Proteomic analysis of inflammatory protein expression patterns in cell culture and transgenic animal models for Alzheimer's disease

Inauguraldissertation

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Zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Augé Christelle

Aus France

Basel, 2004

Genehmigt von der Philosophisch Naturwissenschaftlichen Fakultät auf Antrag von

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Basel , 19th of October, 2004.

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Acknowledgments

Part of this work was performed in the Institute of Physiology, University of Basel and part of the work was performed in F. Hoffmann- La Roche AG, Basel, Switzerland.

First of all, I would like to express my deepest gratitude to my principal supervisors Professor Dr. U. Otten and PD Dr. D. Kunz for their expert guidance and support throughout my work.

In addition I would like to thank the entire Physiology group and particularly Dr. Pia März-Weiss and Dimitriades Beatrice for their friendly support.

I am indebted to PD Dr. H. Langen for his reception into the proteomics group at the Roche Center for Medical Genomics of F. Hoffmann -La Roche AG, and I would like to thank Dr. Peter Bernd for his expert help in his group.

I am greatly indebted to Gaby Walker, my supervisor in F. Hoffmann -La Roche AG, for her understanding and her scientific guidance through this work.

I wish to thank in F. Hoffmann -La Roche AG: Dr.Laurence Ozmen, Patrick Biry, and Marie-Thérese Miss for their helpful collaboration with the dissection of mice.
Dr. Manfred Brockhaus for providing the antibody against tyrosine nitrated proteins, Roland Rémy for his contribution in chromatography technologies and Bernd Müller for LC-MS/MS.

I owe my warmer thanks to Dr. Pol Harvengt for his constructive advice, Julie Eggemann and Anita De Vivo for their helpful and excellent technical assistance. They were very important friends of mine during these years.

I wish to extend my thanks to all my colleagues in F. Hoffmann-La Roche AG with whom I spend really good hours during these years.

Finally, I wish to thank my family and my dear Cédric for their love, support and understanding over these years.

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Abbreviations

Aβ 40 and 42	Amyloid beta 40 and 42
AD	Alzheimer's disease
ACh	Acetylcholine
ACTB	Actin beta
ADAM	Adamalysin family
ALFC	Aldolase C
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BACE	βAPP cleaving enzyme
BBB	Blood brain barrier
BSA	Bovine serum albumin
CDK4	Cell division kinase 4
CNS	Central Nervous System
CJD	Creutzfeld Jakob Disease
Crry	Complement receptor-related protein-y
CSF	Cerebrospinal fluid
DMEM	Dulbecco's modified Eagle's medium
DPY2	Dihydropyrimidinase related protein 2
DS	Down's Syndrome
DTE	Dithioerythreitol
EOFAD	Early onset familial Alzheimer's disease
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FCS	Fetal calf serum
GDIA	Rab-gdp dissociation inhibitor alpha
HPLC	High Performance Liquid Chromatography
IEF	Isoelectric focussing
IF2A	Eukaryotic initiation factor-2 alpha
IL-1	Interleukin-1
IL-6	Interleukin-6
IPG	Immobilized pH gradients
LTD	Long-term depression
LTP	Long-term potentiation

MAP	Microtubule-associated protein
MALDI	Matrix Assisted Laser Desorption Ionisation
MKKs	MAP kinase kinases
MS	Mass- spectrometry
MT	Metallothionein
NDKA	Nucleoside diphosphate kinase A
NFT	Neurofibrillary tangles
NGF	Nerve growth factor
NO	Nitric oxide
NOS	NO synthase
NP40	Nonidet p40
NSO cells	Mouse myeloma cell line
PBEF	Pre-B-cell enhancing factor
PBS	Phosphate buffered saline
PDX	Peroxiredoxin
PHF	Paired helical filament
PP2A	Serine/threonine phosphatase 2A
PS1 and PS2	Presenilin 1 and 2
ROS	Reactive oxygen species
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TACE	Tumor necrosis factor α- convertase
TBA1	Tubulin alpha 1chain
TBB5	Tubulin beta 5
TCP-1	T-complex protein 1
TFA	Trifluoroacetic acid
TKT	Transketolase
TNF-α	Tumor necrosis factor-alpha
TOF	Time of flight
TPIS	Triosephate isomerase
SNAP	N-ethylmaleimide-sensitive factor attachment protein

SUMMARY

Dementia is a syndrome characterized by failure of recent memory and other cognitive functions that is usually insidious in onset but steadily progresses with age. Alzheimer's disease (AD) is the most common form of senile dementia. It is neuropathologically characterized by extracellular and perivascular aggregation of amyloid β (Aβ) peptide, by the generation of intracellular neurofibrillary tangles due to a hyperphosphorylation of tau protein and by an increased rate of neuronal degeneration. The degenerative process starts 20-30 years before the clinical onset of the disease. Clinical diagnosis of AD is difficult but possible, but can only be confirmed by biopsy or autopsy. At present, no biological marker exists for early diagnosis of AD during life. Therefore, identification of biomarkers for AD would be of great value for clinical diagnosis of incipient AD.

Recent studies have proven the involvement of inflammatory processes in the neurodegenerative events in AD. Inflammation may not be the first event in the progression of the disease, but it involves activation of glia cells including microglia and astrocytes and subsequent release of proinflammatory mediators. Cytokines released such as IL-1, TNF-α and IL-6 are the main proinflammatory cytokines that can modulate inflammatory responses as well as glial proliferation and activation. Oxidative stress triggered by inflammatory processes causes changes in proteins such as tyrosine nitration or lipid peroxidation. Aβ deposits, tau hyperphosphorylation, inflammation and oxidative stress may finally lead to changes in synaptic connectivity and efficacy including perturbation of long-term potentiation (LTP), important in the formation of memory.

Proteomic technology used in these studies is a recent technology which is a two step process: separation of proteins and their subsequent analysis by mass spectrometry. Moreover, this technology can provide new information concerning the expression level, post-translational modification of specific proteins as well as their conformational changes during disease progression. In our study, this technology was modified and improved, e.g by the miniaturization of the complete process. Proteomic technology was also used in parallel with other methods such as chromatography in order to increase the sensitivity of detection by mass spectrometry.

This study aimed:

1) To establish that cytokine treatment of human microglia cells is an efficient method to study certain aspects of AD pathogenesis. For this analysis, a map of protein expression in normal and in treated microglia cells was made.

2) To map protein expression in APP/PS2 transgenic mice, a model for human AD, in order to compare human AD brain with murine models.

3) To identify highly nitrated proteins in brains of transgenic animals. Several proteins were found to be modified after injury.

4) To provide evidence for instability of synapses in AD brains. To start with this study, the technologies used to map mouse brain cytosolic proteins were improved.

5) To isolate synaptosomal membranes from the whole brain and to analyse it by mass-spectrometry. For mapping synaptic membrane protein expression in controls or transgenic mouse models, the technology was miniaturizated and optimized. This study is still in progress.

I- INTRODUCTION

I.1- INFLAMMATION AND ALZHEIMER'S DISEASE

I.1.1- Alzheimer's disease

Neuropathological changes in brains of patients with age-related dementia have first been described in 1906 by Alois Alzheimer, a Bavarian psychiatrist. This disorder, which was later named Alzheimer's disease (AD), was for a long time not recognized as different from a common dementia. Studies since the 1980's have, however, revealed specific genetic and molecular mechanisms underlying AD pathogenesis (Selkoe, 2002). Actually, AD is the most common form of dementia affecting about 18 millions persons worldwide. It is one of the major public health problems during the 21st century because of the increasing age of the elderly population. The incidence of the disease has been shown to rise sharply with age, from about 10% at 65 to 50% of the population at 85 (Heinz, 2002).

The two major histological features described originally are still characteristic for the disease namely: extracellular deposits of amyloid (Aβ) peptides that form senile amyloid plaques and intraneuronal neurofibrillary tangles (NFT) comprising phosphorylated forms of microtubule-associated protein tau. Altered processing of amyloid protein from its precursor APP is now recognized as the key event to the pathogenesis of AD.

The pathogenesis of AD is generally divided into three stages (i) mild, (ii) moderate and (iii) severe according to the progression of symptoms: (i) in the first stage, patients show progressive loss of recent memory; (ii) moderately affected patients display defective comprehension and orientation associated with hallucinations; (iii) during the severe stage, patients exhibit very limited language capabilities, daily living behavioral impairments and increased aggressiveness. Finally, this disorder leads to death about 12 years after the first symptoms appear.

The complexity of the disease arises from the different risks factors that are involved in the development of AD. The major risk factor is age. Ageing leads itself to neuron loss and reduction of synaptic plasticity following injury including the capability of neurons to support long-term potentiation (LTP) processes which are involved in memory.

Other risk factors have been described and help in the characterization of AD according to the age of the onset. For example, AD patients older than the age of 65 are considered to have late onset disease which accounts for more than 90% of all

cases of AD with different additional risk factors such as: severe head trauma, low level of education (Stern et al., 1994), increased alcohol consumption (Ruitenberg et al.,2002), depression as well as vascular factors (Kivipelto et al.,2001; Mayeux et al.,2003) and female gender. Indeed, the incidence of disease is higher in women probably because of their longer life expectancy and because of the postmenopausal oestrogen deficiency suppressing the potential of plasticity of axons (Bhavnani, 2003).

In contrast, early onset familial AD (EOFAD) which accounts for less than 10% of all AD cases is more related to genetic mutations such as (i) mutation of the Amyloid Precursor Protein (APP) gene on chromosome 21. This APP gene locus has been suggested to play a role in Down's syndrome (trisomie 21). Studies have shown that trisomic patients show an increased risk for AD caused by an extra copy of chromosome 21. (ii) Missense mutations of the Presenilin 1 and 2 (PS1and PS2) genes, respectively, located on chromosomes 14 and 1, seem to be the more common form of autosomal dominant AD (Selkoe, 2001). (iii) Mutations of the apolipoprotein E (Apo E) gene on chromosome 19. This cholesterol transport protein has commonly three different alleles ε2, ε3 and ε4 which encode three different isoforms of apolipoprotein E (E2, E3 and E4). The presence of allele ε 4 is the most important genetic risk factor for AD. The mechanism whereby E4 promotes AD is not understood, but there is evidence that E4 enhances Aβ aggregation and reduces Aβ clearance. ApoE has been shown to be associated with synaptosomes. Synaptosomes deficient in ApoE appear to be more susceptible to oxidative stress. Impairment of ApoE4 function inhibits neurite outgrowth and dendritic plasticity triggered through a loss of neuron's protection against oxidative stress (Lauterbach et al., 2002).

In addition, AD has been characterized by the progressive degeneration of neurons, particularly cholinergic neurons. Loss of neurons and synapses have been suggested to be correlated with the degree of dementia. Based on neurodegenerative changes, a stage model of progression of the disease has been proposed by Braak and coworkers starting in entorhinal cortex, then extending to limbic regions including hippocampus and finally to neocortex and the basal nucleus of Meynert (Braak et al. 1995).

Actually, although molecular and cellular aspects of the disease process are well described, the early diagnosis of AD by laboratory tests is still unreliable. Indeed, there is a great clinical need for biomarkers to identify incipient AD in patients with mild cognitive impairment. First, because some of the features or symptoms of AD

can also be found in other disorders with memory impairment such as Parkinson's or Huntington's disease. For this reason, all the disorders with similar symptoms must be excluded by differential diagnosis (National Institute of Health). At present, accurate diagnosis of AD cannot be done in the early stage of the disease and still requires a confirmation in postmortem brain. One possibility to improve the early onset-diagnosis of AD would be to find biological markers. Early diagnosis of AD would help to initiate effective drug treatment (Frank et al., 2003) with the aim to slow or to stop the degenerative processes (Blennow et al., 2003).

I.1.2-. Pathophysiology

Neurophathological changes

The brains of AD patients undergo many neuropathological changes associated with decrease in language function, loss of memory, visuospatial process impairment and cholinergic neuron loss. Characteristic morphological changes are atrophy of parietal, temporal and frontal cortex, basal forebrain system and hippocampus. In AD, atrophy occurs early and progresses independently of the age of patients. An index of brain atrophy has been described and was correlated with the severity of cognitive impairment (Barber et al., 2001). Additional morphologic changes found in AD brain include shrinkage of the gyri, thickening of leptomeninges and enlargement of the ventricles as shown in Fig 1 (Barber et al., 2001).

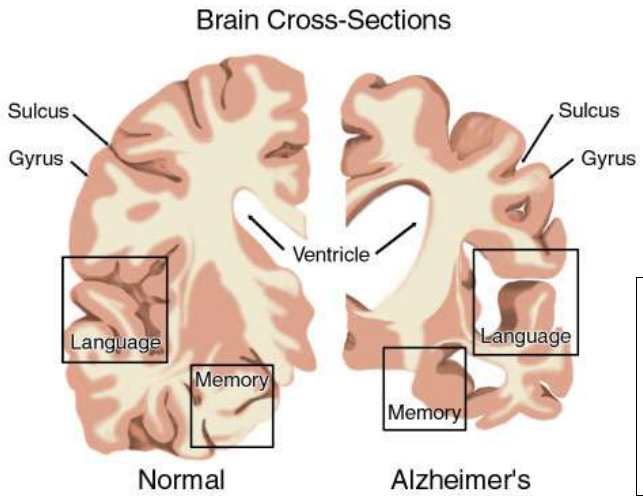


Fig 1: Description of atrophy that can be visualized in AD brain (right) in comparison to normal brain (left). This figure shows the degeneration of basal forebrain and temporal cortex involved in memory and language functionality

The two characteristic microscopic features of the disorder are senile plaques and neurofibrillary tangles (NFT). Seniles plaques are nearly spherical extracellular depositions as shown in Fig 2. They are generally found in the limbic system and in association cortices (Selkoe, 2001).

NFT are abnormal fiber aggregations in the cell body and axons of neurons. They are predominantly observed in the large pyramidal cells of the hippocampus, in the entorhinal cortex, and in the granular layers of the associative cortices. In contrast, primary sensory and motor cortices are relatively spared.

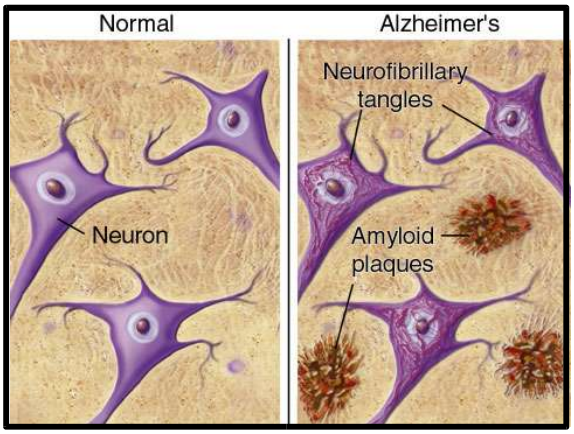


Fig 2: Left: Normal neurons, Right: Insoluble amyloid β aggregates on extracellular part of neurons and neurofibrillary tangles composed of hyperphosphorylated tau proteins deposits in neurons

Senile Plaques, APP protein and Aβ

A fundamental abnormality that plays a key role in the dysfunction and death of neurons in AD is altered proteolytic processing of APP resulting in increased production and accumulation in the brain of neurotoxic forms of Aβ. The evidence for the amyloid hypothesis of AD is extensive and has been recently reviewed (Selkoe, 2002; Mattson, 2004) (Fig4). Amyloid plaques are caused by the extracellular deposits of β-amyloid protein (Aβ) that occur principally in a filamentous form. It has been shown that these plaques appear in the early onset of the disease as diffuse plaques and develop later into senile plaques with a dense core.

The β amyloid peptides are formed by the proteolytical miscleavage of APP as shown in Fig 3. APP is a transmembrane protein, which has an extracellular N-terminal and a short intracellular C-terminal domain. (Cognos, 2002). APP is ubiquitously expressed in many human cells including CNS neurons.

Differential splicing of APP mRNA generates isoforms with different size (695,751 and 770 amino acids). The biological function of this protein is still unknown, but it appears evident that APP contributes to neuroprotective effects such as formation and maintenance of synapses, modulation of long-term potentiation (LTP) as well as protection of neurons against oxidative stress.

APP can undergo a non amyloidogenic cleavage by α-secretase releasing a soluble part sAPPα (Fig 3). Release of this soluble fragment from neurons can have a role in neurite outgrowth and functions as serine protease inhibitor (Selkoe and Shenk, 2003). A second cleavage by γ-secretase can generate a small p3 peptide. The identity of α-secretase remains unclear, althrough tumor necrosis factor α-convertase (TACE) (enzyme that cleave TNF receptor family at the cell surface) and Adamalysin family of proteins (ADAM), notably ADAM 9 and 10, are candidates (Allinson et al., 2003; Dewachter et al., 2002).

Amyloidogenic cleavage of APP within the Aβ domain starts by β-secretase cleavage, a βAPP cleaving enzyme (BACE), that releases a soluble sAPPβ. The second part of this pathogenic cleavage is made by γ-secretases (Fig3). The identity of γ-secretases remains controversial but is most probably composed out of a complex of 4 membrane proteins: presenilin 1 (PS1) and its close homologue presenilin 2 (PS2); nicastrin; aph-1 and pen-2 (Wolfe et al., 2001; Selkoe, 2001; Kimberly et al.; 2003). Presenilins seem to be key mediators of the Notch signaling pathway which is vital for cell fate decision during development (Selkoe, 2001).

Cleavage of APP by β- and γ-secretases leads to formation of Aβ fragments consisting of 39 to 43 amino acids. Two predominant Aβ peptides are present in the senile plaques: Aβ1-40 and Aβ1-42. Aβ1-40 is more frequent but less toxic than Aβ1-42 which aggregates more easily probably because of its longer size. Proteolytic cleavage of APP can occur at different sites during its transport from endoplasmic reticulum (ER) to the cell surface. These cleavage sites induce different fates for the Aβ peptides. The proteolytic cleavage of APP into ER membrane produces a significant amount of Aβ1-42 which is not secreted but remains inside the cell. In contrast, when the cleavage occurs in the Golgi membrane, both Aβ1-40 and Aβ1-42 peptides are produced and secreted. Small amounts of Aβ occur at the cell surface or in the lysosomal system (Wilson et al., 2003).

Aβ is a 4 kDa protein that aggregates first as dimer or trimer and condenses with unfibrillar Aβ peptides to finally form the protofibrils present in diffuse plaques. Later, Aβ1-40 starts to co-deposit to the preexisting Aβ1-42 deposits (Selkoe, 2001).

Diffuse plaques are the earliest detectable lesions in the AD brain and predate the neuritic plaques. In addition, it has been shown that Aβ peptides accumulate in the small blood vessels of meninges and cerebral cortex. The contribution of this accumulation in the pathology of AD is at present unclear. However, clinical complications due to cerebral hemorrhage are very rare in AD.

It remains uncertain whether diffuse plaques alone account for most of the neuronal loss in AD since characteristic amyloid plaques also occur in normal aging brain without memory disturbances.

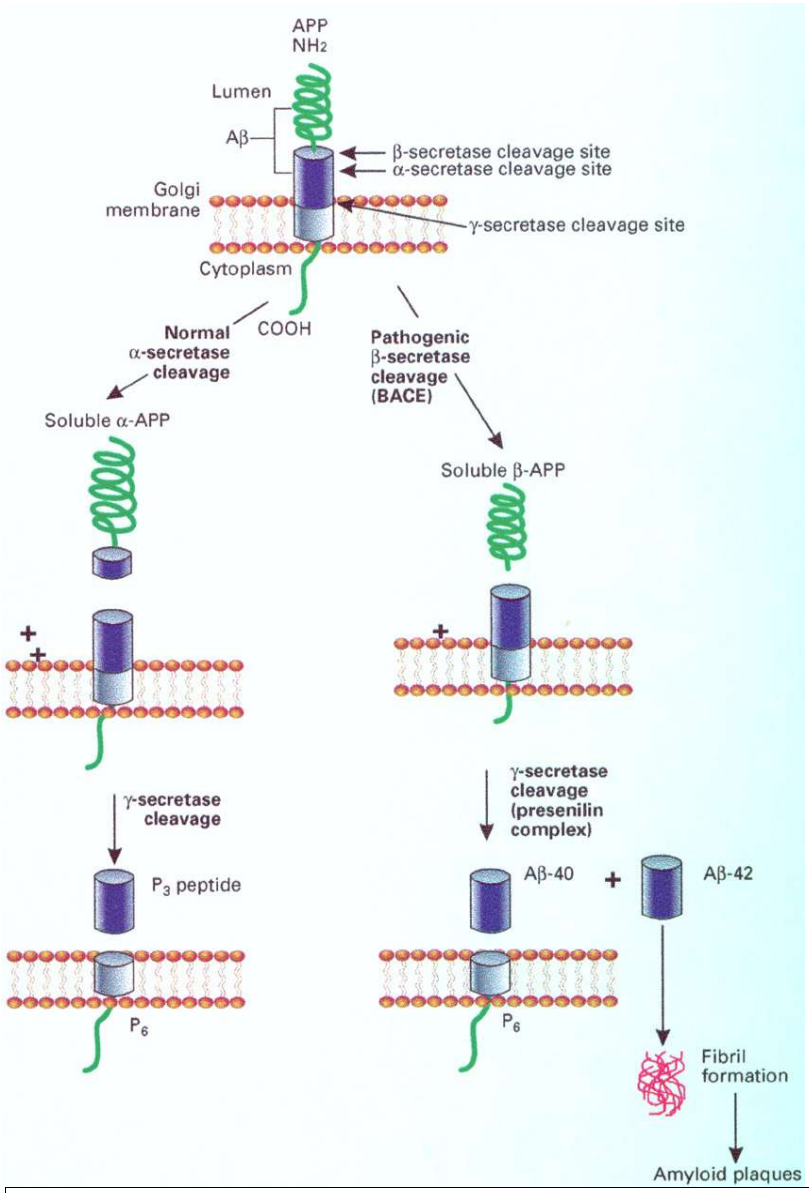


Fig 3: Physiological and pathological cleavage of amyloid precursor protein (APP)

Tau protein and Neurofibrillary tangles

Many affected neurons in AD brains contain intracellular fibrillar aggregates of the microtubule-associated protein tau that exhibit hyperphosphorylation and oxidative modifications. These intraneuronal cytoskeletal filaments (neurofibrillary tangles; NFT) are consistently found in AD in addition to senile plaques. NFT lesion can occur independently of neuritic plaques. Tangles composed of tau aggregates biochemically similar have been found in patients without dementia as well as in diverse neurodegenerative disorders such as Down's syndrome, Pick's disease, amyotrophic lateral sclerosis and Parkinson's disease (Buee et al., 2000).

NFT have been found in the entire brain: during disease progression, the deposition of NFT starts in the transentorhinal cortex, progresses to limbic regions particularly hippocampus and eventually invades the neocortex.

NFT are formed by paired helical filaments (PHF) composed of hyperphosphorylated tau protein (τ), the major microtubule associated protein (MAP). In the human organism, tau and proteins of the MAP family are found in neurons, glia, heart, kidney, lung, muscles and fibroblasts. The human tau gene, located on chromosome 17, constitutes a family of six different tau isoforms from 352 to 441 amino acids generated by alternative splicing (Buee et al., 2000). Much of the tau protein in tangles is present in highly insoluble filaments.

A variety of kinases have been shown to be capable of phosphorylating tau but it is still not clear whether one or several kinases are responsible for hyperphosphorylation initiation (Selkoe, 2001). In contrast, tau protein can be quickly dephosphorylated by endogenous phosphatases such as Ser/Thr phosphatase protein 1, 2A, 2B and 2C. The degree of phosphorylation of tau crucially regulates its biological activity. Phosphorylation of tau is of importance during development, for cell sorting and assembly and structural stability of microtubules. Healthy microtubules are essential for the structure and function of neuronal cells (Cognos, 2002). Tau hyperphosphorylation in AD leads to aggregation in the neuronal cell body and axons and finally to impairment of synaptic transmission. Aggregation of NFT is associated with increased neuronal degeneration in AD (Ramirez-Chamond et al., 1999). Programmed cell death clearly occurs in neurodegenerative disorders, it remains, however, uncertain whether it accounts for the most of the neuronal loss in AD. Appearance of an aberrant cell cycle has been proven in AD and appears to have a particular role in the CNS neurodegeneration (Hamdane et al., 2003).

The Aβ hypothesis

Hallmarks of AD are Aβ deposits and tangle formation. Further studies proved that mutations of PS1 and PS2, ApoE 4 and APP are involved in AD pathology. Currently, the idea that Aβ accumulation is the first event in AD (as shown in Fig 4) and that the following processes such as tau-tangle formation or inflammation result from a primary imbalance between Aβ production and Aβ clearance is generally accepted (Hardy and Selkoe, 2002).

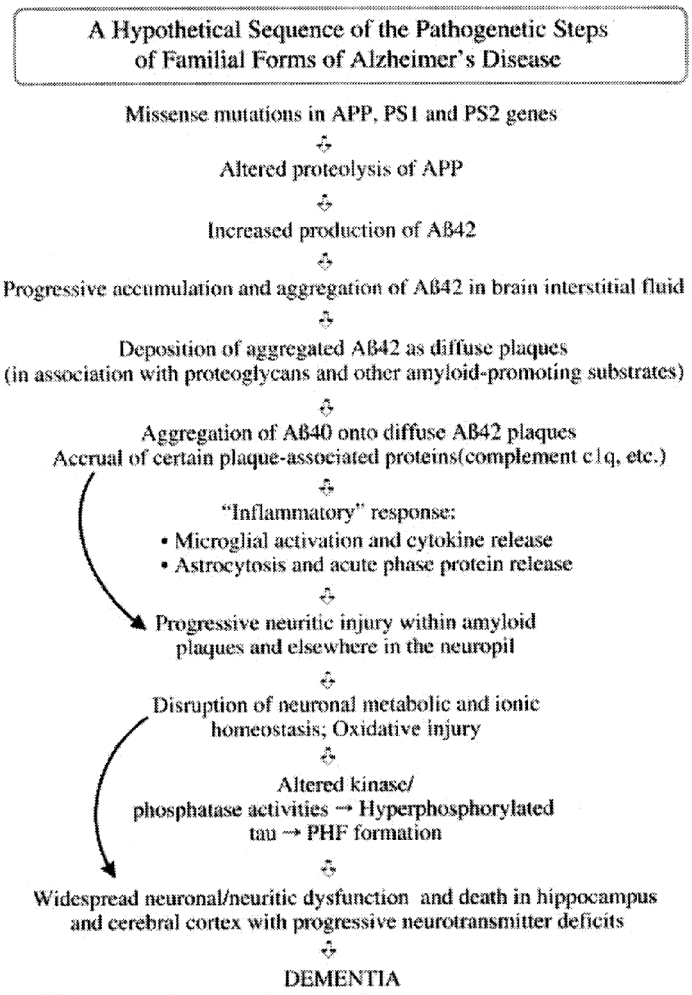


Fig 4: Representation of the Aβ hypothesis

Inflammation is included in this pathogenic cascade as Aβ fibrils are intimately surrounded by dystrophic axons and dendrites, reactive astrocytes and activated microglia. This key role of chronic inflammation in AD is now accepted, but the mechanisms involved in neuronal death and synaptic failure are still under discussion.

I.2- INFLAMMATION

I.2.1- Inflammation in Alzheimer's disease

Generally, inflammatory processes appear after diverse insults including invading microbes such as viruses and bacteria as well as injurious chemicals, physical insults, immune mechanisms, hypoxia, stress, age and tissue damage in order to keep a normal balance in organism homeostasis (Wyss-Coray et al., 2002).

The role of inflammation in the CNS is still unclear and the mechanisms of action of this immune response have been deduced from what is known in autoimmune disorders.

Involvement of inflammation in the CNS and particularly in AD has been for a long time unknown or underestimated. The principle ideas were based on the fact that the brain has a protection system against attacks, the blood brain barrier (BBB). In auto-immune diseases or stroke, the BBB is breached and inflammation in the CNS was then described as adaptative immune response triggered by invasion of lymphocytes or monocytes into the brain in order to detect and reduce the injury (Mc Geer et al., 2003). In contrast, the BBB in AD was never found breached and for this reason the view had to be extended. Finally it has been shown that the inflammation in AD brain is an innate immune response with activation of glia cells including microglia and astrocytes (Akiyama et al., 2000). The role of astrocytes and microglia cells in AD has been postulated since decades. They were thought to be involved primarily in removal of Aβ deposits by phagocytosis. Finally, it has been shown that microglia cells are actively involved in the inflammatory response triggered by Aβ (Meda et al., 2001).

Increasing experimental evidence indicates that inflammation significantly contributes to AD pathogenesis: First, amyloid plaque deposition in the AD brain leads to local inflammation by permanent activation of microglia. Second, the accumulation of activated microglia cells occurs close to the senile plaques. Even if neuroinflammation is not considered as the primary event in the development of AD, the activation of these brain specific macrophages has been described as an early event in the developing disease (Vehmas et al., 2003). Reactive astroglia that surround senile plaques release inflammatory mediators and effectors to orchestrate defense mechanisms and to initiate the removal of the pathogenic trigger molecules (Liu et al 2003). Indeed, numerous markers of inflammation have been found in AD brain: (i) accumulation of proinflammatory cytokines such as TNF-α, IL1-β, or IL-6

released by activated microglia cells and astrocytes; (ii) strong activation of the complement system; (iii) increased oxidative stress with uncrontrolled production of highly reactive oxygen radicals contribute to the lesions in AD brain.

Whether activation of microglia and astrocytes can either promote or counteract neurodegenerative processes is still unclear (Rogers et al., 2002). Activation of glia should help to eliminate the invaders but there may be a risk of misdirection of the immune reaction that can initiate autoimmune responses against host proteins.

This disregulation can lead to the production of an autotoxic loop as shown in Fig 5 (Mc Geer et al., 2002).

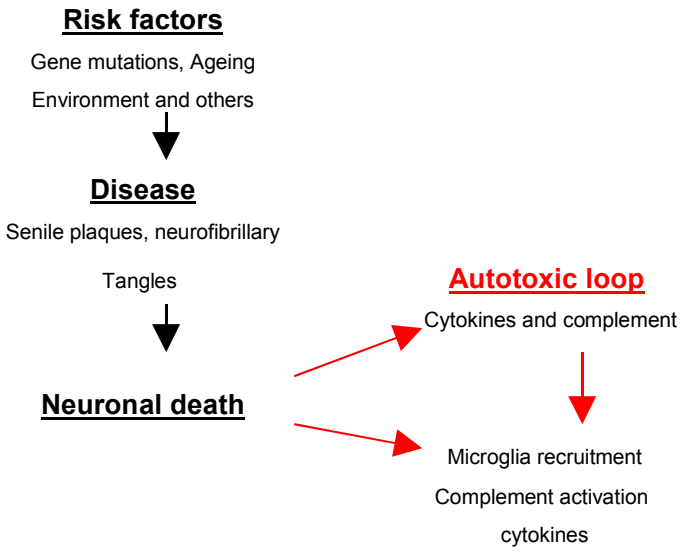


Fig 5 : Autotoxic loop of the central nervous system degeneration

Because all the mechanisms involved in AD can play an opposite role, the immune response can be of benefit in CNS injury and these events could possibly lead to a future immunotherapy for AD (Monsonego et al., 2003). Actually, the most recent studied therapeutic intervention is vaccination with Aβ (Selkoe, 2002).

I.2.2- Complement activation in Alzheimer's disease

Activation of the complement pathway is an essential part of the inflammatory response and is deeply implicated in autodestruction of neurons. Activation of the complement system destroys invaders and helps their elimination by phagocytosis. In AD, aggregated Aβ has been shown to be a strong complement activator (Rogers

et al., 1992). Activation of the complement system by the classical pathway with C1q binding, and the alternative pathway has been supposed to exacerbate the pathology of AD suggesting that complement inhibitors could be used as potential therapeutic agents (McGeer and McGeer, 2001, 2003). Both classical and alternative pathways lead to the formation of protease complexes that can easily be detected by microglia or phagocytic receptors in order to be removed (McGeer et al., 2002; Farkas, 2003). The complement system has to be precisely regulated in order to protect cells from abnormal activation. An inhibitor of complement activation has been found in mice: complement receptor-related protein y (Crry). Studies on transgenic mice expressing a human APP mutation and soluble Crry (sCrry) have shown an increase in extracellular deposition of Aβ peptides and reduced activation of microglia. This points to a neuroprotective role of complement in Aβ induced neurodegeneration and clearance of amyloid. However, these findings do not exclude the possibility that complement activation in neurodegenerative conditions may also have neurotoxic consequences (Wyss-Coray et al., 2002).

I.2.3- Cytokine pathways

Cytokines are a heterogenous group of small molecules that can participate to innate or acquired immunity in an autocrine or paracrine fashion. Cytokines are secreted and released by activated microglia cells after injury. These proteins can have pro- or anti- inflammatory activities. Some of the cytokines have been suggested to have an impact in AD such as TNF-α, IL1-β, and IL-6 which have been detected as overexpressed proteins in AD brains.

IL-1 is an immunoregulatory cytokine and its expression is increased in the early phase of the plaque formation. IL-1 exerts a direct action on the formation of senile plaques via an upregulation of βAPP and a stimulation of its processing (Neuroinflammation Working Group, 2000).

IL-6 is a pleiotrophic cytokine that can mediate various processes in the CNS including cell growth and differentiation. IL-6 is produced and expressed at low levels in normal adult CNS but is strongly induced under pathological conditions (Vanden Berghe et al., 2000). IL-6 can be produced by microglia cells, astrocytes, neurons and endothelial cells. Its overexpression is generally associated with detrimental effects. Moreover, IL-6 may have anti-inflammatory, immunosuppressive and neuroprotective actions probably via neurotrophins such as Nerve growth factor

(NGF) actions (Tarkowski, 2002). Because of its polymorphism, IL-6 has been described as a genetic factor for AD (Papassotiropoulos et al., 2001).
TNF-α appears to have completely opposite actions in AD: normal levels of TNF-α have been reported to have a beneficial effect on hippocampal neurons of rats whereas overexpression of this cytokine resulted in human cortical neuron death (Perry et al., 2001).

I.2.4- Oxidative stress in Alzheimer's disease

Increased oxidative stress (enhanced production of highly reactive oxygen radicals) can be triggered by several stimuli. First, ageing is a condition of increased production of free radicals. In addition, inflammatory responses have been shown to initiate oxidative stress.
A key molecule in oxidative stress is nitric oxide (NO). NO produced from L-arginine is a diffusible free radical with multiple functions in mammalian cells. NO is produced by NO synthase (NOS). There are 3 different NOS including neuronal (nNOS) and endothelial (eNOS) enzymes, both expressed constitutively, as well as inducible NOS (iNOS). Induction of iNOS can produce high levels of NO which is known to be a component of nonspecific immune responses. The biological fate of NO is influenced by its own concentration and the surrounding conditions such as pH, CO_2 concentration or redox state (Boje, 2004).
NO is highly reactive to other oxygen components and formes easily neurotoxic effectors with reactive oxygen species (ROS) including superoxide anion $O_2^{\cdot -}$, hydrogen peroxide H_2O_2 and hydroxylyl radicals $OH^{\cdot}$ resulting in reactive nitrogen species (RNS) such as peroxynitrite anion $ONOO^-$ which finally causes cell death by inducing DNA damage protein oxidation and nitration.
NO has two different kinds of interaction: (i) oxidation not involving attachment of NO groups to target molecules. These oxidations lead the formation of modified proteins with loss of function which then affect multiple homeostatic mechanisms in the brain. (ii) covalent modifications of proteins notably S- or N- nitrosylation: tyrosine is more often nitrated than the two other aromatic amino acids, tryptophane and phenylalanine, because of its mild hydrophobicity and its surface localization in proteins (Ischiropoulos, 2003). The most toxic mechanism of NO is the reaction between $NO^{\cdot}$ and superoxide $O^{\cdot -}_2$ that produces peroxynitrite anion $ONOO^-$.

This reaction has pathophysiological consequences including DNA damage, lipid oxidation and protein modifications via formation of 3-nitro tyrosine (Jiao et al., 2001).

Interestingly, NO can also function as a protective antioxidant at physiological concentrations. Physiological levels of NO influence synaptic efficacy by regulating neurotransmitter release. In contrast, excess of NO causes excitotoxic brain injury (Meda et al. 2001). Moreover, NO which is produced by certain CNS cells can elicit neurotoxic effects on the same cells: e.g neurons, astrocytes, activated microglia or endothelial cells (Boje, 2004).

Oxidative stress in neuroinflammation has been shown to contribute to various neurodegenerative diseases such as Amyotrophic Lateral Sclerosis, Huntington's disease or AD. Even if it is not a primary event, oxidative stress seems to appear very early in AD pathogenesis (Nunomura., 2001).

Overexpression of NO (Monsonego et al., 2003) and presence of proteins containing 3-nitrotyrosine have been shown in several inflammatory diseases, cancer and neurological disorders including AD (Pignatelli et al., 2001). Aberrant expression of the three NOS isoforms has been found in different cell types of the AD brain and was associated with high level of colocalized nitrotyrosine (Lüth et al., 2002). The respiratory system of activated microglia is involved in free radical attack of AD brains via the production of oxygen free radicals.

Treatment with drugs interfering with NO and oxidative stress may be used as new therapeutic approaches of AD. For example, an antioxidant substrate like X34 has been shown to bind to Aβ deposits and to decrease oxidative stress related to Aβ deposition in synaptosomal and hippocampal neurons (Kanski et al., 2003).

I.2.5- Synaptic failure in Alzheimer's disease

Presence of neuritic plaques and NFT in the septal region of hippocampus and basal forebrain neocortex leads to the destruction particularly of cholinergic neurons of the basal forebrain in AD brain. The deficits in cholinergic transmission are early symptoms for the disease. For these reasons, AD has been suggested to be first triggered by a specific impairment of synaptic transmission (Small et al., 2001; Selkoe, 2002).

It was first presumed that neurotoxicity of Aβ is mediated by changes in cellular calcium signaling but recent data demonstrate that Aβ directly interacts with neuronal membranes (Yamaguchi et al., 2000). Aβ peptide induces ultrastructural changes in

synaptosomes (Mungarro-Menchaca et al., 2002). Then, according to the Aβ hypothesis, deficits in synaptic transmission such as LTP occur a long time before Aβ deposits become detectable (Selkoe, 2002).

Initiation of the neurodegenerative cascade has been hypothesized to occur in synapses and, in fact, the loss of the synaptic-associated protein synaptophysin has been suggested as an early marker of neurodegeneration (Mungarro-Menchaca et al., 2002).

Moreover, the oxidation that occurs in AD brain triggered by amyloid β peptide leads to lipid peroxidation of different membranes (Lauderback et al, 2001; Butterfield et al., 2002). Lauderback et al. (2002) have shown that ApoE is associated with synaptosomes. Moreover, deficient synaptosomes are more susceptible to a Aβ-induced oxidation suggesting a specific antioxidant role for ApoE.

III.3- PROTEOMIC TECHNOLOGY

Proteomic analysis aims the high throughput analysis of total proteins expressed in an organism (proteome). Then, it provides valuable informations about the expression level of proteins and post translational modifications, as well as changes of these parameters due to diseases or external factors. It is a recent techology already routinely used for clinical diagnosis and for investigation of infectious diseases, cancer or neurological disorders (Fountoulakis, 2001). A proteomic analysis comprises two major steps : separation of the protein mixture and identification of these separated proteins by mass-spectrometry.

As every technology, proteomic analysis has some limits: i.e the detection of hydrophobic or membrane proteins is difficult due to their low solubility. Proteins have to be brought and kept in solution during the whole process of proteomic analysis for an efficient detection. Another limiting factor is the size of proteins, e.g. proteins smaller than 10 KDa or bigger than 120KDa are rarely detected (Fountoulakis, 2004).

Despite these draw-backs, the technology has been proven to be powerful for studing changes in bacterial proteome or in various cell lines. Analysis of tissues such as brain is actually possible but is difficult due to the involvement of multiple factors: sex, age, and medical treatment for human samples (Fountoulakis, 2001).

A major advantage to use proteomics instead of other analytical techniques is that with the development of mass-spectrometry, automation and availability of informatic software this technique allows the analysis of large amounts of informations.
This new technology was used and improved depending on the specific requirements for this study.

AIM OF PROJECT

Aim of this study was

(i) To prove the involvement of inflammation in AD pathogenesis with the final goal to identify markers for the early onset-stages of this disorder.

(ii) To identify and characterize proteins involved in AD inflammation using defined cell culture systems. The results obtained *in vitro* will be compared with an APP/PS2 transgenic mouse model for human AD and with post-mortem human AD brain.

(iii) To monitor tyrosine nitrated proteins in the transgenic mouse model.

(iv) To establish a detailed map of cytosolic proteins in mouse brain.

(v) To isolate synaptosomes of brain with the goal of mapping synaptic membranes.

For these studies, proteomic technologies such as Matrix assisted laser desorption ionization (MALDI) coupled with time of flight (TOF) analyzer were used. Internal softwares programs from F Hoffmann-La Roche AG were available to analyse data.

II- MATERIALS AND METHODS

II.1- MATERIAL

II.1.1- Material

Dulbecco′s modified Eagle′s medium (DMEM), William E medium, fetal calf serum (FCS) and other cell culture supplements were purchased from GibcoBRL (Basel, Switzerland).

Immobiline dry strips (pH 3-10; Non linear; 18 cm) were purchased from Amersham Pharmacia Biotechnology (Düdendorf, Switzerland). ZOOM IPG-strips (pH 3-10 or pH 4-7; 7cm) for the ZOOM IPG-runner system, were obtained from Invitrogen life technologies.

Hybond ECL nitrocellulose membranes used for Western blotting were purchased from Amersham Pharmacia Biotech.

Acrylamide was obtained from Serva (Wallisellen, Switzerland) and the reagents for the SDS-PAGE were purchased from Bio-Rad laboratories (Reinach, Switzerland). Carrier ampholytes (resolyte 3.5-10) were bought by BDH Laboratory Supplies (Poole, UK). CHAPS, Thiourea and Iodoacetamine were from Sigma (Buchs, Switzerland), Urea and Dithioerythreitol (DTE) were obtained from Merck (Dietikon, Switzerland). Trypsin was first obtained from Promega (Wallisellen, Switzerland) and then directly from F. Hoffmann- La Roche (Basel, Switzerland).

II.2 METHODS

II.2.1- Cell culture and treatment

The human microglia cell line (hMC-3) (Janabi et al., 1995) was grown in DMEM containing 10% FCS, 2% Penicillin-streptomycin and incubated at 37°C in humidified atmosphere containing 10% CO_2. Prior to stimulation, cells were washed with phosphate buffered saline (PBS) and preincubated in William's E medium without FCS for 2 hours. For stimulation, cells were either untreated or incubated with 1nM human Interleukin 1 (IL-1) plus 1nM Tumor necrosis factor –α (TNFα) for 24 hours in William's E medium. Subsequently, the cells were harvested in mannitol buffer (0.29M mannitol, 10 mM triethylamine, 10mM acetic acid, 1mM EDTA and protease inhibitor (Boehringer-Mannheim, Germany) and centrifuged for 2 min at 200 xg at 4° C. Thereafter the pellet was resuspended in 1 ml mannitol buffer and lysed by homogenization in a douncer homogenizer (20 strokes). The extract was further purified by differential centrifugation, first at 750xg for 15 min at 4°C, then the supernatant or cytosol purification was followed by at 15'000xg for 20min at 4°C and 50'000xg for 30 min at 4°C.

II.2.2- Transgenic animal model

The following transgenic (tg) mouse model APP/PS2 (generated by Dr Ozmen Laurence, PRBD, Roche, Basel) was used:

- overexpression of a cDNA carrying the Swedish double mutation K570N, M671L of human APP under Thy 1 neuron specific promoter.
- overexpression of a cDNA carrying the mutation N141I of human PS2 under prion promoter.

The most important pathological characteristic feature of these mice is the accelerated development of fibrillar Aβ deposits in cerebral cortex and hippocampus as compared to APP tg mice. Behavioral studies of the transgenes revealed significant changes in cognitive functions in the early phase of the disease long time before plaque formation occurs.

II.2.3- Protein determination with Bradford method

A Bio-Rad protein microassay, adapted from the Bradford dye-binding procedure (Bradford., 1976.), was used in order to determine low concentrations of proteins.
The standard curve was achieved with BSA standards at the concentration of: 1.5mg/ml; 1mg/ml; 750 µg/ml; 500 µg/ml; 250 µg/ml; 125µg/ml; 25 µg/ml and 5 µg/ml.
25 µl of each standard was mixed with 1 ml of Bio-Rad protein assay solution diluted 1 to 5 times. The same dilution with the Bio-Rad dye was made for the samples. Measurement of the color change of Coomassie Brilliant Blue G 250 dye was performed at 595λ using a Microtiterplate scanning spectrophotometer Power Wave 200 from Bio-Tek Instruments.

II.2.4- Western blot analysis

Samples were merged with NuPAGE sample buffer containing 50mM DTE at a final concentration. The mixture was boiled at 95 °C, loaded on 4-20% Tris-Glycine gels or on 4-12% NuPAGE gel, run first at 75V for half an hour and then at 130V for 2 hours.
For the transfer of proteins from SDS gels to nitrocellulose membrane, a semi-dry transfer cell chamber (Bio-Rad) was used. First, 6 Whatman papers and 1 nitrocellulose membrane were moistened in transfer buffer. For one gel, the packing into the apparatus consisted out of 3 Whatman papers, then the membrane, followed by the gel and 3 Whatman papers. Transfer buffer was either Towbin buffer (191mM Glycin; 25mM Tris and 20% (v/v) Methanol) for Tris-Glycine SDS gels or NuPAGE transfer buffer from Invitrogen.
Blotting ** was achieved at 20V for 70 min. In order to detect any transfer artefacts, membranes were quickly stained with Ponceau S from Sigma.Then membranes were blocked with Superblock blocking buffer from Pierce Biotechnology for 30 min. Unspecific binding sites of the membranes for antibodies were blocked by addition of 5% Milk T-PBS (non fat dry milk, BIO-RAD and 0.1% Tween-PBS). The membrane was quickly washed with T-PBS. The first antibody was left overnight on membranes in 0.5%milk, T-PBS. Membranes were then washed 2 times quickly and 2 times for 5 minutes with T-PBS. Thereafter, the second antibody in 0.5%milk, T-PBS was added for a minimum 2 h. Dilution of the antibodies depended on the antibodies used. Membrane was washed again 2 times quickly and 2 times for 5 minutes with T-

PBS. Detection of Antibodies was performed with Lumi-light Western blotting substrate from Roche Diagnostic Corp. The maximal incubation time of this substrate on membranes amounted to 5 min.

** Western blot analysis of criterion gels for nitrated proteins. For this particular case, the gels were pre-equilibrated after the second dimension with Towbin buffer for a minimum of 1 hour and a wet transfer was achieved. Transfer was performed at 100V during 2 hours in Bio-Rad criterion cassette with nitrocellulose membranes into Tris Glycine SDS buffer (25 mM Tris, 192 mM Glycine, 0.1% w/v SDS and pH 8.3).

II.2.5- Co-Immunoprecipitation

Dialysis of 3 mg of protein from mice brain cytosol extract against Ripa buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% Deoxycholate, 0.1% SDS, 50mM Tris pH 8 plus proteases inhibitors was done overnight at 4 °C. Dialysis was performed in Slide-A-Lyser 3.5K dialysis cassettes from Pierce. These are 3,500 MWCO cassettes with a maximum capacity of 0.5 ml.
The samples were divided into equal portions and diluted up to 500 µl total volume with Ripa buffer. Each portion contains about 300 mg of protein. To each tube an appropriate concentration of antibody was added. The incubation of the antibody was left overnight on a wheel at 4°C.
Equal amounts of protein A and protein G Sepharose were mixed in PBS. This mixture was washed 5 times with 5 volumes of PBS and 1 time with 2 volumes of Ripa buffer. Finally 1 volume of Ripa buffer was added to this mixture.
50 µl of the 1:1 slurry preparation of protein A/G Sepharose in Ripa buffer, was added to each 500 µl portion samples and incubated 2 hours at 4°C. Incubated samples were washed 6 times with 5 volumes of PBS before 1 volume of NuPAGE sample buffer was added. This preparation can then be loaded on a gel and run as described before.

II.2.6- Purification of aldolase C with a size-exclusion column

In order to isolate aldolase C from the cytosolic fraction of mice brain extract, a high performance liquid chromatography (HPLC) analysis was performed.
A size exclusion column Superose 6HR 10/30 from Amersham Pharmacia Biotech was used to enrich and purify the protein. The column was first washed with Milli-Q

water. Further equilibration was done with two column volumes of 320mM Sucrose, 5mM Hepes, pH 7.4 buffer. All buffers were prefiltered before use. The maximum capacity of the column is 5 mg of proteins in 240 µl total volume.
The sample was loaded on the column at a flow rate of 0.3 ml/min. 1ml fractions were collected and fractions containing the aldolase C were detected by Western blot analysis. The apparatus used for this purpose is an AKTA prime from Amersham Biosciences.

II.2.7- Homogenisation of total mouse brain and cortex

After dissection of the brain, total brain or specific brain regions such as cortex were frozen in liquid nitrogen. The samples were homogenised using a motor-driven pestle and then resuspended in 1 ml of 50mM Tris pH8, 1%NP40, 1mM MgCl2, 5mM EGTA, 5mM DTT; 100mM NaCl plus protease inhibitor. The samples were further sonicated 20 times at level 9 and 20-30cycles. A centrifugation step was applied at 50000xg for 30 min at 4 °C and the resulting supernatant used for 2D gel analysis.

II.2.8- Synaptosome extraction protocol (Gordon-Weeks et al. 1987; Huttner et al. 1983)

Mice C57Bl/6J were killed by decapitation and brains were softly homogenized with a glass tube potter (12 times up and down at 800 rpm) in a cold room 4°C. Homogenization was done in buffer A: 320 mM sucrose 5 mM Hepes buffer pH7.4 (0.1% w/v).
The extract was purified by differential centrifugation, first at 800xg for 10 min at 4°C. The supernatant was further centrifugated at 10000xg for 15min at 4°C. The pellet was resuspended in 10ml of buffer A and centrifuged again at 10000xg for 15min at 4°C. The resulting pellet was resuspended in 2 ml of buffer A and merged with 6 ml of 8.5% Percoll. The Percoll gradient ranges from the bottom of the tube: (i) 4 ml of 16% Percoll; (ii) 3 ml of 10% Percoll and (iii) 6 ml of the probe mixtures was applied to the top of the gradient. Gradient centrifugation was performed at 15000xg at 4°C for 1h30 with a swing-out rotor. The crude synaptosomal fraction was localized in the interphase between 16% and 10 % and was diluted 1:1 with isotonic Krebs buffer (20 mM Hepes, 10 mM Glucose, 145 mM NaCl, 5 mM KCl, 1.2 mM CaCl2, 1.3 mM MgCl2, and 1.2 mM NaH2PO4) and re-centrifuged at 72000 xg for 1 h at 4°C.

The pellet was resuspended in 1 ml of 5mM Tris-HCl pH 8.1, dounced 6 times with a 1 ml homogenisator and left on ice for 30 min in order to lyse the synaptosomes. After lysis, 1ml of isotonic Krebs buffer with 5 mM additional NaCl was added to the lysed sample. This mixture was then centrifuged at 25000xg for 20 min at 4°C and the supernatant further purified at 200000xg for 2 h at 4°C. These two centrifugation steps lead to the separation of synaptic organelles in the supernatant (S3) and synaptic membranes in pellet (P3) and finally to separation of the synaptosomal vesicle membranes (P4) and their cytosol (S4).

II.2.9- Proteomic analysis

Sample preparation

Soluble protein extracts obtained were mixed with equal volume of IEF sample buffer (Urea 7M, Thiourea 2M, 50 mM Tris-HCl pH7.5, 2 %(w/v) CHAPS, 0.4 %(w/v) DTE, 0.5 %(v/v) resolyte and traces of bromophenol blue) and concentrated in centrifugal filter tubes (Millipore corporation, Volketswil, Switzerland) at 2'000xg, 4°C for 20 min. This procedure was repeated three times. Protein concentration was determined by the Bradford method (1).
Gel strips were rehydrated overnight by placing them side-down in a immobiline DryStrip Reswelling Tray (Amersham Pharmacia Biotech.) containing rehydration buffer (8M Urea, 2%(w/v), CHAPS, 0.4 %(w/v), DTE. 0.5 %(v/v), resolyte and traces of bromophenol blue) covered with paraffin oil.

Two dimensional (2D-) gel electrophoresis

Total soluble protein amounts of 1mg were applied on 18 cm IPG-strip and were subjected to isoelectric focusing. The focusing program with the IPG phor cup loading system (Amersham Pharmacia Biotechnology) is composed of five steps including: (i) start at 300 V for 3h; (ii), a step from 300V to 600V for 4 h; (iii), a step from 600 V to 1000V for 5h, and (iv), a step from 1000V to 8000V for 3 h, and finally, at 8000V for 3 h.
Prior to second dimension electrophoresis, IPG gel strips were equilibrated for 15 min in a reducing solution (6M Urea, 50mM Tris-HCl pH8.8, 30% Glycerol, 2% SDS, 0.03 M Dithioerythreitol) followed, for 15 min in a alkylation solution (6M Urea, 50mM Tris-HCl pH8.8, 30% Glycerol, 2% SDS, 0.23M Iodoacetamine). The second

dimension separation was carried out in a Protean MultiCell (160×299×1.5mm) system (Bio-Rad) for 6 h at 40 mA per gel in a solution of 25mM Tris, 192 mM Glycine and 0.1% (w/v) SDS.

Gel staining, imaging and protein digestion

The protein content of the gel was determined by the Coomassie blue method (mixture done according to the kit) (Novex, Invitrogen, Basel, Switzerland). Coomassie blue-stained gels were destained in water and scanned in an Agfa DUOSCAN densitometer. Electronic images of the gels were recorded using photoshop software (Adobe). Then, a software program Fragment 21 and later Scimagic were used to identify and save the coordinates of the visible spots. The corresponding spots were automatically cut out using an automated gelpicker (Sisyphus, Roche, Basel).
Picked spots were placed into 96-well microtiter plates, destained with a solution containing 50mM Ammonium hydrogen carbonate and 30% Acetonitril, and finally dried in speedvac evaporator for 20 minutes. Digestion of the proteins was performed overnight with 5µl/spot with aqueous trypsin (15µg/ml) solution at room temperature.

Matrix assisted laser desorption ionization- mass spectroscopy (MALDI-MS)

After digestion, 20 µl of a solution containing 50% Acetonitrile, 0.1% Trifluoroacetic acid (TFA) was added to each spot and the samples were incubated at room temperature for 15 min.
The Bruker target was washed previously with deionised water and then sonicated for 10 minutes with absolute ethanol. The sample application onto targets was performed by a Cybio apparatus (Roche, Basel). The robot applied simultaneously 1.5µl of the sample mixture and 1µl of matrix, consisting of 0.25mg/ml α-Cyano-4-hydroxycinnamic acid (Sigma) in buffer containing65% Ethanol, 32% Acetonitrile and 0.03% TFA.
Two standards were added to the matrix for an internal calibration of the MS: Bradykinin (10pmol/µl) (FW 904.4681) and ACTH (41pmol/µl) (FW 2465.1989).
After drying of the loaded targets at room temperature in darkness, they were transferred for 10 minutes to 4°C. Then a recristallisation step was performed with

the same robot which applied 0.8µl of a recristallisation solution containing 65% Ethanol, 32% Acetonitrile and 0.03% TFA solution. At the end, we spotted by hand onto the target: 1.5µl of human serum albumin (100pmol) in 1µl of 5mg/ml matrix, to calibrate the apparatus.
Samples were analyzed in a time-of- flight mass spectrometer (Ultraflex, Bruker). Samples and matrix were submitted to a laser ray. The time of flight of the peptides after laser shots is correlated with the mass of the peptide which are represented by a spectrum related to the peptide mass fingerprinting of the global protein. The peptide masses were compared to the theoretical peptide masses of all available proteins databases from all species (see: Westermeier R. Proteomics in practices, 2002).

Description of MALDI/TOF

A mass spectrometer is composed out of an ion source which is the matrix, a mass analyzer which is a TOF analyzer that separates ions according to the mass charge ratio (m/z) and a detector that delivers a mass spectrum. The laser employed is a N2-laser operating at a wavelength of 337 nm with pulse durations of 1-5 ns. Shots of laser into matrix and sample create a beam of gaseous ions which are accelerated from sample to analyzer. Then the time between acceleration and detection of ions on the detector is measured by the analyzer. Detectors used are micro-channel plates which are porous solid cores containing millions of tiny holes (ø 5-10µm and 0.5-0.8mm length) and play a role as multiplier. Finally, micro-channel plate detectors delivered voltage outputs which have to be digitized and sent to a PC for analysis.
The machine used were first Autoflex with a sensitivity of 2-3 femtomol and later the Ultraflex system which has a sensitivity of 100 attomol, both in a total mass range of 5000 Da (Bruker Daltonics. 2002).

II.2.10- Miniaturization of all proteomic technologies

Invitrogen ZOOM IPG runner system

Minisystem (Invitrogen Life Technologies) was first used in order to minimize the quantity of protein consumption which has to be applied in the first electrophoresis dimension (60 to 90 µg used instead of 500 µg to 1 mg). Another advantage is the facility of handling and the time of processing.

Desalting of samples is a key event and final salt concentration should be less than 5mM. The desalting column used for this purpose is the Micro BioSpin 6 Chromatography Column from Bio-Rad. The column had to be equilibrated with 500µl of the IEF sample buffer followed by centrifugation at 1000 xg for 1 min. This was repeated 3 times. Sample was applied on the column, centrifuged for 4 min at 1000 xg. The eluate collected at the end contains the desalted samples.

7cm ZOOM IPG strips (pH3-10) from Invitrogen were placed overnight at room temperature into a ZOOM IPG runner cassette (Invitrogen) with gel-side up. The total volume applied on these strips (sample + rehydration buffer) is 155µl. To limit evaporation, two papers were placed on the two entries of the cassette limited by white plastic. After overnight rehydration, the strips were left in the cassette and only the two white plastic were removed and replaced by 2 absorbant papers: one was moistened with a diluted 1: 200 cathode buffer (stock solution kept at 4°C) and the other with 175X dilution of anode buffer (stock solution kept at RT). Then, the cassette is placed into a mini-cell chamber filled with 650 ml of Milli-Q water (according to Invitrogen protocols). The isoelectric focusing was done with an Electrophoresis Power Supply EPS 3501XL from Amersham Bioscience. The program on the Mini system is composed of four steps including: (i) start at 200 V for 20 min; (ii), then at 450V for 15 min; (iii), then 750V for 15 min, and finally (iv), a step at 2000V for 2 hours.

After focusing, strips were equilibrated for 15 min in a reducing solution (6M Urea, 50mM Tris-HCl pH8.8, 30% Glycerol, 2% SDS, 0.03 M Dithioerytheitol) followed, for 15 min by an alkylation solution (6M Urea, 50mM Tris-HCl pH8.8, 30% Glycerol, 2% SDS, 0.23M Iodoacetamine).

The second dimension was performed into “X-cell sure lock chamber” from Novex with ZOOM gels 4-12% NuPAGE with MES buffer. Then the gel can be directly stained with Coomassie blue and spots can be picked (as described before).

Biorad minisystem used for nitrosylation

11 cm Criterion strips from Bio-Rad were rehydrated as described before and were focused for the first dimension with a multiphor apparatus from Amersham. The programme includes two steps: 1) increase of voltage up to 5000V for 5 h and 2) maintenance of voltage at 5000V for 2 hours. The second dimension was performed into a Biorad multi-chamber filled with Tris-Glycine buffer. The strips were loaded on 4-20% Tris-Glycine gels. Then the gels were stained with Coomassie blue and picked as described before.

II.2.11-DEAE-ion exchange chromatography

Mouse brains were homogenized in a Potter homogenizer with 12 strokes at 800rpm. The homogenate was centrifugated at 800xg for 10 min at 4°C. The resulting supernantant was further centrifugated at 10000xg for 15min at 4°C. Finally, the supernatant was centrifugated at 50000xg for 60 min at 4°C. The cytosolic part was either directly applied onto 10% SDS Polyacrylamide gels or further fractionated by DEAE-ion exchange column. For the fractionation, the sample was first filtrated and desalted in HiTrap desalting column (3 times 5 ml column), thereafter 15 mg of brain cytosol was loaded on DEAE column and was eluted by a salt gradient up to 500mM NaCl.

The DEAE column used for this study was a 5PW Glass column (internal diameter 8 mm and 7.5cm length) from TOSOH BIOSEP, with a TSK polymer gel.

III.1-ROLE OF ACTIVATED MICROGLIA CELLS IN INFLAMMATOTY PROCESSES

II.1.1- 2D proteins map

Proteomic analysis using 2D-gel electrophoresis and matrix assisted laser desorption-mass spectrometry (MALDI-MS) for protein separation and identification was applied to purified cytosolic extracts from microglia cells that were either untreated (control) or were stimulated for 24 hours with a mixture of cytokines including 1 nM IL-1β and 1nM TNF-α. A total of 3 identically run 2D-gels with extracts from control and cytokine-stimulated cells were used for the study. Only spots which appeared on all gels were picked and analysed. Thus, a total of 345 different proteins were identified with isoelectric point values (pI) ranging from 4.5 to 10.4 and molecular weights in the range of Mw 14.9 KDa and Mw 123.7 KDa.

For a correct analysis of the data, a normalization of values has been performed. Indeed, different numbers of spots were picked on each gel. The total number of spots picked in control and treated samples has to be the same for a correct appreciation of a change in protein levels. For this calculation, the control values were used as a baseline and a correlation coefficient according to the total number of spot was applied on the others values. With this system, 53 of the 354 identified proteins were found to be upregulated with a range of ≥1.5 fold increase (Table 1). Table 2 shows 82 proteins that were found to be down regulated (≥1.5 fold decreased).

After the MS measurement, up- or down-regulated proteins were grouped according to their functions as shown in Fig 6 and 7. Up-regulated proteins have been classified into 10 groups (Fig 6) including proteins involved in stress, structural constituents of cytoskeleton, transport processes, hydrolase activity, oxydoreductases, transferases, or other kinase activities, proteins involved in signal transduction as well as proteins involved in cell communication and organization.

Down-regulated proteins were classified in 16 groups (Fig 7): the same spectrum as described above plus metal ion binding proteins; proteins with kinase inhibitor activities; proteins involved in metabolism; ubiquitin conjugating enzyme; proteins involved in development and proteins with unknown function.

Table 1

List of up-regulated proteins					
swiss prot	pi	Mr(kDa)	fold induction	protein description	
sw:TKT_HUMAN	7,62	67,8	14,4	transketolase	
sw:ALBU_HUMAN	6,2	71,3	5,4	serum albumin precursor	
sw:TPM4_HUMAN	4,48	28,5	4,8	tropomyosin alpha 4 chain	
sw:ENPL_HUMAN	4,6	92,7	4,2	endoplasmin precursor	
sw:PRO1_HUMAN	8,35	14,9	4,2	profilin i	
sw:UAP1_HUMAN	6,3	58,8	4,2	udp-n-acetylhexosamine pyrophosphorylase	
sw:TCPA_HUMAN	6	60,3	4,2	t-complex protein 1, alpha subunit	
sw:PDI_HUMAN	4,6	57,1	3,6	protein disulfide isomerase precursor	
sw:VINC_HUMAN	5,43	123,7	3,6	vinculin	
sw:CH60_HUMAN	5,64	61	3,6	60 kda heat shock protein, mitochondrial precursor	
sw:SERA_HUMAN	6,68	56,6	3,6	d-3-phosphoglycerate dehydrogenase	
sw:SERC_HUMAN	7,65	40,4	3,6	phosphoserine aminotransferase	
sw:TBA4_HUMAN	4,8	49,9	3,03	tubulin alpha-4 chain	
sw:AAC4_HUMAN	5,21	104,8	3	alpha-actinin 4	
sw:AMPL_HUMAN	6,71	52,6	3	cytosol aminopeptidase	
sw:MYH9_HUMAN	5,4	22,6	3	myosin heavy chain, nonmuscle type a	
sw:MX1_HUMAN	5,68	75,4	2,6	interferon-regulated resistance gtp-binding protein mxa	
sw:FSC1_HUMAN	7,2	54,3	2,55	fascin	
sw:CYPH_HUMAN	//	//	2,4	peptidyl-prolyl cis-trans isomerase a	
sw:G6PD_HUMAN	6,9	59,1	2,4	glucose-6-phosphate 1-dehydrogenase	
sw:TPM2_HUMAN	4,47	32,8	2,4	tropomyosin beta chain	
sw:ACTS_HUMAN	5,15	42	2,4	actin, alpha skeletal muscle	
sw:LDHA_HUMAN	8,34	36,5	2,34	l-lactate dehydrogenase a chain	
sw:AAC1_HUMAN	5,14	103	2,25	alpha-actinin 1	
sw:TBB1_HUMAN	4,6	49,7	2,22	tubulin beta-1 chain	
sw:HS27_HUMAN	6,37	22,7	1,92	heat shock 27 kda protein	
sw:UGDH_HUMAN	7,08	55	1,8	udp-glucose 6-dehydrogenase	
sw:143E_HUMAN	4,5	29,1	1,8	14-3-3 protein epsilon	
sw:CAZ2_HUMAN	5,7	32,8	1,8	f-actin capping protein	
sw:COF2_HUMAN	8,1	18,7	1,8	cofilin, muscle isoform	
sw:DDH2_HUMAN	5,92	29,6	1,8	ng,ng-dimethylarginine dimethylaminohydrolase 2	
sw:IEFS_HUMAN	6,8	62,6	1,8	stress-induced-phosphoprotein 1	
sw:PBEF_HUMAN	7,16	55,5	1,8	pre-b cell enhancing factor precursor	
sw:PUR9_HUMAN	6,7	64,6	1,8	bifunctional purine biosynthesis protein purh	
sw:TCPZ_HUMAN	6,7	58,4	1,8	t-complex protein 1, zeta subunit	
humangp:CHR3-Q9Y3F5	7,1	//	1,8	tr:q9y3f5: a6 related protein	
sw:143T_HUMAN	4,51	27,7	1,8	14-3-3 protein tau	
sw:IRE1_HUMAN	6,66	98,4	1,8	iron-responsive element binding protein 1	
sw:KPR1_HUMAN	6,9	34,7	1,8	ribose-phosphate pyrophosphokinase i	
sw:PSA1_HUMAN	6,6	29,5	1,8	proteasome subunit alpha type 1	
sw:SODM_HUMAN	8,4	24,8	1,8	superoxide dismutase	
sw:TCPG_HUMAN	6,6	60,4	1,8	t-complex protein 1, gamma subunit	
sw:RAN_HUMAN	7,52	24,4	1,68	gtp-binding nuclear protein ran	
sw:HS71_HUMAN	5,4	70	1,61	heat shock 70 kda protein 1	
sw:ALDR_HUMAN	7	35,7	1,6	aldose reductase	
sw:143Z_HUMAN	4,54	27,7	1,6	14-3-3 protein zeta/delta	
sw:KPY1_HUMAN	7,6	57,8	1,52	pyruvate kinase	
sw:LDHB_HUMAN	5,96	36,5	1,5	l-lactate dehydrogenase b chain	
sw:CAP1_HUMAN	8,09	51,5	1,5	adenylyl cyclase-associated protein 1	
sw:CNE1_HUMAN	5,66	59	1,5	copine i	
sw:GBLP_HUMAN	7,64	35	1,5	guanine nucleotide-binding protein beta subunit-like protein 12.3	
sw:SPSY_HUMAN	4,71	41,2	1,5	spermine synthase	
sw:TI47_HUMAN	5,21	47	1,5	cargo selection protein tip47	

Table2

List of down-regulated proteins					
swiss protein number	Pi	MW(KDa)	fold induction	protein identification	
sw:GR75_HUMAN	6	73.6	10	stress-70 protein, mitochondrial precursor	
sw:K1CI_HUMAN	5	61.9	9	keratin, type i cytoskeletal 9	
sw:IPYR_HUMAN	5.7	32.6	8.33	inorganic pyrophosphatase	
sw:NDKA_HUMAN	6.1	17.3	6.66	nucleoside diphosphate kinase a	
sw:GTP_HUMAN	5.37	23.2	5.55	glutathione s-transferase p	
humangp:CHR6-Q8TDM4	/	/	5	tr:q8tdm4: acetyl coa transferase-like protein.	
sw:CRKL_HUMAN	6.73	33.7	5	crk-like protein.	
sw:DUT_HUMAN	10.4	26.7	5	deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial precursor	
sw:PSB1_HUMAN	8.19	26.5	5	proteasome subunit beta type 1	
sw:2AAA_HUMAN	4.81	65	4	serine/threonine protein phosphatase 2a, 65 kda	
sw:CLI4_HUMAN	5.36	28.7	4	chloride intracellular channel protein 4	
sw:PRSX_HUMAN	7.5	44.2	4	26s protease regulatory subunit s10b	
sw:PSA4_HUMAN	7.73	29.5	4	proteasome subunit alpha type 4	
sw:PSB2_HUMAN	7.02	22.8	3.33	proteasome subunit beta type 2	
sw:6PGL_HUMAN	5.97	27.5	3.33	6-phosphogluconolactonase	
sw:ACTC_HUMAN	5.15	42	3.33	actin, alpha cardiac.	
sw:GDIR_HUMAN	4.85	23.2	3.33	rho gdp-dissociation inhibitor 1	
sw:IMB1_HUMAN	4.51	97.1	3.33	importin beta-1 subunit	
sw:LGUL_HUMAN	5.15	20.6	3.33	lactoylglutathione lyase.	
sw:PSD9_HUMAN	6.95	24.6	3.33	26s proteasome non-ATPase regulatory subunit 9	
sw:PTD4_HUMAN	7.89	44.7	3.33	putative gtp-binding protein	
sw:RINI_HUMAN	4.54	49.8	3.33	placental ribonuclease inhibitor	
sw:RSP4_HUMAN	4.62	32.8	3.33	40s ribosomal protein sa	
sw:TF1B_HUMAN	5.62	88.5	3.33	transcription intermediary factor 1-beta .	
sw:PUR4_HUMAN	5.81	144.6	3.3	phosphoribosylformylglycinamidine synthase	
sw:FAAA_HUMAN	6.93	46.4	3	fumarylacetoacetase	
sw:K1CR_HUMAN	5.22	47.9	3	keratin, type i cytoskeletal 18	
sw:PSE1_HUMAN	5.9	28.7	2.77	proteasome activator complex subunit 1	
sw:PDX1_HUMAN	8.18	22.1	2.5	peroxiredoxin 1	
sw:BID_HUMAN	5.19	21.9	2.5	bh3 interacting domain death agonist (bid).	
sw:PNPH_HUMAN	6.94	32.1	2.5	purine nucleoside phosphorylase	
sw:PSA2_HUMAN	7.51	25.7	2.5	proteasome subunit alpha type 2	
sw:UBL1_HUMAN	5.28	24.8	2.5	ubiquitin carboxyl-terminal hydrolase isozyme l1	
sw:PGK1_HUMAN	8.1	44.6	2.3	phosphoglycerate kinase 1	
sw:KCRB_HUMAN	5.37	42.6	2.22	creatine kinase, b chain.	
sw:PDX2_HUMAN	5.8	21.9	2.08	peroxiredoxin 2	
sw:ANX2_HUMAN	7.82	38.5	2	annexin a2	
humangp:CHR1-ENOA	/	/	2	sw:enoa_human: alpha enolase	
sw:AKA1_HUMAN	6.78	36.4	2	alcohol dehydrogenase	
sw:APT_HUMAN	5.88	19.5	2	adenine phosphoribosyltransferase	
sw:AR72_HUMAN	7.1	39.6	2	aflatoxin b1 aldehyde reductase 1	
sw:CRK_HUMAN	5.52	33.8	2	proto-oncogene c-crk (p38)	
sw:GRB2_HUMAN	6.26	25.2	2	growth factor receptor-bound protein 2	
sw:IF2A_HUMAN	4.84	36	2	eukaryotic translation initiation factor 2 subunit 1	
sw:K22E_HUMAN	8.08	65.8	2	keratin, type ii cytoskeletal 2 epidermal	
sw:METK_HUMAN	6.45	43.6	2	s-adenosylmethionine synthetase gamma form	
sw:P2AA_HUMAN	5.3	35.6	2	serine/threonine protein phosphatase 2a,	
sw:PDA6_HUMAN	6.71	27.4	2	protein disulfide isomerase a6 precursor	
sw:RBB7_HUMAN	4.77	47.8	2	histone acetyltransferase type b subunit 2	
sw:SWS1_HUMAN	4.97	26.7	2	swiprosin 1.	
sw:TBBX_HUMAN	4.65	49.6	2	tubulin beta-5 chain	

sw:TEBP_HUMAN	4.16	18.7	2	telomerase-binding protein p23	
sw:UNRI_HUMAN	4.85	38.4	2	unr-interacting protein	
sw:TAG2_HUMAN	8.34	22.4	1.66	transgelin 2	
sw:PDX6_HUMAN	6.31	25	1.66	peroxiredoxin 6	
sw:143B_HUMAN	4.58	27.9	1.66	14-3-3 protein beta/alpha .	
sw:TCPH_HUMAN	7.61	59.4	1.66	t-complex protein 1, eta subunit	
sw:KPR2_HUMAN	6.59	34.6	1.66	ribose-phosphate pyrophosphokinase ii	
sw:RUV1_HUMAN	6.38	50.2	1.66	ruvb-like 1	
sw:UBA1_HUMAN	5.57	117.8	1.66	ubiquitin-activating enzyme e1	
sw:GUAA_HUMAN	6.85	76.7	1.66	gmp synthase	
sw:IF32_HUMAN	5.44	36.5	1.66	eukaryotic translation initiation factor 3 subunit 2	
sw:KC21_HUMAN	7.84	45.1	1.66	casein kinase ii, alpha chain).	
sw:KCY_HUMAN	7.84	22.2	1.66	ump-cmp kinase	
sw:PUR6_HUMAN	7.17	47	1.66	multifunctional protein ade2	
sw:TCPE_HUMAN	5.42	59.7	1.66	t-complex protein 1, epsilon subunit	
humangp:CHR19-O95717	/	/	1.66	tr:o95717: sumo-1-activating enzyme e1 n subunit.	
sw:ACY1_HUMAN	6.13	45.8	1.66	aminoacylase-1	
sw:ANX3_HUMAN	5.78	36.3	1.66	annexin a3	
sw:AR34_HUMAN	7.3	34.3	1.66	arp2/3 complex 34 kda subunit	
sw:CDK4_HUMAN	7	33.7	1.66	cell division protein kinase 4	
sw:EF1D_HUMAN	4.7	31.1	1.66	elongation factor 1-delta	
sw:HS72_HUMAN	5.51	70	1.66	heat shock-related 70 kda protein 2	
sw:PDX4_HUMAN	6.25	30.5	1.66	peroxiredoxin 4 .	
sw:PRS4_HUMAN	6.1	49.1	1.66	26s protease regulatory subunit 4 (p26s4).	
sw:PSA7_HUMAN	8.73	27.8	1.66	proteasome subunit alpha type 7	
sw:PUR2_HUMAN	6.68	107.7	1.66	trifunctional purine biosynthetic protein adenosine-3	
sw:ROK_HUMAN	5.28	50.9	1.66	heterogeneous nuclear ribonucleoprotein k	
sw:SPEE_HUMAN	5.26	33.8	1.66	spermidine synthase	
sw:TPM1_HUMAN	4.5	32.7	1.66	tropomyosin 1 alpha chain	
sw:UBC7_HUMAN	8.75	17.8	1.66	ubiquitin-conjugating enzyme e2-18 kda ubch7	

The representation of up- and down-regulated proteins (Fig. 6 and 7) demonstrates a different repartition of proteins (The colors used in tables for each function have been maintained for the representation). For example, the cytoskeleton binding protein group is composed of more up- than down-regulated proteins: 11 proteins compared to only one protein, respectively. These findings point to an important role of cytoskeleton proteins during inflammatory processes. In contrast, proteins such as hydrolase and transferase activities are preferentially down-regulated.

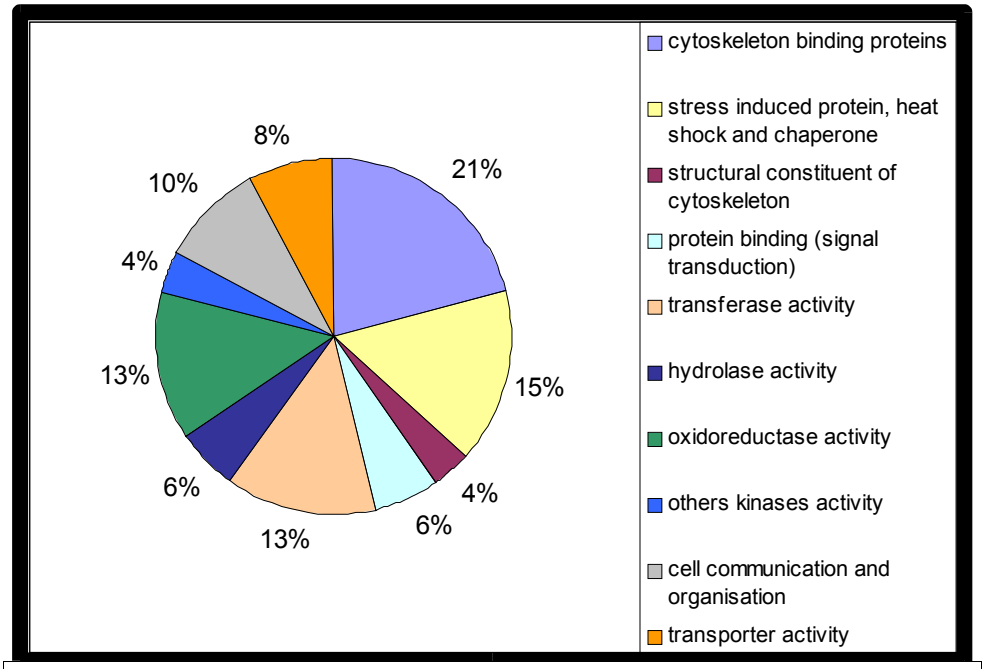


Fig 6: Representation of the repartition of up-regulated proteins according to their functions. Repartition in 10 different groups

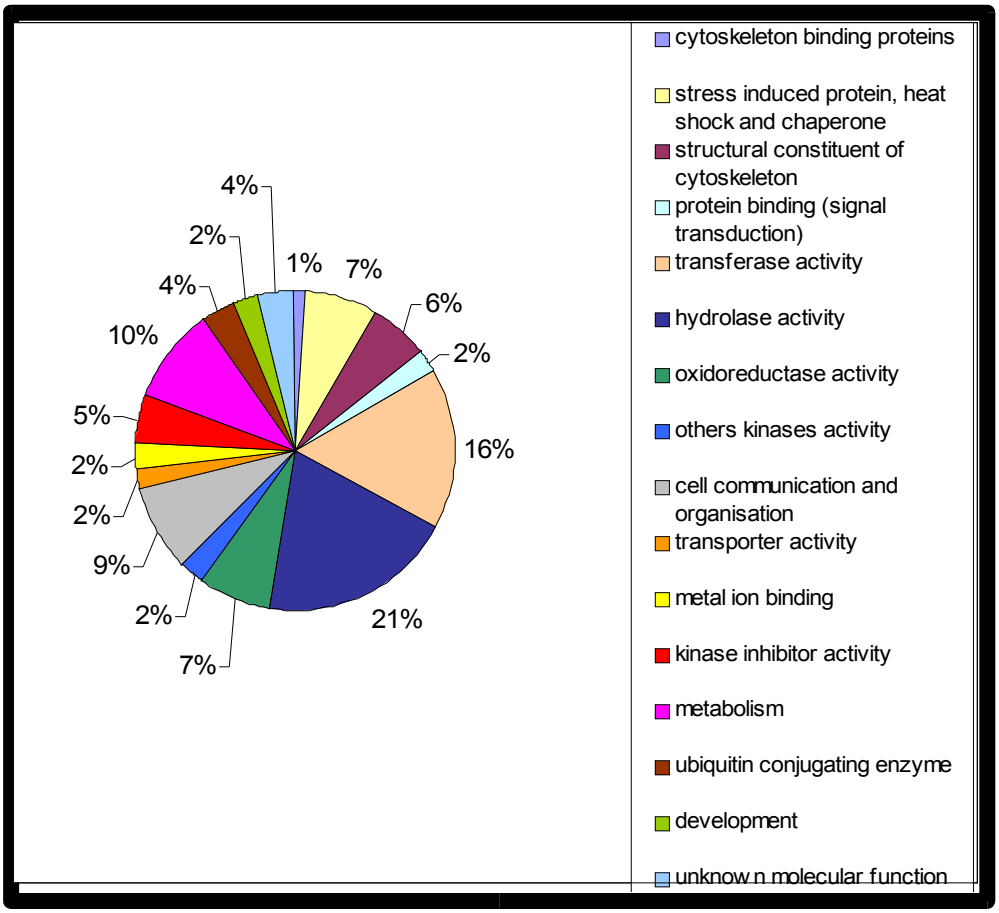


Fig 7: Representation of the repartition of down-regulated proteins according to their functions. Repartition in 16 groups

Impaired cellular energy metabolism as well as increased oxidative stress are typical features of many age-related diseases such as AD. Proteins involved in kinase inhibition, metabolism and ubiquitin-conjugating enzyme activity are exclusively found to be down-regulated. We found two differents ubiquitin-conjugating enzyme E1 and E2-18Kda down-regulated. Loss of function of ubiquitynation, co-factor of ATP-dependent proteolysis, has been shown to be sufficient to cause familial forms of neurodegenerative diseases by stopping the clearance of gamma-secretase (BACE) or playing a role in Aβ neurotoxicity. This supports the idea that metabolism and mostly glycolysis and proteolysis are down-regulated mechanisms during inflammation in age-related neurodegenerative diseases.

III.1.2- Identification of up and down –regulated proteins

III.1.2-1- U*p-regulated proteins*

In our studies, the most up-regulated protein is Transketolase (TKT) (Table 1, Fig. 8-A) that is 14-fold induced by the cytokine treatment. TKT has a transferase activity and is a key protein in the pentose pathway. The involvement of TKT in AD brain has been discussed and may be related to its anti-oxidant functions.

Several cytoskeleton binding proteins were found to be up-regulated by inflammatory cytokines. This group includes proteins such as Profilin and Cofilin, which are up-regulated by 4.2 and 1.8 fold, respectively. Impaired cytoskeletal functions might contribute to neuronal or synaptic damage that occurs in AD. For example, cofilin was shown to initiate or to enhance the progression of different neurodegenerative disorders such as AD.

Interestingly, proteins involved in signal transduction, including several subunits of 14-3-3 protein: Epsilon (143E), Tau (143T) and Zeta (143Z), respectively are increased by 1.8, 1.8 and 1.6 fold, and have been shown to be involved in neurodegenerative diseases. The subunit Zeta has clearly been identified as a tau protein effector and may trigger or modulate abnormal phosphorylation of tau protein.

A protein classified in the chaperone group, T-complex protein-1(TCP-1) has three different subunits found to be up-regulated: Alpha (TCP-A) by 4.2 fold, Gamma (TCP-G) by 1.8 and zeta (TCP-Z) by 1.8 fold. The tubulin beta-1 (TBB1), up-regulated 2.2 fold, and classified in the same chaperone group, is the more specific substrate of TCP-1Z. A dysregulation of these proteins has been found in AD and may be associated with the misfolding of proteins during AD. In contrast, two other subunits of TCP-1 were found to be down-regulated: Epsilon (TCPE) and Eta (TCPH), both by 1.6 fold. TCP-1E has been shown to be involved in cell cycle regulation and is thought to play a role in neurodegenerative disorders.

Pre-B-cell enhancing factor (PBEF), involved in cell communication and organization, was found to be upregulated by 1.8 fold. Surprisingly, this protein which is involved in the maturation of B-cell precursors has been shown to be highly up-regulated in presence of cytokines such as IL-1β and TNF-α.

III.1.2-2- Down-regulated proteins

Proteins with hydrolase activity are the most down-regulated proteins following cytokine treatment (Table 2). In addition, one of the most down-regulated proteins is nucleoside diphosphate kinase a (NDKA) (Table 2, Fig. 8-B) which has a transferase activity. This protein is down-regulated by 6.6 fold upon cytokine stimulation of microglial cells.

Another protein known to play a role in degenerative disorders is cell division kinase 4 (CDK4). In our study, this protein is classified in the group of protein with transferase activity and was found to be down-regulated (1.6 fold). Cdks have been shown to be suppressed in neurodegenerative disorders such as AD or Parkinson's disease.

Proteins such as Ser/Threonine phosphatase 2A (2AAA) play a role in tau phosphorylation and its impairment has been shown to contribute to abnormal hyperphosphorylation of tau. Inhibition of this phosphatase delays ERK inactivation which leads to increased tau phosphorylation. In our *in-vitro* model system, the cytokine-stimulated microglial cells, 2AAA was found to be markedly down-regulated (4-fold).

Phosphorylation of eukaryotic initiation factor-2 alpha (IF2A) via RNA dependent protein kinase (PKR) has been shown to be pro-apoptotic. The phosphorylation of this factor that appears in AD inhibits protein translation and promotes neuronal loss by apoptosis. In our study, IF2A has been found to be down-regulated by 2 fold.

Peroxiredoxins are antioxidant enzymes that have been hypothesized to play an important role in the pathogenesis of diverse diseases associated with neurodegeneration such as Pick's disease, Down syndrome and AD. Actually, in our study, peroxiredoxins 1, 2, 4 and 6 were found down-regulated (Table 2).

In conclusion, these *in-vitro* results obtained in a human microglia cell line, support the important contribution of inflammatory responses as well as a role of activated microglia cells in CNS degeneration processes. Further studies have to prove whether the proteins found with changes in their expression level could be potential drug targets. Moreover, the results obtained with activated microglial cells form the brain for subsequent biochemical analyses of transgenic mouse models for AD.

Fig 8- A:

1- TKT- control

2-*TKT*-inflammatory cytokine-treated

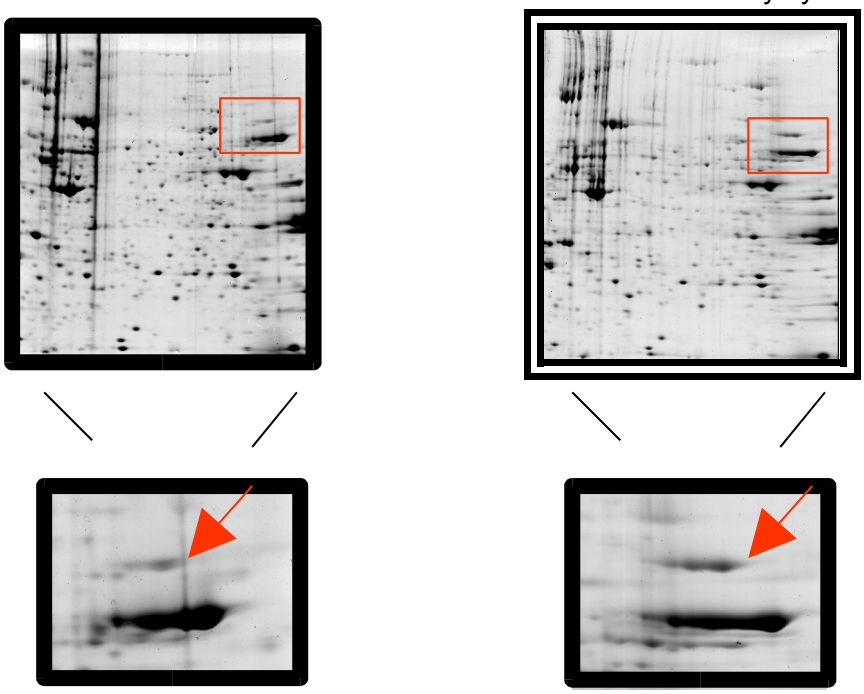


Fig 8- B:

1- NDKA control

2- NDKA - inflammatory cytokine-treated

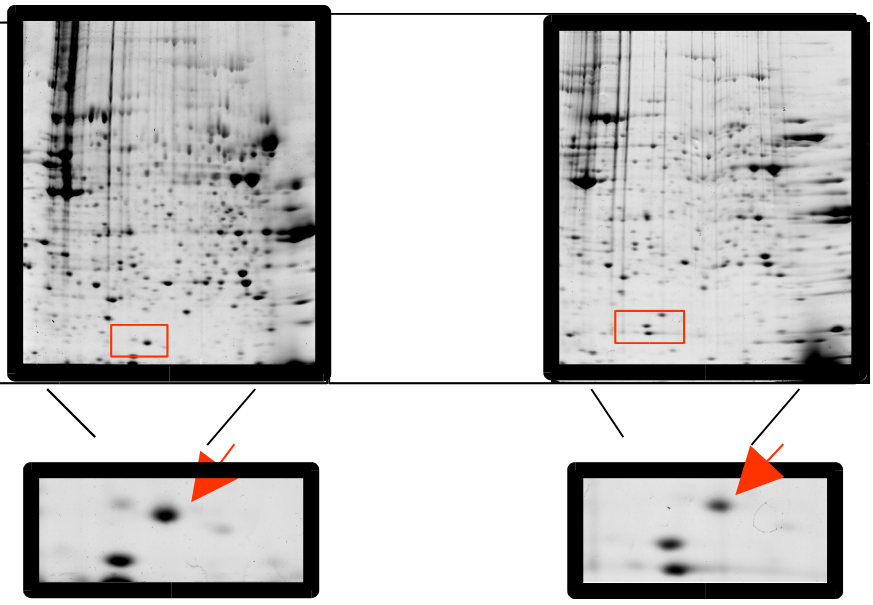


Fig 8: Representative 2D gels displaying up-regulated or down-regulated proteins. Fig8-A shows the up-regulation of the expression of transketolase (TKT) after cytokine treatment of microglial cells (2-) as compared to control level (1-). The protein spot on 2D gel is marked by the red arrows in the enlarged pictures
Fig 8-B shows the down-regulation of the expression of nucleoside diphosphate kinase-A (NDKA) after cytokine treatment of microglial cells (2-) as compared to controls (1-). NDKA protein corresponding spot on the 2D gel is marked by the red arrows on the enlarged pictures

III.2- TRANSGENIC APP/PS2 MOUSE MODEL

These studies were performed with C57BL/6 control and APP/PS2 transgenic mice provided by Dr. L. Ozmen, F.Hoffman-La Roche, Basel. A total of 26 SDS-PAGE were analysed including 13 gels from control and 13 gels from transgenic mice.
In summary, a total amount of 497 different proteins was detected. In general, all proteins identified in these experiments were also found in cytokine-stimulated microglia.
70 proteins were found to be up-regulated (up to 1.5 fold, Table 3). Among those, six proteins found to be up-regulated in mouse brain were also found up-regulated in microglial cell (Table 3, noted by Y). These proteins include heat shock 70KDa protein, 14-3-3 protein tau isoform, endoplasmin precursor, protein disulfide isomerase precursor, gtp-binding nuclear protein ran and cytosol aminopeptidase.
Some proteins such as copine, 14-3-3 eta, actin, or beta actin which are up-regulated in brain are very similar or are members of the same family of proteins which have been found up-regulated in activated microglia (Table 3, noted by S).
On the other hand, three further proteins were found to be up-regulated in brains of transgenic mice but down-regulated in cytokine-activated microglial cell. (Table 3, noted by O): Ubiquitin- activating enzyme e1, 14-3-3 beta/alpha and unr- interacting protein.
Other proteins such as TKT, TCPG, 14.3.3 Zeta, or Fascin which were found induced after cytokine treatment of microglia cells, were only slightly up-regulated (TKT and fascin) or not changed in their expression as in the case of TCPG and 14.3.3 Zeta in the transgenic mouse model (data not shown).

In addition, 45 proteins were found to be down-regulated in the brain of the transgenic animal model of AD (Table 4). Notably, proteins such as actin alpha cardiac, rho gdp-dissociation inhibitor, lactoylglutathione kyase, peroxiredoxin 1 and 6 and ser/thr protein phosphatase found to be down-regulated in transgenic animal model were also found down-regulated in the microglia study. (Table 4 noted by Y).
Proteins such as tropomyosin, heterogeneous nuclear ribonucleoproteins a2, elongation factor, and annexin 7 detected as down-regulated in mice brain are members of the same family of proteins found to be down-regulated in activated microglia cells (Table 4 noted by S).

Finally, changes of proteins such as l-lactate dehydrogenase, superoxide dismutase, actin alpha from muscle, and 14.3.3 epsilon found in the AD mice were in the opposite direction as compared to the other study (Table 4 noted by O).

Table 3: List of up-regulated proteins			
Swiss prot name	fold induction	Protein description	Microglia
SW:HS71_MOUSE	13.65	heat shock 70 kda protein 1	Y
SW:GB02_MOUSE	7.3	guanine nucleotide-binding protein g	
SW:UBA1_MOUSE	6.37	ubiquitin-activating enzyme e1 1.	O
SW:DPY5_MOUSE	6.37	dihydropyrimidinase related protein-5	
SW:GTP2_MOUSE	6.37	glutathione s-transferase p 2	
SW:CN37_MOUSE	5.46	2',3'-cyclic nucleotide 3'-phosphodiesterase	
SW:HXK1_MOUSE	5.46	hexokinase, type i	
SW:WDR1_MOUSE	4.55	wd-repeat protein 1	
SW:VATE_MOUSE	4.55	vacuolar atp synthase subunit e	
SW:OAT_MOUSE	4.55	ornithine aminotransferase,	
SW:UCR2_MOUSE	3.6	ubiquinol-cytochrome c reductase complex core protein 2,	
SW:IDHG_MOUSE	3.6	isocitrate dehydrogenase subunit gamma	
SW:OS94_MOUSE	3.3	osmotic stress protein 94	
SW:P2AB_HUMAN	3.2	serine/threonine protein phosphatase 2a	
SW:NFM_MOUSE	3.2	neurofilament triplet m protein	
SW:SYS_MOUSE	3.1	seryl-trna synthetase	
SW:PPIA_MOUSE	3	peptidyl-prolyl cis-trans isomerase a	
SW:SYN1_MOUSE	2.9	synapsin i.	
SW:TERA_MOUSE	2.73	transitional endoplasmic reticulum atpase	
SW:PAC1_MOUSE	2.73	protein kinase c and casein kinase	
SW:143B_MOUSE	2.73	14-3-3 protein beta/alpha	O
SW:COF1_MOUSE	2.73	cofilin, non-muscle isoform.	
SW:FUMH_MOUSE	2.73	fumarate hydratase, mitochondrial precursor	
SW:VIME_MOUSE	2.73	vimentin.	
SW:143T_MOUSE	2.73	14-3-3 protein tau	Y
SW:HBB1_MOUSE	2.7	hemoglobin beta-1 chain	
SW:ENPL_MOUSE	2.7	endoplasmin precursor	Y
SW:DHAM_MOUSE	2.7	aldehyde dehydrogenase, mitochondrial precursor	
SW:ACY2_MOUSE	2.7	aspartoacylase	
SW:CNE6_MOUSE	2.7	copine vi	S
SW:OTB1_MOUSE	2.42	ubiquitin thiolesterase protein otub1	
SW:TRFE_MOUSE	2.28	serotransferrin precursor	
SW:HS9B_MOUSE	2.2	heat shock protein hsp 90-beta	
SW:HS9A_MOUSE	2.16	heat shock protein hsp 90-alpha	
SW:NSF_MOUSE	2.12	vesicle-fusing atpase	
SW:HS72_MOUSE	2	heat shock-related 70 kda protein 2 (	
SW:MDHM_MOUSE	1.97	malate dehydrogenase, mitochondrial precurso	
SW:GBB2_HUMAN	1.82	guanine nucleotide-binding protein	
SW:PEBP_MOUSE	1.82	phosphatidylethanolamine-binding protein	
SW:HS74_MOUSE	1.82	heat shock 70-related protein apg-2.	
SW:PDX2_MOUSE	1.82	peroxiredoxin 2	
SW:DPY3_MOUSE	1.82	dihydropyrimidinase related protein-3	
SW:CRYM_MOUSE	1.82	mu-crystallin homolog.	
SW:PDI_MOUSE	1.82	protein disulfide isomerase precursor	Y
SW:RAN_HUMAN	1.82	gtp-binding nuclear protein ran	Y
SW:CGL1_MOUSE	1.82	cytosolic nonspecific dipeptidase	
SW:UNRI_MOUSE	1.82	unr-interacting protein	O
SW:MYOP_MOUSE	1.82	inositol-1(or 4)-monophosphatase	
SW:PYC_MOUSE	1.82	pyruvate carboxylase, mitochondrial precursor	
SW:PRS7_MOUSE	1.82	26s protease regulatory subunit 7	
SW:UBP5_MOUSE	1.82	ubiquitin carboxyl-terminal hydrolase 5	
SW:NDR1_MOUSE	1.82	ndrg1 protein	
SW:AHA1_MOUSE	1.82	activator of 90 kda heat shock protein	
SW:LIS1_MOUSE	1.82	platelet-activating factor acetylhydrolase ib alpha subunit	
SW:CAH8_MOUSE	1.82	carbonic anhydrase-related protein	
SW:143F_MOUSE	1.82	14-3-3 protein eta	S
SW:CO1C_MOUSE	1.82	coronin 1c	
SW:FKB4_MOUSE	1.82	fk506-binding protein 4	
MOUSEGP:MCHR11-079188	1.82	tr:q8bfz3: actin.	S
SW:APA1_MOUSE	1.82	apolipoprotein a-i precursor	
SW:ACT1_HELER	1.82	actin, cytoplasmic cyi.	S
TR_ROD:Q91ZK5	1.82	beta actin (fragment)	S
SW:SYG_MOUSE	1.82	glycyl-trna synthetase	
SW:AMPL_MOUSE	1.82	cytosol aminopeptidase	Y
SW:PSA_MOUSE	1.82	puromycin-sensitive aminopeptidase	
SW:MK03_MOUSE	1.82	mitogen-activated protein kinase 3	
SW:FTDH_MOUSE	1.82	10-formyltetrahydrofolate dehydrogenase	
SW:ARS1_MOUSE	1.82	arsenical pump-driving atpase	
SW:VA0D_MOUSE	1.82	vacuolar atp synthase subunit d	
SW:ANX5_MOUSE	1.64	annexin a5	

Table 4: List of down-regulated proteins.			
Swiss prot name	fold induction	Protein description	Microglia
SW:ACTC_HUMAN	6.5	actin, alpha cardiac	Y
SW:SEP6_MOUSE	5.5	septin 6.	
SW:TPM3_MOUSE	4.5	tropomyosin alpha 3 chain	S
SW:SEP5_MOUSE	4.5	septin 5	
SW:K1CI_HUMAN	3.3	keratin, type i cytoskeletal 9	
SW:ACT3_FUGRU	3.3	actin, cytoplasmic 3	
SW:GDIR_MOUSE	2.7	rho gdp-dissociation inhibitor 1	Y
SW:ROA2_MOUSE	2.7	heterogeneous nuclear ribonucleoproteins a2/b1	S
SW:NFM_PIG	2.2	neurofilament triplet m protein	
SW:LGUL_MOUSE	2.2	lactoylglutathione lyase	Y
SW:ATPD_MOUSE	2.2	atp synthase d chain	
SW:EF2_MOUSE	2.2	elongation factor 2	S
SW:NUDM_MOUSE	2.2	nadh-ubiquinone oxidoreductase 42 kda subunit	
SW:FRIH_MOUSE	2.2	ferritin heavy chain	
SW:PSA1_MOUSE	2.2	proteasome subunit alpha type 1	
SW:MPK1_MOUSE	2.2	dual specificity mitogen-activated protein kinase kinase 1	
SW:PDX1_MOUSE	2.2	peroxiredoxin 1	Y
SW:TLIP_MOUSE	2.2	toll-interacting protein	
SW:P2AA_MOUSE	2.2	serine/threonine protein phosphatase 2a	Y
SW:P2BA_MOUSE	2.2	serine/threonine protein phosphatase 2b	
SW:LDHA_MOUSE	2.2	l-lactate dehydrogenase a chain	O
SW:PD6I_MOUSE	2.2	programmed cell death 6 interacting protein	
SW:MBP_MOUSE	2.2	myelin basic protein	
SW:SODM_MOUSE	2.2	superoxide dismutase	O
SW:SEP2_MOUSE	2.2	septin 2	
SW:ACTB_HUMAN	2.1	actin, cytoplasmic 1	
SW:TBA8_MOUSE	2	tubulin alpha-8 chain	
SW:SH32_MOUSE	2	sh3-containing grb2-like protein 2	
SW:CABV_MOUSE	2	calbindin	
SW:DHCA_MOUSE	2	carbonyl reductase	
SW:ACT1_ECHGR	2	actin 1	
SW:SYW_MOUSE	2	tryptophanyl-trna synthetase	
SW:GBLP_HUMAN	2	guanine nucleotide-binding protein beta subunit-like protein	
SW:ANX7_MOUSE	2	annexin a7	S
SW:LAS1_MOUSE	2	lim and sh3 domain protein 1	
TR_ROD:O35248	2	beta actin	
SW:MDHC_MOUSE	1.8	malate dehydrogenase, cytoplasmic	
SW:ACTG_EMENI	1.8	actin, gamma.	
SW:ACTM_PISOC	1.75	actin, muscle.	
SW:G3P_MOUSE	1.6	glyceraldehyde 3-phosphate dehydrogenase	
SW:NUHM_MOUSE	1.6	nadh-ubiquinone oxidoreductase 24 kda subunit,	
SW:PDX6_MOUSE	1.6	peroxiredoxin 6	Y
SW:IMMT_MOUSE	1.6	mitochondrial inner membrane protein	
SW:ACTS_HUMAN	1.6	actin, alpha skeletal muscle	O
SW:143E_HUMAN	1.55	14-3-3 protein epsilon	O

Tables 3 and 4 represent a summary of up- and down-regulated proteins, respectively, detected in the brain of transgenic mouse model of human AD. Comparison of these proteins with those obtained from the human microglia cell study, proteins noted with “Y” were found in transgenic mice and microglia model; protein members of the same family and detected in both studies were noted “S”; and expression of proteins found increased in the microglia study and decreased in the transgenic animal model (or opposite) were noted “O”

Former studies were performed using human postmortem brain material in order to generate a human map of proteins. Results obtained with the transgenic mouse model of AD clearly show a similar pattern of identified proteins with studies made with cytokine-stimulated microglia cells and human postmortem brain material (Langen et al,. 1999).

A comparison of results with AD human brain studies allows to distinguish some proteins with significant differences in expression levels in control versus AD brains.

Increased levels of 14.3.3 gamma and epsilon were found in several brain regions of AD and Down's Syndrome (DS) brains. However, isoform 14.3.3 gamma was not detected in all our studies. In contrast, 14-3-3 epsilon was found to be induced in all three models of AD (Table 5; Fountoulakis et al., 1999).

Proteins including NDKA (down-regulated by 6.6 fold in the microglia study) was found only slightly decreased in the transgenic APP/PS2 mice study (Table 5). However, in human AD brains, NDKA levels- similar to the microglia data- were found to be down-regulated (Kim et al., 2002).

Proteins such as Peroxiredoxin (PDX-) composed of different subunits were differently expressed in our models analysed. In human AD brain, PDX-1 expression was found unchanged whereas PDX-2 was increased and PDX-3 and PDX-4 were found either increased or decreased in Down Syndrome and Pick's disease. PDX-6 failed to show any changes in human AD brains (Krapfenbauer et al., 2003; Kim et al., J Neural Transm. Suppl., 2001). In contrast, in our study on induced-microglia cells, PDX-1; -2 and -6 were found to be down-regulated but only PDX-1 and -6 were also down-regulated in the transgenic mouse model. Thus, our studies reveal a different regulation of these proteins as compared to human AD brains.

NADH ubiquinone oxidoreductase 24 KDa and 75 KDa have been found down-regulated in human AD brain (Kim et al. Life Sci., 2001). Our results on the transgenic mice model show a decreased level (1.6 fold) of the 24 KDa subunit. However, the 75 KDa complex was not detected whereas another subunit 42 KDa was down-regulated (2.2 fold; Table 5).

In human AD brains levels of 2', 3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) were found to be decreased only in the frontal cortex (Vlkolinsky et al. 2001). Our results on mouse brains, however, show an up-regulation by 5.4 fold.

Studies on chaperones are of importance for AD and their expression level has been shown to be aberrant according to the brain region analyzed (Yoo et al., 2001). In the course of our study, we identified also different stress proteins, which were either up-or down-regulated.

Beta-soluble N-ethylmaleimide-sensitive factor attachment protein (beta-SNAP) was found significantly reduced in the temporal cortex of human AD brain whereas gamma-SNAP was unchanged (Yoo et al.,2001). Our results performed on a transgenic mouse model as compared to control mice show opposite effects with no difference in beta-SNAP and slightly decreased levels of gamma-SNAP (1.4 fold).

Decreased of Stathmin expression was observed in frontal and temporal cortices of AD brains (Cheon et al., 2001). Stathmin binds to two tubulin heterodimers and prevents their addition on to the end of microtubules thereby inhibiting tubulin polymerization. However, Stathmin was rarely detected in cytokine-stimulated human microglial cells.

Proteins such as the voltage-dependent anion selective channel that were described by Yoo and co-workers as a protein with significant expression changes in AD, have not been identified during our studies. (Yoo et al., Electrophoresis. 2001). Likewise Aldehyde reductase (ALDH) and Carbonyl reductase (CBR) that were found to be up-regulated in human AD brains (Balcz et al., 2001) could not be identified in any of our models investigated.

In summary, a large number of proteins involved in neurodegenerative diseases identified in human brains, have also been found in cytokine-stimulated microglia or in a transgenic mouse model of AD. Results of our studies suggest that the cellular source of the proteins analyzed is to a large extent activated microglia.

Table 5 : Representation of changes in protein expression in three different experimental models: microglia cell cultures, transgenic mouse model and human post mortem AD brain were used

Proteins	**Microglia cell culture**	**Transgenic mouse model**	**Human Post-mortem AD brain**
14.3.3 ε	Up-regulated	Down-regulated	Up-regulated
NDKA	Down-regulated	Non significant down regulation	Down-regulated
Peroxiredoxin-1	Down-regulated	Down-regulated	No change
Peroxiredoxin-2	Down-regulated	Not detected	Up-regulated
Peroxiredoxin-6	Down-regulated	Down-regulated	No change
NADH ubiquinone oxidoreductase 24KDa	Not detected	Down-regulated	Down-regulated
β-SNAP	Not detected	No change	Down-regulated
Stathmin	No change	Not detected	Down-regulated

III.3- ANALYSIS OF NITRATED PROTEINS AND THEIR POTENTIAL ROLE IN ALZHEIMER'S DISEASE

III.3.1- Characterization of an antibody against nitrated tyrosine residues

III.3.1-1- Generation of the NOY-1 antibody

For this project, a specific antibody directed against tyrosine nitrated proteins was required. The antibody NOY-1 was generated by Dr. Manfred Brockhaus from F. Hoffmann-La Roche AG, Basel. Antiserum was generated by NOY-1 immunisation of mice using *in vitro* nitrated ovalbumin. The hybridoma was selected for reactivity against nitrated BSA and lack of reactivity against non-nitrated OVA and BSA. The main specificity was expected to be against nitro-tyrosine residues, but, like for all hapten specific antibodies, crossreactivities cannot be ruled out. For this reason, we performed detailed experiments to characterize the NOY-1 antibody specificity.

III.3.1-2- Specificity of the antibody

In order to prove the capacity of the antibody to recognize tyrosine-nitrated proteins, we used nitrated human albumin. Nitration was performed *in vitro* by application of Tetranitromethane (Crow et al., 1996). The albumin was first dialysed against Tris buffer (pH 8) overnight. Then 10mM of Tetranitromethane was added to the albumin for 30 min at room temperature. Five successive washes with the Tris- buffer were done to eliminate excess of Tetranitromethane. Finally, the albumin can be loaded onto a SDS-PAGE .

Different amounts of nitrated human albumin (40µg and 20µg, lines 1,2 in Fig 11) and non-nitrated human albumin (40µg and 20µg, lines 3,4 in Fig 11) were loaded on 4-20% SDS-PAGE and used for Western-blot analysis.

The results presented in Fig. 9 clearly show that the antibody specifically recognizes nitrated proteins (lanes 1 and 2).

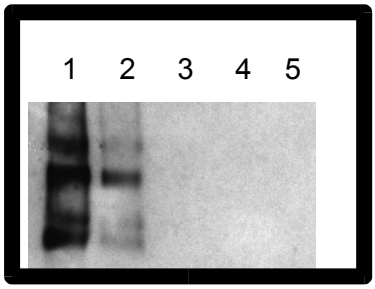


Fig 9: Western blot analysis of nitrated human albumin using the antibody NOY-1.
1- 40µg of in vitro nitrated human albumin
2- 20µg of in vitro nitrated human albumin
3- 40µg of human albumin
4- 20µg of human albumin
5- 40µg of in vitro nitrated human albumin quenched.
Lanes 1 and 2 show the detection of nitrated human albumin (Mol. Wt. 66 Kda)

To check the ability of the antibody to recognize exclusively nitrated proteins and to eliminate albumin crossreactivity, three other purified proteins with different molecular weights were selected: DnaK (heat shock protein 69 KDa); GrpE (10.4 KDa) and MurD (46 KDa) and 100ng of each were separated on SDS-PAGE (Fig 10).

Western blot analysis revealed that the antibody recognized specifically the nitrated proteins (lines 2, 4, 6 and 8), but not the non-nitrated proteins (lines 1, 3, 5, and 7).

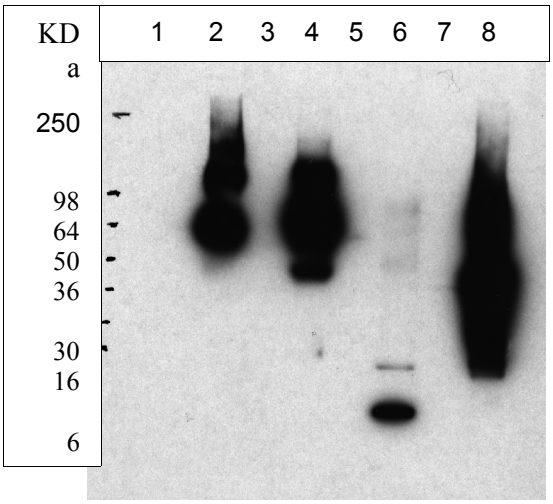


Fig 10: Western-blot analysis with NOY-1 antibody of proteins with different molecular weights:
1-100ng of human albumin; **2-**100ng of human albumin nitrated; **3**-100ng of DnaK; **4**-100ng of DnaK nitrated; **5**-100ng of GrpE; **6**-100ng of GrpE nitrated; **7**-100ng of MurD; **8**-100ng of MurD nitrated

For comparison of the obtained results, a commercially available antibody (JURO) against tyrosine nitrated proteins was used for parallel experiments. In these experiments, the identical amounts of nitrated proteins were used for SDS-PAGE and Western-blot analysis. However, this antibody, as compared to NOY-1 antibody, detected the nitrated proteins with a much lesser signal intensity (data not shown).

III.3.1-3- Quantification of the detection limit of the NOY-1 antibody

In a next step of characterization of our antibody, experiments were performed to determine the minimal amount of nitrated proteins that can be detected with the antibody. Different amounts of nitrated human albumin ranging from 1 pg to 100 ng were applied to SDS-PAGE and Western blot analyses using NOY-1. Our results show that a protein amount as low as 100 ng e.g nitrated albumin is sufficient for a correct reproducible detection by NOY-1 (Fig. 11).

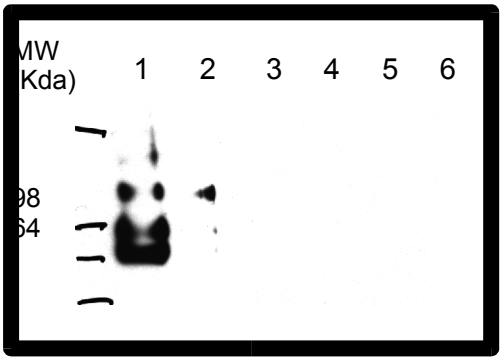


Fig 11: Determination of the detection limit of NOY-1 antibody:
1- 100ng of nitrated human albumin; 2-10ng of nitrated human albumin; 3- 1ng of nitrated human albumin;
4- 100pg of nitrated human albumin; 5- 10pg of nitrated human albumin; 6- 1pg of nitrated human albumin.
Lane 1 show that 100ng of nitrated human albumin (66 KDa) is an minimum amount clearly detectable by the NOY-1 antibody

III.3.2- Analysis of nitrated proteins with MALDI-MS

For further analysis by MALDI, theoretical molecular data are required. Nitration on tyrosine leads to addition of a NO2 group linked by an Hydrogen (H) on the protein. This additional group triggers a shift of 45 Da in the total molecular weight of the protein fragment. This change into the mass has been observed and quantified by MS (Fig.12).

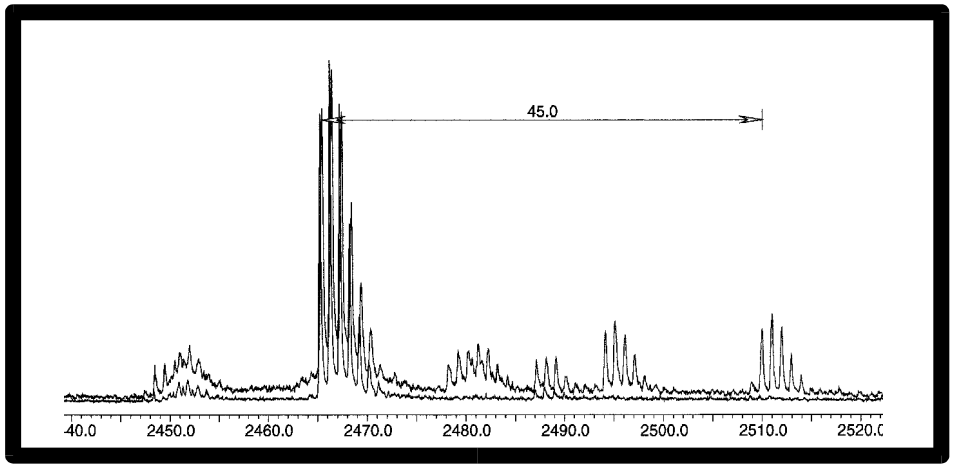


Fig 12: Mass spectrum showing the 45 Da shift obtained during a measurement of a nitrated protein by MALDI- TOF apparatus

III.3.3- Identification of nitrated peptides with Poseidon software

Supernatants from the cortex of control and APP/PS2 mice were obtained by centrifugation steps as described in Material and Methods. These supernatants were focussed with the minisystem from Invitrogen as described in Material and Methods. The second dimension was done with 10% Bio-Rad gels.

Two gels were transferred onto nitrocellulose membranes and were immunostained with NOY-1 antibody whereas two other gels were stained with Coomassie blue. On

these Commassie gels, the protein spots that correspond to antibody stained spots were picked as described in Material and Methods. Only nitrated proteins were picked with this method (data not shown).
MS measurement of these samples are of help to obtain a map of proteins. Then, a software (Poseidon, F. Hoffmann-La Roche AG) was used which permitted to analyse all peptides containing tyrosine residues and to select proteins which display different nitration patterns in AD than in control cortex, including: triosephosphate isomerase (TPIS); Beta actin (ACTB), tubulin beta 5 chain (TBB5), dihydropyrimidinase related protein 2 (DPY2); rab gdp dissociation inhibitor alpha (GDIA); tubulin alpha 1 chain (TBA1) and aldolase C (ALFC).

Table 6 demonstrates that the expression level of these proteins in AD cortex as compared to controls was different. For example, in AD brains ACTB is clearly down-regulated by 1.8 fold whereas TBA1 is highly up-regulated (5 fold). On the other hand, ALFC expression is only slightly increased in AD samples (1.2 fold).

	control	AD
TPIS	33	30
ACTB	135	75
TBB5	68	42
DPY2	126	114
GDIA	10	15
TBA1	2	10
ALFC	38	48

Table 6: Number of identification of each protein in 2D gels of cortex of control and in AD transgenic mouse. The analysis was performed with MALDI-TOF

Nitration of these proteins was tested with the help of internal software from F.Hoffmann-La Roche. For example, this theoretical measurement revealed aldolase C (ALFC) as the protein with the most pronounced changes in peptide nitration. Aldolase C, a molecule of 39 kDa, is a key enzyme of the glycolytic pathway and is specifically expressed in brain.
The other proteins listed in Table 6 are not further analyzed yet but could be potential candidates as marker molecules for inflammatory degeneration. Indeed, TBB5, TBA1 and ACTB are cytoskeleton binding proteins, and their functions are impaired in AD. DPY2, a protein of interest in axonal guidance, has been already

described to be involved in neurodegenerative events such as AD. GDIA is a molecule participating in intracellular trafficking and a loss of function of this protein can finally leads to alteration of vesicle exocytosis.

III.3.3-1- Identification of Y-nitrated peptides in ALFC sequence

Five different nitrated peptides from ALFC protein have been identified by mass spectrometry (Fig 13).

1- **YSPEEIAMATVTALRR**
2- **CIGGVIFFHETLYQK**
3- **AEMNGLAAQGRYEGSGDGGAAAQSLYIANHAY**
4- **PHSYPALSAEQKK**
5- **CPLPRPWALFFSYGR.**

Fig 13: List of peptides derived from aldolase C (ALFC) that were nitrated acording to MS analysis.

These five peptides represent different parts of the ALFC- protein (Fig 14).

1 **PHSYPALSAE QKK**ELSDIAL RIVTPGKGIL AADESVGSMA KRLSQIGVEN
51 TEENRRLYRQ VLFSADDRVK K**CIGGVIFFH ETLYQK**DDNG VPFVRTIQDK
101 GILVGIKVDK GVVPLAGTDG ETTTQGLDGL LERCAQYKKD GADFAKWRCV
151 LKISDRTPSA LAILENANVL ARYASICQQN GIVPIVEPEI LPDGDHDLKR
201 CQYVTEKVLA AVYKALSDHH VYLEGTLLKP NMVTPGHACP I**KYSPEEIAM**
251 **ATVTALRR**TV PPAVPGVTFL SGGQSEEEAS LNLNAINR**CP LPRPWALTFS**
301 **YGR**ALQASAL NAWRGQRDNA GAATEEFIKR **AEMNGLAAQG RYEGSGDGGA**
351 **AAQSLYIANH AY**

Fig 14: Representation of the amino acid composition of ALFC protein. Peptides of interest are represented in color

Total number of nitrated peptides amounted to 29 in control and to 42 in APP/PS2 mice. These results demonstrate that there are more nitrated ALFC-peptides in APP/PS2 mice than in controls and that this level is increased by about 1.4 fold. The most important peptide is **AEMNGLAAQG RYEGSGDGGAAAQSLYIANH AY,** because nitration of the tyrosine amino acid (Y) at the carbon terminal is a key event that leads to a loss of function of the protein (Fig 14).

Since the data are based on theoretical analysis, results have to be confirmed by experimental studies. Our goal is to visualize this increase in nitration of the ALFC-protein by immunocytochemistry. Immunohistochemical studies with a specific antibody that could detect the ALFC protein is needed.

	control	**AD**
YSPEEIAMATVTALRR	21	25
CIGGVIFFHETLYQK	6	11
AEMNGLAAQGRYEGSGDGGAAAQSLYIANHA Y	1	-
PHSYPALSAEQKK	-	1
CPLPRPWALFFSYGR.	1	5
TOTAL of the nitrated peptides	29	42

Table 7: Number of peptides of ALFC displaying Y-nitration found in control or APP/PS2 transgenic mice

III.3.3-2- Detection of ALFC protein by antibodies

For this project, the cytosolic part of homogenates from control mice brain was loaded onto 4-12% NuPAGE gels. The proteins were transferred to nitrocellulose membranes for Western blot analysis using ALFC antibody. First, two commercially available antibodies from Santa Cruz Biotechnology were used. One was directed against the N-terminal part (N14) and the second antibody was directed against an internal part (D14) of the protein. The more specific antibody was D14 that detected the protein ALFC at 39 KDa with less background than N14 (data not shown). The detection of ALFC by D14 is shown in Fig 15 by the green arrow.

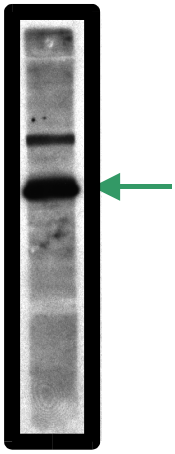


Fig 15: Western-blot analysis of cytosolic extracts from control mice brain with the D14 antibody. The arrow show the detection of aldolase C by the antibody (Mol. Wt. 39 KDa)

III.3.3-3- Co-Immunoprecipitation

An important goal of our study was to verify a change in the nitration of ALFC in AD brain. For this purpose, we have performed co-immunoprecipitations of control and AD samples with either the ALFC antibody D14 , NOY-1 antibody or Cytochrome-C antibody. The samples were loaded in 4-12 % NuPAGE gels, and Western blot analysis was performed, using the three antibodies. The results are represented in Fig 16.

On Figs 16-A and -C, lanes 1 and 2 were loaded with immunoprecipitates from control samples, and lanes 3 and 4 from the cytosololic fractions of APP/PS2 transgenic mice brain. On lanes 1 and 3, the immunoprecipitations were performed with the ALFC antibody and on lanes 2 and 4 with the NOY-1 antibody. The Western blot analysis was performed by using ALFC antibody in Fig 16-A and NOY-1 antibody in Fig16-C.

Our results show a positive signal about 39 KDa as indicated by the green arrow (Fig 16-A and -C) which corresponds to ALFC protein. This indicates, that a co-immunoprecipitation of ALFC protein with ALFC antibody and NOY-1 antibody is effective. There was no difference in the level of ALFC expression (Fig 16-A, line 1 and 3) and no difference in the level of protein nitration (Fig 16-C, line 2 and 4).

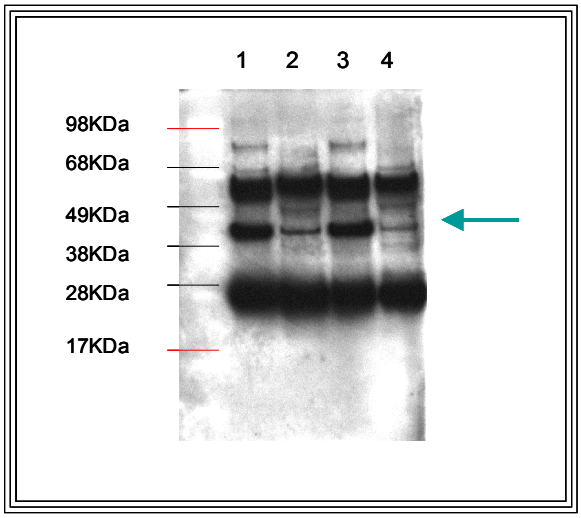
Fig 16-B, -D and -E represent gels loaded on lane 1 with control samples and on lane 2 with an AD sample, immunoprecipitated with Cytochrome C antibody for a control. Western blot analyses were performed using ALFC , NOY-1 and Cytochrome C antibodies, respectively. The black-arrows (Fig 16-B, -D and –E) indicate the bands representing the Cytochrome C signal. Only very weak staining of ALFC bound to cytochrome C antibody (Green-arrow) was found with the ALFC antibody (Fig. 16-B) and with NOY-1antibody (Fig 16-E).

In conclusion, even with the help of co-immunoprecipitation, no significant differences in ALFC expression levels were detected between control and AD samples. Moreover, no difference in the level of nitration was detected.

Currently, similar experiments are performed on proteins exhibiting a change in Y-nitration and a more pronounced change in expression between control and APP/PS2 mice samples. One of the proteins currently under investigation is TBA1 which is highly induced (about 5 fold) in AD brains compared to controls as shown in Table 6.

Fig 16: Coimmunoprecipitation from control and APP/PS2 mice with ALFC, NOY-1 and Cytochrome C antibodies
Fig 16-A represents a coimmunoprecipitation of control (lanes 1 and 2) and AD samples (lanes 3 and 4) with ALFC (lanes 1 and 3) and NOY-1 antibodies (lanes 2 and 4). Western blotting was performed with ALFC antibody
Fig 16-B represents a coimmunoprecipitation of control (lane 1) and AD (lane 2) samples with Cytochrome C antibody and analysis by Western blotting with ALFC antibody
Fig 16-C represents a coimmunoprecipitation of control (lanes 1 and 2)and AD (lanes 3 and 4) samples with ALFC (lanes 1 and 3) and NOY-1 antibodies (lanes 2 and 4). The western blotting was performed with NOY-1 antibody
Fig 16-D represents a coimmunoprecipitation of control (lane 1) and AD (lane 2) samples with Cytochrome C antibody and analysis by Western blotting with Cytochrome C antibody
Fig 16-E represents a coimmunoprecipitation of control (lane 1) and AD (lane 2) samples with Cytochrome C antibody and analysis by Western blotting with NOY-1 antibody
The green arrows on each Fig represent the detection of the aldolase C (39 Kda), the blacks arrows on Fig16-B,-D and –E represent the Cytochrom C signal

16-A



1- Immunoprecipitation of control sample with ALFC antibody

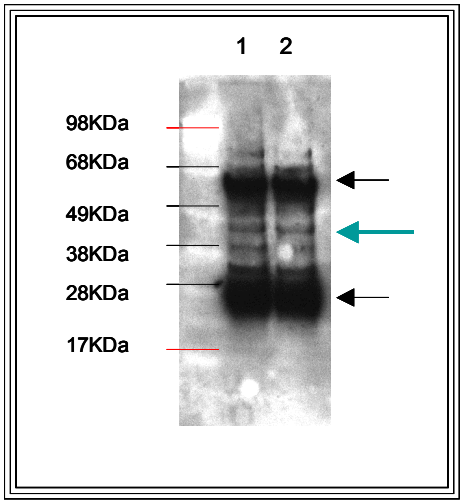
2- Immunoprecipitation of control sample with NOY antibody

3- Immunoprecipitation of AD sample with ALFC antibody

4- Immunoprecipitation of AD sample with NOY antibody

The Western blotting was performed with an antibody against ALFC

16-B

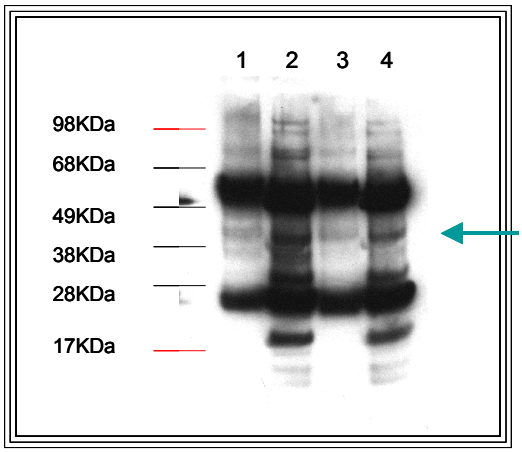


1- Immunoprecipitation of control sample with CytochromeC antibody

2- Immunoprecipitation of AD sample with CytochromeC antibody

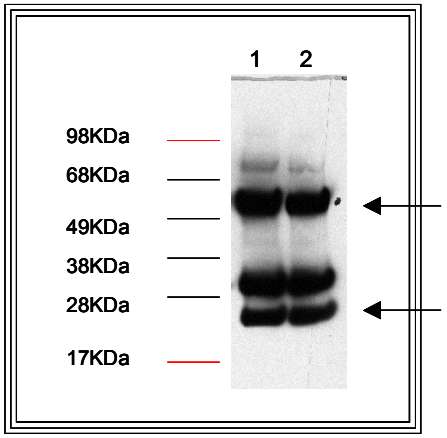
The western blotting was performed with an antibody against ALFC

16-C



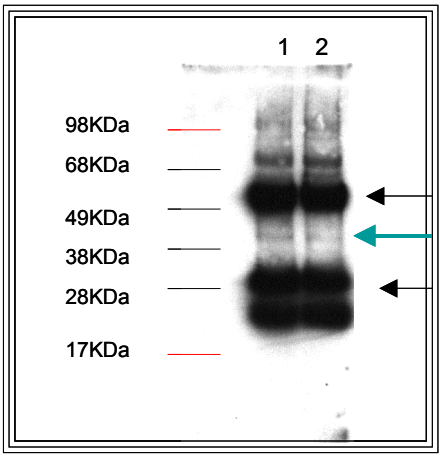
1- Immunoprecipitation of control sample with ALFC antibody
2- Immunoprecipitation of control sample with NOY antibody
3- Immunoprecipitation of AD sample with ALFC antibody
4- Immunoprecipitation of AD sample with NOY antibody
The western blotting was performed with an antibody against NOY

16-D



1- Immunoprecipitation of control sample with CytochromeC antibody
2- Immunoprecipitation of AD sample with Cytochrome C antibody
The western blotting was performed with a antibody against CytochromeC

16-E



1- Immunoprecipitation of control sample with Cytochrom C antibody

2- Immunoprecipitation of AD sample with Cytochrom C antibody

Western blotting was performed with an antibody against NOY

In order to proceed with this study, HPLC fractionation was performed to isolate ALFC from the cytosolic part of mice brain. This HPLC analysis was performed with a size exclusion column superose as described in Material and Methods. Eluated proteins were then collected according to their molecular weight into fractions. The flowthrough of proteins is represented by an elution profile (Fig 17). The bigger pic in the elution profile represents the fractions in which most of the proteins from the initial solution were eluated. These fractions were analyzed using an anti-ALFC antibody (Fig 18).

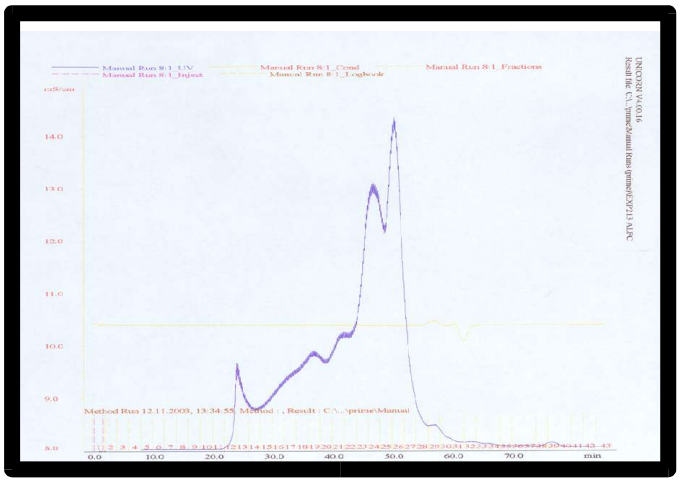


Fig 17: HPLC analysis of cytosolic extracts of brain from AD mice. This elution profile represents the flowthrough of the proteins according to their size. "y" axis represent mS/cm (conductivity of the column) and "x" axis represent the time expressed in minutes

Fractions 21 to 27 were loaded on 4-12% NuPAGE gels and were analysed by Western-blotting.

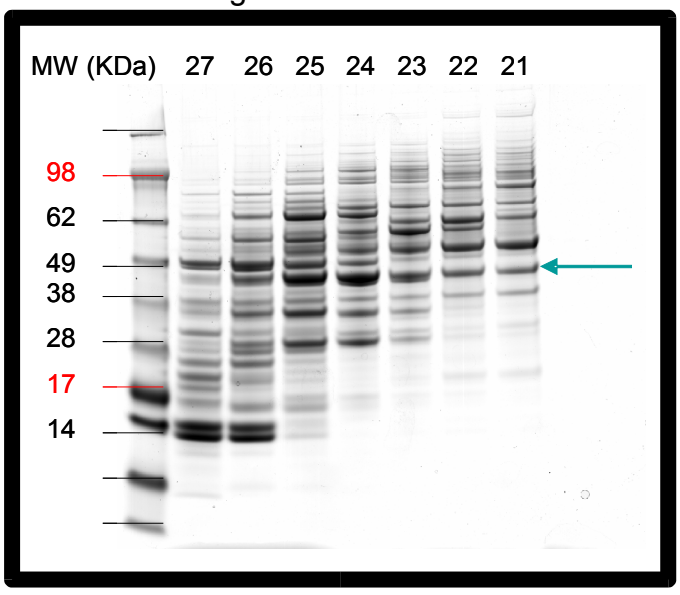


Fig 18: Western-blot analysis of the different fractions of interest obtained by HPLC

ALFC could be detected in fractions 23 to 26 as shown by the green arrow in Fig 18.

A confirmation whether higher levels of ALFC nitration are indeed found in affected AD samples is still in progress. For this purpose LC-MS/MS, performed in collaboration with Dr. Bernd Müller (F.Hoffmann-La Roche, Basel), will be used.
LC-MS/MS is a combination of liquid chromatography and electrospray tandem mass spectrometry that allows to eliminate the two-dimensional electrophoresis. After protein digestion of the fraction by trypsin, the resultant peptides are directly eluted from a Reverse Phase HPLC (RP HPLC) column into mass spectrometer.

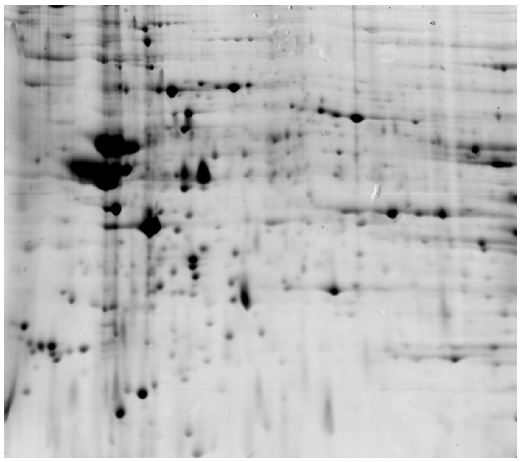
III.4- PROTEOMIC ANALYSIS OF SOLUBLE PROTEINS OF MICE BRAIN

These studies were performed using a control mouse model C57BL/6J (F-Hoffmann-La Roche, Basel) The major goal was to compare brains from control mice with transgenic animal models of AD and to provide evidence of synaptic membrane instability in the pathology of AD. First the technologies for the detection of synaptic proteins had to be improved and then in a following step, the MS sensitivity was improved by miniaturization of the entire system.

III.4.1- Detailed protein map of cytosolic brain

The first aim of this project was to generate a detailed map of proteins of mouse brain cytosol. For this purpose, fractions of samples obtained by sequential centrifugation were submitted to DEAE-ion exchange chromatography in order to increase the amount of proteins which have been separated by 2D-gel electrophoresis . The chromatographic fractionation method employed increases the probability to detect low abundant proteins by reducing the complexity of protein solution loaded. The ion-exchange chromatography used was adapted according to the specific requirements of the experiment (Krapfenbauer et al., 2001).

Brains of C57Bl/6J mice were homogenized as described in “Material and Methods”, the cytosolic fractions obtained by differential centrifugation were worked up differently:
The cytosolic part of the mouse brain was prepared and subjected to 10% SDS-PAGE using the established proteomic technology. A Coomassie blue stained gel corresponding to these samples is shown in Fig 19A. With this method, about 625 different proteins could be identified and the repartition of the identified proteins in 16 different functional groups is shown in Fig 19B.



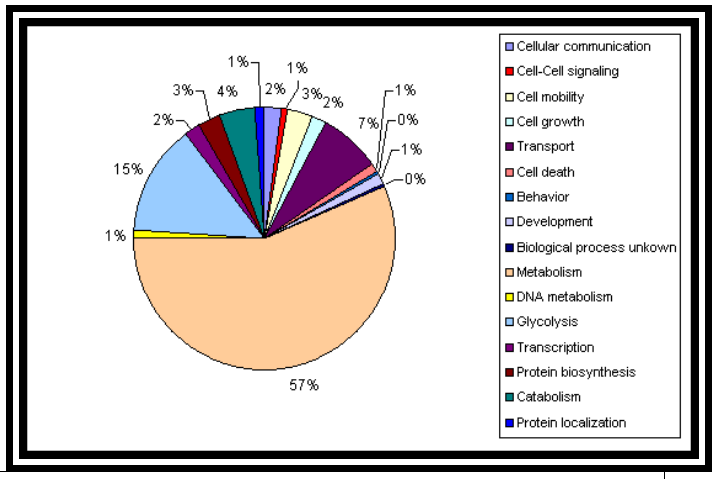


Fig 19A: Coomassie blue stained gel obtained from cytosolic extract derived from mouse brain
Fig 19B: Distribution of proteins according to their function. The grouping was performed following MS measurement

On the other hand, half of cytosolic brain samples were first fractionated by DEAE-ion exchange chromatography, performed by Remy Roland (F.Hoffmann-La Roche, Basel) as described in “Material and Methods”. Proteins were eluated from the column according to their conductivity. The entire spectrum obtained, representing a different pattern of proteins, was separated into several equal parts. Fifteen fractions displaying completely different expression patterns were selected and used for 2D gels as shown in Fig 20.

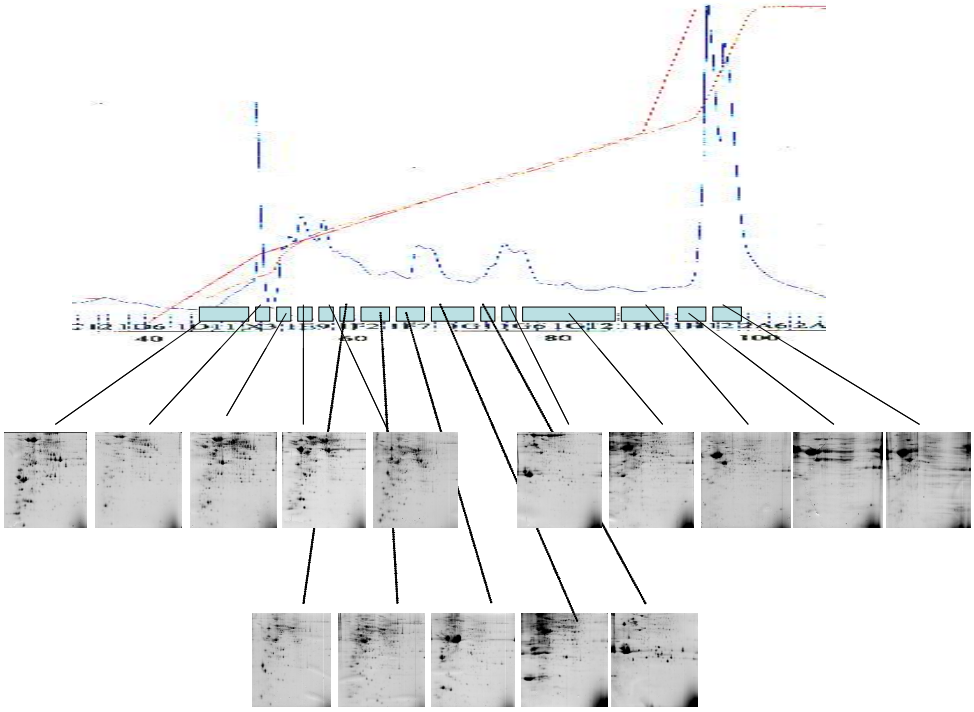


Fig 20: Representative gels obtained from 15 fractions collected by DEAE-Chromatography

The combined use of chromatography and proteomic technology allowed the excision of 12'600 spots in total. 9'425 spots were identified by MALDI-TOF. From these, 1'240 were identified as different proteins. This is double the amount of protein which could be identified by the established proteomic procedure.
Identified proteins were classified in 16 groups according to their function as shown in Fig 21. All the percentages indicated in Fig 19B and Fig 21 are according to the total number of identified protein in each case. However, we do not exclude the possibility that one protein could be classified in several different groups.

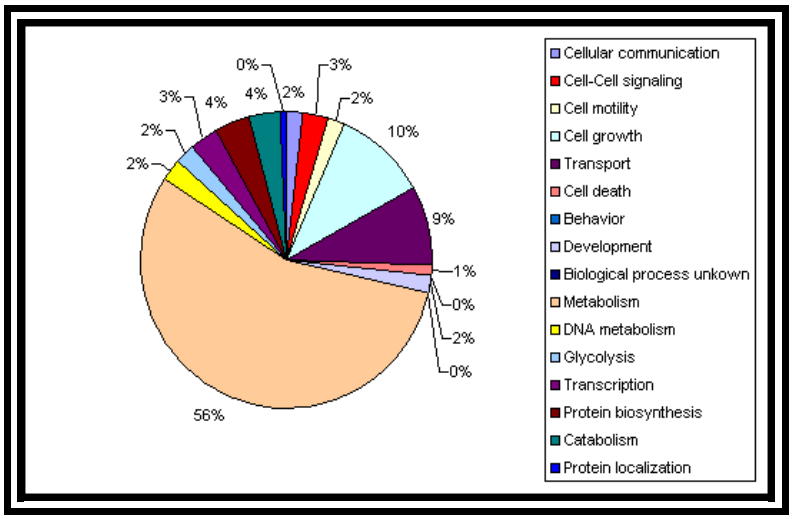


Fig 21: Protein classes detected after Chromatography

The combination of DEAE chromatography and MS analysis allowed the identification of various proteins involved in signal transduction such as MAP kinase kinase kinase (MEKK); extracellular signal related kinase (ERK1 and 2) , MAP kinase kinase (MAKK1, MAKK6), ras-related protein rab (RAB), gtp binding nuclear protein ran (RAN), RAS and rho-protein; Protein kinases such as c-AMP-dependent protein kinase (KAP1 and 2), Ser/Threonine kinase (AKT2), IKKB, Ser/Threonine kinase pak (PAK 1 and 3), src, CRK6; G protein-coupled receptor such as NMDA-receptor or GABA-A receptor subunit IV; molecules involved in synaptic transmission such as neuronal tropomodulin (TMO2) ; molecules involved in neurotransmitter metabolism and secretion such as catechol-O-methyltransferase (COMT) and synapsin1 (SYN1).

Using mass-spectrometry, it is the first time that downstream kinases such as ERK 1 and 2, which phosphorylate a variety of downstream proteins including other kinases as well as gene regulatory proteins in the nucleus, could be identified. This increased level of detection could not be reached without the previous chromatography purification step.

This part of our study revealed a drastic improvement of the proteomic technology. We could clearly achieve a significant increase in the sensitivity of MALDI-MS analysis by the pre-fractionation procedure of the samples using DEAE-ion exchange chromatography.

This improvement is a major progress in the investigation of very low abundant proteins. However, it should be mentioned that the chromatographic approaches may have certain limits: (i) an overlap of the protein spots can occur; (ii) since high- and low-abundant proteins are enriched, expression of high aboundant proteins can mask the detection of low expression proteins in a 2D gel; (iii) proteins eluted from columns usually require removal of salts used for their elution. This disalting process can affect the composition of the mixture.

III.5- ISOLATION OF SYNAPTOSOMES

Loss of synapses has been correlated with the degree of dementia (Callahan 2002). Thus, it is important to investigate the changes in specific protein expression patterns in synaptic terminals in the central nervous system of AD patients as compared to healthy controls.

Thus, we focused on the development of protocols for the isolation of synaptosomal membranes from mouse brains. The experiments were initially performed on control C57/B1/6 mice brain (provided by Dr. Laurence Ozmen, Basel). Whole brains of mice were homogenized and fractionated according to the method described in Material and Methods (summurized in Fig 22).

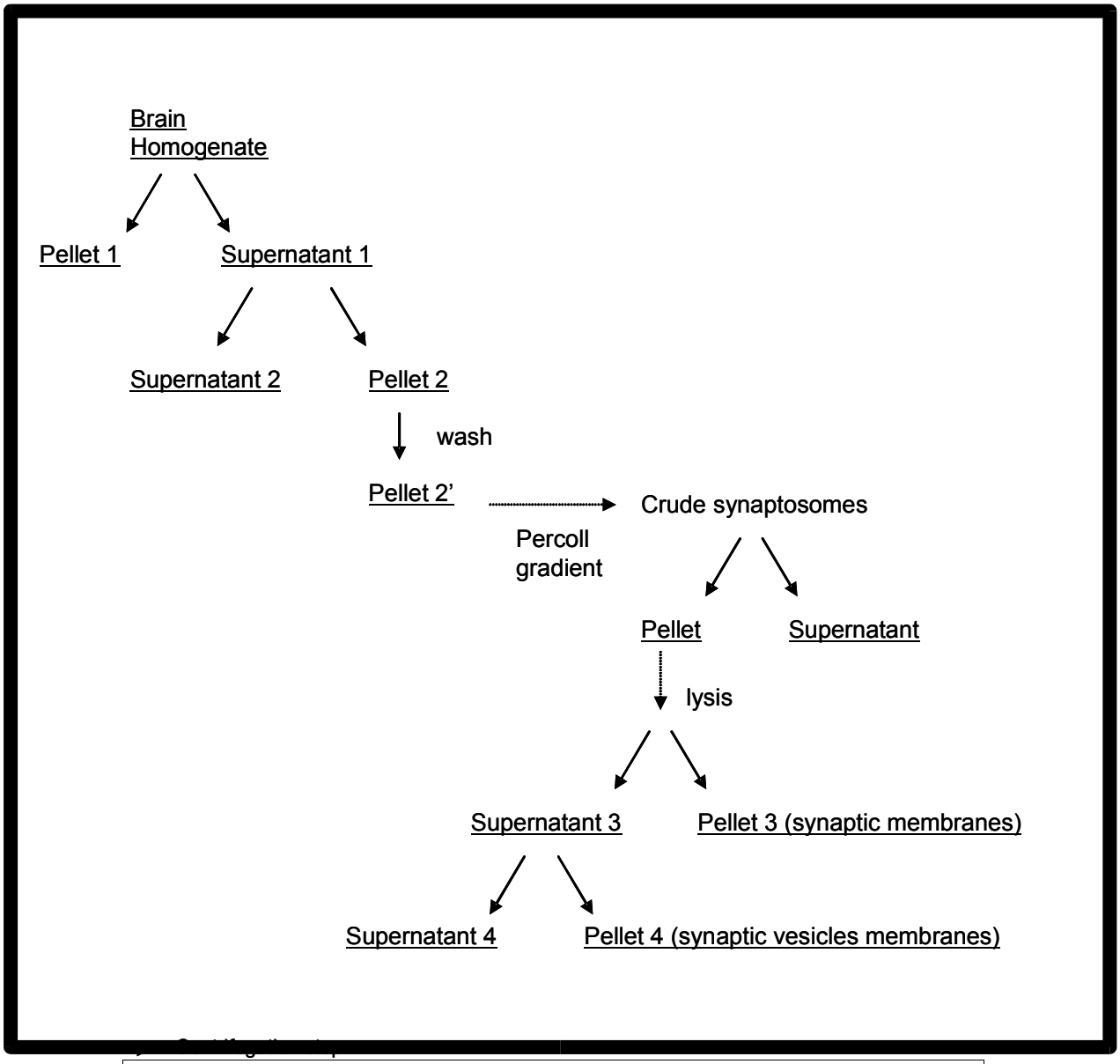


Fig 22: Representative protocol for the isolation of brain synaptic membranes

The purification steps of synaptosomes were monitored using Western-blots immunostained with an antibody against synaptophysin, a vesicle-membrane protein as shown in Fig 23.

The synaptic membranes, characterized by the positive signal of the synaptophysin antibody, are present in all fractions (lanes 1 to 10). After a first crude separation of the synaptosomes by the Percoll gradient, two additionnal centrifugation steps were performed to separate the membranes. These membranes are significantly enriched in the two last pellets P3 and P4, representing the synapses and synaptic vesicles and were not present in the cytosolic fractions S3 and S4 (lanes 14 and 16).

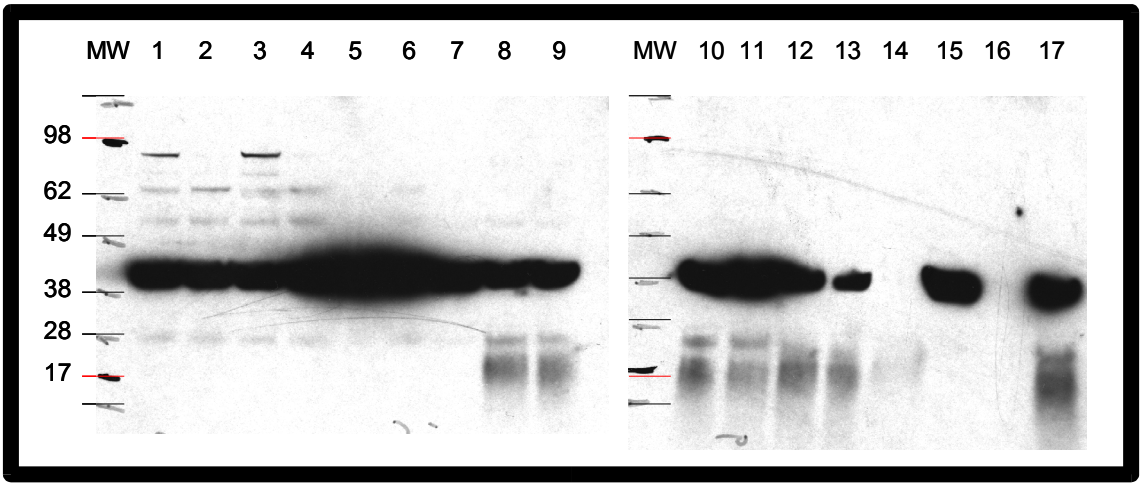


Fig 23: Western-blot analysis of the different fractions obtained. The antibody used is anti-synaptophysin. 1- Homogenate; 2- Supernatant 1 (S1); 3- Pellet 1 (P1); 4- S2; 5- P2; 6- P2 washed; 7- P2'; 8- upper part of the Percoll gradient; 9- second part of the Percoll gradient; 10- crude synaptosomes; 11 and 12- phase below; 13- bottom pellet of the Percoll gradient; 14- S3; 15- P3; 16- S4; 17- P4. (Molecular weight is expressed in KDa)

The isolation of synaptic membranes has been shown to be efficient. In a next step, MS analysis was performed in order to generate a map of proteins present in synaptic membrane fractions. Generally, the quantity of proteins isolated by this protocol was not sufficient to perform a normal 2D gel. Indeed, purified synaptosomal membranes represented low-protein samples and only 70 µg of proteins was obtained after preparation and fractionation of two total mouse brains. Therefore we established the mini-system technology from Invitrogen which is a miniaturization of all proteomic techniques and is of particular value for low-protein samples.

The improvement of this technology involved desalting of samples, use of 7 cm IPG-strips, isoelectric focusing in 3 hours and a second dimension on NuPAGE-gels

(8.5mm, 9mm, 1mm). These 2D gels after staining with Coomassie-blue were further picked and analysed by the usual apparatus used (data not shown).

This mini-system technology is now optimized to be used for MS measurement. Studies in our laboratory are in progress to proceed with this map of synaptic membranes proteins of control and transgenic mice. Isolation of synaptosomes from brains of AD mice bas been performed and the samples are available for further studies.

IV.1- Characterization of cytokine treated microglia cells

IV.1.1- Up- regulated proteins

IV.1.1-1-Role of transketolase

In order to search for possible biomarker molecules that could be of value for an early diagnosis of AD, we investigated changes in proteins expression patterns that were induced upon cytokine treatment of microglia cells.

The major finding was the upregulation of identified proteins known to be involved in AD or other neurodegenerative disorders. Following stimulation of microglia cell cultures with IL-1β and TNF-α, the most upregulated protein found (up to 14 fold) is Transketolase (TKT), a thiamine diphosphate dependent enzyme (Schenk et al., 1998). TKT was originally isolated from different sources such as mouse brain, human leukocytes or erythrocytes. This ubiquitous enzyme catalyses two steps of the non oxidative pentose pathway. Its biological role is to transfer the glucoaldehyde group from xylulose-5-phosphate to ribose-5-phosphate, or erythrose-4-phosphate to form sedoheptulose-7-phosphate and fructose-6-phosphate, respectively (Calingasan et al.,1995). In addition, the TKT gene has been shown to be a metallothionein (MT) regulator-gene in MT-null mice. MT proteins, regulated by glucocortocoids, are known to modulate DNA binding of transcriptional factors and are induced under various conditions such as cytokine-induced stress, and are thought to be involved in the tissue protection against generation of free radicals. Thus, in the MT-null mice, TKT was found to be up-regulated as a compensation of MT proteins lack for oxidative stress protection (Kimura et al., 2000).

The involvement of TKT in AD has been investigated mainly because neurodegenerative diseases are often associated with proteolysis that amplifies tissue damage (Paoletti et al., 1997 and 1998).

TKT has an heterogenous expression throughout the brain and its activity has been shown to be decreased in postmortem brains of AD patients (Calingasan et al.,1995). This decrease in enzyme activity is probably caused by reduced thiamine concentration. Indeed, the levels of thiamine were decreased in plasma but not in CSF of AD patients (Schenk et al., 1998; Molina et al., 2002).

Most of the earlier studies were done on postmortem brain materials or with fibroblast cultures. In contrast, our study was performed with pure human microglia

cell cultures. Thus, it can be hypothesized that TKT levels are increased at sites of cytokine-activated microglia, probably in order to reduce oxidation.

IV.1.1-2- Role of cytoskeletal proteins

Proteins involved in the cytoskeleton structure are of particular interest in AD because of the typical synaptic changes that occur in this disorder. The most important protein of the cytoskeleton is actin that has the ability to bind several proteins. The dynamics of this assembly is involved in a variety of cellular functions including cell motility, vesicle transport and membrane turnover.

Actin assembly is regulated by the complex actin depolymerizing factor (ADF) conjugated with cofilin which has been discovered in embryonic chicken brain (Dos Remedios et al., 2002). This complex ADF/cofilin regulates actin dynamics. In response to stress or injury, ADF/cofilin binds to actin and forms inclusions in the nucleus. By immunocytochemistry, transient and persistent inclusions containing ADF-cofilin have been co-localized with amyloid plaques in hippocampus and frontal cortex of human AD brains (Minamide et al., 2000 ; Bamburg et al., 2002). These rod like inclusions within neurites could be involved in synaptic loss by inhibiting the intracellular transport processes.

In our study, cofilin was found to be up-regulated and supports the idea that cytoskeleton changes could also occur in microglia cells. The loss of synapses can be due to changes in cytoskeleton and of neuron loss and subsequent loss of communication with surrounding cells.

Profilin 1, a further actin binding protein, is a regulator of the microfilament system and is involved in diverse signaling pathways (Wittenmayer et al., 2004). This low molecular weight protein is involved in actin polymerization at the cell surface and is necessary for synaptic morphology (Dos Remedios et al., 2002; Ackermann et al., 2003). Studies on dendritic spines have shown that enrichment of profilin and its targeting into the cytoplasm is activity-dependent (Ackermann et al. 2003). A high production of profilin has been shown to stabilize spine morphology. Profilin was first studied in order to understand how the activation of postsynaptic NMDA-receptors is transduced to long-term morphological changes. It is known that long-term potentiation (LTP) and long term depression (LTD) may induce profilin targeting as well as actin stabilization. In our study, profilin was found up-regulated 4.2 fold supporting the idea that profilin is overexpressed to stabilize cell morphology after injury until the consolidation of the cell is complete (Ackermann et al., 2003).

IV.1.1-3- Proteins Involved in signal transduction

Another protein of interest is the 14-3-3 protein which consists of 7 isoforms: β,γ, ε, η, σ, τ , and ξ. Two other isoforms (α and δ) represent phosphorylated forms of beta β and zeta ξ (Green., 2002). This low molecular weight dimeric protein has been localized to several brain regions and diverse cell types. For example: the zeta subunit is more present in cortical neurons and in cerebellar Purkinje cells. In contrast, the epsilon subunit is predominantly expressed in astrocytes (Richard et al., 2003).

14-3-3 proteins are known to play a role in the regulation of signal transduction, cell cycle pathways, apoptosis and may play a role as molecular chaperones (Richard et al 2003; Dev et al., 2003; Fig 24).

Increased levels of 14-3-3 proteins have been described in Creutzfeld Jakob Disease (CJD; Zerr et al., 2002; Huang et al., 2003) and at present 14-3-3 proteins are useful marker molecules that diagnostically distinguish between CJD and other neurodegenerative diseases, especially AD (Riemenschneider et al., 2003). However, increased levels of 14-3-3 in CSF of CJD patients are believed to be due to an acute neuronal damage and thus are most likely not specific for this disorder but the level of 14-3-3 in CSF is less detectable in AD brain (Green et al.,2002).

In our study, the isoforms epsilon ε, tau τ and zeta ξ were found 1.8, 1.8, and 1.6 fold induced, respectively. Up to date, presence of 14-3-3 ξ in AD amyloid plaque formation has not been confirmed, but it has been hypothesized that 14-3-3 ξ is an effector of tau protein phosphorylation and is involved in the accumulation of tau in AD (Hashigushi et al., 2000). No former results contradict the presence of 14-3-3 ξ in microglia cells and, perhaps, our data could confirm the idea of its chaperone function developed after a cytokine stress.

On the other hand, the Epsilon subunit is claimed to be with the γ-subunit specific for CJD. Epsilon and zeta subunits have a significantly different sequence, but

14-3-3 ε has been also found to be increased in CSF of CJD patients. The elevated levels of this isoform are clearly due to astrocyte activation. Our results suggest a mimetic effect due to microglia activation.

Two other isoforms, 14-3-3-β and 14-3-3-α, were found down-regulated (1.6 fold) in our study. However, these isoforms have not been yet described to have a particular impact in AD.

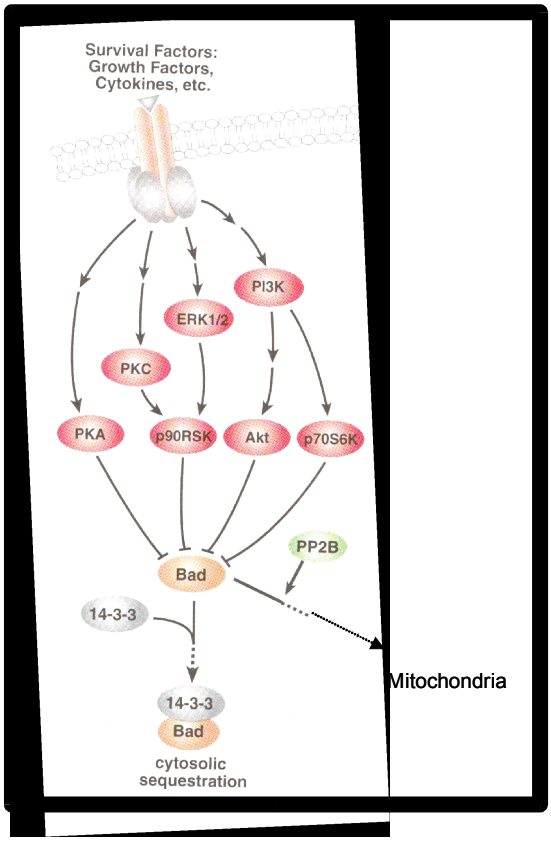


Fig 24: Representation of signalling cascades involving 14-3-3 protein

IV.1.1-4- Role of chaperones

The T-complex protein-1 (TCP-1) is classified in the chaperone group. This protein of 60 KDa represents an assembly of several polypeptides and is abundant in the cytosol of all eukaryotic cell. (Wagner et al., 2004). Studies in human erythrocytes have shown that a translocation of TCP-1 from the cytosol to the cytoskeleton occurs upon heat-stress. Moreover, TCP-1 is involved in microtubule growth, tubulin assembly and in actin polymerization. Tubulin and actin were described as the major substrates and the more specific substrate is Tubulin β-1 (TBB1; Grantham et al., 2002).

Our studies demonstrate that three subunits of TCP-1 are up-regulated in cytokine-stimulated microglia cells: TCP-1 Zeta (ξ), alpha (α) and gamma (γ). This is in contradiction with former studies from Schuller and co-workers (Schuller et al., 2001). The authors found the TCP-1 Zeta expression (TCP-1Z) is decreased in AD as well as the ratio of TCP/TBB1. These controversial results could be best explained by the different tissues analyzed in the various studies: the Schuller studies were done in six different brain regions (temporal, frontal, occipital, parietal cortex, cerebellum and thalamus) whereas our study was performed in cytokine-stimulated human microglia cells. The inflammatory response produced in our experiments leads to a proliferation of microglia cells that was not evaluated in the

total brain. TBB1, a cytoskeletal protein with chaperone functions is probably more expressed in stressed-microglia cells in order to support their proliferation, and this could be associated with a higher level of complex TCP-1/TBB1. In the total brain, up-regulation of TCP-1 in microglia is probably non-significant as compared to the massive neuronal cell loss leading a decrease of TBB1 and TCP-1.
Our studies show a down-regulation of other TCP-1 subunits: Epsilon (ε) and Eta (H). These results are in line with former studies by Yoo and co-workers on human parietal cortex (Yoo et al., 2001). These authors found decreased levels of the TCP-1 subunit ε in AD brain. TCP-1 ε is involved in the regulation of the cell cycle and decreased levels of TCP-1 ε may stop the cell cycle of brain cells in the G2/M phase. Thus apoptosis may be initiated by p53/Bax pathway.

IV.1.1-5- Role of proteins involved in cell communication and organization

Pre B-cell enhancing factor (PBEF) was first isolated from activated lymphocytes and has been proved to be involved in maturation of B-cells precursors (Ognjanovic et al., 2001). PBEF-gene sequence contains several regulatory elements, suggesting that this gene may be chemically and mechanistically responsive to inducers of transcription. Studies on human fetal membranes have shown that PBEF is expressed in normal cells and is highly increased after stimulation with cytokines such as IL-1β and TNF-α.

The results of our studies have also shown an up-regulation of PBEF (1.8 fold) after cytokines treatment of microglia cells.

IV.1.2- Down-regulated proteins

IV.1.2-1- Role of nucleoside diphosphate kinase A

One of the most down-regulated proteins found after cytokine treatment is nucleoside diphosphate kinase A (NDKA).
NDKA, a protein of 17.3 KDa, is highly conserved through evolution. The function of NDKA is related to cell proliferation and differentiation with an important role in the cell cycle (Kim et al., 2002). NDKA has been shown to be co-localized with microtubules present at synapses, and to be essential for normal presynaptic functions. The human A isoform is encoded by the nm23-H1 gene. In our study the

expression of NDKA was decreased by 6.6 fold. This finding is in line with former studies by Kim and co-workers (Kim et al., 2002). The authors reported that NDKA activity is decreased in frontal, occipital and parietal cortices of AD and Down syndrome patients. Modification of the kinase by oxidation has been reported as an important functional regulator of inactivation of the NDKA kinase activity (Kim et al., 2002). This supports the theory of an important involvement of oxidation in dementias like AD. Another participation of NDKA in AD and also in Down Syndrome is by its effects on neuronal differentiation such as neurite outgrowth. Decreased activity of NDKA may lead to altered expression of cytoskeletal proteins which participate in aberrant proliferation as described before (Kim et al., 2002).

IV.1.2-2- Role of cyclin-dependent kinases

CdK4 is a member of cyclin-dependent kinases (CdKs) that regulate cell cycle progression and may also be involved in neurodegenerative disorders via deregulation of programmed cell death (Lim el al., 2003).
CdK4 is a major catalytic partner protein of D type cyclin in the phase G1/S. It has multiple functions such as cell proliferation, survival, death and tumorigenesis. The complex CdK4/cyclin has been shown to be induced in neuronal apoptosis (Tsujiota et al., 1999).
Former studies revealed that CdK4 could only be detected in NFT bearing neurons in AD brains, but Tsujiota and co-workers (Tsujiota et al., 1999) found it also in diverse cells such as astrocytes, neurons, oligodendrocytes and microglia. Moreover, these studies reported higher levels of CdK4-mRNA expression in AD brains.
Currently, these is no explanation for the contradictory results, except that the role of CdK4 in apoptosis was confirmed and its expression appears to be more pronounced in neurons than in other cell types.

IV.1.2-3- Role of phosphatase 2A

The serine/threonine phosphatase 2A (PP2A) has been suggested to be involved in AD due to its regulatory role of tau and MAP kinase dephosphorylation. Former studies in cultured fibroblasts have shown that impairment of PP2A in AD prevents

the ability to reverse the hyperphosphorylation of tau protein and, in addition, to delay the ERK1/2 inactivation leading to tau phosphorylation (Zhao et al., 2003).
Our results correlate with previous findings, because in microglia cells, PP2A was found to be clearly down-regulated by pro-inflammatory cytokines.

IV.1.2-4- Role of proteins involved in metabolism

IF2A is a protein involved in translation of proteins as shown on Fig 25. In AD, phosphorylation of IF2A has been found to inhibit protein translation and subsequent induction of apoptosis. Studies made on human brain sections have shown a high presence of phosphorylated IF2A in AD (Chang et al., 2002). However, our results show a 2-fold down-regulation of IF2A, but its identification by mass spectrometry did not reveal whether this protein is phosphorylated or not. As described previously, phosphorylation of a protein changes its molecular weight: non-phosphorylated IF2A was down-regulated whereas phosphorylated IF2A was not detected. This discrepancy between published data and our findings may be due to the different cellular models used.

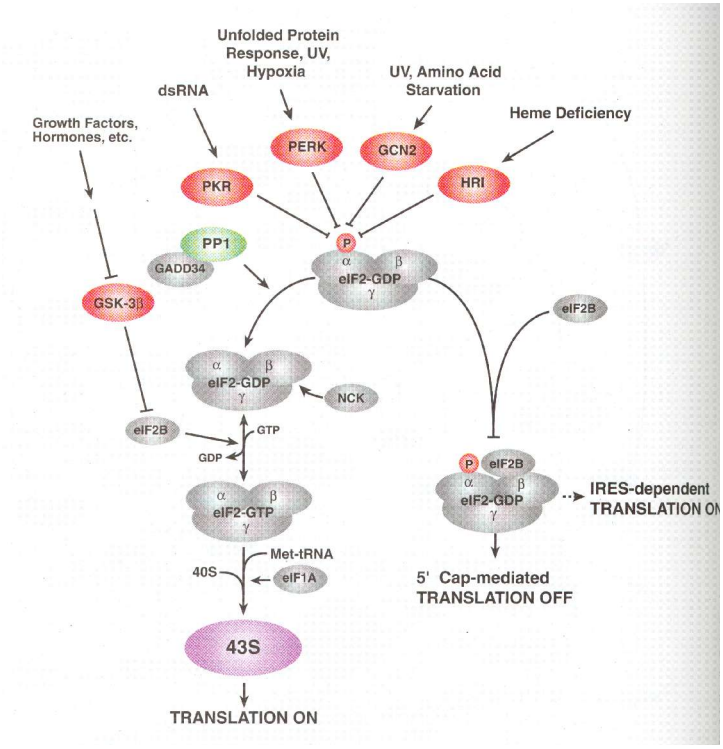


Fig 25: Cascade representation of the translation control of proteins. Involvement of the eukaryotic initiation factor 2 alpha (eIF2A)

An interesting finding of our study is that two ubiquitin-conjugating enzymes, E1 and E2, are found to be down-regulated in cytokine-stimulated microglia cells. Downregulation of ubiquitin proteasomes which are involved in the degradation of secretases such as BACE (Quing et al., 2004) may be of key importance for Aβ-mediated neurotoxocity and thus could play a major role in the pathogenesis of AD

(Song et al., 2003). Our results confirm former findings of down-regulation of ubiquitin-conjugating enzyme E1 and E2 by Layfield and collaborators (Layfield et al., 2003).

IV.1.2-5- Role of proteins with oxidoreductase activity

Peroxiredoxins (PRX) are antioxidant enzymes found in six different isoforms in mammalians: PRX (I-VI). For example, levels of peroxiredoxin 2 and 6 were increased in human postmortem AD brains, whereas no change was observed in Peroxiredoxin-1 (PRX1) expression (Krapfenbauer et al. 2003). Our results demonstrate that PRX-1, -2, and -3 expression was decreased in the cytokine-treated microglia cells. At present no explanation can be given to unsderstand these controversial data.

To sum up, our results obtained with cytokine-stimulated microglia cells confirm a significant contribution of inflammatory responses and a key role of activated microglia cells in the neurodegenerative process of AD. Moreover, most of the proteins found with altered expression levels have a functional role in neurotransmission, in axonal transport, in signal transduction, or are involved in metabolism pathways. Thus, it is an attractive hypothesis to state that among these proteins may be potential new drug targets.

After comparison with the transgenic mice model and human postmortem brain, the conclusion of this first project is that the human microglia cell line represents a valuable cellular model to mimic and to study inflammation processes occuring in AD brains. This study allowed the identification and the description of several proteins which are of particular interest in AD pathogenesis and which could be used as biological marker molecules and possibly help in the early diagnosis of the disease.

VI.2- CHARACTERIZATION OF NITRATED PROTEINS IN A TRANSGENIC MOUSE MODEL

The participation of nitrative oxidation has been clearly demonstrated both in neurodegenerative and inflammatory disorders (Sarchielli et al., 2003). Nitration has been reported to affect protein structure and function. Former studies have been performed by mass spectrometry in order to isolate nitrated proteins by isolation of the amino acid sequence (Nikov et al., 2003; Butterfield et al., 2003; Marcotte et al., 2003). A future aim would be to show the revelance of nitration for the development and progression of neurodegenerative diseases (Sarchielli et al., 2003).

In our study, previous software analyses have shown a much higher number of nitrated peptides for fructose 1-6 biphosphate aldolase than for a variety of other proteins. In mammals, three different fructose 1-6 biphosphate aldolase have been described: aldolase A, B, and C. The aldolase B, present in liver, is involved in the fructose utilization pathway. Aldolase A is more ubiquitous and is also present in brain. Aldolase C has been described as brain-specific.

Aldolase C has first been found to be expressed in brain of rodents and in the Purkinje cells of the rat cerebellum. In human CNS, mRNA of this protein was found to be expressed at low levels in brain cortex. Levels of mRNA gradually increase extending from frontal and parietal cortex to the occipital cortex. Aldolase C is also expressed in Purkinje cells of human cerebellum (Buono et al., 2001).

Aldolase is a glycolytic enzyme catalyzing the conversion of D-fructose 1,6 bisphosphate into glycerone phosphate and D glyceraldehydes 3 phosphate (Kanski et al., 2003). An additional role of aldolase C as an actin binding protein, including a role in actin polymerization has been described. Binding of aldolase C to microtubules has been reported to occur via binding to α-tubulin. This binding decreases the enzyme activity by about 90% (Schindler et al., 2001).

First of all, nitration of proteins has been correlated with ageing. In tissues of elderly people, nitrated proteins accumulate faster mostly because the degradation by proteasomes is decreased with age (Kanski et al., 2003).

There is also evidence that aldolase suffers nitration after nitric oxide-induced inflammation: studies on aldolase A have shown that tyrosine nitration of aldolase A may contribute to an impairment of the glycolytic activity (Koeck et al., 2004); more recent studies on Purkinje cells by Welsh and coworkers (Welsh et al., 2002) revealed that after a global brain ischemia, these neurons dy more easily because of

a deficiency in aldolase C. This finding strongly suggests a potent role of Aldolase C on neuronal survival in response to injury such as hypoxia (Welsh et al., 2002).

At present, the involvement of Adolase C in AD is only speculative. Little is known about the metabolic events correlated with cerebral aging or dementia disorders. During AD, there is a severe reduction of glucose comsumption. A key regulatory enzyme for glycolysis is 6-phosphofructokinase (PKF). Postmortem studies of Bigl and colleagues (Bigl et al., 1996) have shown that the PKF activity was significantly increased in frontal and temporal cortex of AD patients. Authors found that aldolase activity which converts the product of the PKF reaction, fructose 1, 6-biphosphate, was unchanged.

The theoretical part of our study showed an increased level of ALFC in AD by 1.26 fold that could not be shown by the Western blot analysis performed. This slight up-regulation of the expression of the protein is a border line effect.

We postulate an increased levels of nitrated ALFC in AD as compared to control by theoretical studies (1.4 fold), but the difference in level of nitration could not be demonstrated by the Co-immunoprecipitation performed. The observation of regulation of the nitration level in the neurodegenerative process should be amplified by increasing or decreasing the levels of protein expression. Our results indicate that further studies should be performed with proteins exhibiting a higher expression change in AD in comparison with in control samples. In line with this, are recent studies with the analysis of the ATPase synthase shown to be 10-fold induced in AD brains as compared to controls.

Nitration of the last residues of ALFC protein may be the most important functional change that can occur: studies on insects done by Gavilanes and coworkers (Gavilanes et al., 1982) have shown that C terminal residues are essential for maintenance of the catalytic conformation of Aldolase. However, this modification was detected only once in our studies, probably due to the fact that the different peptides do not have the same facility to be detected during the analysis by MALDI/TOF. This may depend on their affinity to the matrix used in the proteomic analyses. In conclusion, proteomic technology has proven as an extraordinary valuable methology which allows the detection and analysis of each protein sequence.

Moreover, during our study other nitrated proteins were detected. These proteins could be potential biomarkers molecules to identify AD in patients. Proteins such as tubulin beta 5, tubulin alpha1 chain or actin B may play a role in neurodegenerative diseases such as AD due to their functional role in microtubule stability. Proteins

such as the dihydropyrimidinase 2 (DPY2), involved in axonal growth and guidance in the brain, have been shown to play a role in AD. Indeed in AD brains, DPY2 has been found in a more oxidized form than in controls leading to neuritic degeneration probably due to a lack of neuroprotective mechanisms in the brains (Castegna et al., 2002). The protein, rab-gtp dissociation inhibitor alpha (GDIA) is another protein of interest. GDIA is one the proteins controlling the activity of small GTPases of the rab family involved in functional vesicle fusion and intracellular trafficking. Alterations of GDIA expression can lead to impairment of synaptic vesicle exocytosis, a synaptic dysfunction that occurs in the brain in AD (D'Adamo et al., 2002).

In summary, our studies confirm that post-translational changes such as nitration of proteins can be detected and analysed with proteomic technologies. Additionaly, application of this technology can help to monitor other diseases-related changes in functional proteins, for example phosphorylation and oxidation.

VI.3- Loss of synaptic connections in Alzheimer's disease

VI.3.1- Map of cytosolic proteins of mouse brain

These experiments were performed with the cytosolic part of brains of control mice. DEAE chromatography and MS were used in order to improve the sensitivity of detection of proteins involved in diverse pathways.

Recent studies have shown the importance of abnormal signaling pathways in the pathogenesis of AD. For example, it has been demonstrated that the p38/mitogen activated protein (MAP) kinase is one of the pathways overactivated in AD (Johnson et al., 2003). Currently four distinct groups of MAP kinases are known: (i) the extracellular signal regulated kinases (ERKs); (ii) the c-jun N-terminal protein kinases (JNKs); (iii) the big MAP kinases named BMK1 or ERK5 and (iv) the p38 MAP kinases. The p38 enzyme is activated by MAP kinase kinases (MKKs) such as the MKK6 that phosphorylate all isoforms of p38. These MKKs are themselves activated by MKK kinases such as MEKK4.

In AD, oligomeric Aβ is thought to activate signaling pathways and to increase p38 activity (Johnson et al., 2003). p38 may also play a role in Tau phosphorylation. In fact, recent findings indicate that tau protein is a downstream target of kinases activated by p38. Previous studies revealed that inhibition of p38, significantly decreases brain injury and neurological deficits. Ongoing research supports the view that p38 is a potential candidate for therapeutic treatment of AD.

Another pathway has been correlated with the Aβ neurotoxicity; the serin-threonine kinase (Akt)/ GSK-3β (Suhura et al.,2003; Zhang et al.,2001). Akt is crucial for survival of neuronal and endothelial cells, growth processes and glucose metabolism. NGF activates trkA and signaling proteins of the rap1/Erk1,2; p38MAPK and Pi3K pathways (Delcroix J.D. 2003). Neurotrophins such as NGF activate Akt kinase which in turn promotes neuronal survival and inhibits apoptosis. In AD, injury such as deposition of Aβ or lack of neurotrophic factors such as NGF lead to a disturbed Akt activation consistent with increased apoptosis of neurons.

In our study, the parallel use of chromatography and Mass spectrometry allowed the detection of diverse proteins from different levels of the signaling pathways such as Akt, MEKK and ERK. The detection of ERK 1 and ERK 2 are of considerable interest because they belong to downstream kinases of the signaling pathway (Fig 26), such sensitivity level has never been reached before.

Identification of kinases represented approximately 45% of all identified proteins in the brain extracts. The first identification by mass-spectrometry of specific kinases such as ERK 1 and 2 which are downstream kinases in the signal transduction pathway, is the result of a significant improvement of the sensitivity of the technologies used. Indeed, the real protein amount for a MALDI-TOF detection is in the attomolar range. However, there is loss due to peptide extraction from gel and dilution to have enough volume for an automation of the process, the visible spots on Coomassie-blue stained gel were represented about 10-25ng of protein. This extremly low amount for a downstream protein of the signaling pathways can be achieved only by chromatography enrichment.

The functional implication of some of these proteins is represented in the cascade shown below (Fig 26). This cascade is just a part of the growth factor signaling pathway, from the binding of the growth factor to a specific membrane receptor leading to modulation of gene expression.

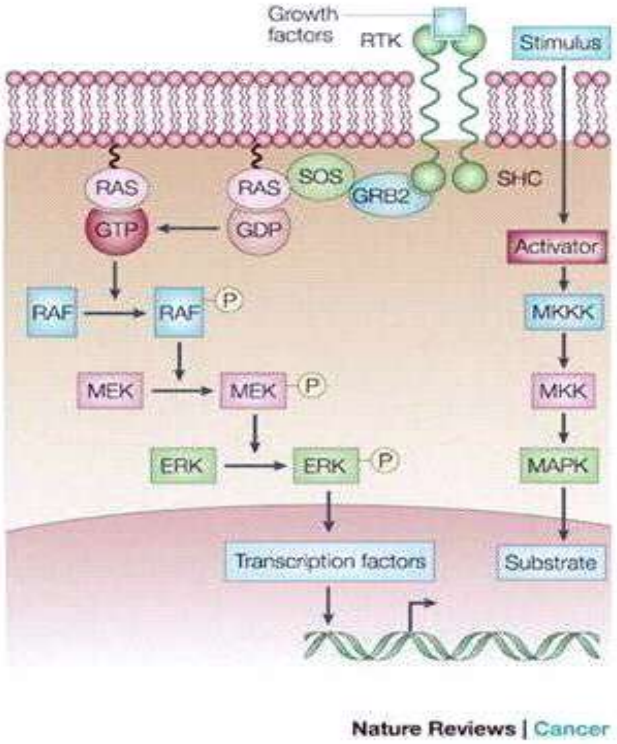


Fig 26: Representation of signaling pathways of growth factors

Our study proved that proteins of the signaling pathways possibly involved in AD are detectable by proteomic technologies and that the sensitivity of detection is

significantly improved by chromatography. This demonstrates that these technologies will help to a better understanding of the biochemical processes contributing to dysfunction and death of neurons in AD. Further investigation aims to identify the different modifications that can occur in several signaling pathways known to be involved in AD such as p38/MAP kinase pathway.

VI.4- ROLE OF SYNAPTOSOMES IN AD

Loss of neurons and synaptic connections has been shown in the pathology of AD and correlates with the degree of dementia. For example, the synaptic density has been shown to be decreased by 25 to 35% in AD cortex (Selkoe., 2002). Cholinergic and glutamatergic neurons appear to be particularly affected. However, little is known about the neurotoxic mechanisms involved in disease progression.

Evidence has been provided that impaired synaptic transmission in LTP occurs long time before Aβ deposition. Moreover, synaptic dysfunction and subsequent neuron loss starts to occur early during the presymptomatic phase of the human disease (Bigio et al., 2002). Further studies by Rowan and co-workers provided evidence that soluble Aβ peptides affect synaptic transmission, via interactions with the intracellular signaling pathways, long time before the occurence of fibrillar Aβ peptides (Rowan et al. 2003). Aβ inhibits cholinergic transmission by decreasing the synthesis of acetylcholine (ACh) or modulating of its release and degradation via activation of the ERK pathway. Based on the studies on the role of Aβ in diminishing LTP and increasing LTD, Rowan hypothesized a model for the pathogenetic role of Aβ, as shown in Fig 27.

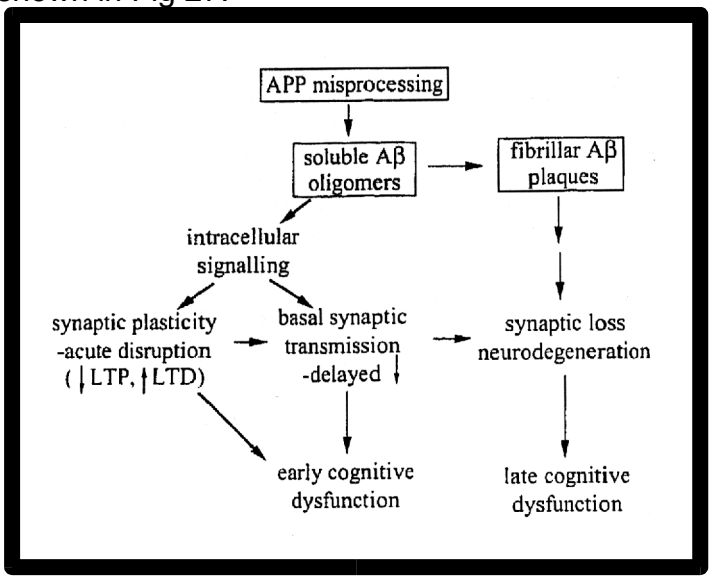


Fig 27: Representation of the role of Aβ peptide in early and late cognitive dysfunctions

Protein patterns of synaptic membranes and vesicle membranes required for a functional neurotransmission are important. Characterization of changes in receptor expression, e.g neurotrophin receptors or in cytoskeletal proteins that lead to a decreased neurotransmission should be performed in order to understand and to define the site of the impairment of synaptic function in AD.

Our study clearly demonstrates that isolation of synaptosomes of brain is possible by differential fractionation. Separation of cellular components was confirmed by an antibody against synaptophysin , a specific vesicle membrane-protein.

In addition, we optimized the sensitivity of the proteomic technology to study samples where proteins are expressed at low level and to have an efficient identification level by the development of minisystem technology. Another methodological challenge was to separate membrane proteins on 2D-gels. The development of the proteomic technology including treatment of protein solutions with specific reagents such as DTE or iodoacetamine, lead to a more and more efficient separation of these membrane proteins. The future goal will be to create a map of synaptic membrane proteins. This will be continued by a comparison of protein patterns in control and in AD synaptic membranes. In addition, further studies will aim to perform a kinetic analysis of these changes, in order to better understand when specific modifications in brain develop and when the synaptic failure in AD occurs.

V- OUTLOOK

Optimization of the techniques employed in these studies and the results on the *in vitro* experiments form the basis for continuation of this project. A triple transgenic mouse model APP/PS2/IL-6 is being developed. These mice should open up the possibility of studying the role of interleukin-6 (IL-6) in inflammatory processes as well as in amyloid β deposition in brains. Furthermore, these transgenic animals could help to distinguish between proteins involved in neurodegenerative processes as compared to normal ageing.

In the future, it will be interesting to study other nitrated proteins found by theoretical analysis. This project is still ongoing and an interesting protein candidate is ATPase synthase, which is highly overexpressed (10 fold) in diseased AD brains.
Other proteins such as TBB5, TBA1 or ACTB which are cytoskeleton binding proteins could play a role in neurodegeneration and their modifications during disease progression would be of interest. This technology could also be used for identification of other conformational protein changes due to inflammation, phosphorylation or oxidation.

These improvements in technology should help to detect diverse proteins at low expression levels in brain cells. The miniaturization of proteomic technology should improve the separation and detection of membrane proteins. The latter were previously difficult to identify in a 2D gel.
The major goal will be to map the proteins in synaptosomal membranes of control and AD transgenic mice and to extend these studies to neurons from diseased AD brains. The methodologies developed in the course of this study form the basis for focussed molecular and cellular analyses of abnormalities in neurodegenerative processes.

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VII- APPENDIX

VII.1-Curriculum vitae

Personal details

Name	Augé Christelle
Date of birth	October 10, 1977
Place of birth	Perpignan
Nationnality	French
Marital status	Unmarried
Languages	French, English, German notions.
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Education experience

2001-present	PhD thesis Institute of Physiology, University of Basel F. Hoffmann- La Roche AG, Basel.
2000-2001	Research project (Montpellier, France)
1999-2000	D.E.A in Neuroscience (Montpellier, France)
1998-1999	Master in Physiology (Montpellier, France)
1997-1998	Licence in Physiology (Montpellier, France)
1995-1997	DEUG in General Biology (Montpellier, France)

VII.2- Publications

Vestibular Schwann cells are a distinct subpopulation of peripheral glia with specific sensitivity to growth factors and extracellular matrix components.
Sylvain Bartolami, Christelle Augé, Cécile Travo, Stéphanie Ventéo, Marlies Knipper and Alain Sans. Journal of neurobiology, Volume 57, Issue 3 (p 270-290) 2003.

Proteomics analysis of protein after cytokines stimulation of human microglia cells.
Christelle Augé, Gaby Walker, Peter Berndt, Uwe Otten, Dieter Kunz.
Manuscript in preparation

Gene expression profiling in Hyper-IL-6- stimulated PC 12 cells: Increased expression of mRNAs coding for Reg/PAP proteins.
Dieter Kunz,*, Christelle Auge, Gaby Walker, Marc Bedoucha, Pia März, Beatrice Dimitridis-Schmutz and Uwe Otten. Manuscript in preparation

Role of Hsc/Hsp 70, CHIP, Bag-1 in degradation and prevention of heat induced aggregates, and identification of Hsc/Hsp 70 substrates.
Thuy-Anh tran thi, Thomas wiederkehr, Christelle Augé, Dieter Kunz., Peter Bukau.
Manuscript in preparation.

VII-3. Poster

Detailed protein map of cytosolic brain extract from C57Bl/6 Mice.
Gaby Walker, Christelle Augé, Rémy Roland, Stefan Evers, Daniel Roeder and Peter Berndt.
Presented into the Swiss proteomics society congress. Basel 2 -4, December 2003.
Underline = co-author.

Thanks to people involved in my formation:

PD. Dr. D. Kunz
Prof. Dr. U. Otten