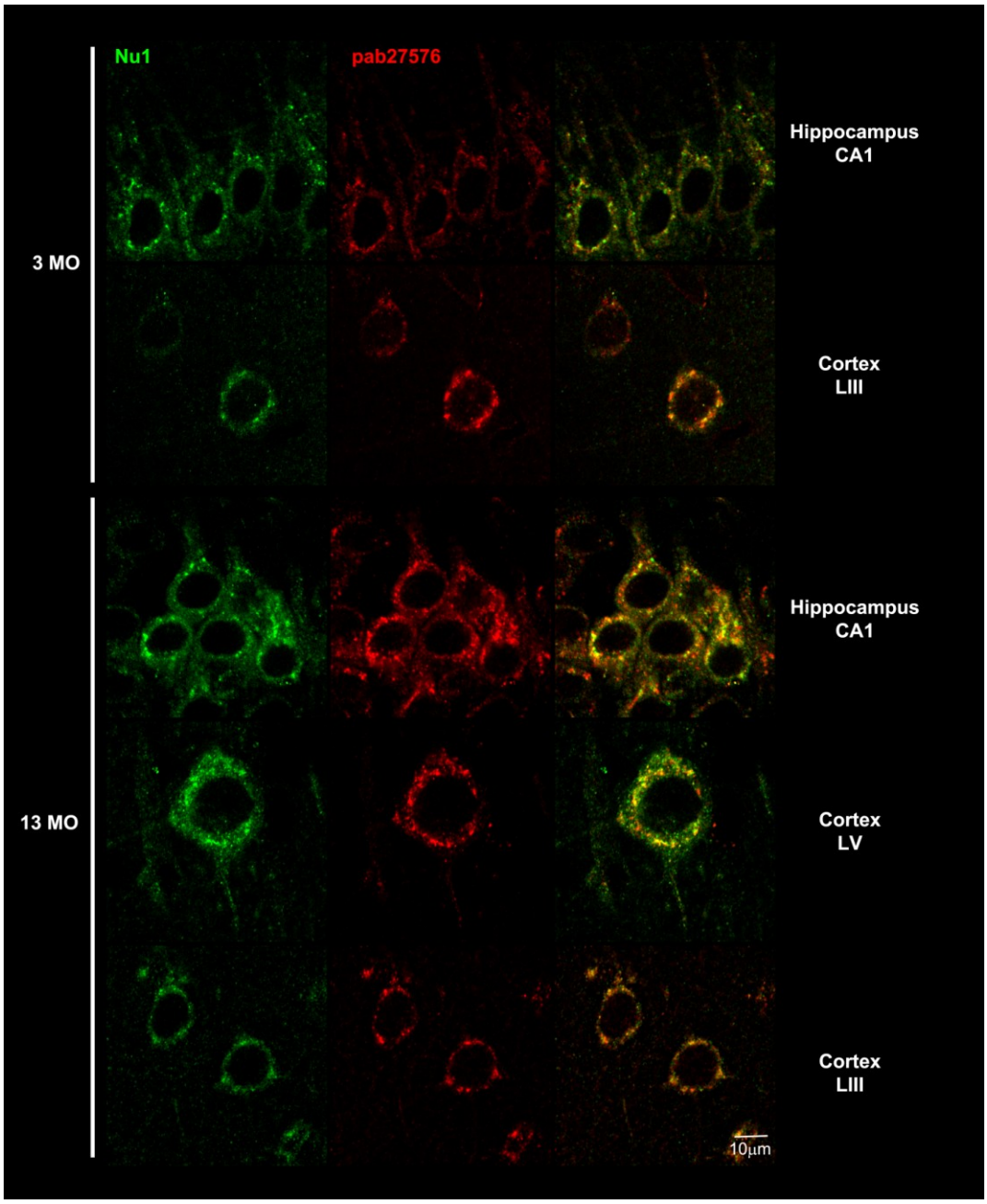

Figure 4-S3. Double immunolabeling with Nu1 and pab27576. Representative high-magnification confocal micrographs depicting lack of complete co-localization between pab27576 (APP/CTF-specific sites, red) and Nu1 (Aβ oligomer-specific sites, green). These images illustrate CA1 neurons of the hippocampus and neurons of lamina V and III of the parietal cortex from animals aged 3 and 13 months. Scale bar = 10 µm.

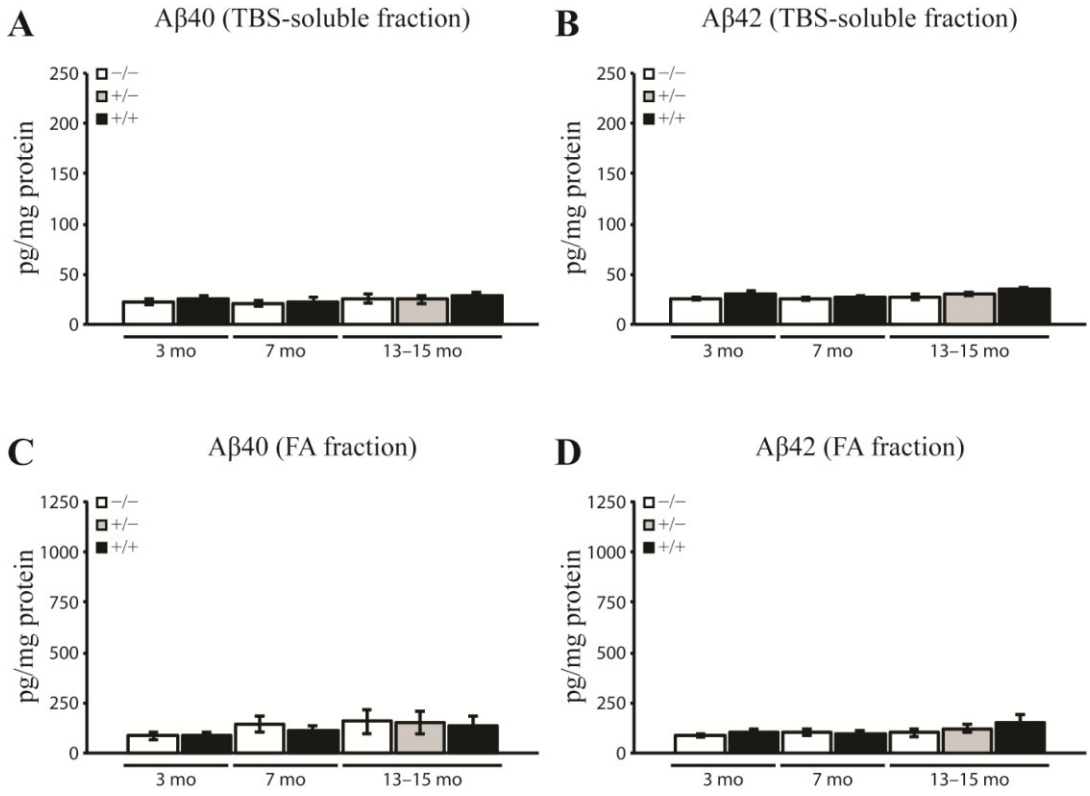

Figure 4-S4. Quantification of $A\beta_{40}$ and $A\beta_{42}$ levels by ELISA in cerebellum. $A\beta_{40}$ and $A\beta_{42}$ levels in cerebellum homogenates from non-transgenic (−/−), heterozygous (+/−) and homozygous (+/+) transgenic rats at different ages (3 months, 7 months and 13-15 months) were quantified with specific G2-10/W0-2 and G2-13/W0-2 sandwich ELISAs, respectively. Values were normalized to total protein concentration and expressed as means ± SEM. One-way ANOVA, followed by Dunnett's post-hoc test.

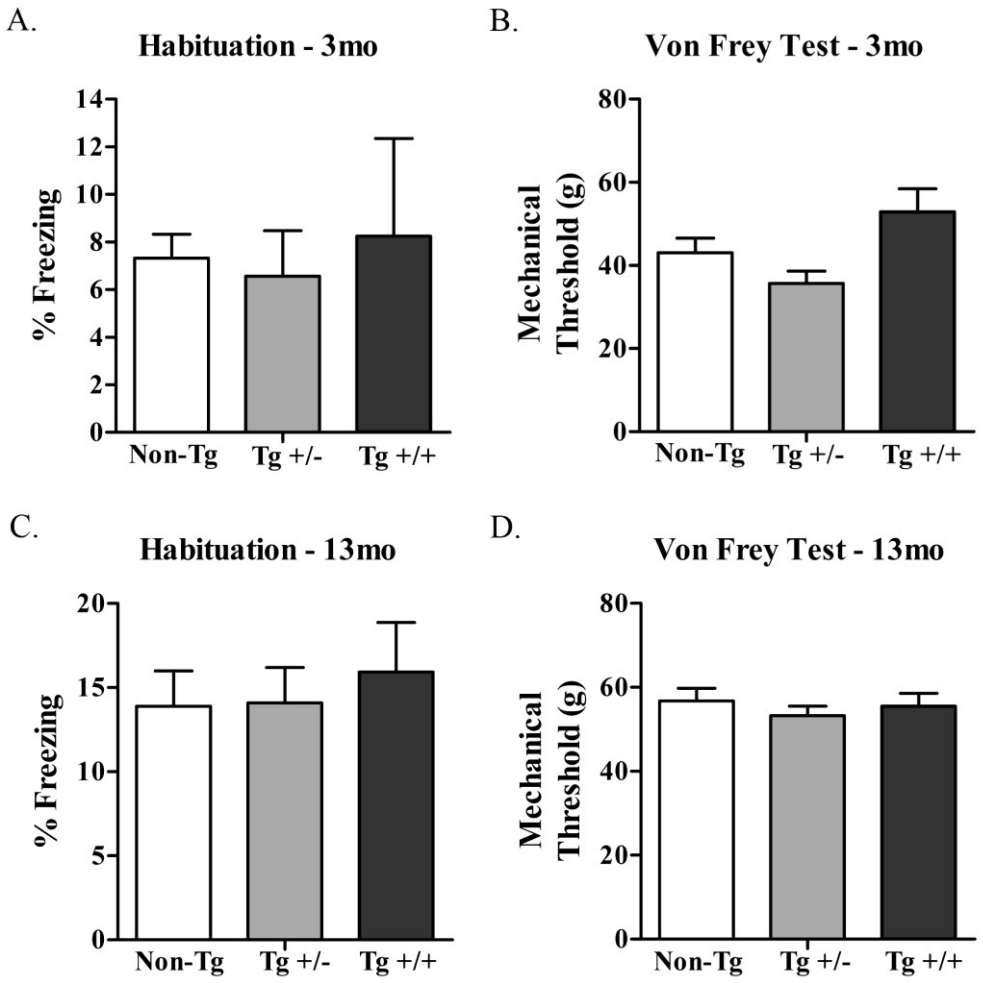

Figure 4-S5. Analysis of locomotor activity and pain sensitivity in McGill transgenic rats. A, C) Freezing responses recorded during a 5 min exploratory session in the testing environment. The animals were allowed to explore the arena and returned to their home cages. No stimuli were presented. B, D) Analysis of tactile sensitivity in the rat hind paw using Von Frey filaments of increasing force. The graph depicts the average withdrawal thresholds to mechanical stimulation, expressed in grams of pressure. Data is expressed as mean ± SEM. One-Way ANOVA, followed by Bonferroni post-hoc tests.

CHAPTER 5

General Discussion

5.1. Summary of thesis objectives and main findings

Alzheimer's disease is a serious, worldwide health concern. In Canada about ~500,000 people suffer from AD and other dementias; and this number is expected to escalate if the aged sectors of the population continue to increase and no effective therapies emerge (Alzheimer Society of Canada, 2010). Medical and cultural advances have also improved the survival and life expectancy of adults with DS. While this is certainly an encouraging outcome, individuals with DS are now more exposed to health issues associated with aging. Particularly, they are at an increased risk of developing AD (5-6 times higher), compared to the normal population (Zigman *et al*, 2008).

The recent failure of major Alzheimer trials would indicate that the best strategy to achieve disease modification should be early intervention (Sperling *et al*, 2011b). Unfortunately, current diagnostic methods cannot *unequivocally* identify individuals with "latent" AD prior to dementia onset. In contrast, DS brains offer the unique opportunity to investigate the temporal sequence of AD pathology progression. Understanding the events that precede amyloid plaque formation or neurodegeneration may provide new vistas for the identification of novel biomarkers, which could be combined with current Aβ and tau assessments. Given the importance of early intervention, biomarkers will be central to monitor the efficacy of treatments or to discriminate who may be at risk of disease progression.

Based on the above, the main goal of this doctoral Thesis was to gain a deeper understanding of the molecular changes that occur in the brain during the earliest

stages of AD pathology development. First, we have focused on the study of NGF metabolism in adult subjects with DS and dementia, as well as in fetal cortex tissue. Second, we have explored whether alterations in NGF-metabolic markers are reflected in CSF from AD and MCI patients. Finally, using a transgenic APP rat developed in our laboratory, we have characterized the early intracellular Aβ pathology and investigated the associated cognitive impairments, several months prior to amyloid plaque deposition.

Our investigations have revealed for the first time a compromised extracellular NGF metabolism in adult DS brains (Iulita *et al*, 2014), similar to that previously reported in AD. Alterations in proNGF, MMP-9 and tPA activity were further detected in primary cultures derived from DS fetal cortex tissue, suggesting that the early accumulation of Aβ peptides may be sufficient to unleash a dysfunction in NGF metabolism, prior to the onset of dementia (Chapter 2).

We have subsequently identified alterations in matrix metallo-protease 3 (MMP-3) and tissue plasminogen activator (tPA) in CSF from AD patients; with significant correlations between the levels of these analytes in CSF and clinico-pathological parameters (Hanzel *et al*, 2014a). Although these levels were normal in individuals with MCI, other CSF changes were detected in this clinical group, particularly, an increase in plasminogen, neuroserpin and TIMP-1, compared to control subjects (Chapter 3).

Finally, we have demonstrated that several months prior to amyloid plaque deposition, intracellular Aβ is present within pyramidal neurons of the cortex and

hippocampus and that Aβ peptides can also be detected in the rat CSF (Iulita *et al*, submitted). At this early stage, the increase in soluble $A\beta_{40}$ and $A\beta_{42}$ paralleled the presence of cognitive impairments; deficits which progressed at later stages with the evolution of the amyloid pathology and deposition of plaques (Chapter 4).

Overall, the results of these investigations support the concept that the early accumulation of Aβ peptides is sufficient to induce CNS damage (e.g. NGF dysfunction in DS; learning and memory deficits in transgenic models), before the development of full-blown AD pathology. They further provide new clues for the development of cholinergic neuroprotective therapies for AD and DS, and hint at potential new biomarkers signalling the presence of a CNS trophic disconnection and evolving inflammation.

5.2. Pre-symptomatic Alzheimer's disease: hypothesis or theory?

Research diagnostic criteria for the staging of AD have been recently revisited by the US National Institute on Aging and Alzheimer's Association (Sperling *et al*, 2011a). Briefly, these guidelines redefined AD as a continuum of clinical and pathological changes that begin with a decades-long pre-symptomatic phase. This period is subdivided into four stages ranging from 0 - 3, according to the gradual, ordered accumulation of AD pathological hallmarks. The pre-clinical stage is followed by the presence of objective memory impairments (beyond what is expected for the person's age and education but in the absence of dementia); a condition which is known as mild cognitive impairment. Finally, as the pathology

advances, additional cognitive and functional deficits appear leading to a stage referred as clinical (or symptomatic) AD.

The concept of pre-clinical disease should not be too unfamiliar in medicine. For instance, cancer can be detected at an early stage before significant metastasis has occurred and, similarly, high cholesterol levels are often a good predictor of future risk or myocardial infarct. In other words, other human diseases can be detected and treated before the emergence of symptoms. To translate this to the management of AD, the goal would be to identify individuals with biomarker positivity and begin treatment before progression to clinical disease. But how strong is the evidence that individuals with Aβ or tau changes are at increased risk of transitioning to dementia later in life?

In individuals with MCI, biomarkers of brain amyloidosis (e.g. low CSF $A\beta_{42}$ levels or high retention of the tracer ^{11}C-PIB) have been associated with a higher likelihood (3-5 times greater) of dementia progression (Hansson *et al*, 2006; Buchhave *et al*, 2012). Similarly, healthy older adults with evidence of pre-clinical AD (stages 1-3), developed clinical symptoms of MCI or AD in greater proportion than amyloid or tau-negative individuals; a likelihood that increased across stages (Knopman *et al*, 2012; Vos *et al*, 2013). Complementing these observations, asymptomatic subjects bearing familial AD mutations, also display similar temporal biomarker changes preceding expected symptom onset (Bateman *et al*, 2012). Importantly, besides the increased likelihood of transitioning to MCI or AD, asymptomatic subjects with Aβ biomarker positivity exhibit functional and structural brain abnormalities and subtle deficits in episodic memory, compared to

amyloid-free individuals (Pike *et al*, 2007; Sperling *et al*, 2009; Lim *et al*, 2012; Sperling *et al*, 2013a; Villemagne *et al*, 2013; Lim *et al*, 2014). Therefore, an increasing number of studies would support the existence and pathological relevance of a pre-symptomatic AD stage.

A corollary of this assertion is the hypothesis that clearing Aβ at this early stage should prevent or slow the emergence of AD symptoms. Further to it, prospective longitudinal studies and the recent failure of anti-Aβ immunotherapy in symptomatic AD indicate that although Aβ may initiate the cascade of events that lead to AD, the disease becomes disconnected from Aβ at late stages, given that amyloid accumulation slows down and reaches a plateau (Villemagne *et al*, 2013). At this point, the resultant neurodegenerative changes (e.g. synaptic and neuronal loss, reduced brain glucose metabolism) are the dominant substrate of the cognitive dysfunction; explaining why Aβ would not be a suitable target at advanced disease stages.

Whether Aβ is indeed the initial driving factor in AD pathogenesis will be tested in three ongoing secondary prevention immunotherapy trials which have been recently launched (2012-2013) for pre-symptomatic AD. For a review about the rationale behind these studies see (Carrillo *et al*, 2013). Secondary prevention refers to delaying or arresting symptom onset in individuals with biomarker evidence for pre-clinical disease.

The Anti-Amyloid Treatment in Asymptomatic AD Trial, also known as A4, will study clinical normal older adults, age 70 or older, with evidence of brain

amyloidosis (as determined by PET imaging). These subjects will be treated with a monoclonal antibody against soluble Aβ (Solanezumab, developed by Elli Lilly) in a double-blind, placebo-controlled study during three years, including a two-year clinical follow-up upon trial completion. Therefore, this intervention will test whether targeting Aβ at early stages will prevent downstream neurodegenerative lesions and preserve cognition.

The Dominantly Inherited Alzheimer Network (DIAN) Trial will treat asymptomatic individuals that carry a genetic mutation for AD and who are approximately 10 years before expected symptom onset. The first aim will be to compare three putative disease-modifying drugs, Solanezumab, Gantenerumab (against fibrillar Aβ, developed by Roche) and a BACE inhibitor (developed by Elli Lilly) against a shared placebo, in terms of biomarker indications of efficacy during a two-year treatment. Cognitive outcomes will be further evaluated with the most promising drug(s) during three subsequent years of treatment.

Finally, the Alzheimer Prevention Initiative (API) will test the anti-Aβ antibody Crenezumab (developed by Genentech) in two groups of at-risk individuals: one will consist of asymptomatic subjects (age 35+) bearing PSEN1 mutations, belonging to large Colombian pedigree. The second group will be formed by asymptomatic individuals (age 50+) who are homozygous for the APOE ε4 allele (and for that reason, potentially at risk). These two groups of subjects will be treated for two years, with drug or placebo, and primary outcome measures will include cognitive testing as well fluid and imaging biomarker monitoring.

To further illustrate the increasing acceptance that the redefinition of the AD trajectory has had, the Food and Drug Administration (FDA) has recently issued a new regulatory framework to accelerate the approval of early stage Alzheimer drugs (Kozauer & Katz, 2013). In its traditional criteria, the FDA requested evidence of both cognitive and functional improvement for a new Alzheimer drug to be approved. However, given the increased shift toward preventive therapies, these guidelines would be difficult to follow in patients with pre-clinical disease or MCI, who only show mild (or little) evidence of cognitive decline and carry out a normal life. Therefore, the new FDA criteria will allow a new Alzheimer drug to be approved on the basis of cognitive improvement alone in patients with compromised memory but who do not present dementia, with the condition that subsequent studies will confirm the clinical benefit. This is an important advance in the field of AD drug development because it means that a public health institution recognizes that earlier treatment might be the clue for therapeutic success and that treating patients at the dementia stage may be too late to arrest disease progression.

Despite the increasing number of studies on pre-clinical AD (>2000 hits in PubMed), this concept is still very recent and our understanding of this stage is poor. One of the biggest challenges that we face is that with current biomarkers, it is yet difficult to ascertain with certainty who will be at risk of progressing to MCI or dementia, at an individual basis. Therefore, future clinical longitudinal studies are needed before the translation of current pre-clinical AD criteria into formal diagnostic guidelines used in a doctor's office. Equally important for earlier

diagnosis is to deepen our understanding of the mechanisms of AD onset and progression, and basic research is central to generate that knowledge.

5.3. Alzheimer's disease pathology in Down's syndrome: similarities and differences

It is well established that nearly all adults with DS will develop the neuropathological changes of AD, including amyloid plaques, neurofibrillary tangles and overt gliosis (Jervis, 1948; Wisniewski *et al*, 1985; Mann, 1988b; Lemere *et al*, 1996). Given the increased life expectancy of adults with DS, the prevalence of dementia and other age-related disorders has gained prominence in this population. Therefore, the study of DS should give valuable insight into the temporal progression of AD pathology; a knowledge that could be used to better understand the onset of clinical AD in the general population. However, is the pathology in DS similar to that in individuals with AD?

Several studies support that, the morphological and biochemical properties of amyloid-β plaques and neurofibrillary tangles are virtually identical between AD and DS. The 4 kDa, β-pleated protein extracted from vascular and parenchymal amyloid deposits has an equal amino acid composition in both conditions (Glenner & Wong, 1984a; Masters *et al*, 1985). Even post-translational changes to Aβ are similar between AD and DS, such as pyroglutamate modifications (Frost *et al*, 2013). Likewise, paired helical filaments are constituted of abnormally phosphorylated tau protein filaments in adult DS subjects (Hanger *et al*, 1991).

Furthermore, the topographical distribution and development of Alzheimer-type changes in DS closely follows the pattern of brain areas affected in AD (Mann *et al*, 1986). There is a prominent presence of amyloid plaques and tangles in the frontal, temporal and parietal cortices as well as in the hippocampus and amygdala. The latter regions are the first brain areas to be affected by AD pathology, in both conditions. Conversely, the occipital cortex and cerebellum are usually spared of neuritic plaques or tangle formation. Deficits in transmitter systems and their respective neuronal populations are also similar between AD and DS; with prominent pathological alterations in neurons of the nucleus basalis (cholinergic), locus coeruleus (noradrenergic) and raphe nuclei (serotonergic) (Yates *et al*, 1980; Yates *et al*, 1981; Yates *et al*, 1986).

There is more controversy in the literature regarding the density of Aβ and tau pathology in AD versus DS. While some studies indicate that amyloid plaque and neurofibrillary tangle burden is comparable in cortex and hippocampus (Ball & Nuttall, 1980; Ropper & Williams, 1980); other reports indicate that DS brains exhibit higher densities of neuritic plaque and tangle pathology than elderly subjects with AD (Mann *et al*, 1986; Mann *et al*, 1987; Hof *et al*, 1995). A possible explanation for these differences in plaque and tangle burden is that in DS, the AD pathology develops over many years, beginning very early in life, driven by APP overexpression.

Regarding the clinical presentation, the prevalence of AD also increases with age in both conditions (Lai & Williams, 1989). Although the initial signs of dementia in DS often involve changes in behavior, short-term memory is also affected early,

similar to AD. However, this may be more difficult to notice due to the underlying cognitive difficulties that DS subjects exhibit. In both conditions, the presence and progressive development of AD neuropathology occurs over many years preceding the onset of dementia; and likewise not all DS subjects will progress to develop clinical AD. Many live on well beyond their 50s and die with substantial evidence of AD neuropathology but without dementia.

In sum, the AD pathology and clinical syndrome in adults with DS is considerably similar to that of AD in the general population. Therefore, investigating early disease mechanisms in DS may generate important knowledge that could be extrapolated to find better treatments for AD patients and vice versa. An example of such cooperative effort is the recent clinical pilot study named the Down Syndrome Biomarker Initiative (DSBI), which has been launched in early 2013 with the goal of discovering novel biomarkers of dementia in individuals with DS (Ness *et al*, 2012). This private-public program (Janssen Pharmaceuticals, NJ, USA and the Down Syndrome Center for Research and Treatment at University of California, San Diego) has enrolled 12 DS participants aged 30-60 years and will examine novel AD indicators (including imaging, blood-based biomarkers and neuropsychological evaluations) over three years. This initial study could provide valuable information on the temporal order of biomarker changes during AD progression, and bring new insights for the development of preventive therapies to arrest the onset of dementia both in AD and DS individuals. Supporting this effort, the Down Syndrome Consortium Registry (DS-Connect), is a recently created online database (sponsored by NIH), which will allow the exchange of information

about ongoing clinical studies between researchers, Down syndrome sufferers and their caregivers.

5.4. Evidence for NGF metabolic dysfunction during early stages of Alzheimer's disease

Previous studies from the Cuello lab have confirmed the occurrence of an up-regulation of proNGF levels in AD cerebral cortex (Fahnestock *et al*, 2001; Pedraza *et al*, 2005a), and further demonstrated that the increased concentration the NGF precursor is accompanied by a strong reduction in tPA, plasminogen and plasmin (Bruno *et al*, 2009a). Moreover, analysis of the zymogenic activity of MMP-9, further revealed a robust increase in MMP-9 activity in AD, compared to control cases (Bruno *et al*, 2009a). These observations provided the fundamental grounds for demonstrating that the availability of NGF to basal forebrain cholinergic neurons is compromised at two levels in AD. First, the extracellular maturation of proNGF is impaired and second, the increase in MMP-9 activity would anticipate an exacerbated degradation of the mature NGF molecule in basal forebrain target tissue.

Our subsequent studies in DS (presented in Chapter 2 of this Thesis) have further demonstrated that a similar NGF metabolic imbalance occurs in this condition, at stages of advanced pathology and when dementia is present. This observation is of great relevance because it indicates that similar pathological mechanisms leading to cholinergic neuron atrophy may operate in AD and DS. It further invites to investigate NGF-metabolic deficits in DS subjects who are not yet demented.

Therefore, an important question arising from these studies is: how early in the disease process does NGF dysfunction manifest? Interestingly, increased cortical proNGF levels and elevated MMP-9 activity have been reported in parietal and frontal cortex tissue from MCI subjects (age range 66-98 years) (Peng *et al*, 2004; Bruno *et al*, 2009b). In both cases, proNGF levels and MMP-9 activity in MCI negatively correlated with neuropsychological measures of cognitive decline. Whether neuroserpin, plasminogen and tPA levels are altered in MCI brains remains to be determined; however, our studies in CSF (Chapter 3 of this Thesis) would anticipate AD-like CNS changes in these proteins at this stage.

The fact that elevated proNGF and MMP-9 activity appear as early as in MCI and correlate with the degree of antemortem cognitive impairment strongly suggests that NGF dysfunction is an early, relevant feature of β-amyloid pathologies. Supporting this case, our studies in DS fetal cortex (Chapter 2) showed elevations in proNGF and MMP-9 in DS cultures, accompanied by increased levels of sAPP-β, $A\beta_{40}$ and $A\beta_{42}$ peptides. The significance of this finding is twofold. First, it provides an additional solid argument for the involvement of Aβ peptides in NGF-dysfunction. Second, it hints that an early deposition of Aβ in the human brain, even in the absence of tau pathology, may be sufficient to trigger NGF metabolic dysfunction during the pre-symptomatic phases of AD. The fact that the intra-hippocampal injection of soluble synthetic Aβ oligomers in young rats was sufficient to increase proNGF levels and MMP-9 activity, as well as to induce the up-regulation of classical pro-inflammatory molecules, e.g. CD40 and iNOS, (Bruno *et al*, 2009a) further strengthens such hypothesis.

Future studies to investigate this important question are warranted; both in younger DS subjects without dementia (ideally examining different age groups) as well as in asymptomatic individuals with suspected latent AD. Preliminary observations (presented in Appendix I and II of this Thesis) would support the possibility that NGF-deregulation is altered early in the course of AD.

First, we have examined cortical levels of proNGF in a large population (n = 37) of cognitively healthy adults (age range 69-93 years) participating in the Religious Orders Study (ROS). The ROS is a longitudinal study of aging and AD with an extensive cognitive and neuropathological database, involving older clergy who agreed to a yearly medical evaluation and brain donation after death. We had access to quantitative measures of global Aβ and neurofibrillary tangle pathology examined from six brain regions, following previously reported procedures (Bennett *et al*, 2004). The cognitive status of each individual had been investigated with the MMSE and the Global Cognitive Score, the latter which consists of a battery of 19 tests measuring memory, perceptual speed and visuo-spatial abilities, expressed as a z score. We have used the pathological information to segregate non-cognitively impaired (NCI) subjects into cases with and without substantial evidence for AD pathology, using a cut-off score obtained from the average amyloid and tangle load of MCI and AD cases. The NCI cohort was thus subdivided into NCI-H ("healthy") and NCI-AD (representing asymptomatic individuals with AD-like pathological changes); for demographic details see Table A1. Following this segregation into two groups, we confirmed that the NCI-AD group exhibited significantly higher Aβ and tangle burden than NCI-H subjects

(Figure A1; Appendix I). In line with this observation, the ApoE ε4 allele was more prevalent in individuals within the NCI-AD group (Table A1; Appendix I).

Our preliminary investigation would indicate that cortical (frontal cortex) levels of proNGF are higher in asymptomatic adults with evidence of AD neuropathology (NCI-AD), compared to NCI-H individuals (Figure A1; Appendix I). Importantly, NCI individuals with substantial AD pathology and higher proNGF levels also exhibited lower Global Cognitive Score values, compared to asymptomatic subjects with little to no pathology (Figure A1; Appendix I). These preliminary observations support our hypothesis that NGF dysfunction may occur early in the course of AD, as early as in the pre-symptomatic stages of the disease. This analysis will be extended and validated in subsequent investigations with ROS post-mortem brain material; also to examine other members of the NGF pathway (i.e. MMP-9, neuroserpin, plasminogen and tPA).

A second promising observation we have gathered involved the preliminary investigation of proNGF and MMP-9 levels in post-mortem frontal cortex tissue from a small pilot cohort of DS newborns (age range 1 – 13 months) and age-matched control brains (Appendix II of this Thesis). Although we could not do a quantitative analysis due to the small sample size, examination of proNGF (via Western blotting) and MMP-9 mRNA levels (by qRT-PCR) in frontal cortex homogenates revealed higher proNGF levels in DS brains at 1-3 months of age compared to a control case of similar age (Figure A2, Appendix II). This difference was also evident at a later time point (9-13 months), except for one DS case with lower proNGF expression. Records from the brain bank indicate an unusually high

post-mortem interval (>80 h) for such case, suggesting that protein degradation may have occurred (Figure A2, Appendix II). It would be of paramount importance to extend this study to a larger cohort of DS newborns and to include infants and adolescents in the analysis of NGF-metabolic deficits.

In brief, the evidence for NGF dysfunction in AD, MCI and DS, together with the above-mentioned preliminary observations support the possibility that alterations in NGF metabolism and in basal forebrain cholinergic neurons occur early in the course of AD pathology development and therefore, justify future investigations on this topic. This possibility is also strengthened by recent *in vivo* MRI data revealing reduced basal forebrain volume in individuals with MCI (which correlated with amyloid deposition and cognitive impairment); and higher annual rates of basal forebrain atrophy in cognitively healthy subjects who later declined to clinical AD (Grothe *et al*, 2010; Grothe *et al*, 2013; Grothe *et al*, 2014).

5.5. Elevated proNGF: a signature of β-amyloid pathologies?

A growing number of studies, as discussed previously, indicate that accumulation of proNGF accompanies the presence of AD neuropathology in AD, MCI and DS. The fact that cortical proNGF levels correlated with the degree of cognitive decline in MCI and AD would make this neurotrophin an attractive candidate biomarker signalling the presence of AD neuropathology and a CNS trophic disconnection.

Analysis of NGF levels in CSF has revealed a significant increase in this neurotrophin in AD compared to CSF derived from control subjects. (Hock *et al*, 2000; Blasko *et al*, 2006; Mashayekhi & Salehin, 2006). However, these studies

were conducted using NGF sandwich ELISAs, which quantify both mature and proNGF molecules without distinction. It is likely that the reported NGF elevation in AD CSF reflects the accumulation of proNGF in the brain. However, future studies are warranted to investigate whether proNGF is indeed elevated in AD or MCI CSF and whether levels of proNGF in CSF correlate with cognitive function. Additionally, it will be relevant to investigate whether proNGF up-regulation is capable of distinguishing AD from other neurodegenerative diseases, such as Parkinson's, multiple sclerosis, tau-dementias, etc.

In this regard, a recent investigation led by Fahnestock and collaborators has provided novel evidence indicating that elevations in proNGF may occur in other CNS diseases, in the absence of Aβ. Specifically, they have found a significant elevation of proNGF in parietal cortex from individuals with Pick's disease (Belrose *et al*, 2014), which is another type of dementing disorder caused by aggregation of tau within neurons, predominantly affecting the fronto-temporal lobes (Cairns *et al*, 2007). However, the elevation of proNGF was not observed in other degenerative tauopathies, such as progressive supranuclear palsy and corticobasal degeneration (Belrose *et al*, 2014).

These observations reinforce the need for further studies to investigate the involvement of proNGF in different CNS diseases. However, they do not rule out the potential of proNGF as biomarker for AD. A possible strategy could be to combine the assessment of proNGF in a multiplexing platform with Aβ and tau and/or with other -yet undefined- biomarkers. For instance, the group of Dr. David Juncker at the Biomedical Engineering Department of McGill University has

recently developed a novel multiplexing approach called the antibody co-localization microarray, which allows the quantitative analysis of multiple targets (up to ~50) in complex samples (e.g. CSF, plasma). This procedure has the advantage of eliminating potential cross-reactivity between mixed reagents, as both capture and detection antibodies are co-localized to the same spot (Pla-Roca *et al*, 2012). This is an example of a potential diagnostic assay, which could be customized to accommodate multiple targets of interest to AD research, such as Aβ and tau (core AD pathology indicators); proNGF, MMP-9 and tPA (markers of NGF dysmetabolism), MMP-3 (marker of inflammation, microglial activation), etc.

5.6. Current status of Alzheimer's disease biomarkers: why looking for new candidates?

Having access to reliable biomarkers can have a significant impact on the management of AD. Firstly, they may help to design clinical trials with the right populations of subjects to receive disease-modifying therapies (i.e. individuals with advanced AD pathology, or with early indicators of disease onset). Secondly, they may help to monitor the efficacy and target engagement of novel pharmacological compounds. Thirdly, they may improve the accuracy of early AD detection and help to predict disease prognosis. The most common, validated AD biomarkers used in clinical research are listed in Table 5-1. Some of these markers reflect signature pathological processes of AD while others are indicators of brain degeneration, such as regional brain atrophy and reduced brain glucose utilization. For a comprehensive review on the leading fluid and imaging biomarkers in AD see (Hampel *et al*, 2008).

Table 5-1. Leading candidate biomarkers in Alzheimer's disease

Markers of brain amyloid accumulation
• $A\beta_{42}$ levels in CSF
• ^{11}C-PiB PET imaging
Markers of neurodegeneration and neuronal dysfunction
• Total tau or p-Tau in CSF
• ^{18}F-fluorodeoxyglucose PET (brain glucose metabolism)
• Volumetric MRI (regional brain atrophy)

Adapted from Sperling RA, et al., Nat Neurol 2013 (9) 54-58.

As reported by Hampel and colleagues, approximately 20 studies have been conducted on ~2000 individuals, reporting a consistent reduction of $A\beta_{42}$ levels in AD CSF (in the magnitude of 50%), compared to healthy aged adults of similar age (Clark *et al*, 2003; Fagan *et al*, 2006). Although its diagnostic sensitivity and specificity was high (80-90%) with respect to the non-demented group; the concentration of $A\beta_{42}$ in CSF may not be specific enough to distinguish AD from other types of dementias (Hampel *et al*, 2008). However, when $A\beta_{42}$ levels in CSF were compared with ^{11}C-PiB retention measures or with post-mortem analysis of brain amyloid load; it was found that virtually all individuals with low concentrations of $A\beta_{42}$ in CSF exhibited neuritic (or diffuse) CNS amyloid deposits (Strozyk *et al*, 2003; Fagan *et al*, 2006). Therefore, assessing the levels of Aβ in CSF may provide a good indication of brain amyloid deposition.

Although numerous studies have consistently found significant t-tau increases in AD CSF (in the magnitude of 300%) with high sensitivity, this determination alone may not be specific enough to provide a differential AD diagnosis. As a marker of neuronal degeneration, elevations in t-tau levels in CSF may occur in other neurodegenerative dementias, such as Creutzfeldt–Jakob disease (Buerger *et al*, 2006). Conversely, the assessment of specific phosphorylated forms of tau (p-tau) in CSF; such as p_{231}-tau (in threonine 231) or p_{181}-tau (in threonine 181), has been reported as highly specific to detect AD versus cognitively healthy subjects, and those suffering from other dementias (Hampel *et al*, 2004). A better diagnostic segregation may further be achieved by the combined assessment of $A\beta_{42}$ and p-tau in CSF; as discussed by (Thal *et al*, 2006).

Despite the promising aspects of Aβ and tau biomarker analysis some issues remain to be addressed. First, the predictive value of these biomarkers has not been firmly established at an individual basis. Second, standardization of laboratory assays and cut-off values will be key for the translation of biomarkers to the clinic: How much p-tau or $A\beta_{42}$ (e.g. in ng/L CSF) is too much, or too little? Third, although safe, lumbar puncture is yet an invasive procedure; sometimes associated with infections, or side effects such as headaches and pain. Therefore, blood biomarkers would be ideal because they are cost-effective (compared to the more sophisticated equipment needed for brain imaging) and they are easy to obtain and to examine, with minor complications. However, studies investigating Aβ levels in plasma have been mostly contradictory, or have reported no associations between Aβ plasma levels and those in CSF, as reviewed by (Hampel *et al*, 2008). In that sense, plasma may be a difficult source of AD biomarkers, as any biochemical change in the CNS will be diluted in the large blood volume. This highlights the advantage of the CSF which is in direct equilibrium with the extracellular CNS milieu and may thus offer reliable indicators of brain pathology.

However, a recent elegant investigation by Mapstone and colleagues proposes that the study of the blood proteome or metabolome may hold promise over Aβ and tau assessments for early pre-clinical AD diagnosis (Mapstone *et al*, 2014). Briefly, they described the identification of changes in ten phospholipids in plasma though the use of mass spectrometry, which was able to distinguish between cognitively stable older adults and those who converted to symptomatic disease (MCI / AD) within a period of 2-3 years (Mapstone *et al*, 2014). Although this approach was

highly sensitive (>90% accuracy), it remains to be determined whether a similar altered lipid profile occurs in other diseases. However, the results of Mapstone and colleagues may have great impact on future biomarker research studies; inviting the examination of novel markers in plasma (such as lipids) rather than only Aβ or tau. This is especially relevant for individuals with DS, where robust biomarkers signalling the presence of AD pathology, or the forthcoming conversion to dementia, have yet to be discovered.

5.7. Intracellular Aβ and cognitive impairments: what can we learn about early disease mechanisms from transgenic rats?

Transgenic animals models continue to be valuable tools in AD research. Despite their limitations in not fully modeling all aspects of the human condition (e.g. not developing neurofibrillary tangles and neuronal loss), they do exhibit important pathological disease hallmarks, such as amyloid plaques, dystrophic neurites, inflammation, synaptic loss and progressive cognitive impairments. Considering that all animals will invariably develop AD-like neuropathologic lesions, they offer an important investigative platform to examine the progression of the pathology at its earliest time points. This is particularly relevant when considering that, at present, there is no diagnostic evaluation that can detect the onset of AD with certainty in humans, prior its clinical manifestation.

In Chapter 4 of this Thesis we have investigated the evolution of the AD-like amyloid pathology, in a novel transgenic rat model of AD, developed in our laboratory. Transgenic rats offer important advantages over mice for modelling

AD; for a review see (Do Carmo & Cuello, 2013). Firstly, rats are genetically and physiologically closer to humans, compared to mice (Lin, 1995; Gibbs *et al*, 2004). Secondly, both rats and humans (as opposed to mice) exhibit a post-natal brain development, leading to an increased number and size of neurons and more complex synaptic networks (Whishaw *et al*, 2001; Snyder *et al*, 2009). Particularly important for modeling AD is the fact that rats display a richer behavioural and social phenotype than mice; being able to perform more complex cognitive tasks, involving spatial navigation skills, accurate motor coordination and visual discrimination (Whishaw *et al*, 2001; Horner *et al*, 2013). These are relevant advantages for modelling a disease with a central cognitive component.

The McGill-R-Thy1-APP transgenic rat is unique compared to many other rodent AD models. First, the AD-like amyloid pathology has been achieved with a single mutated human APP transgene insertion site per allele. Conversely, virtually all other mouse (and newer rat) models of AD exhibit multiple transgene copies or combinations of more than one transgene (e.g. APP and PSEN1) (Hall & Roberson, 2012), resulting in more aggressive phenotypes which are not common in familial AD cases. In addition, the only mouse model that exhibits amyloid plaque deposition together with NFT formation co-expresses (besides APP and PSEN1) a human tau mutant protein, which has not been observed in AD brains but is common in fronto-temporal dementia (Spires & Hyman, 2005). Therefore, the presence of a single APP transgene per allele makes the McGill-R-Thy1-APP transgenic rat the closest model to the human disease condition. Moreover, the topographical distribution of amyloid plaques in McGill transgenic rats is fairly

similar to that of human AD. The first incidental amyloid plaques appear in the hippocampal formation, particularly in the subiculum, followed by area CA1 and later by the rest of the neocortex. Similar to the human pathology, mature amyloid plaques are surrounded by distrophic neurites and activated microglia at advanced stages (Leon *et al*, 2010). The profile of Aβ peptides in CSF is also comparable between both species. For these reasons, the McGill-R-Thy1-APP transgenic rat line stands out as a valuable model for AD research.

Using McGill transgenic rats and high magnification microscopy we have shown that Aβ accumulates intracellularly within pyramidal neurons of the cortex and hippocampus; and that this immunoreactivity is distinguished from that deriving from APP or its C-terminal fragments. These results contribute to resolve the controversy regarding the nature of the intracellularly accumulated material in AD transgenic models, with a quantitative high-resolution microscopic analysis. Given the relevance of this knowledge to the human pathology, this work offers new opportunities to translate this methodology to examine human brain tissue of AD/MCI patients as well as of individuals with the earliest indications of pre-clinical pathology.

Further to it, the accumulation of intracellular Aβ in McGill transgenic rats was evident several months before amyloid plaques appear. At this time point, soluble $A\beta_{40}$ and $A\beta_{42}$ species were highly elevated, coincidental with the manifestation of cognitive impairments. However, in humans, the clinical manifestation of AD is often associated with advanced pathology, namely the presence of neuritic amyloid plaques, neurofibrillary tangles, synaptic loss and eventually neuronal death.

Therefore, why do cognitive deficits appear so early in transgenic animal models? Several possibilities can be reconciled. First, the complex neuronal connectivity of the human brain may allow it to withstand substantial pathology, compared to rodents. In other words, the more complex mental functions and cognitive activities in which humans engage may confer more plasticity to the human brain that allow it to compensate for damaged circuits; a concept that is known as cognitive reserve (Stern, 2002). An alternative explanation is that the earliest accumulation of Aβ pathology is also detrimental in humans, however we may as yet lack the tools to detect such subtle cognitive deficits. Therefore, a more accurate reflection of decline may be to establish individual cognitive baselines, rather than population-based assessments. This also reinforces the idea that the combination of several biomarkers may be needed to identify the earliest indication of a silent AD pathology in non-demented individuals.

This concept is currently being translated into an ongoing research diagnostic project known as "PredictAD", supported by top-class European research institutions (Karolinska Institute, Imperial College London, University of Eastern Finland, the VTT Technical Research Centre of Finland, The Memory Disorders Research Group at Rigshospitalet in Copenhagen and the Università degli Studi di Milano) and industry (GE Healthcare UK and Nexstim Ltd., Finland). The PredictAD project, which has run for 3,5 years, aimed to examine several AD biomarkers (including blood-based markers and MRI, PiB-PET, FDG-PET) and to investigate how to integrate data from such assessments and psychometric tests into a software tool that a physician could use to provide an AD diagnosis at the

earliest possible stage (based on biomarker data), in a standardized and objective manner (Antila *et al*, 2013). The tool may also prove useful to improve the monitoring of disease progression and to help select patients for clinical trials. More information can be found in the PredictAD website (http://www.predictad.eu).

5.8. Management of Alzheimer's disease in the near future

5.8.1. Is there promise for anti-Aβ therapies?

AD is a complex neurodegenerative disorder that evolves over many years. The recent failure of anti-Aβ immunotherapies has stirred scepticism on Aβ as a target and mainly on the validity of the amyloid hypothesis, as discussed by (Giacobini & Gold, 2013). However, the argument that Aβ is not a proper target may be true (or not) depending on the stage of the disease that it refers to.

The amyloid hypothesis centers the accumulation of Aβ as the initial event in AD pathogenesis. While Aβ may trigger the disease, this does not imply that following "onset", Aβ is the driver of neurodegeneration. In fact, in AD patients, there is only a weak correlation between the burden of amyloid plaques and the severity of dementia (Terry *et al*, 1991). Moreover, longitudinal studies have demonstrated that the rate of amyloid deposition slows down at late disease stages, when neurodegeneration is more pronounced (Villemagne *et al*, 2013). This would suggest that the initial CNS accumulation of Aβ may trigger downstream processes that are responsible for driving neurodegeneration and cognitive decline. Thus, clearing Aβ at these advanced stages should have little impact on cognition.

Therefore, the argument that Aβ is not a proper target may only apply at late-disease stages, when the pathology has become Aβ-independent. Importantly, despite the absence of cognitive improvement, recent immunotherapy trials have shown evidence of efficacy and target engagement (i.e. reduced brain amyloid burden and lower tau levels in CSF), indicating that we have biologically active drugs that work. The field will thus eagerly await the outcome of the three ongoing secondary prevention trials, which target the amyloid pathology at the earliest possible stages (discussed in Section 5.2).

5.8.2. Thinking beyond Aβ

Although the amyloid hypothesis is supported by solid evidence (as discussed in Chapter 1), it must be acknowledged that AD is a multifactorial disorder where other multiple pathways may contribute to disease, beyond the damage cause by Aβ. In this sense, the pathological role of inflammation and microglial activation, particularly at early disease stages, is gaining increasing attention among AD researchers.

In a recent genetic study, a novel, rare gene variant of TREM2 (triggering receptor expressed on myeloid cells 2) was reported to be associated with a significant increase in the risk of AD (Guerreiro *et al*, 2013). TREM2 is a membrane protein that forms signalling complexes with a tyrosine kinase receptor and triggers the activation of immune responses (Takahashi *et al*, 2007). In microglia, proper TREM2 function is associated with phagocytic activity and suppression of inflammatory cytokine production (Takahashi *et al*, 2007). The TREM2 variants

reported in the study by Guerreiro, St George-Hyslop, Hardy and colleagues, are associated with a loss of TREM2 function, suggesting that inflammation may play an important role in AD pathogenesis. In line with this concept, additional genome-wide studies have identified other inflammation-related susceptibility loci (besides TREM2) associated with increased risk of AD (i.e. the macrophage cell-surface lectin CD33, and the receptor for complement factor 3b (CR1)) (Lambert *et al*, 2009; Bradshaw *et al*, 2013).

Recent investigations by the Cuello lab have further supported that a pro-inflammatory process may occur very early during disease progression as a consequence of the accumulation of Aβ oligomers in the brain (Ferretti & Cuello, 2011; Ferretti *et al*, 2012a; Ferretti *et al*, 2012b). This finding is of high relevance, particularly when considering the potential link between inflammation and NGF deregulation. For instance, pro-inflammatory mediators such as interleukin-1β, TNF-α and nitric oxide (NO) are known to be potent stimulators of MMP-9 production and activation (Gottschall & Yu, 1995; Gottschall *et al*, 1995; Gu *et al*, 2002). Further to it, in the McGill-R-Thy1-APP transgenic mouse model of AD-like amyloid pathology, there is evidence for proNGF accumulation, increased MMP-9 activity and an early inflammatory process preceding the development of amyloid plaques (Bruno *et al*, 2009a; Ferretti *et al*, 2011). Administration of an the anti-inflammatory drug minocycline corrected learning and memory deficits in McGill transgenic mice and importantly, restored proNGF, iNOS and MMP-9 activity to values comparable to non-transgenic animals. It remains to be elucidated whether an early pro-inflammatory process occurs in the pre-symptomatic stage of

AD; particularly because numerous studies support an association between inflammation activation and advanced amyloid pathology (McGeer *et al*, 1987; Eikelenboom *et al*, 1998; Stoltzner *et al*, 2000; Okello *et al*, 2009).

Following our evidence indicating an NGF-metabolic compromise in adult DS subjects, it would be of interest, as a continuation of the presented studies, to investigate the involvement of inflammation and NGF-metabolic deficits at younger ages, in particular, several years before the onset of dementia. Given the presence of triplicated inflammatory genes in chromosome 21 (Wilcock, 2012), their increased expression would anticipate an early, exacerbated pro-inflammatory response in DS. The proposed investigations should therefore reveal the temporal order of AD pathology accumulation, NGF metabolic deficits and appearance of inflammation. Based on previous studies, a hypothetical sequence of such changes is depicted in figure 5-1.

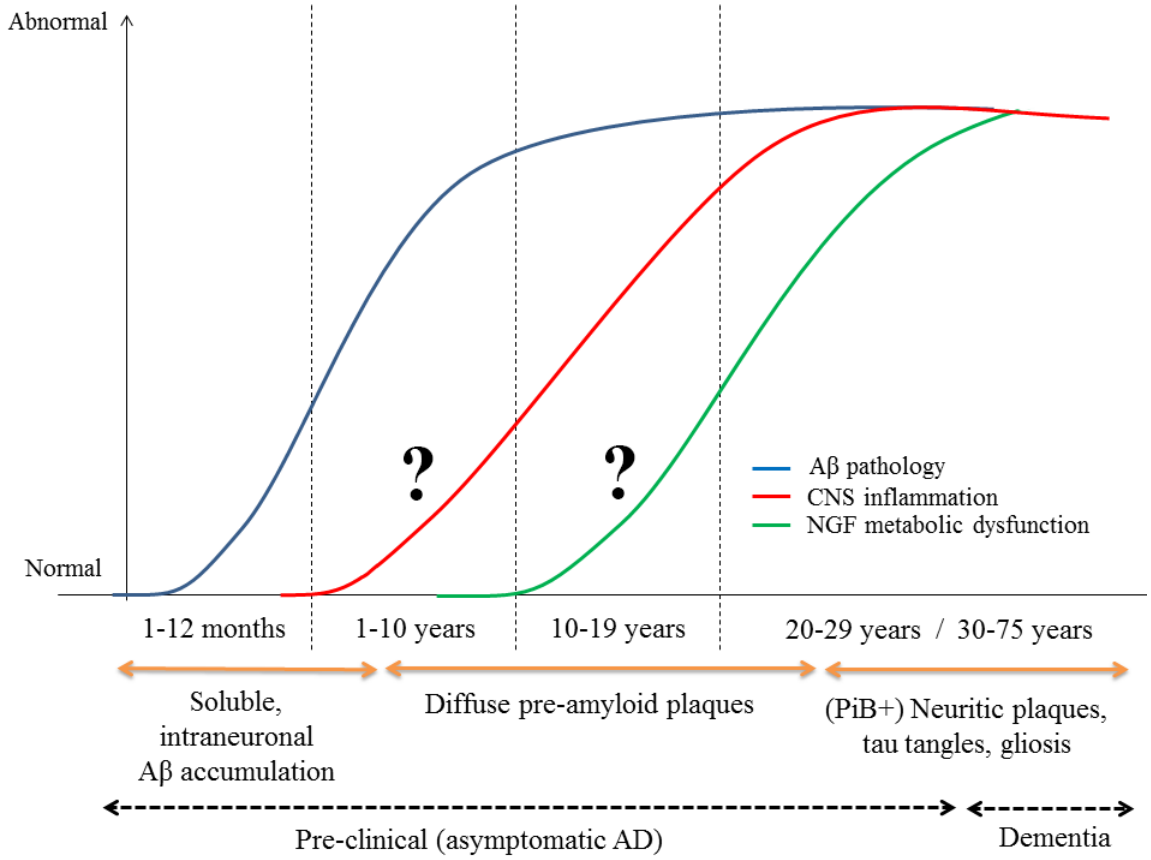

Figure 5-1. Hypothetical sequence of the development of Aβ pathology, CNS inflammation and NGF pathway alterations in Down's syndrome. The graph depicts the magnitude of abnormality (*Y axis*) vs. the clinical and pathological progression of AD in DS (*X axis*). The underlying hypothesis is that the early, gradual accumulation of Aβ peptides will unleash a CNS pro-inflammatory reaction, which will be followed by signs of NGF metabolic dysfunction. The graph is only hypothetical and it may be subject to modifications upon completion of the proposed investigations. From Iulita MF and Cuello AC 2014, unpublished.

5.8.3. Alzheimer's disease prevention: is there something we can do?

The 2010 report by the US Alzheimer's Association, entitled "Changing the Trajectory of Alzheimer's Disease: A National Imperative" estimated that a hypothetical intervention that delayed the onset of AD by 5 years would diminish both the number of AD sufferers and the associated healthcare costs by half. This is not a negligible benefit.

Growing evidence from epidemiological and interventional studies indicates that certain modifiable risk and protective factors may impact the onset of AD dementia (Fratiglioni & Qiu, 2011). For instance, elevated blood pressure, high cholesterol levels, smoking and physical inactivity have been attributed to increase the susceptibility of dementia later in life; as reviewed by (Mangialasche *et al*, 2012). Conversely, high education, an engaging social network, regular physical exercise and a healthy diet (antioxidants, low fat, fish consumption) have all been linked –in combination or alone- to reduce the risk of dementia (Mangialasche *et al*, 2012). It is important to note that there might be a certain time window when these factors have a stronger effect. It seems unlikely that a good diet and physical exercise will prevent disease progression in a person with clinical AD, when extensive brain damage has already occurred. At present, there are a number of ongoing long-term (~5-7 years) interventional studies in non-demented older adults investigating the impact of multifactorial lifestyle interventions (diet, exercise, cognitive training) on the onset of dementia (Mangialasche *et al*, 2012). The evidence that will originate from these studies will be paramount to develop strategies to delay or prevent the

onset of dementia and subsequently promote awareness and education in the population.

5.9. Concluding remarks: investigating pre-clinical Alzheimer's disease

The current view of AD recognizes that its neuropathological lesions develop over many years before the onset of cognitive decline and dementia. Therefore, our goal was to gain a deeper understanding of the molecular events that accompany the development of AD pathological hallmarks.

The work presented in this doctoral Thesis provides multidisciplinary evidence to support that the early development of AD pathology has detrimental consequences in the CNS (e.g. NGF-metabolic deregulation and learning and memory deficits). It also reinforces the concept that biomarker combinations may hold promise for the identification of individuals at different disease stages (e.g. pre-clinical, MCI, AD).

Furthermore, our studies present novel angles to explore early brain alterations in asymptomatic individuals with "latent AD", for instance by examining markers of NGF dysfunction or inflammation. Our investigations may thus bring new clues for the development of novel cholinergic neuroprotective treatments, such as aiming at correcting NGF-metabolic deficits.

Lastly, our work reinforces the importance of studies focusing on the convergence between AD and DS; particularly for the better understanding of early pathological events in individuals at high risk of progressing to clinical AD.

Original Contributions

The work comprised in this doctoral Thesis provides several contributions to original knowledge.

First, we have discovered that adult Down's syndrome subjects with AD dementia exhibit deficits in the extracellular metabolism of NGF, similar to those previously reported in brains of patients affected with Alzheimer's disease. We have further detected such CNS NGF alterations in a widely used animal model of DS, the Ts65Dn mouse line, as well as in primary cultures derived from DS fetal cortex. This work, described in Chapter 2 of this Thesis, has helped to resolve the paradox that basal forebrain cholinergic neurons become atrophic in a context of abundant levels of the NGF precursor, proNGF. The results of this project also strengthen the evidence for an NGF trophic disconnection in AD and DS brains and provide a novel, mechanistic rationale for the preferential atrophy of cholinergic neurons in these neurodegenerative diseases.

Second, we have identified that AD and MCI patients exhibit distinct signature changes in the CSF with respect to markers of NGF dysmetabolism. As specified in Chapter 3, we have found that MMP-3 is elevated and tPA diminished in CSF from AD subjects. In both cases, we have found correlations between the levels of these analytes in CSF and clinical and pathological parameters (i.e. MMSE scores, Aβ, t-tau and p-tau concentrations in CSF). Instead, we have found that the CSF of subjects with MCI was characterized by elevations in plasminogen, TIMP-1 and neuroserpin, compared to control subjects. The results of this work thus invite

future investigations to evaluate the specificity of these novel markers to discriminate between AD and MCI versus other causes of cognitive decline.

Third, we have used a multi-dimensional approach to investigate the intraneuronal amyloid pathology in APP transgenic rats, including high-magnification and super-resoultion microscopy, ELISA and MALDI-MS analysis of Aβ peptides in brain and CSF from transgenic rats. This study allowed to resolve the controversy that Aβ peptides accumulate intraneuronally and also highlighted the concept that the species which exist inside pyramidal neurons include Aβ peptides, as well as APP and its C-terminal fragments. The immunoprecipitation of Aβ from the rat CSF followed by MALDI-MS analysis demonstrated that it is possible to monitor the amyloid pathology in the rat CSF. This protocol thus offers a novel method to evaluate the efficacy of experimental therapeutics in biological fluids of transgenic animals. Further to it, the detailed behavioral characterization we have done at early and late time points permitted us to establish that the development of the soluble Aβ pathology was accompanied by CNS deficits, before the progression of full-blown AD pathological hallmarks. For such analysis we have defined a novel, cognitive composite, denominated “cognitive index”, indicative of the global cognitive performance of the rats tested. Therefore, this study invites to reconsider the need for more sensitive cognitive tests to detect subtle signs of CNS dysfunction in humans, at the earliest stages of the disease. Together, the techniques and analysis presented in this work may also be highly valuable for the study of other neurodegenerative diseases of protein aggregation.

List of Publications

1. Nerve Growth Factor Metabolic Dysfunction in Down's Syndrome Brains. **MF Iulita**, S Do Carmo, AK Ower, AM Fortress, L Flores Aguilar, M Hanna, TM Wisniewski, AC Granholm, M Buhusi, J Busciglio and AC Cuello. *Brain* 2014, Mar; 137 (Pt 3): 860-72. doi: 10.1093/brain/awt372. Epub 2014 Feb 11.

2. Analysis of Matrix Metallo-Proteases and the Plasminogen System in Mild Cognitive Impairment and Alzheimer's Disease Cerebro-Spinal Fluid. CE Hanzel*, **MF Iulita***, H Eyjolfsdottir, E Hjorth, M Eriksdotter and AC Cuello. *The Journal of Alzheimer's Disease* 2014, Feb 14. [Epub ahead of print] *co-first authors.

3. Intracellular Aβ Pathology and Early Cognitive Impairments in a Transgenic Rat Model Overexpressing Human Amyloid Precursor Protein: A Multidimensional Study. **MF Iulita***, S Allard*, L Richter, LM Münter, A Ducatenzeiler, C Weise, S Do Carmo, WL Klein, G Multhaup and AC Cuello. Acta Neuropathol Commun. 2014 Jun 5;2(1):61. *co-first authors.

4. Nerve growth factor metabolic dysfunction in Alzheimer's disease and Down's syndrome. **MF Iulita** and AC Cuello, *Trends in Pharmacological Sciences*, 2014 Jun 21. pii: S0165-6147(14)00072-8

5. Neuronal-driven pre-plaque neuroinflammation in a transgenic rat model of Alzheimer's disease. CE Hanzel, A Pichet-Binette, L Pimentel, **MF Iulita**, S Allard, A Ducatenzeiler, S Do Carmo and AC Cuello. *Neurobiology of Aging* 2014 Mar 28. pii: S0197-4580(14)00283-8.

6. Abeta-induced Inflammation and NGF Deregulation in Transgenic Models of Alzheimer's Disease-Like Amyloid Pathology. AC Cuello, MT Ferretti and **MF Iulita**, *Neurodegener Dis* 2012; 10(1-4): 104-7.

7. Early-Stage Inflammation and Experimental Therapy in Transgenic Models of the Alzheimer-Like Amyloid Pathology. AC Cuello , MT Ferretti, WC Leon, **MF Iulita**, T Melis, A Ducatenzeiler , MA Bruno, F Canneva, *Neurodegener Dis*. 2010; 7(1-3): 96-98.

8. Cholinergic involvement in Alzheimer's Disease. A link with NGF maturation and degradation. AC Cuello, MA Bruno, S Allard, WC Leon, **MF Iulita**, *J Mol Neurosci*, 2010; 40 (1-2):230-5.

Appendices

Appendix I

Investigation of proNGF levels in asymptomatic individuals with pathological evidence of latent Alzheimer's disease

Table A1. Demographics of the NCI population studied

		NCI-H (*n* = 21)	NCI-AD (*n* = 16)	*t* test
Age	Mean ± SD	80.85 ± 8.933	84.46 ± 8.548	$P > 0.05$
Gender	F / M	10 F / 11 M	9 F / 7 M	N/A
Education	Mean ± SD	17.62 ± 4.353	18.13 ± 2.553	$P > 0.05$
ApoE genotype	ε4/ total	2 / 21 (9.5 %)	6 / 16 (37.5 %)	N/A

NCI = non-cognitively impaired

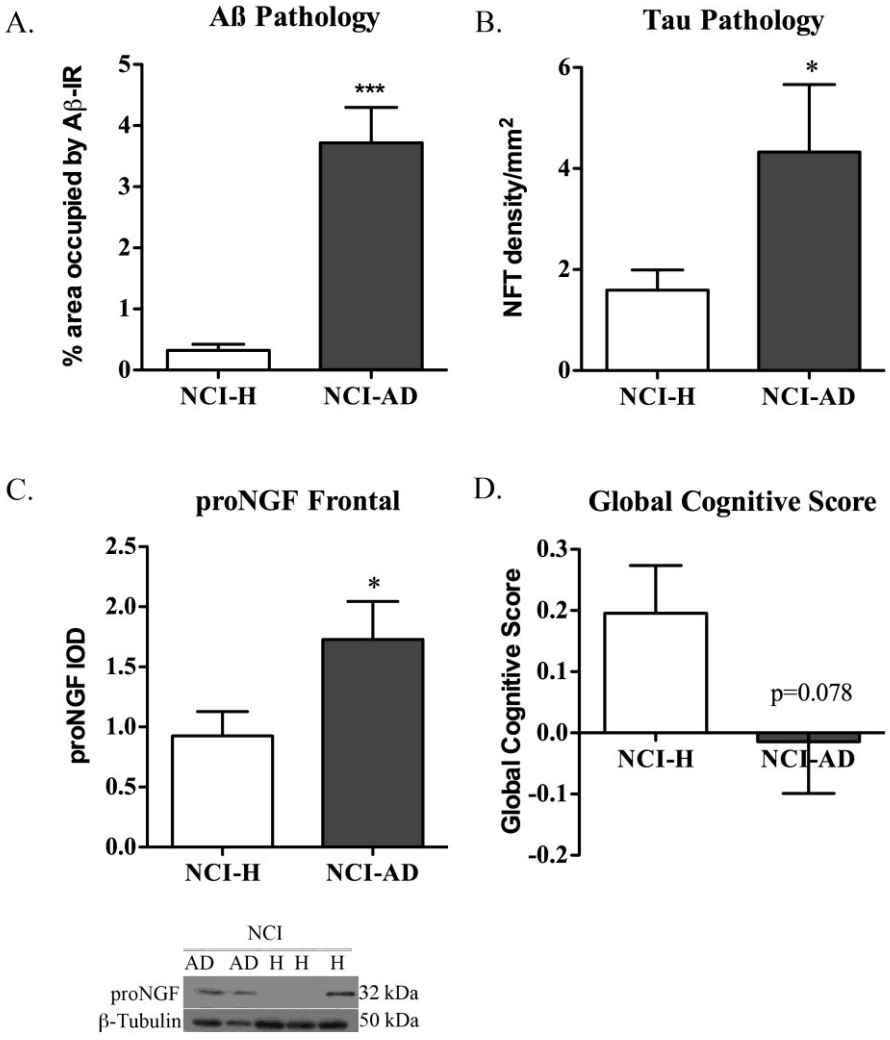

Figure A1. Segregation of NCI individuals with pathological evidence of Alzheimer's disease. A) A quantitative measure of global Aβ burden was obtained from six different brain regions (entorhinal cortex, CA1/subiculum, frontal, temporal, parietal and primary visual cortex) and expressed as percentage area occupied by Aβ-immunoreactivity. B) Tau pathology is expressed as NFT density per square millimeter. C) proNGF levels were measured from frontal cortex homogenates by Western blotting with a polyclonal antibody specific for the NGF precursor protein. D) The Global Cognitive Score is expressed as a z score, from 19 different cognitive tests. Graphs represent mean ± SEM; Student's *t* test; **P*<0.05; ****P*<0.001. Iulita MF, Bennett DA, Cuello AC 2014, unpublished.

Appendix II

Analysis of proNGF and MMP-9 in frontal cortex from Down's syndrome newborns and infants

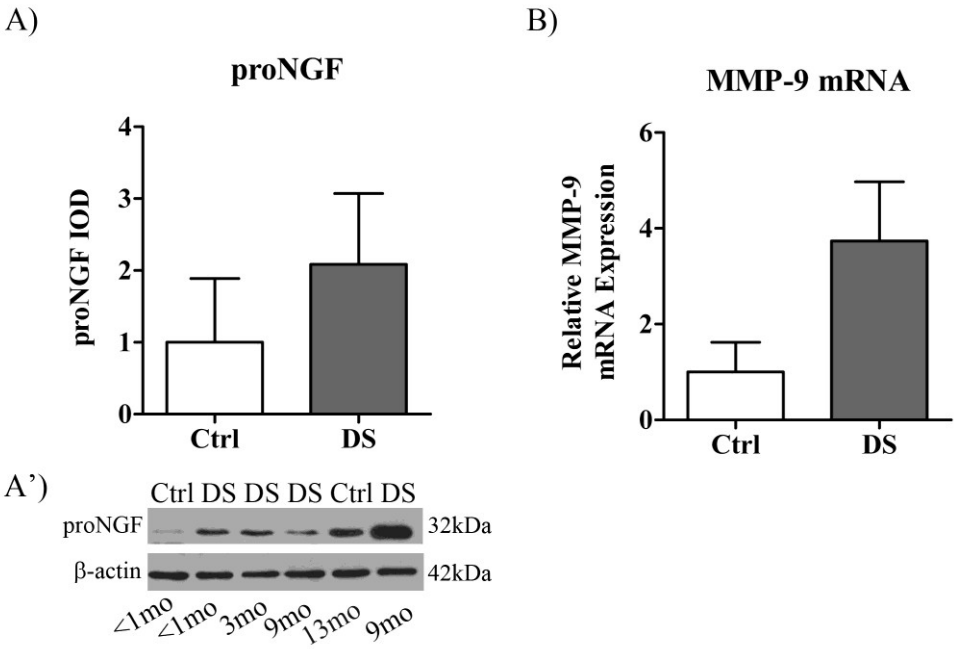

Figure A2. Early signs of NGF dysfunction in Down's syndrome. Preliminary analysis of proNGF (Western blotting) and MMP-9 mRNA levels (qRT-PCR) in frontal cortex homogenates from newborns (<1 month of age) and infants (3-13 months of age) with and without DS. A) There was a trend reflecting higher proNGF levels in DS brains (pooling all ages together). A') At 1mo-3mo proNGF was higher in DS brains compared to a control case of similar age (1mo). This difference was also evident at a later time point (9-13 mo), except for one DS case with lower proNGF expression. Records from the NYU brain bank indicate an unusually high post-mortem interval (PMI) (>80 h) for such case, suggesting that protein degradation could have occurred. B) A similar trend reflecting increased MMP-9 mRNA levels was observed in DS brains between 1-9 months of age. Iulita MF, Wisniewski T, Cuello AC 2014, unpublished.

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