UNIVERSITY OF VERONA

DEPARTMENT OF

Neurological, Neuropsychological, Morphological and Movement Sciences

DOCTORAL SCHOOL OF
Science, Engineering and Medicine

DOCTORATE
Neuroscience

CICLO XXIV

DOCTORAL THESIS'S TITLE

# "Role of Chitin in Alzheimer's disease: a new cytotoxic pathway"

S.S.D. MED/26

Coordinator: Prof. Michele Tinazzi

Tutor: Prof. Bruno Bonetti

Student: Dott.ssa Ermanna Turano

# ABSTRACT

The pathogenesis of Alzheimer's disease (AD) is generally attributed to the abnormal production and accumulation of β-amyloid protein, in association with neurofibrillary tangle (NTF) formation. The production and subsequently accumulation of β-amyloid protein in AD brains finally results in direct neuronal toxicity and in microglial activation which, through the production of inflammatory mediators, contributes to neuronal damage.

In recent years the scientific community has raised doubts regarding the exclusive pathological role of amyloid. Familiar AD, where amyloid deposition is supposed to play a prevalent pathogenetic role, represents a condition confirming this hypothesis, but the vast majority of AD cases are sporadic and in this condition the scenario is complicated by the possible role of additional components/pathways involved. In fact, a wide range of molecules are present in AD plaques, whose significance has not been clearly characterized. Among these, previous studies have identified chitin, an insoluble polymer of N-acetyl-glucosamine, in close association with β-amyloid in autoptic sporadic AD brains. Chitin was detected by Calcofluor staining both in amyloid plaques and within the cytoplasm of surrounding microglia.

The aim of this study was to investigate whether chitin has a pathogenetic role in AD by assessing its biological effects on two important players: neurons and microglia.

First of all, we have found chitin deposits only in sporadic AD but not in familiar AD and Down syndrome, emphasizing the complexity of amyloid-related pathology. Then we performed in vitro experiments, in which the exposure of microglial cultures to chitin showed that the cells were able to phagocyte small chitin particles, and the process was significantly inhibited by β-amyloid. Similarly to what described with β-amyloid, phagocytosis of chitin activated microglial cells.

In addition, experiments with neuronal cultures clearly showed a significant cytotoxic effect induced by chitin on neurons to levels comparable to β-amyloid.

A central point of this research concerned the production of chitin by mammalian cells, which lack chitin synthase. In sporadic AD glucose metabolism is frequently impaired with activation of the exosamine pathway with consequent production of N-acetyl-glucosamine. Previous studies suggested that, under such condition, the absence of a chitin synthesizing enzyme may be overcome by hyaluronan synthase-1 (HAS-1), that has been shown to convert UDP-N-acetyl-glucosamine to chito-oligosaccharides in vitro.

We demonstrated that in the presence of UDP-N-acetyl-glucosamine, both microglia and neurons are able to produce chitin-like deposits that HPLC-MS analysis confirmed to be "new-formed" chitin-like compounds. Such treatment leads to activation of microglia as well as significant neuronal cytotoxicity, mimicking the effect of exogenous chitin.

Our results indicate that in particular conditions of altered glucose metabolism both microglia and neurons produce chitin-like polymers, which may trigger a neurotoxic effect either by direct neuronal toxicity and by microglia activation. Moreover, preliminary experiments suggest that synaptic transmission is affected in murine hippocampal slice cultures treated with UDP-N-acetyl-glucosamine.

Taken together, these results suggest a cytotoxic role of chitin-like molecules in AD and offer new insights in the understanding the complex pathogenesis of AD.

# INDEX

# INTRODUCTION

## 1. ALZHEIMER DISEASE

### 1.1 Historical and social background

Alzheimer disease (AD) is the most common and complex neurodegenerative disease that is estimated to affect approximately 15 million of people worldwide (Alzheimer's Association 2010). It is the most common form of dementia that accounts for 50 to 80 percent of total dementia cases. It is characterized by deficit in memory, language, executive functions and other intellectual abilities serious enough to interfere with daily life. The greatest known risk factor is increasing age, and the majority of people with Alzheimer's are 65 and older. But Alzheimer's is not just a disease of old age; up to 5 percent of people with the disease have early onset Alzheimer's (also known as younger-onset), which often appears when someone is in their 40s or 50s.

Alzheimer's disease was first described in 1906 by the German psychiatrist and neuropathologist Alois Alzheimer and was named after him by Emil Kraepelin (Kraepelin 1910; Berchtold and Cotman 1998). Alois Alzheimer identified the first case of what we know as Alzheimer's disease in a fifty-year-old woman that he called *Auguste D* with strange behavioral symptoms, including a loss of short-term memory. The autopsy of the patient showed the presence of neurofibrillary tangles (NFTs) and amyloid plaques that are today the key histopathological hallmarks of AD.

Although the causes of AD are not yet known, most experts agree that, like other common chronic conditions, it probably develops as a result of multiple factors rather than a single cause.

Unfortunately AD is reaching epidemic proportions and a cure is not yet available with a social and economic impact on all society.

### 1.2 Epidemiology and genetic

Cohort longitudinal studies (studies where a disease-free population is followed over the years) provide rates between 10 and 15 per thousand person–years for all dementias and 5–8 for AD (Di Carlo et al. 2002), which means that half of new dementia cases each year are AD. Advancing age is a primary risk factor for the disease: every five years after the age of 65, the risk of acquiring the disease approximately doubles. There are also sex differences in the incidence rates, women having a higher risk of developing AD particularly in the population older than 85 (Andersen et al. 1999).

A small percentage of AD cases, around 5 percent, is caused by genetic mutations found in a small number of families worldwide. In these inherited forms of Alzheimer's, the disease usually develops before age 65, sometimes in individuals as young as 30.

A genetic factor in late-onset AD (older than 65) is apolipoprotein E- ε4 (APOE-ε4). APOE-ε4 is one of three common forms of the APOE gene, with an important role in the catabolism of triglyceride-rich lipoprotein constituent. APOE-ε4 acts as a pathological chaperone that promotes deposition of Aβ (Reiman et al. 2009) and the phosphorylation of tau (Holtzman et al. 2000), increasing the risk to develop AD.

The gene for amyloid precursor protein (APP) is on chromosome 21. Down syndrome, characterized by duplication of chromosome 21 provides a clear mechanism for Aβ deposition: persons affected with this condition develop one and an half times as much APP as normal people, resulting in susceptibility to Alzheimer's dementia at early age (Busciglio et al. 2002).

There are also familial forms of AD supported by genetic alterations leading to increased production of Aβ42: the presenilin is a component of the γ-secretase complex involved in the cleavage of APP. The main consequence of the mutation of presenilins leads to increase levels of Aβ42 (Rothman and Olney1995).

Other proteins recently considered genetic risk factors identified are: Apolipoprotein J (Lambert et al. 2009), translocase of outer mitochondrial membrane 40 homolog (yeast) TOMM40 (a transport of proteins across the mitochondrial membrane) and a neuronal sortilin-related receptor (SORL1), involved in an APP-recycling pathway whose levels are greatly reduced in patients with AD and mild cognitive impairment (Sager et al. 2007; Rogaeva et al. 2007).

### 1.3 Clinical diagnosis

AD is a progressive dementia with multiple memory deficits as the major clinical manifestation. Although AD develops differently for every individual, there are many common symptoms. Cortical signs and symptoms such as apraxia, aphasia, agnosia and visuo-spatial dysfunction may become apparent over the course of the disease. Disturbance of language and behavioural problems emerge throughout the various stages of the disease together with mood disturbances such as depression, anxiety, apathy, hallucinations and psychosis. In advanced stages of AD, patients might exhibit extrapyramidal signs such as tremor and gait

disturbance, urinary incontinence, and myoclonus. Gradually, body functions are lost, ultimately leading to death (Tabert et al. 2005).

In 1984, the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Diseases Association (NINCDS-ADRDA) established diagnostic criteria designed for research purposes and clinical definition (McKhann et al. 1984) extensively updated in 2007 and still valid (Dubois et al. 2007). These criteria require that the presence of cognitive impairment and a suspected dementia, to be confirmed by neuropsychological testing such as the "mini-mental state examination" (MMSE), even if the confirmation of diagnosis is possible only with autoptic histopathologic evidence.

The use of imaging studies and laboratory analysis can be used to predict AD: functional imaging studies used in clinical research include positron emission tomography (PET) and single-photon emission computed tomography scans (SPECT), which demonstrate hypometabolism and hypoperfusion, respectively, in the temporal-parietal regions bilaterally. In addition, routine chemistry panels, blood counts, metabolic panels, spinal fluid analyses, and inflammatory markers are used as instruments of early diagnosis.

### 1.4 Pharmacological treatments

The cause and progression of Alzheimer are not well understood, probably because some pathogenetic aspects and the related biochemical and molecular mechanisms are still not clear. Current treatments are palliative and no treatments that stop or reverse the progression of the disease are available. Up to 2008, more than 500 clinical trials have been conducted, but without relevant conclusion (Abbott 2008).

Currently the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved five drugs to treat the cognitive manifestations of AD: four are acetylcholinesterase inhibitors (*Tacrine, Rivastigmine, Galantamin* and *Donepezil*) and the other (*Memantine*) is an NMDA receptor antagonist. Acetylcholinesterase inhibitors may improve some cognitive aspects and slow cognitive decline in patients with AD. Reduction in the activity of the cholinergic neurons is a well-known feature of AD (Geula and Mesulam 1995). Acetylcholinesterase inhibitors are employed to reduce the rate at which acetylcholine (ACh) is broken down, thereby increasing the concentration of ACh in the brain and contrasting its reduction caused by cholinergic neurons loss (Stahl 2000). Clinical studies show conflicting results: some studies supported their efficacy (Birks et al. 2009), but their use is ineffective in delaying the onset of AD in patients affected with mild cognitive impairment (CMI) (Raschetti et al. 2007).

**1.5 Neuropathology**

From the macroscopic point of view, AD brains show a widening of cerebral sulci due to diffuse atrophy with a compensatory ventricular dilatation. The cortical atrophy is more important in hippocampus and medial temporal regions, but may involve also frontal, parietal and occipital regions (Figure 1)

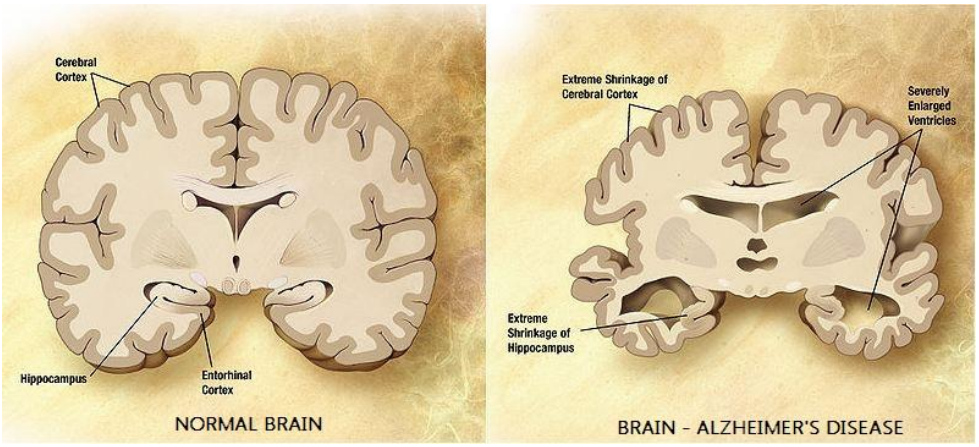


**Figure 1.** Macroscopic difference between normal and AD brains, with the latter showing decreased brain weight and volume due to marked expansion of cortical atrophy as grooves and flattening of the convolutions.

Senile Plaques (SPs) and neurofibrillary tangles (NTFs) are the key histopathological hallmarks of AD described by Alois Alzheimer over a century ago (Figure 2). SPs are spherical lesions in the cerebal cortex, measuring up to 100 μm. In their fully developed stage, the neuritic plaque have a central core of extracellular amyloid protein surrounded by a halo of dystrophic neuronal processes with neurofibrillary degeneration. Reactive astrocytes and microglia may appear around the periphery of these plaques. The presence of plaques is detected in the hippocampus but also in the neocortex. The core of the plaques consists primarily of a small peptide known as β-amyloid (Aβ) which is derived from the larger amyloid precursor protein (APP) as well as many different Aβ-associated factors, such as heparan sulfate proteoglycans (O' Callaghan et al. 2008), apolipoproteins, and complement factors. These factors may all influence Aβ deposition, aggregation and clearance and therefore seem important in the development of human Aβ deposits (Timmer et al. 2010).

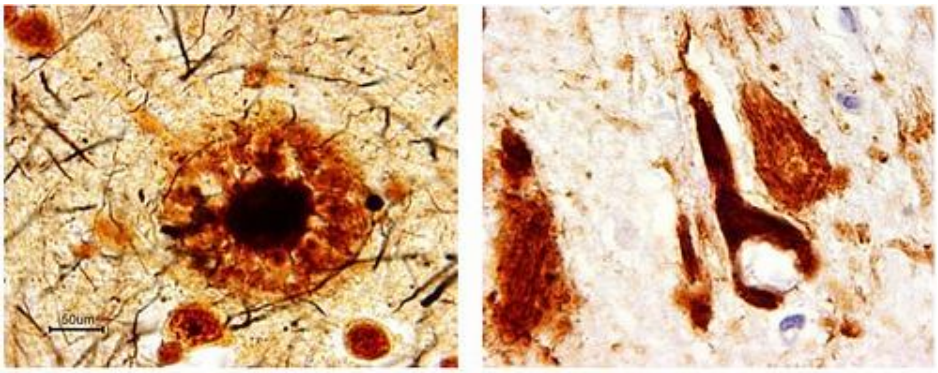


**Figure 2.** Histological hallmarks of AD brain: amyloid senile plaque (left) and neurofibrillary tangles (right).

The amyloid core has a fibrillary structure and is birifringent at Congo Red staining. Each SPs represents a focus of damage of the neuropil that includes axon terminals and dendrites of several neurons and probably thousands of synapses. Plaques that have the amyloid proteins but lack the neuritic processes are known as diffuse plaques. Diffuse plaques do not disrupt the neuropil, and are seen sometimes in large numbers in old, non demented persons and are not associated with dementia. Many AD patients have also a cerebral amyloid angiopathy and granolovacuolar degeneration (Mirra et al. 1993; Perl 2000).

NFTs are deposits of tau protein filaments in the neuronal body. Similar deposits are present in the dystrophic processes that surround the amyloid core of SPs and in dendrites (neuropil threads-NTs). In severe AD, the hippocampus often contains extracellular NFTs embedded in the neuropil. The mechanism of accumulation of tau in NFTs is unclear as it is unclear the relationship between tau and Aβ. Cognitive decline correlates more strongly with NFTs load rather than with the number of SPs. NFTs in the hippocampus and entorhinal cortex correlate with memory impairment, while neocortical NFTs correlate with cognitive decline. NFTs are found in many neurodegenerative diseases besides AD, including the fronto-temporal dementia, dementia pugilistica, myotonic

dystrophy, and prion diseases. These evidences indicate that NFTs can cause neurodegeneration indepenently of Aβ deposition. On the other hand, neuritic plaques are only found in AD. Most cases of AD show a combination of SPs and NFTs, but some cases have a predominance of one or the other.

## 1.6. Pathogenesis

### 1.6.1. Amyloid cascade hypotheses

The most popular pathogenic hypothesis in AD is the "amyloid cascade hypothesis" proposed by John Hardy and David Allsop in 1991. This hypothesis suggests that the mismetabolism of amyloid precursor protein (APP) is the initiating event in AD pathogenesis, subsequently leading to the aggregation of Aβ, specifically Aβ42. The formation of neuritic plaques would is a further pathological step with the formation of NFTs and disruption of synaptic connections, which lead to a reduction in neurotransmitters, death of neurons and dementia (Hardy and Allsop 1991 ). The APP gene, located on chromosome 21, encoding a protein of neuronal membrane that can be expressed in a variety of tissues. Aβ peptides are natural products of metabolism consisting of 36-43 aminoacids and derive from the proteolisis of the transmembrane protein APP by sequential enzymatic actions of enzymes: beta-site amyloid precursor protein cleaving enzyme-1 (BACE-1), beta-secretase and gamma secretase, a protein complex with presenilin-1 at site catalytic core (Querfurth and La Ferla 2010). The result of the action of these enzymes is the formation of two fragments of 40 and 42 amino acids that are secreted into the extracellular space (Figure 3).

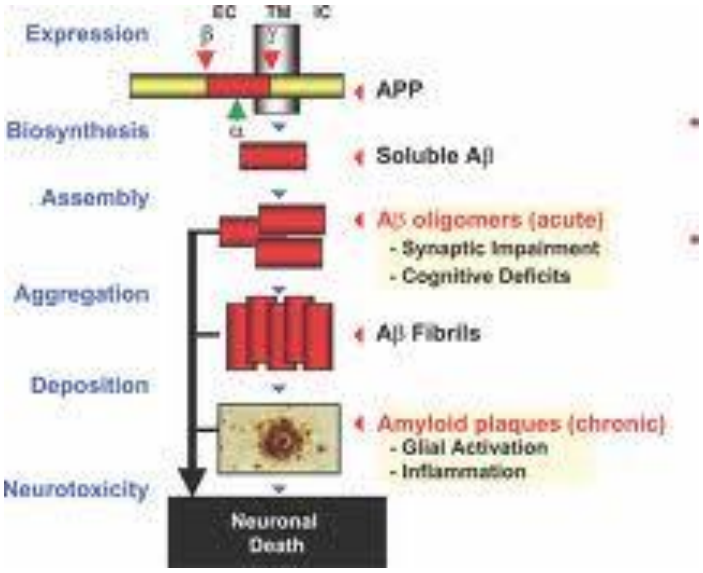


**Figure 3.** Amyloid cascade hypothesis: The cleavage of APP from β and γ secretases lead to the formation of Aβ peptides in oligomeric and fibrillar forms that can aggregate in plaques and lead to neurotoxicity.

These peptides are found in low concentrations in plasma and cerebrospinal fluid (CSF) under physiological conditions. An imbalanced clearance of Aβ from aberrant cleavage of APP and other mechanisms results in its accumulation up to reach a critical concentration of polymerization (Selkoe 1999). Insoluble fragments such as Aβ42 precipitate as amyloid fibrils to form the core of AD-plaques.

Aβ aggregates spontaneously in various forms: soluble oligomers (2 to 6 peptides), which assemble into intermediate forms of amyloid (Kayed et al. 2003; Klein et al. 2001) or insoluble fibrils with characteristic β-sheets folding, which are found in advanced AD; this latter form binds Congo red and appears birefringent when viewed at polarized light microscopy. The severity of cognitive impairment correlates with the levels of oligomers rather than the total level of Aβ in the brain (Lue et al. 1999). The accumulation of amyloid is considered a feature necessary but not sufficient for disease development, because the quantity does

not correlate with the degree of dementia and is present (especially in the form of diffuse plaques) in an high percentage of non-demented aged subjects.

Consistent with this hypothesis, Aβ is neurotoxic for neuronal cells (Goodman and Mattson 1994) through a variety of mechanisms: disruption of mitochondrial function via binding of the Aβ-binding alcohol dehydrogenase protein (ABAD) (Lustbader et al. 2004), induction of apoptotic genes through inhibition of Wnt (Caricasole et al. 2003 ) and insulin signalling (Xie et al. 2002 ), formation of ion channels (Kagan et al. 2002), triggering loss of calcium homeostasis (Goodman and Mattson 1994), stimulation of the JNK/SAPK pathway (Kim et al. 2004 ) or activation of microglia cells leading to the expression of pro-inflammatory genes and increase of reactive oxygen species with eventual neuronal toxicity and death (Bamberger and Landreth 2001).

### 1.6.2 Role of tau protein

The second histopatological hallmark of AD are neurofibrillary lesions mainly composed of highly phosphorylated, aggregated assemblies of the protein tau. Tau belongs to the family of microtubule-associated proteins (MAPs) and it is present principally in the axons in soluble form (Ittner et al. 2010), where stabilizes microtubules. Tau contains an unusually high number of putative phosphorilation sites. Under pathological conditions some sites are phosphorilated to an higher degree than in the healthy brains with a consequent dissociation from microtubules, causing axonal collapse.

High levels of total and phosphorilated tau in the CSF of patients correlate with a reduction of cognitive performances in cognitive tests. The analysis of total and phosphorilated tau levels in the CSF is today an important diagnostic marker to predict the onset of AD in patients with MCI (Mattson et al 2009). The

relationship between tau and Aβ in AD is not yet fully understood and is a matter of discussion. Experimental evidence indicates that Aβ accumulation precedes and drives tau aggregation (Oddo et al 2003; Lewis et al. 2001). Moreover, Aβ-induced degeneration of cultured neurons and cognitive deficits in mice with an Alzheimer's disease–like illness require the presence of endogenous tau (Roberson et al. 2007; Rapoport et al 2002).

### 1.6.3 Neuronal loss and synaptic dysfunction in AD

The AD brain is characterized by areas of neuronal and synaptic loss. The death of cholinergic neurons in the nucleus basalis of Meynert is correlated with a deficit in acetylcholine (Ach), a major transmitter believed to be involved in memory. In addition, loss of serotoninergic neurons in the median raphe and adrenergic neurons in the locus coeruleus lead to deficits in serotonin and norepinephrine, respectively. Several studies have examined the relationship between cognitive impairment and plaque and tangle burden; although in general the number of NFTs correlates better with severity of dementia than amyloid plaques, the most robust correlation in the staging of dementia and early AD is the magnitude of synaptic loss (Davies et al. 1987 Scheff et al. 2007). Indeed, synaptic degeneration appears to be an early event in pathogenesis being evident in patients with early AD and MCI (Masliah et al. 2001; Scheff et al 2006, 2007). In recent years, biochemical analysis of AD brain have revealed a correlation between soluble Aβ levels and the extent of synaptic loss and severity of cognitive impairment (Lemere et al 2002; Wang et al. 1999).

The oligomeric forms of Aβ are more potent in causing synaptic dysfunction (Klein and Krafft 2001; Cleary et al. 2005; Lesné et al. 2006; Shankar et al. 2007, 2008; Cheng et al. 2007; Selkoe 2008; Walsh and Selkoe 2007;

Tomiyama et al. 2010). Aβ oligomers appear to reduce the strength and plasticity of glutamatergic synaptic transmission (Hsia et al. 1999; Chapman et al. 1999; Mucke et al. 2000; Walsh et al. 2002; Kamenetz et al. 2003), by reducing the number of AMPA and NMDA surface receptors. Synaptic dysfunction may be considered a response to excessive neuronal excitability, in fact, an increase of neuronal activity increases the production of Aβ (Shankar et al. 2007).

Experimental application of Aβ oligomers impair synaptic plasticity by altering the balance between long-term potentiation (LTP) and long-term depression (LTD) and reducing the numbers of dendritic spines. LTP and LTD are two widely used mechanisms of learning and memory and such processes are believed to play important roles in neural circuits of the brain (Morris et al. 2003, Lynch et al. 2004; Malenka et al. 2004; Whitlock et al. 2006). The term LTP is defined as a long-term increase in the transmission of signal between two neurons stimulated synchronously. The induction of hippocampal LTP requires a burst of action potentials leading to release of glutamate from the presynaptic terminal (produced experimentally with a tetanic stimulation). This implicates the opening of AMPA receptor channels in the postsynaptic membrane of ions with influx of sodium and potassium in the cells, which induces an excitatory postsynaptic potential and release of magnesium ions from the receptors to the NMDA receptor.

The LTD is a rather long-lasting decrease in synaptic efficacy after sustained electrical stimulation in the hippocampus. Similarly to LTP, LTD depends critically on the NMDA receptors, with calcium playing again a central role. LTD is induced in response to continuous low frequency stimulation: postsynaptic repeated entry of small amounts of calcium through

NMDAR activates the calcium dependent phosphatase, which removes phosphate groups.

High concentrations of Aβ oligomers have been shown to suppress basal synaptic transmission facilitating the endocytosis of NMDA and AMPA receptors and leading to an increase in glutamate concentration at the synaptic level (Li et al. 2009). This involves initially an increase of NMDA receptors followed by a receptor desensitization with a consequent synaptic depression (Hsieh et al. 2006; Liu et al. 2009). Aβ can have also an influence at the presynaptic level depending on its concentration: physiological levels of Aβ enhance synaptic activity (Abram et al. 2009; Puzzo et al. 2008), while pathological levels induce an increase in postsynaptic LTD and excessive loss of spines dendritic cells.

### 1.6.4 Neuroinflammation and immune system activation in AD

A number of evidences suggests an involvement of inflammatory events and immune mechanisms in the pathogenesis of AD, since Aβ activates microglia and astrocytes in vitro and in situ around fibrillar plaques (Wyss-Coray and Mucke 2002). The central player of neuroinflammation in AD is principally activated microglial cells, probably with a dual role: initially, microglia phagocyte and degrade Aβ thus protecting neurons from the cytotoxic effect of Aβ. However, chronic exposure to Aβ activates microglia, which release chemokines and proinflammatory molecules, with increase of nitric oxide synthase and hence free radicals causing dysfunction and neuronal death (Schultzberg et al. 2007). Moreover microglia express receptors for advanced glycation end products (RAGE), which bind Aβ, thereby amplifying the generation of cytokines, glutamate, nitric oxide and mediate influx of vascular Aβ. Fibrillar Aβ and glial activation also stimulate the classic complement pathway (McGeer and McGeer.

2001). The inflammatory milieu provokes neuritic changes and breakdown of the vascular blood–brain barrier.

In line with this evidence, pharmacological studies demonstrated that patients taking non-steroidal anti-inflammatory drugs had a lower risk of AD as compared to age-matched controls (Lleò et al. 2004; Weggen et al. 2003). However, recent randomized trials failed to show evidence of reduced risk of AD by non steroidal anti-inflammatory agents (Szekely 2008).

### 1.7 Other contributing factors

The role of other factors in the pathogenesis of AD is now beginning to be explored. Environment, diet, state of health, oxidative stress, glycation of proteins, presence of diabetes can contribute to the loss of neurons and synapses. The production of free radicals, by mithocondrial disfunction and oxidative stress is accelerated by the action of Aβ and activated microglia in AD. The exposure to Aβ inhibits key mitochondrial enzymes in the brain and in isolated mitochondria. Cytochrome c oxidase is specifically down-regulated (Caspersen et al. 2005). Consequently, electron transport, ATP production, oxygen consumption, and mitochondrial membrane potential all become impaired. Another metabolic disturbance of emerging importance in AD is type 2 diabetes that is considered another risk factor for AD and dementia (Arvanitakis et al. 2004).

### 1.8 Controversy on amyloid cascade hypothesis

For over 100 years the neuroscience community has confirmed the pathogenetic role of Aβ, although there is no proof that it is the only causative agent of AD. Although a high number of important studies have been made in understanding the molecular and pathological bases of AD, there have been few

successes in the clinic and a number of fundamental questions remains unanswered.

The amyloid cascade hypothesis has been invaluable in elucidating the physiology of Aβ metabolism, but it has failed in equal measure to produce any tangible treatment benefit (Haass 2010; Castellani and Smith 2011). The amyloid hypothesis well explain FAD, where genetically mediated high levels of amyloid play a prevalent pathogenetic role. In fact, most mutations in the APP and presenilin genes on chromosome 21 increase the production of Aβ42, which is the main component of senile plaques. Moreover people with Down Syndrome who have an extra gene copy almost universally exhibit AD by 40 years of age. In addition in vivo studies on transgenic mice that express a mutant form of the human APP gene develop fibrillar amyloid plaques and Alzheimer's-like brain pathology with spatial learning deficits (Pietropaolo et al. 2011; Lalonde et al. 2002; Games et al. 1995). All these evidences taken together have promoted the "amyloid cascade hypothesis" invaluable in elucidating several molecular mechanisms in the pathogenesis of AD. Such hypothesis, however, has been deeply challenged by the substantial failure of a number of clinical trials to improve cognition with agents aiming to decrease β-amyloid burden in AD (Holmes et al. 2008, Lemere et al 2010). Indeed the vast majority of cases of AD are sporadic, a condition where several factors and additional components/pathways are probably involved.

Not only the substantial differences between familiar and sporadic AD raises doubts on the amyloid theory, but also a number of other considerations. Firstly, the presence of senile plaques in cognitively normal individuals the poor correlation between Aβ plaque burden and disease severity (memory decline)

raise questions about the specific and exclusive role of Aβ in AD. In addition, the cytotoxicity of Aβ oligomers has not been demonstrated in vivo, since these molecules are identified with isolation laboratory procedures in vitro and are difficult to characterize *in vivo* (Castellani and Smith 2011).

A further complication derives from the spatial and temporal relation between tau and Aβ: inhibition of tau expression blocks seizures induced by Aβ (through overstimulation of NMDA receptors) and improves survival in a transgenic mouse model of Alzheimer's disease (Roberson et al. 2007). Synaptic damages, formation of dendritic spines and Aβ-mediated toxicity are events that apparently occur before NFTs are formed, thus arguing against their relevance in neuronal cell death and indeed recent studies have provided evidence that neuronal cell death can occur independently of NFTs formation (de Calignon et al. 2010, Paquet et al. 2009).

Finally, the poor concordance between the pathological findings and clinical phenotype in transgenic mice complicates even more the situation. These experimental models mimicking a genetic conditions in fact reproduce only the major neuropathological aspects of AD with extensive deposition of amyloid plaques and increased soluble levels of Aβ1–42, but do not display concomitant neuronal loss (Hsiao et al. 1996). This is in agreement with earlier data showing that amyloid injections in rat brain had no long-term behavioral or neuropathological effects (Winkler et al. 1994; Stephenson et al. 1992). Although some of the APP transgenic animals with amyloid deposition have been reported to display behavioural deficits, it is not clear whether these deficits are due to amyloid deposition or to the overexpression of APP.

To verify the pathogenicity of amyloid as the unique agents to determine AD, one should assess whether Aβ is able to induce spreading or propagation of the disease and initiate a neurodegenerative cascade. In this regard, recent studies demonstrated that brain homogenates containing Aβ were sufficient to induce AD–like pathology, but not spreading, suggesting that additional cofactors may be needed (Meyer-Luehmann et al. 2006). In this directions there are recent studies of the group of Aguzzi that hypothesizes that many amyloid proteins are able of amplifying themselves via conformational alterations in a similar manner to prion protein (Aguzzi and Rajendram 2009).

Finally, we have to keep in mind that other molecules beside Aβ have been identified in AD plaques, whose significance is not yet clearly characterized (Strittmatter et al. 1996; Bronfman et al. 1996; Selkoe 2001) and their interactions with β-amyloid contributes to complicate the scenario of AD pathogenesis.

## 2. CHITIN

### 2.1 Chitin: structure and features

Chitin is the main component of the fungal cell walls and it is present in the exoskeleton of arthropods and insects and the microfilaria sheath of nematodes, acting as a protective layer against the harsh conditions that may be endured by the pathogen or arthropod (Glaser 1957; Nishimura et al. 1984; Roncero 2002; Banks 2005). Chitin is a linear polymer of N-acetyl-glucosamine units connected through β1-4 glycosidic linkage (Figure 4). It is very similar to cellulose, differing for the presence of an acetamido group that participates in the formation of intermolecular hydrogen binding, leading to a stiff crystal insoluble in almost all solvents. Chitin is the second most abundant glycopolymer on earth,

with an estimated 1010 tonnes produced each year. It is generally assumed that mammals lack the ability to produce chitin because they do not synthesize the enzyme chitin synthase, although display the enzyme responsible for its degradation: chitotriosidase.

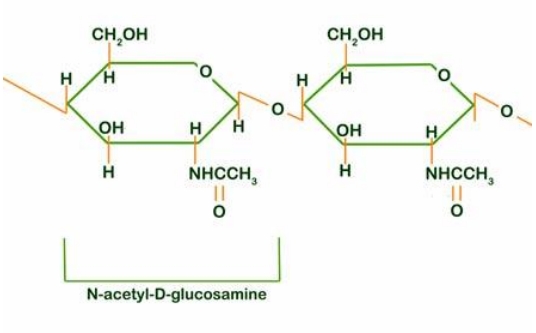


**Figure 4**. Chemical structure of chitin.

Chitotriosidase is a protein of 50 kDa, member of chitinase family secreted by activated macrophages and neutrophils, whose function in humans is unknown; in this regard, it may be a relic of an archaic response against chitin-containing pathogens (Sotgiu et al. 2005; 2006, 2007; Barone et al. 2007). Nevertheless, chitotriosidase has been identified as a potential biomarker because high levels of protein have been correlated with storage diseases (Gaucher's disease), but also in some CNS diseases as AD, Stroke and MS (Kumar et al. 1991; Ishii et al. 1998; Nunomura et al. 2001).

A commercial derivate of chitin, chitosan, is obtained by deacetylation of chitin. A large number of prostheses such as artificial skin, contact lenses, and surgical stitches have been produced from chitin derivates, known to be non toxic, non allergenic, not biodegradable and biocompatible and are widely used in medicinal practice (Muzzarelli 1997). It is very common for humans to be exposed to chitin/chitin derivatives in daily life

The synthesis of chitin is an energy-dependent process requiring N-acetylglucosamyniltransferase using the uridine diphosphate (UDP)-activated monomer as sugar donor (Glaser and Brown 1957). Even if no definitive mammalian chitin synthase has been documented, the pathogenetic role of these oligosaccharides has been reported in some studies (Semino et al. 1996; Bakkers et al. 1997). Previous works have demonstrated that another enzyme like Hyaluronan synthase-1 (HAS-1) is able to convert activated glucosamine to chito-oligosaccharides in vitro using HAS-1 gene product (Bakkers et al. 1997; Semino et al. 1996; Yoshida et al. 2002). The presence of DG42 protein (involved in chito-oligosaccarid synthesis in Xenopus and also found in zebrafish and mouse during embryogenesis) has been demonstrated to produce chito-oligomers capable to act as primers in the synthesis of hyaluronan. Overexpression of DG42 in mouse cells leads to the synthesis of chito oligomers, and hyaluronan synthase preparations also contain chitin synthase activity. Thus, it is conceivable that chito-oligomers can act as templates for hyaluronan synthesis (Varki et al. 1996; Bakkers et al. 1997).

**2.2 Glucose metabolism in AD**

Brain glucose utilization decreases with age (Ivancevic et al. 2000) and this decline is further accelerated in AD. Extensive studies have established an impaired glucose metabolism and utilization in the AD brain (McGeer et al. 1989, 1990; Heiss et al. 1991; Smith et al. 1992; Minoshima et al. 1995), which occurred prior to the appearance of clinical symptoms and in MCI (Pietrini et al. 1993; Mielke et al. 1994; de Leon et al. 2001; Drzezga et al. 2003, 2005; Mosconi et al. 2004).

The use of positron emission tomography (PET) and single photon emission computed tomography (SPECT) in AD has demonstrated bilateral temporo-parietal and medial temporal hypoperfusion with subsequent decreased oxygen metabolism (Kumar et al. 1991; Ishii et al. 1996, 1997, 1998; Nunomura et al. 2001). An impaired glucose metabolism is also evidenced by a reduced concentration of glucose transporters 1 and 3 (GLUT1) and (GLUT3) in different areas of the cerebral cortices of AD (Simpson et al. 1994; Simpson et al. 1994). In this regard, it has been suggested that the altered glucose passage through the blood brain barrier may be related to congophylic angiopathy, due to increased thickness of capillary (Piert et al. 1996). This glucose impairment leads to intracellular hyperglycemia which a consequent "shift" in the amount of glucose to the hexosamine biosynthesis pathway (HBP), as suggested by the diabetic model. Approximately 2–5% of total glucose feeds into the HBP to produce glucosamine-6-phosphate and, finally, UDP-Nacetylglucosamine (UDP-GlcNAc) (Love and Hanover, 2005). It is possible that upregulation of the hexosamine pathway leads to the synthesis of glucosamine polymers (Castellani et al. 2005). Some authors have hypothesized that the intracellular hyperglycemia and increased glucosamine levels are secondary to hexosamine pathway activation due to impaired glucose metabolism and might have glucose and glucosamine polymers as end products (Figure 5)

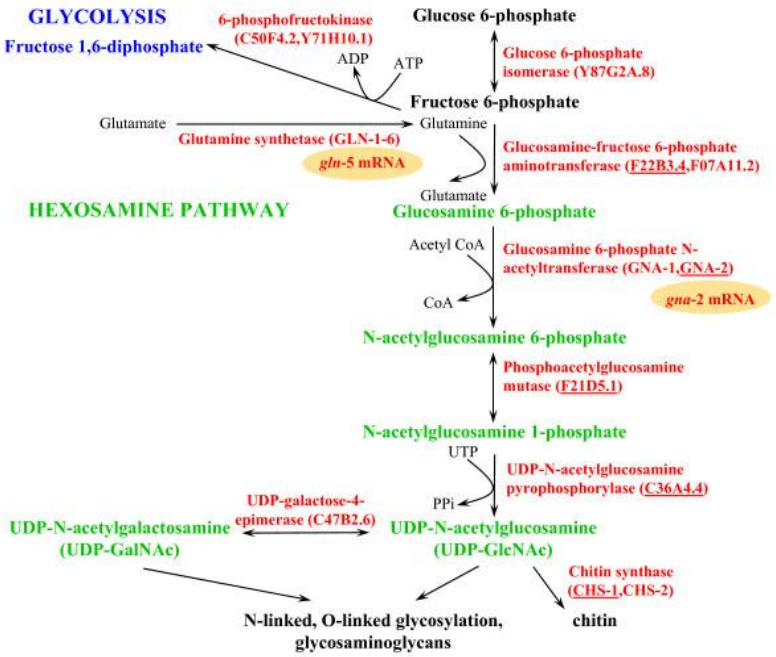


**Figure 5.** Hexosamine pathway. Approximately 2-5% of total glucose feeds into HBP to produce glucosamine-6-phosphate and UDP-N-Acetyl-Glucosamine. This last product may be a primer to form chitin-like compounds.

### 2.3 Role of carbohydrates in AD

The contribution of carbohydrates in AD pathogenesis has largely been ignored even if controversy over the role of carbohydrates in amyloidosis has existed since the initial recognition of amyloid. Historical studies conduced in 1854 by Virchow have already introduced the term “corpora amylacea” to describe the microscopic intracellular lesions in the CNS of patients with amyloidosis (Rottkamp et al. 2001; Smith et al. 2002; Castellani et al. 2005, 2007). This definition was clarified and improved five years later by Friedreich and Kehule. Recently, several emerging evidences indicate that the interaction of amyloid with polysaccharides derived from the impaired glucose utilization is one of the key event in AD pathogenesis. The presence of several deposits derived from impared glucose metabolism such as proteoglycans (PGs), are the object of different studies. These compound share with Aβ characteristics like relative

insolubility, fibrillary structure and β-sheet secondary conformation associated with aggregation or polymerization. Those deposits are intrinsic components of plaques and NFTs (Wisniewski et al. 1997; Selkoe 2000; Verdier and Penke 2004; Ariga et al. 2008). PGs are heterogeneous macromolecules consisting of a protein core that is covalently linked to glycosaminoglicans (GAGs). These GAGs side chains are composed of repeated disaccharide units of 1-4 linked iduronic acid (IdoA)/glucuronic acid (GlcA) and N-acetyl-glucosamine. Amyloid deposits containing PGs or GAGs play a clear role in the pathogenesis of AD (Castillo et al. 1997; van Horssen et al. 2003; Gruys et al. 2006) enhancing both amyloid aggregation and fibril formation (Fraser et al. 1992; Sipe and Cohen 2000; Cohlberg et al. 2002). Heparan sulphate proteoglycans (HSPGs) were also found in microglial and astroglial cells surrounding senile plaques (O'Callaghan et al. 2008). Co-deposition of HSPGs with Aβ was observed in the Tg2576 mice brain, in which glypican-1 and syndecan-3 were expressed in glial cells associated with Aβ deposits (O'Callaghan et al. 2008). These complex macromolecules have also been implicated in other neurodegenerative diseases, including Gerstmann-Straussler syndrome (Snow et al. 1990), Creutzfeldt-Jakob disease, scrapie, (Snow et al. 1990) mucopolysaccharidoses (Ginsberg et al. 1999), Parkinson's disease (Liu et al. 2005), and other neuromuscular diseases (Peat et al. 2008). Castellani (Castellani et al. 2005, 2007) and subsequently our group (Sotgiu et al. 2008) showed the presence of glucosamine polymers, in particular chitin in association with amyloid deposits in AD.

**2.4 Role of chitin-like polymers in AD**

An impared glucose metabolism in AD increases the synthesis of glucose and of glucosamine through the activation of HBP. High levels of both glucose

and glucosamine are found in AD (Brownlee 2001; Castellani et al. 2005). The presence of chitin-likepolymers in AD brains has been detected by Calcofluor fluorescence histochemistry (Castellani et al. 2005; Sotgiu et al. 2007). Calcofluor white is a fluorescent dye with great affinity for β1-4 linkage and a very useful tool to detect chitin in tissues (Klis et al. 2002; Castellani et al. 2005, 2007; Sotgiu et al. 2008). Calcofluor is used in the paper industry, in washing powders and for detection of fungal elements in clinical and biological specimens, that fluoresce when expose to UV light.

The staining with Calcofluor (Figure 6) showed the presence of chitin (Castellani 2004) in all type of plaques as well as in blood vessels affected by amyloid angiopathy, whereas but no signal has been detected in pathological conditions without amyloid deposits, such as multiple sclerosis (Sotgiu et al. 2008), suggesting a strict relationship between chitin and Aβ in amyloidotic conditions.

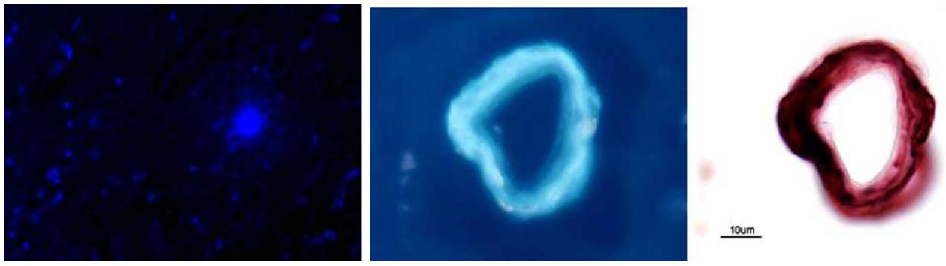


**Figure 6.** Calcofluor histochemistry on brain tissue from AD patient demonstrates intense labelling of amyloid plaques (Sotgiu et al. 2008) and of blood vessel affected by amyloid angiopathy (Castellani et al. 2005).

In addition, the treatment of AD sections with chitinase (which degrades chitin to chitobiose) and β-N-acetylglucosaminidase significantly diminished Calcofluor fluorescence, therefore suggesting that chitin-like polysaccharides are indeed present in pathognomonic lesions of AD (i.e. senile plaques and amyloid angiopathy) (Castellani et al. 2005, 2007).

Although the two molecules have many characteristics in common (the birefringence to polarized light, the insolubility, the resistance to protease activity), the relationship between chitin and Aβ is poorly investigated.

**2.5 Chitin in immune response**

The interest on chitin in human pathology has been focused for its effects on the innate and adaptive immune responses both in vivo and in vitro. In fact, chitin activates peritoneal macrophages and NK cells to express a number of pro-inflammatory cytokines such as IL-1 β, colony stimulating factor (CSF) and IFN-γ (Shibata et al. 1997, 2000). The intravenous administration of fractionated chitin particles into the lung of mice activated alveolar macrophages to express cytokines such as IL-12, TNF-α, and IL-18 leading to INFγ production mainly by NK cells (Shibata et al. 1997). Chitin can regulate type 2 immune responses (Gavett et al. 1995; Shibata et al. 2001; Strong et al. 2002) stimulating macrophages and other innate immune cells. Considering the ability of chitin to stimulate the production of type I cytokines, and the known ability of the type I cytokines to inhibit type 2 inflammation (Sur et al. 1996, Gavett et al. 1995), there are evidences suggesting that chitin could negatively modulate type 2 immune responses (Lee 2008).

Chitin has a size-dependent role in immune response: large chitin polymers are biologically inert, while polymers of intermediate size (40-70 nm) are PAMPS (pathogen-associated molecular patterns) that trigger immune response and cytokine production (IL-17, TNF, IL-23) through the recognition receptor TLR-2 signaling pathway and the MYD-88 (Da Silva et al.2008). The smaller fragments instead (< 40 nm) stimulate the production of IL-10 by macrophages through a

signaling pathway involving NF-κB, Dectin-1 and Syk kinase (Da Silva et al. 2008).

# MATERIALS AND METHODS

## CELL CULTURES

### Cell lines and primary cultures

Murine N9 microglial cells were cultured routinely in RPMI media supplemented with 10% fetal bovine serum (FBS) (all from Euroclone). 5H-SY5Y human neuroblastoma cells were grown in RPMI media supplemented with 10% FBS. Human fibroblasts from muscle biopsy were grown in DMEM (Euroclone) 10% supplemented with FBS. All cell lines were supplemented with 100 U/ml penicillin/streptomycin (P/S) (Euroclone) and were grown in a humidified atmosphere with 5% $CO_2$ in 75 $cm^2$ cell culture flasks (Corning). Cells were grown to ~90% confluency and passaged every 4 days. For immunocytochemical experiments cells were seeded onto 12$mm^2$ glass coverslips and plated at different densities in 24-well ($5 \times 10^3$ cells per well for N9, $1 \times 10^4$ cells per well for SY5Y and $1 \times 10^3$ cells per well fibroblasts).

New-born BALB/c mice were used for the preparation of primary cultures. All animals, purchased from Harlan Italy (S. Pietro di Natisone, Italy), were housed in pathogen free conditions and treated according to the guidelines of Animal Ethical Committee of the University of Study of Verona. Primary microglial cultures were prepared from E16-E18 up to 2 days old mice. Briefly, whole brains were removed and carefully cleared from the meninges. Cortices were subsequently minced into small pieces and treated with trypsin (Sigma-Aldrich) in the presence of DNase I (Sigma-Aldrich) and centrifuged at 1000 rpm for 10 min. After digestion, the cell pellet was resuspended in complete medium DMEM, 10% FBS,

100 Uml P/S, and 2 mM glutamine (all Euroclone) and plated. Cells were then incubated at 37 °C with 5% $CO_2$ in an humidified atmosphere. The medium was changed after 3 days. At day 7-8, adherent cells were confluent and consisted of astrocytes and microglia. Microglial cells were removed by mild shaking from the astroglial layer. The non-adhering cells were centrifuged at 1000 rpm for 10 minutes and plated on uncoated plastic wells in DMEM supplemented with 10% FBS. The day after plating, the medium was replaced with fresh medium to remove the non adhering cells.

Primary hippocampal neurons were prepared from E16-E18 up to 2 days old mice. Brains were dissected, the meninges were removed, the hippocampi were isolated and mechanically processed as for microglia. Finally they were plated on glass coverslips in 24-wells grown in Neurobasal media, 10% FBS, 2 mM glutamine, P/S and with B27 supplement (all from Euroclone). The day after plating, the medium was replaced with fresh medium to remove the non adhering cells.

**Preparation of chitin particles**

Chitin fragments were generated according the modified protocol previously described by Shibata et al. 1997. Briefly, chitin powder from shrimp shells (Sigma-Aldrich) was suspended at 10 mg/ml in PBS and sonicated three times for 5 min. The suspension was filtered with 40 μm sterile strainer and autoclaved. Before use, chitin particles were resuspended by brief sonication and incubated on cell cultures.

**Chitin synthesis**

Synthesis of the endogenous chitin was obtained by treating different cells (N9, primary microglial cultures, SY5Y, primary hippocampal neurons and

fibroblasts) with 5 mM N-Acetylglucosamine, 150 μM UDP-N-Acetylglucosamine (all for Sigma) for 72 hours. After this incubation period, immunofluorescence was performed with Calcofluor (see below).

**Patients**

Autoptic CNS tissues from 3 sporadic human AD patients, 4 Down syndrome cases, 2 familiar AD cases with a mutation on PS1 (E280G and DELTA4) and 2 familiar AD patients with a mutation for APP717 (VAL-ILE) were obtained from MRC London Brain Bank for Neurodegeneration disease.

**Immunofluorescence and confocal microscopy**

Immunofluorescence assay was performed on both brain tissues and cell cultures. Brain sections from autoptic CNS tissues were embedded in paraffin and processed for histopathological and histochemical analyses. Section of brains were treated with 0.2% Calcofluor (Sigma-Aldrich) in Tris/HCl buffered solution (0.1 M, pH9) for 1hour at room temperature as previously described (Castellani et al. 2005, Sotgiu et al. 2008). Double immunostainings were performed using Congo Red (Electron Microscopy Sciences) to stain amyloid plaques.

After washing with PBS, sections were viewed on Zeiss Axiolab fluorescent microscope and analyzed with AxioVision LE Rel. 4.5 software.

Cells, grown on coverslips, were fixed in 4% para-formaldehyde and permeabilized with 0.5% Triton. Subsequently, they were incubated with 20% normal goat serum (Vector) and 1% BSA and then incubated with primary antibodies over night at 4 °C: monoclonal rat anti-mouse CD11b (1:100, Serotec), polyclonal rabbit anti β-tubulin class III (1:250, Millipore), monoclonal rat-anti mouse CD68 (1:200 Chemicon).

Double immunostaining were performed using 0.2% Calcofluor (Sigma) for 1 hour at room temperature. Rhodamine–conjugated chitin binding probe (1:500 New England Biolabs) was used like primary antibodies overnight. All staining for chitin detection were confirmed by the use of this specific probe.

After washing with PBS, appropriate biotinilated secondary antibodies (Vector), and fluorophore-conjugated secondary antibodies (Molecular Probes, Invitrogen) were added. Parallel sets of cells were stained with the secondary antibodies with omission of the primary antibodies as negative controls.

Coverslips were mounted on glass slides and observed as above.

**Proliferation, activation and viability assays**

To evaluate cell viability, cells were usually counted on 10 random fields following treatment for 48 or 72 hours with 1 mg/ml chitin and 50 μg/ml β-amyloid fragment 25-35 ($A\beta_{25-35}$, Bachem). Experiments were performed in triplicate and cells blindly counted by two independent investigators. The MTT assay is a colorimetric assay for measuring the activity of enzymes that reduce MTT, a yellow tetrazole, to purple formazan dye. The assay was used to assess the viability, proliferation and activation of living cells. For MTT assay N9 cells, were seeded into a flat bottom 96-well plate and incubated at 37 °C for 48 and 72 hours prior to the addition of 10 μl of MTT solution (Cell proliferation Kit I, Roche Diagnostics GmbH) for 4 hours at 37 °C. Absorbance at 655 nm was measured after 24 hours with a multi-function reader (BioRad).

To evaluate the activation of N9 induced by chitin, the supernatant of cells was collected and used for the quantitative determination of human tumor necrosis factor alpha (TNF-α) according to commercial datasheet protocol (R&D systems). LPS 100 g/ml was used as positive control.

To determine the mitotic activity of N9 cells before and after treatments, cells were exposed to 10 μM BrdU (Sigma) for 4 hours, fixed with methanol for 10 minutes, treated with 2N HCl and then with $Na_2B_4O_7$ (0.1M pH 8,5). Double immunofluorescence was performed for CD11b and BrdU, whose signals were detected with secondary antibodies conjugated with Streptavidine Texas Red (Vector) and anti-mouse Alexa 488 (Invitrogen). Nuclei were stained with DAPI (Abbott Molecular Inc.). Cells were visualized under the Fluorescent microscope (Zeiss MC80) and the rate of mitotic activity was calculated dividing the number of $BrdU^+$ nuclei for the total number of cells.

**Electron microscopy**

Microglial cells incubated with chitin were evaluated by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). For TEM, N9 cells at confluence were treated with 1mg/ml chitin for 48 hours or with 5 mM N-Acetyl Glucosamine and with 150 μM UDP-N-Acetyl Glucosamine. N9 cells were then collected and centrifuged at 150g, washed with HBSS and finally with phosphate buffer. Cells were then fixed with 1.25% glutaraldheide and 0.5% para-formaldeide in phosphate buffer for 1 hour at 4 °C and post-fixed in osmium tetraoxide ($OsO_4$) for 2 hours at 4 °C. Cellular pellet was subsequently dehydrated by increasing concentrations of acetone and included in Spurr resin. The EM photographs were acquired by a scanner at 600 dpi resolution.

For SEM, cells were fixed in glutaraldehyde 2,5% for 1 hour at 4°C and then post-fixed in 1% osmium tetroxide for 1 hour, dehydrated in graded acetone, fixed to stubs with colloidal silver, spattered with gold with a MED 010 coater and examined with DSM 950 Zeiss.

**HPLC-MASS**

Two samples of 5H-SY5Y neuroblastoma cells were cultured up to confluence and treated for 48 hours with 5 mM N-Acetylglucosamine, 150 μM UDP-N-Acetylglucosamine (control group was not subjected to any treatment). Cells were homogenized by pestle and mortar in liquid nitrogen and then placed at -80 °C. The powder obtained was dissolved in a solvent extraction (water and methanol in the ratio 8:20) sonicated for a couple of times to obtain a supernatant and further centrifuged twice at 12000 rpm for 10 minutes. The supernatant was placed in a 1 ml sterile syringe, provided with a suitable filter of cellulose (RC Minisart 4). The elute was collected in a glass tube with inner chamber of 400 ml. For the analysis of HPLC-mass, samples are injected into a system of separation consisting of a reverse phase C18 column (HP Alltime C18 3m measuring 150 mm x 2.1 mm) equipped with a pre-column (HP Alltime C18 5m measuring 7.5 mm x 4.6 mm) and a mass spectrometer connected to an analyzer ion trap with ESI source. Two solvents were used for chromatographic elution: solvent A consisting of 94.5% $H_2O$ + 5% acetronile + 0.5% formic acid and solvent B consisting of 100% acetronile. The spectrophotometer was set to perform an analysis in positive ionization mode and not fragmented to the calculation of quantities. In addiction it was set in positive mode and fragmented analysis for the identification of chitin-like molecules. The data were obtained by analysis in positive mode and were processed using mzMine 2.0 software. This has allowed the creation of a matrix showing final data.

**Hippocampal slices**

The hippocampal slices were obtained from BALB/c mice killed by decapitation around 60 days. The brains were placed in an ice-cold dissection medium and meninges were removed, followed by separation of the temporal lobes of both hemispheres. The temporal lobes were sectioned into slices of 200 μm in thickness using a vibratome. The hippocampal slices were used to form organotypic cultures which were exposed to exogenous or endogenous chitin and subjected to different treatments and subsequently used for Western blotting or histochemistry.

**Organotypic cultures**

The slices were kept for 1 hour at 4°C in HBSS supplemented with 2 mg/ml of D-glucose (Euroclone) and were then transferred into a 6-well plate (Corning). Each well contained 2 ml of N-MEM supplemented with 10% FBS, P/S. The plates were maintained in a humidified atmosphere with 5% $CO_2$ at 37 °C and the next day the cultures were tested with: 1 mg/ml of chitin or 5mM of N-acetylglucosamine plus 150 μM UDP-N-acetylglucosamine. Every 2-3 days the medium was replaced and treatments re-added. This procedure was repeated for a week.

**Organotypic slices embedding**

The organotypic cultures were fixed in 4% para-formaldehyde overnight and then embedded in paraffin. 8 μm thick sections were subjected to immunofluorescence as previously described to detect chitin and microglia (anti-Iba1 antibody 1:200 Abcam).

**Western blot analysis**

Organotypic hippocampal slice cultures were cultured and treated as described above and subsequently used for protein extraction. Tissues were resuspended with 50 µl RIPA Buffer (150 mM NaCl, 50 mM Tris-HCl, 1mM EDTA, 1% NP-40, 0.1% SDS, 0.5% Deoxycolic acid, pH 8.0) containing protease inhibitors (Roche Diagnostics). After 2 hours of homogenization at 4 °C, homogenates were centrifuged at 4000 rpm to discard cellular debris and then the supernatant was collected and stored at -80°C. Protein content was determined by Bradford Assay (Sigma-Aldrich). For western blot analysis, 8µg of total protein lysates were diluted in Laemli buffer, boiled at 90 °C for 5 minutes and then resolved by 12% SDS-PAGE at 100V. Proteins were transferred onto the nitrocellulose membrane (Bio-Rad), which were blocked with TBS and 0.1% Tween20 and 10% non-fat dry milk (GE-Healthcare) at room temperature. Further, proteins were incubated over night at 4 °C with primary antibodies: monoclonal anti-syntaxin antibody (Abcam), polyclonal anti-β-III-tubulin antibody and polyclonal anti-Actin antibody (Sigma-Aldrich).

Anti-mouseor rabbit IgG HRP (GE Healthcare) were added for 1 hour at room temperature and chemiluminescent detection was performed with ECL Plus advanced (Amersham GE Healthcare). Quantitative analysis of the signal obtained was performed by ImageJ software (NIH).

**Statistical analysis**

Statistical comparison of the results was carried out according to the Student's T-test. Differences were considered statistically significant when $p<0.05$.

# RESULTS

## Chitin is present in sporadic AD brains

Chitin-like deposits in amyloid plaques have been observed with Calcofluor or with a specific chitin-binding-probe (CBP) on autoptic AD brains in hippocampus and cerebral cortex of sporadic AD cases, but not in determined genetic conditions such as Down syndrome, familiar AD cases with a mutation on PS1 (E280G and DELTA4), familiar AD with a mutation for APP717 (VAL-ILE) (Figure 1a).

Calcofluor fluorescence in sporadic AD brain was intense with a predominantly plaque pattern that strictly co-localize with Congo Red, the typical dye used to detect Aβ in AD plaques (Figure 1b).

Double immunofluorescence with Calcofluor and microglial markers (CD68) showed the presence of chitin-like deposits not only in the core of AD plaques but also in the cytoplasm of surrounding microglia (Figure 1c).

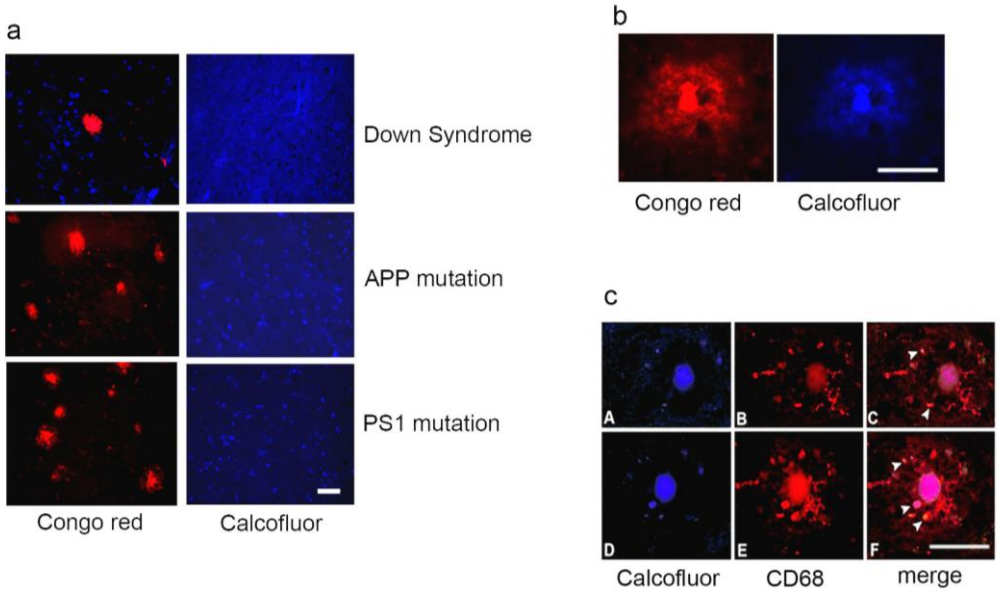


**Figure 1.** Calcofluor staining on AD brains. (a) Genetic-determined conditions didn't show positivity for Calcofluor staining. (b) Calcofluor fluorescence in sporadic AD brain was intense with a predominantly plaque patter that strictly co-localize with the signal of Congo Red. (c) Chitin deposits, positive for Calcofluor (A-D) are present in the core of AD plaques and also in cytoplasm of surrounding microglia $CD68^{+}$ (B-E). Scale bars equal to 50 μn.

**Microglia phagocytes chitin particles in vitro**

The experiments on N9 cell lines and primary microglial cultures showed that, after 48h of treatment with exogenous chitin, microglial cells are able to phagocytes small chitin fragments as shown by Calcofluor (Figure 2a) and CBP (not shown). The phagocytosis of chitin by N9 has been confirmed at electron microscopic levels (Figure 2b and 2c). Chitin deposits were identified in the cytoplasm of about 60-75% of N9 and primary cultures. Interestingly, the treatment with Aβ significantly reduced the phagocytic ability, with only 20-35% of cells containing chitin particles (Figure 2d and 2e ). These experiments clearly demonstrate the ability of microglia to phagocyte chitin polymers, and that process is significantly inhibited by the concomitant presence of □ Aβ.

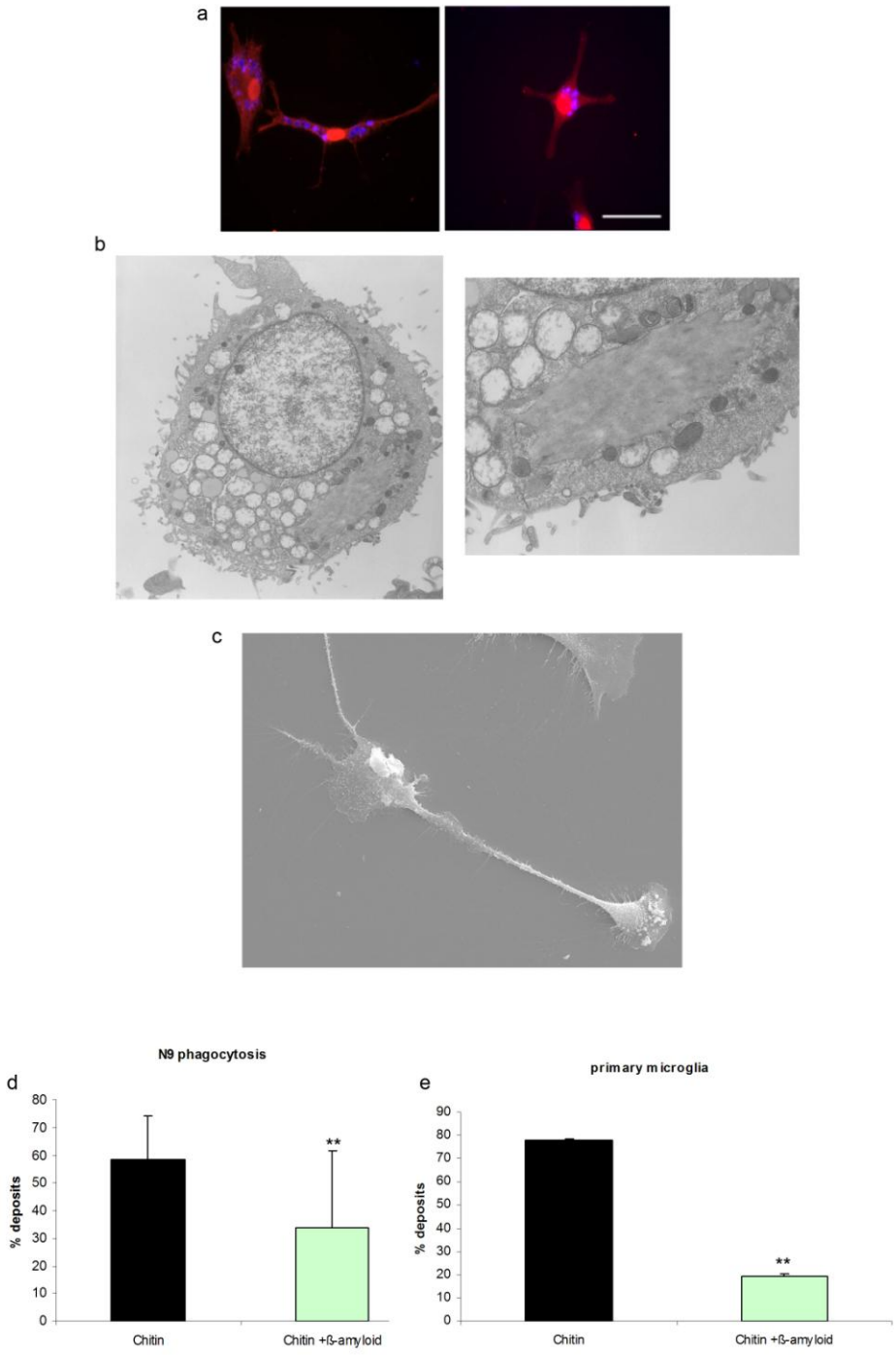


**Figure 2.** (a) Microglial cells are able to phagocyte chitin particles, which appear positive to Calcofluor staining (blue) in the cytoplasm of $CD11b^{+}$ microglial cells (red). By TEM (b) and SEM (c) we observed chitin particles in the cytoplasm of N9. (d) 60% of N9 cells contains chitin deposits but the treatment with Aβ greatly reduces this phagocytic capability with only 30% of the cells containing chitin deposits. (e) The experiment was also performed on primary microglial cultures with similar results. Scale bar equal to 78,75 μn.

**Chitin induces the activation of microglia**

The exposure to chitin for 48h had a clear activatory effect on N9 cell lines (Figure 3a and 3b) similarly to what has been described for Aβ in literature (Wyss-Coray et al 2002; Meda et al. 1995). In particular, we observed a significantly metabolic activity (measured by MTT assay) and TNF-α production, with no changes in their proliferation rate (Figure 3c).

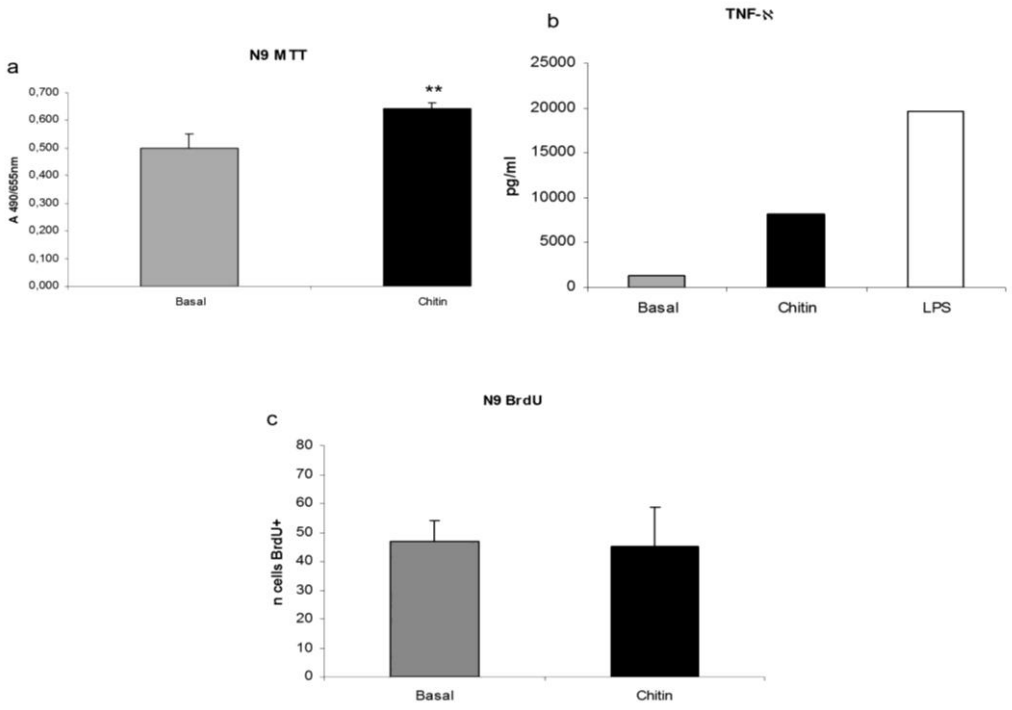


**Figure 3.** Activatory effects of chitin on N9 cells. (a) Activation of cellular metabolism observed with MTT assay and (b) TNF-α production (LPS was used as a positive control). (c) No changes were observed in terms of proliferation evaluated as number of $BrdU^+$ cells.

**Chitin induces neuronal toxicity**

At variance with microglia, neurons cells (SY5Y) were not able to phagocyte to chitin (Figure 4a) for 48h. However we observed a significant cytotoxicity induced by either Aβ and chitin with a reduction of visible cells of 33% (Figure 4b). The same experiment was performed on primary hippocampal

neurons, when we observed a cell reduction of 42% compared to untreated cells (Figure 4c).

To verify whether the cytotoxicity effect observed on neuronal cells was due to a unspecific toxicity of chitin particles, we incubated fibroblast cultures to the same treatment.

Although fibroblast had the ability to phagocytes chitin, no difference of cell viability was observed (Figure 4d), suggesting that chitin induced a selective neuronal toxicity.

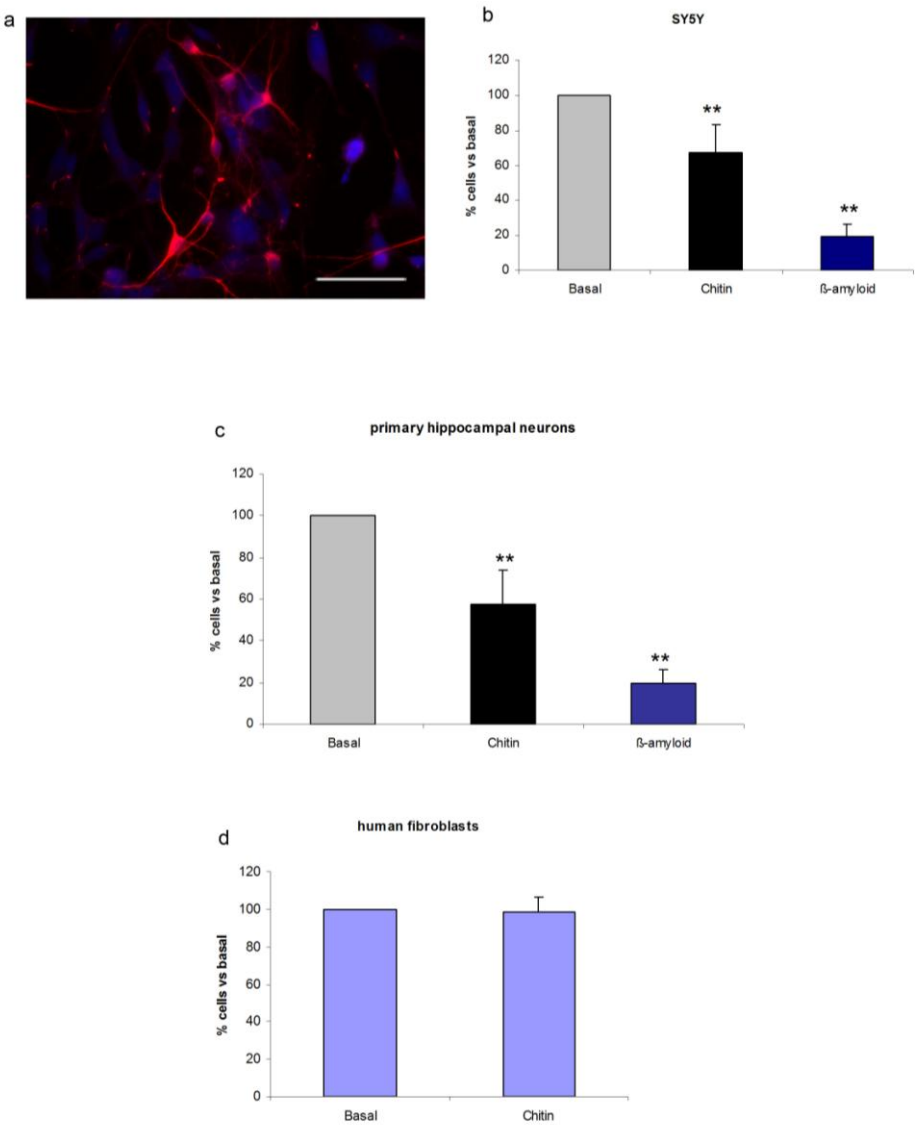


**Figure 4.** (a) No Chitin deposits were detected in β-III-tubulin$^{+}$ neurons (red) after exogenous chitin treatment; (b) such treatment lead

to a significant reduction SY5Y as well as of primary hippocampal neurons (c). A similar cytotoxicity was observed after Aβ treatment. (d) No significant differences were observed on fibroblasts cells after chitin treatment. Scale bar equal to 40μn.

**Microglial cells produce chitin-like polymers**

Similarly to the experiments performed with exogenous chitin, we used microglial cells to verify if an endogenous chitin synthesis was possible in mammalian cells. The treatment for 72h with an excess of N-Acetyl-glucosamine and UDP-N-Acetyl-glucosamine (Uridine diphosphate acts as an energetic substrate for the enzymatic reaction), lead to the detection with Calcofluor of deposits inside the cytoplasm of N9 cells and in the extracellular space (Figure 5a, b); such finding was confirm by SEM, which showed deposits on primary microglial cultures (Figure 5d) with Calcofluor positivity protruding from N9 cells (Figure 5c) deposits present in the cytoplasm of microglia (Figure 5d). Similarly to exogenous chitin the treatment with N-Acetyl-glucosamine and UDP-N-Acetyl-glucosamine lead to activation of N9 cells, shown by MTT assay (Figure 5e).

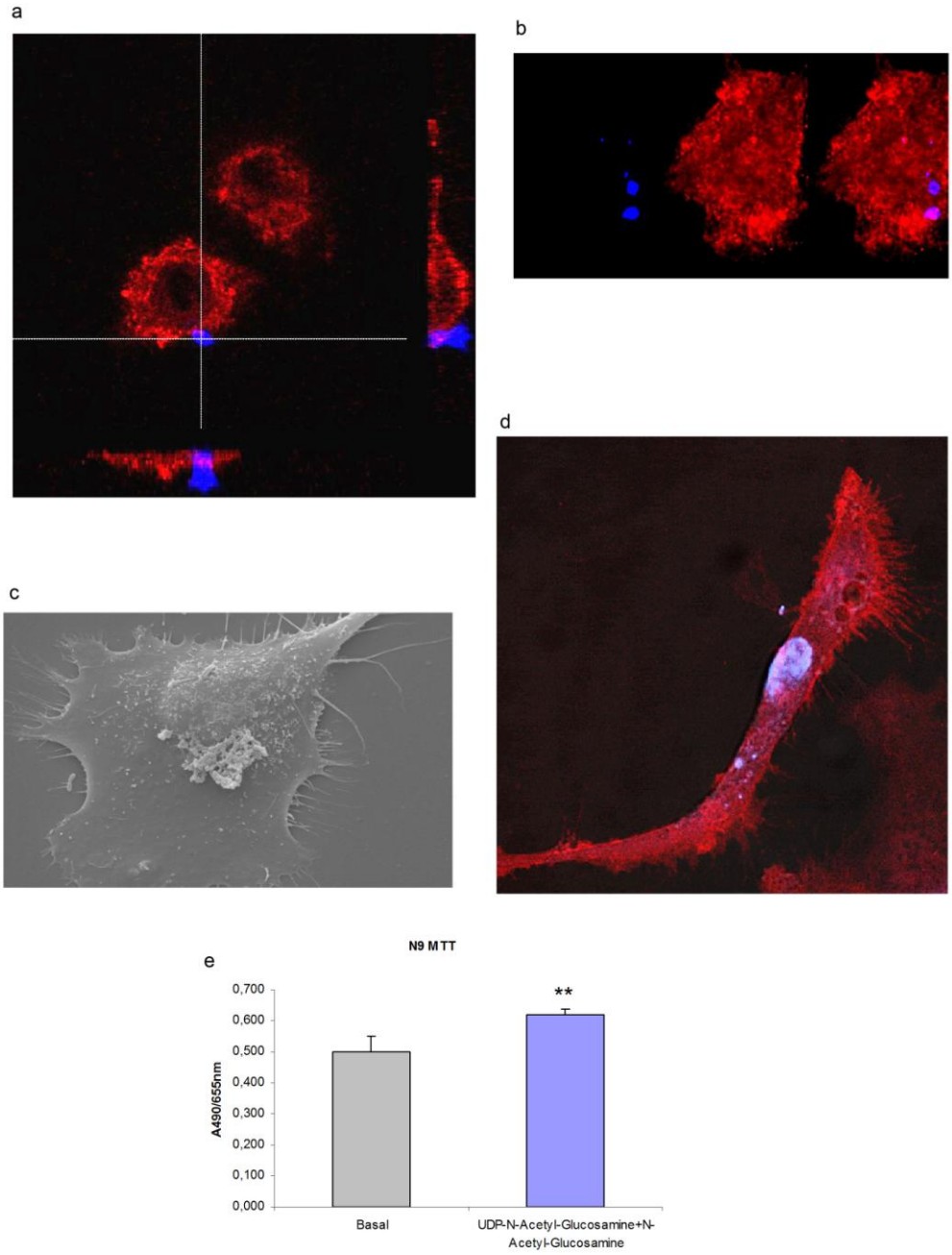


**Figure 5.** (a,b) Confocal images of microglial cells after the treated with N-Acetyl-glucosamine and UDP-N-Acetyl-Glucosamine. Calcofluor staining showed the presence of chitin deposits (blue) in $CD11b^{+}$ (red) microglial cultures. (c) SEM showed new formed chitin-like particles in N9 cells. (d) Primary microglial cells produced intra and extra cellular chitin deposits after treatment with UDP-N-Acetyl-Glucosamine and N-AcetylGlucosamine. (e) Endogenous chitin produces activation of N9 cells in terms of metabolic activity observed by MTT assay.

**Neurons produce chitin-like polymers**

We next assessed if neuronal cultures were also able to produce endogenous chitin. For this purpose SY5Y and primary hippocampal neurons were incubated for 72h with N-Acetyl-glucosamine and UDP-N-Acetyl-glucosamine. At variance with microglia no chitin deposits were detected with Calcofluor staining, albeit a reduction of cell number (43% and 33% for SY5Y and primary cultures respectively) was observed (Figure 6a and 6b).

To assess the presence of small chitin particles (i.e. below the sensitivity of Calcofluor) in neurons, To explain this significant cytotoxicity, we used a powerful and sensible technique, the HPLC-MS, Cellular extracts of SY5Y cells treated or not with N-Acetyl-glucosamine and UDP-N-Acetyl-glucosamine were analyzed by this tool obtaining a typical chromatogram in the treated sample (Figure 6c and 6d). Subsequently mass fragmentation showed typical peaks; each of one representing a new-formed polymers of N-Acetyl-glucosamine with increasing molecular weights (Figure 6e).

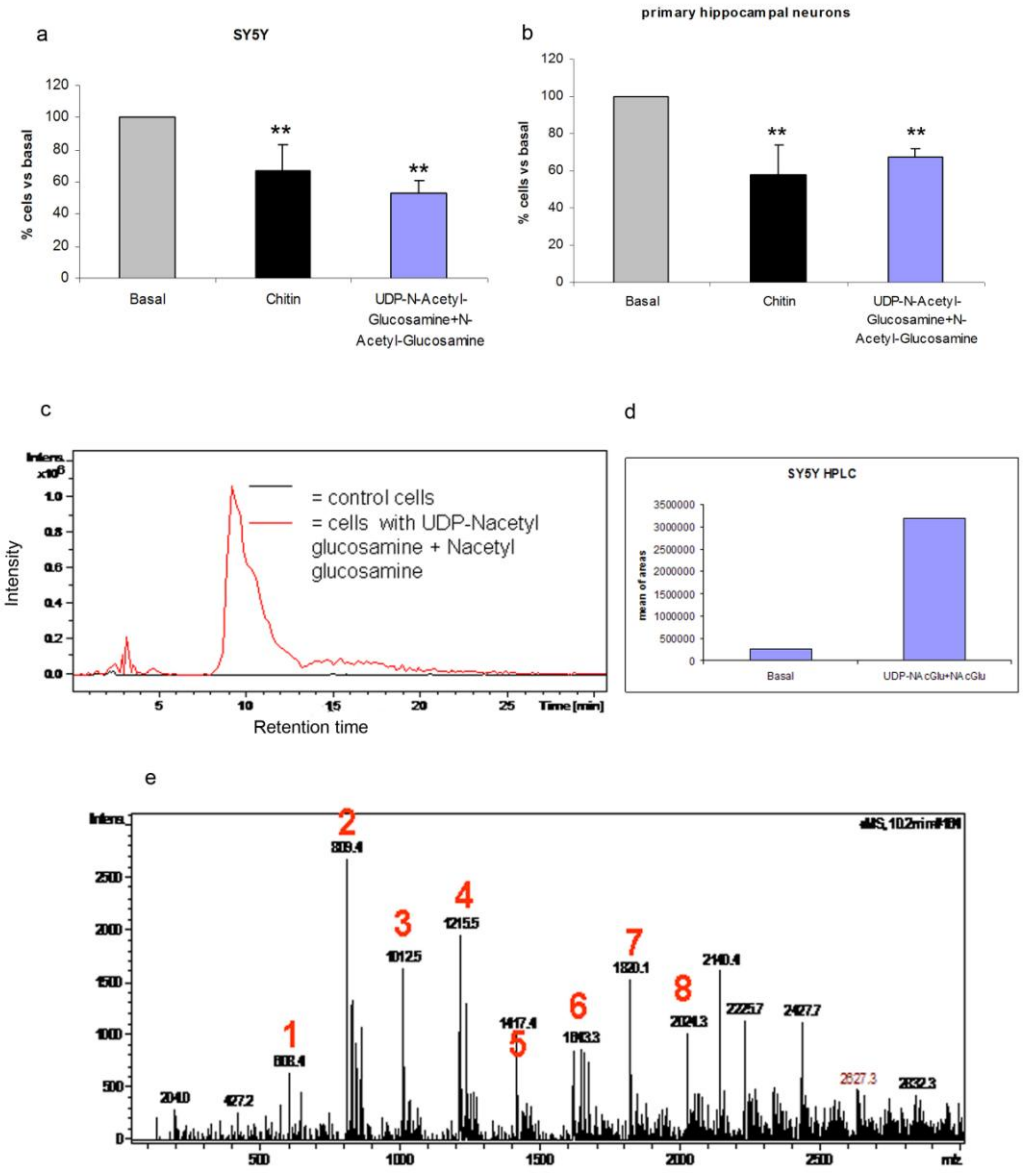


**Figure 6.** (a) Cytotoxicity of both exogenous and endogenous chitin on SY5Y (b) and primary hippocampal cultures. (c) Ion Chromatotogram of SY5Y cell extract after the treatment with UDP-N-Acetylglucosamine and N-Acetylglucosamine showed a signal (in red) absent in untreated cells (in black). (d) The peak from HPLC may be expressed as the area underlying. After separation by chromatography, the treated SY5Y sample was analyzed by MS-fragmentation which showed multiple peaks with molecular weight characteristics for polymers of N-acetyl-glucosamine. In red it is represents the number of multiple units of N-acetyl-glucosamine (monomer $C_8H_{15}NO_6$ MW: 221,2078 g/mol).

**Chitin production by microglia and synaptic impairment in an ex-vivo model.**

A more complex method was used to verify the possibility of an endogenous chitin synthesis in an ex-vivo model. Organotypic hyppocampal cultures, representing a biological system with physiological cellular connections and synapses, were treated repeatedly for a 7 days with N-Acetyl-glucosamine and UDP-N-Acetyl-glucosamine.

After such chronic exposure, Calcofluor staining showed positive signals in treated slices, suggesting that the presence of an excess of N-Acetyl-Glucosamine and its energetic substrate can produce chitin-like compounds in vivo.

Double immunofluorescence performed using antibodies anti-Iba-1, and β-III-tubulin, showed that Calcofluor signals co-localized with microglia, whereas no chitin was found on neurons (Figure 8a). To assess the effect of such new-formed chitin/ endogenous chitin on neuronal function, we performed a western blot analysis on slice cultures homogenates focusing on proteins involved in synaptic transmission and neuronal assembly: Syntaxin and β-III-tubulin (Figure 8b).

Syntaxin 1, also known as HPC1, is an integral membrane protein which along with SNAP25 and VAMP/synaptobrevin plays a role in trafficking and membrane fusion. β-III-tubulin is a protein that constitute the cytoskeleton of microtubules. The subunit β is expressed principally on neuronal terminals (Wang and Nogales 2005).

In line with cytotoxicity effect observed in vitro, preliminary results have revealed an important reduction in Syntaxin and β-III-tubulin levels obtained with both exogenous and endogenous chitin (Figure 8c and 8d).

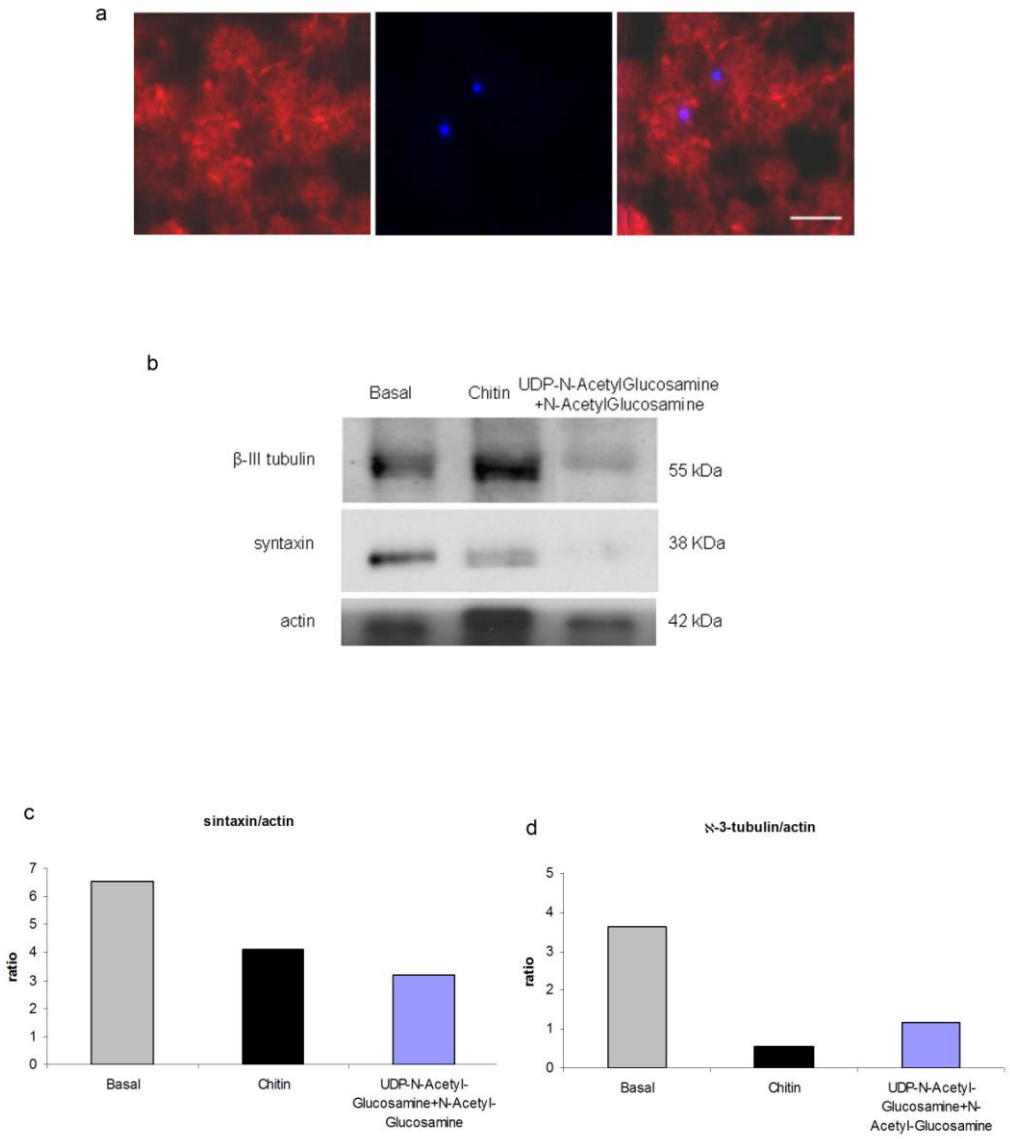


**Figure 8.** (a) Organotypic hippocampal cultures treated with N-Acetyl-glucosamine and UDP-N-Acetyl-glucosamine. Double immunofluorescence showed the presence of new-formed chitin (Iba-1$^{+}$) in microglial cells after chronic treatment. (b) Western blot from treated organotypic slices for Syntaxin and β-III-tubulin showed a reduction in the protein levels of both proteins exerted by either exogenous or endogenous chitin (c-d).

# DISCUSSION

The pathogenesis of AD, the most frequent neurodegenerative disorder, is far to be clear despite thousands of papers published in the last decades. The two major relevant molecules in the pathogenesis of AD are Aβ protein in amyloid plaques and hyperphosphorilated tau in NFTs. According to the so-called "amyloid cascade hypothesis" (Haas and Selkoe 2007), both proteins are required to exert neurotoxicity. The cascade begins from imbalanced production and clearance of Aβ. Aβ accumulation and deposition may trigger a complex downstream cascade that finally resulted in synaptic and neuronal injury leading to progressive dementia. Most information regarding such hypothesis has been confirmed by the pathological analysis of AD patients with mutations involving Aβ-related molecules. The increase of Aβ in genetic AD is due to mutations present mostly in three different genes: APP, PS1 and PS2. The paradigmatic example is the study of patients with Down syndrome which develop early onset dementia associated with Aβ plaques and NFT (Olson and Shwan 1969) as a consequence of the over-expression of the APP gene on 21 chromosome (Kang et al. 1997). Such studies have provided a valid help in elucidating the molecular mechanisms underlying the role of Aβ in AD pathogenesis and have paved the road of the research in the last decades. Most of these results have been then confirmed in transgenic mice carrying the same mutations. However, the translation of such information to sporadic AD did not completely address all the complexity of sporadic AD. For example, a direct correlation of amyloid plaques load with memory loss has not been found in sporadic AD (Winklhofer et al

2008); in addition, amyloid plaques are also found in non-demented old subjects (Haass 2010). Finally, the results from recent clinical trials designed (according to the amyloid cascade) to decrease the burden of Aβ in sporadic AD have been frustrating: even though different pharmaceutical compounds indeed lead to reduced Aβ deposits in the brains, the cognitive decline progressed. Based on these considerations, it is conceivable that the amyloid cascade hypothesis clearly elucidates the physiology of Aβ metabolism and explains several aspects of genetic AD, but it can not be applied tout-court to sporadic AD, where it failed to produce any tangible treatment benefit (Haass 2010; Castellani and Smith 2011).

Epidemiological, clinical and pathological evidences rather suggest that the pathogenesis of sporadic AD is multi-factorial with the variable contribution of environmental and genetic factors, involving additional components/pathways. Beside Aβ, a wide range of molecules have been identified in AD plaques, whose significance and interaction with Aβ are not yet clearly characterized (Strittmatter et al. 1995; Bronfman et al. 1996; Selkoe 2001). For example, heparan sulphate proteoglycans, complement factors, acute phase proteins and other molecules have been described in AD plaques (Timmer et al. 2010). The contribution of these molecules is still a matter of debate: some authors suggest a potential and active role, while others consider these molecules only as co-factors in the pathogenesis of AD.

Previous studies have identified the presence of chitin in close association with Aβ in autoptic AD brains (Castellani et al. 2005, 2007; Sotgiu et al. 2008) by Calcofluor staining both in amyloid plaques and within the cytoplasm of surrounding microglia. The detection of chitin in amyloid plaques of sporadic AD brains prompted us to investigate if chitin could have a role in the pathogenesis of

AD, giving new insights to the complex scenario of the disease. Chitin is a linear and insoluble polymer of N-acetyl-glucosamine units connected through β1-4 glycosidic linkage. It is the main component of the fungal cell walls and is present in the exoskeleton of arthropods and insects and the microfilaria sheath of nematodes, acting as a protective layer against the harsh conditions (Glaser 1957; Nishimura et al. 1984; Roncero 2002; Banks 2005). The detection of chitin-like molecules in humans (albeit only in pathological conditions) is quite surprising, if we consider that mammalian cells lack the gene codifying for chitin synthase.

We first compared sporadic and familiar AD brain sections for the presence of chitin by Calcofluor and CBP. Interestingly, both techniques revealed chitin-like deposits in amyloid plaques only in sporadic AD brains, while no signals have been observed in familiar and Down syndrome cases. In line with this, preliminary results in transgenic mice "5XFAD" (a mouse model that co-express five familiar mutations; Oddo et. al 2003; Oakley et al. 2006) confirmed the absence of chitin.

We then assessed the biological effects of chitin on neural cells in vitro and ex vivo. For this purpose, we first exposed microglial cells (both N9 cell line and primary cultures) to small chitin particles, since large chitin fragments have been shown to be biologically inert (Shibata et al. 1997, Da Silva et. al. 2008). This experiment confirmed the ability of microglia to phagocyte chitin particles and the process was significantly inhibited by the co-incubation with Aβ□. The phagocytosis of chitin by microglial cells is probably mediated by the mannose receptor, as previously described for splenic macrophages (Shibata et al. 1997). Similarly to Aβ, upon chitin phagocytosis microglial cells undergo activation with increased metabolic activity and production of inflammatory cytokines. This

biological effect of chitin on microglia is similar to that described for Aβ and may have relevant implications in AD pathogenesis, since microglia activation may contribute to neuronal damage.

We then evaluated the effect of chitin directly on neurons. At variance with microglia, neurons were not able to phagocyte chitin particles, although the exposure to chitin induced a significant cytotoxicity both on SY5Y cell line and on primary hippocampal neurons. The effect observed was comparable to Aβ-induced toxicity. Interestingly, no significant toxixicity by chitin was observed on fibroblasts cultures, suggesting that chitin induced a selective neuronal toxicity.

A central point of this research concerned the production of chitin by mammalian cells, which lack chitin synthase. In this regard, Semino et al. have shown that the absence of chitin synthesizing enzyme may be overcome by hyaluronan synthase-1, which converts UDP-N-acetyl-glucosamine to chito-oligosaccharides in vitro. Thus, a condition characterized by an excess of UDP-N-acetyl-glucosamine may predispose to chitin formation through alternative pathways. In this regard, it is interesting to note that intracellular glucose metabolism has been demonstrated in AD brains with activation of the hexosamine pathway with consequent accumulation of its end product, N-acetyl-glucosamine. A point that needs to be further investigated concerns the role of this up-regulation of the hexosamine pathway in AD. It will be interesting to assess whether this reflects a mere consequence of the impairment of the glycolitic pathway or whether it may be sustained by a genetic or acquired perturbation of the cerebral glucose metabolism in favour of the hexosamine pathway.

In line with these evidences, we demonstrated that microglia, but not neurons, were able to produce Calcofluor-positive signals after exposure to UDP-N-acetyl-

glucosamine in vitro. The analysis by confocal microscopy confirmed Calcofluor-positive signals in the cytoplasm of microglia; in addition, such technique evidenced the presence of chitin-like deposits in close proximity of the plasma membrane as well as in the extracellular space, suggesting that the excessive production of chitin is followed by its extrusion. The lack of Calcofluor-positive deposits in neurons was probably due to its low sensitivity, since HPLC-MS analysis on treated SY5Y cell line treated with UDP-N-acetyl-glucosamine confirmed the presence of "new-formed" chitin-like compounds also in neurons. We then assessed the effects of "endogenous" chitin on neural cells. We found that the new-formed polymers lead to activation of microglia as well as to significant neuronal cytotoxicity, mimicking the effects observed with exogenous chitin. Thus, our experiments in vitro may recapitulate several aspects of the histochemical findings observed in human AD brains. In fact, chitin-like deposits have been documented both in vitro and in vivo either in the cytoplasm of microglia and in the extracellular space (i. e. amyloid plaques), but not in neurons. We further analyzed the effect of endogenous chitin in a more physiological setting, using the organotypic slice cultures. In such ex vivo model, hippocampal neurons are connected to each other through synapses and are influenced by the surrounding glial cells. As shown in vitro, Calcofluor-positive signals were detected in microglial cells after treatment with UDP-N-acetyl-glucosamine. Moreover, preliminary experiments in murine hippocampal slice cultures suggested that synaptic transmission is impaired following exposure to UDP-N-acetyl-glucosamine, as suggested by a reduction in syntaxin levels by western blotting. In parallel, we observed a decrease of β-III-tubulin levels, indicating a cytoskeleton disruption, confirming the toxic effect of "endogenous" chitin on

neurons, as previously shown in vitro. Preliminary experiments aimed at evaluating the effect of this treatment on synaptic transmission by electrophysiological techniques showed that the treatment with UDP-N-Acetyl-glucosamine on hippocampal slices affects LTP similarly to Aβ (Shankar et al. 2008).

Taken together, our results indicate that chitin-like molecules may contribute to the pathogenesis of sporadic AD. At variance with Castellani who hypothesized that chitin may act as a scaffold for the subsequent deposition of Aβ (Castellani et al. 2004, 2005, 2007), our results support an active role of this polymer in the pathogenesis of AD, due to its neurotoxic effect demonstrated both in vitro and ex vivo by impairment of synaptic transmission and neuronal architecture.

In light of these considerations, future therapeutic strategies for AD should take into account the complexity of the disease with the aim not only to reduce amyloid burden, but also to act on endogenous chitin, inhibiting key steps in the biosynthesis of glucosamine. In this regard, anti-chitin antibodies have been produced (Solomon and Frenkel 2002) and a number of molecules interfering with chitin synthase (Nikkomycins, Polyoxins and micronazoles) may be also considered as additional therapeutic tools in sporadic AD.

# BIBLIOGRAPHY


Abbott A, The plaque plane. Nature 2008 Nov 13; Vol 456.

Abram M, Wegmann M, Fokuhl V, Sonar S, Luger EO, Kerzel S, Radbruch A, Renz H, Zemlin M. Nerve growth factor and neurotrophin-3 mediate survival of pulmonary plasma cells during the allergic airway inflammation. J Immunol. 2009 Apr 15; 182(8): 4705-12.

Aguzzi A, Rajendran L. The transcellular spread of cytosolic amyloids, prions, and prionoids. Neuron. 2009 Dec 24; 64(6): 783-90.

Andersen K, Launer LJ, Dewey ME, Letenneur L, Ott A, Copeland JR, Dartigues JF, Kragh-Sorensen P, Baldereschi M, Brayne C, Lobo A, Martinez-Lage JM, Stijnen T, Hofman A. Gender differences in the incidence of AD and vascular dementia: The EURODEM Studies. EURODEM Incidence Research Group. Neurology. 1999 Dec 10; 53(9): 1992-7.

Ariga T, McDonald MP, Yu RK. Role of ganglioside metabolism in the pathogenesis of Alzheimer's disease--a review. J Lipid Res. 2008 Jun; 49(6): 1157-75.

Arvanitakis Z, Wilson RS, Bienias JL, Evans DA, Bennett DA. Diabetes mellitus and risk of Alzheimer disease and decline in cognitive function. Arch Neurol. 2004 May; 61(5): 661-6.

Bakkers J, Semino CE, Stroband H, Kijne JW, Robbins PW, Spaink HP.: An important developmental role for oligosaccharides during early embryogenesis of cyprinid fish. Proc Natl Acad Sci USA 1997; 94: 7982-6.

Bamberger ME, Landreth GE. Microglial interaction with beta-amyloid: implications for the pathogenesis of Alzheimer's disease. Microsc Res Tech. 2001 Jul 15; 54(2): 59-70.

Banks IR, Specht CA, Donlin MJ, Gerik KJ, Levitz SM, Lodge JK. A chitin synthase and its regulator protein are critical for chitosan production and growth of the fungal pathogen Cryptococcus neoformans Eukaryot Cell. 2005 Nov; 4(11): 1902-12.

Barone R, Sotgiu S, Musumeci S. Plasma chitotriosidase in health and pathology. Clin Lab 2007; 53: 321-333.

Berchtold NC, Cotman CW. Evolution in the conceptualization of dementia and Alzheimer's disease: Greco-Roman period to the 1960s. Neurobiol Aging 1998; 19: 173–89.

Birks J, Grimley Evans J, Iakovidou V, Tsolaki M, Holt FE. Rivastigmine for Alzheimer's disease. Cochrane Database Syst Rev. 2009 Apr 15; (2).

Bronfman FC, Soto C, Tapia L, Tapia V, Inestrosa NC. Extracellular matrix regulates the amount of the beta-amyloid precursor protein and its amyloidogenic fragments. J Cell Physiol 1996; 166: 360-9.

Brownlee M. Biochemistry and molecular cell biology of diabetic complication. Nature 2001; 414: 813-20.

Busciglio J, Pelsman A, Wong C, Pigino G, Yuan M, Mori H, Yankner BA. Altered metabolism of the amyloid beta precursor protein in associated with mitochondrial dysfunction in Down's syndrome. Neuron 2002; 33: 677-88.

Caricasole A, Copani A, Caruso A, Caraci F, Iacovelli L, Sortino MA, Terstappen GC, Nicoletti F. The Wnt pathway, cell-cycle activation and beta-amyloid: novel therapeutic strategies in Alzheimer's disease? Trends Pharmacol Sci. 2003 May; 24(5):233-8.

Caspersen C, Wang N, Yao J, Sosunov A, Chen X, Lustbader JW, Xu HW, Stern D, McKhann G, Yan SD. Mitochondrial Abeta: a potential focal point for neuronal metabolic dysfunction in Alzheimer's disease. FASEB J. 2005 Dec; 19(14): 2040-1.

Castellani RJ, Common R, Perry G, Ghetti B, Smith MA. Calcofluor stains amyloid-beta deposits. J Neuropathology and Exp Neurol 2004; 63: 524.

Castellani RJ, Perry G, Smith MA. The role of novel chitin-like polysaccharides in Alzheimer disease. Neurotox Res 2007; 12: 269-74.

Castellani RJ, Siedlak SL, Fortino AE, Perry G, Ghetti B, Smith MA. Chitin-like polysaccharides in Alzheimer's disease brains. Curr Alzheimer Res 2005; 2: 419-23.

Castellani RJ, Smith MA. Compounding artefacts with uncertainty, and an amyloid cascade hypothesis that is 'too big to fail'. J Pathol. 2011 Jun; 224(2): 147-52.

Castillo GM, Ngo C, Cummings J, Wight TN, Snow AD. Perlecan binds to the beta-amyloid proteins (A beta) of Alzheimer's disease, accelerates A beta fibril formation, and maintains A beta fibril stability. J Neurochem. 1997 Dec; 69(6): 2452-65.

Chapman PF, White GL, Jones MW, Cooper-Blacketer D, Marshall VJ, Irizarry M, Younkin L, Good MA, Bliss TV, Hyman BT, Younkin SG, Hsiao KK. Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. Nat Neurosci. 1999; 2: 271–276.

Cheng IH, Scearce-Leviek, Legleiter J, Palop JJ, Gerstein H, Bien-Ly N, Puoliväli J, Lesné S, Ashe KH, Muchowski PJ, Mucke L. Accelerating amyloid-β fibrillization reduces oligomer levels and functional deficits in Alzheimer disease mouse models. J Biol Chem 2007; 282: 23818–23828.

Cleary JP, Walsh DM, Hofmeister JJ, Shankar GM, Kuskowski MA, Selkoe DJ, Ashe KH. Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function. Nat Neurosci. 2005 Jan; 8(1): 79-84.

Cohlberg JA, Li J, Uversky VN, Fink AL. Heparin and other glycosaminoglycans stimulate the formation of amyloid fibrils from alpha-synuclein in vitro. Biochemistry. 2002 Feb 5; 41(5): 1502-11.

Da Silva CA, Hartl D, Liu W, Lee CG, Elias JA. TLR-2 and IL-17A in chitin-induced macrophage activation and acute inflammation. J Immunol 2008; 181: 4279-4286.

Davies CA, Mann DM, Sumpter PQ, Yates PO. A quantitative morphometric analysis of the neuronal and synaptic content of the frontal and temporal cortex in patients with Alzheimer's disease. J Neurol Sci 1987; 78: 151-164.

De Calignon A, Fox LM, Pitstick R, Carlson GA, Bacskai BJ, Spires-Jones TL, Hyman BT. Caspase activation precedes and leads to tangles. Nature 2010; 464: 1201-1204.

De Leon M, Bobinski M, Convit A, Wolf O, Insausti R. Usefulness of MRI measures of entorhinal cortex versus hippocampus in AD. Neurology. 2001 Mar 27; 56(6): 820-1.

Di Carlo A, Baldereschi M, Amaducci L, Lepore V, Bracco L, Maggi S, Bonaiuto S, Perissinotto E, Scarlato G, Farchi G, Inzitari D; ILSA Working Group. Incidence of dementia, Alzheimer's disease, and vascular dementia in Italy. The ILSA Study. J Am Geriatr Soc. 2002 Jan; 50(1): 41-8.

Drzezga A, Grimmer T, Riemenschneider M, Lautenschlager N, Siebner H, Alexopoulus P, Minoshima S, Schwaiger M, Kurz A. Prediction of individual clinical outcome in MCI by means of genetic assessment and (18)F-FDG PET. J Nucl Med. 2005 Oct; 46(10): 1625-32.

Drzezga A, Lautenschlager N, Siebner H, Riemenschneider M, Willoch F, Minoshima S, Schwaiger M, Kurz A. Cerebral metabolic changes accompanying conversion of mild cognitive impairment into Alzheimer's disease: a PET follow-up study. Eur J Nucl Med Mol Imaging. 2003 Aug; 30(8): 1104-13.

Dubois B, Feldman HH, Jacova C, Dekosky ST, Barberger-Gateau P, Cummings J, Delacourte A, Galasko D, Gauthier S, Jicha G, Meguro K, O'brien J, Pasquier F, Robert P, Rossor M, Salloway S, Stern Y, Visser PJ, Scheltens P. Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria. Lancet Neurol. 2007 Aug; 6 (8): 734-46.

Fraser PE, Nguyen JT, Chin DT, Kirschner DA. Effects of sulfate ions on Alzheimer beta/A4 peptide assemblies: implications for amyloid fibril-proteoglycan interactions. J Neurochem 1992; 59: 1531-40.

Games D, Adams D, Alessandrini R, Barbour R, Berthelette P, Blackwell C, Carr T, Clemens J, Donaldson T, Gillespie F. Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. Nature. 1995 Feb 9; 373(6514): 523-7.

Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Wills-Karp M. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. J Exp Med. 1995 Nov 1; 182(5): 1527-36.

Geula C, Mesulam MM. Cholinesterases and the pathology of Alzheimer disease. Alzheimer Dis Assoc Disord. 1995; 9 Suppl 2: 23-8.

Ginsberg SD, Galvin JE, Lee VM, Rorke LB, Dickson DW, Wolfe JH, Jones MZ, Trojanowski JQ. Accumulation of intracellular amyloid-beta peptide (A beta 1-40) in mucopolysaccharidosis brains. J Neuropathol Exp Neurol. 1999 Aug;58(8):815-24.

Glaser L, Brown DH. The synthesis of chitin in cell-free extracts of Neurospora crassa. J Biol Chem 1957; 228: 729-42.

Goodman Y, Mattson MP. Secreted forms of beta-amyloid precursor protein protect hippocampal neurons against amyloid beta-peptide-induced oxidative injury. Exp Neurol. 1994 Jul;128(1):1-12.

Gruys E, Ultee A, Upragarin N. Glycosaminoglycans are part of amyloid fibrils: ultrastructural evidence in avian AA amyloid stained with cuprolinic blue and labeled with immunogold. Amyloid. 2006 Mar; 13(1): 13-9.

Haass C, Selkoe DJ. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Biol. 2007 Feb; 8(2): 101-12.

Haass C. Initiation and propagation of neurodegeneration. Nat Med. 2010 Nov; 16(11): 1201-4.

Hardy J, Allsop D. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Pharmacol Sci. 1991 Oct; 12(10): 383-8.

Heiss WD, Szelies B, Kessler J, Herholz K. Abnormalities of energy metabolism in Alzheimer's disease studied with PET. Ann N Y Acad Sci 1991; 640: 65-71.

Holmes C, Boche D, Wilkinson D, Yadegarfar G, Hopkins V, Bayer A, Jones RW, Bullock R, Love S, Neal JW, Zotova E, Nicoll JA. Long-term effects of Aβ42 immunisation in Alzheimer's disease: follow-up of a randomised, placebo controlled phase I trial. Lancet 2008; 372: 216-23.

Holtzman DM, Bales KR, Tenkova T, Fagan AM, Parsadanian M, Sartorius LJ, Mackey B, Olney J, McKeel D, Wozniak D, Paul SM. Apolipoprotein E isoform-dependent amyloid deposition and neuritic degeneration in a mouse model of Alzheimer's disease. Proc Natl Acad Sci U S A 2000; 97: 2892-7.

Hsia AY, Masliah E, McConlogue L, Yu GQ, Tatsuno G, Hu K, Kholodenko D, Malenka RC, Nicoll RA, Mucke L. Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. Proc Natl Acad Sci USA 1999; 96: 3228-3233.

Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G. Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. Science. 1996 Oct 4; 274(5284): 99-102.

Hsieh H, Boehm J, Sato C, Iwatsubo T, Tomita T, Sisodia S, Malinow R. AMPAR removal underlies Aβ-induced synaptic depression and dendritic spine loss. Neuron 2006; 52: 831-843.

Ishii K, Inamura T, Sasaki M, Yamaji S, Sakamoto S, Kitagaki H, Hashimoto M, Hirono N, Shimomura T, Mori E. Regional cerebral glucose metabolism in dementia with Lewy bodies and Alzheimer's disease. Neurology 1998; 51: 125-30.

Ishii K, Kitagaki M, Kono M, Mori E. Decresed medial temporal oxygen

metabolism in Alzheimer's disease shown by PET. J Nucl Med 1996; 37: 1159-65.

Ishii K, Sasaki M, Kitagaki H, Sakamoto S, Yamaji S, Maeda K. Regional difference in cerebral blood flow and oxidative metabolism in human cortex. J Nucl Med 1996; 37: 1086-8.

Ishii K, Sasaki M, Kitagaki H, Yamaji S, Sakamoto S, Matsuda K, Mori E. Reduction of cerebellar glucose metabolism in advanced Alzheimer's disease. J Nucl Med 1997; 38: 925-8.

Ishii K, Sasaki M, Yamaji S, Sakamoto S, Kitagaki H, Mori E. Demonstration of decreased posterior cingulate perfusion in mild Alzheimer's disease by means of H215O positron emission tomography. Eur J Nucl Med 1997; 24: 670-3.

Ittner LM, Ke YD, Delerue F, Bi M, Gladbach A, van Eersel J, Wölfing H, Chieng BC, Christie MJ, Napier IA, Eckert A, Staufenbiel M, Hardeman E, Götz J. Dendritic function of tau mediates amyloid-β toxicity in Alzheimer's disease mouse models. Cell 2010; 142: 387-397.

Ivançević V, Alavi A, Souder E, Mozley PD, Gur RE, Bénard F, Munz DL. Regional cerebral glucose metabolism in healthy volunteers determined by fluordeoxyglucose positron emission tomography: appearance and variance in the transaxial, coronal, and sagittal planes. Clin Nucl Med. 2000 Aug; 25(8): 596-602.

Kamenetz F, Tomita T, Hsieh H, Seabrook G, Borchelt D, Iwatsubo T, Sisodia S, Malinow R. APP processing and synaptic function. Neuron 2003; 37: 925-937.

Kang DE, Saitoh T, Chen X, Xia Y, Masliah E, Hansen LA, Thomas RG, Thal LJ, Katzman R. Genetic association of the low-density lipoprotein receptor-related protein gene (LRP), an apolipoprotein E receptor, with late-onset Alzheimer's disease. Neurology. 1997 Jul;49(1):56-61.

Kayed R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, Glabe CG. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science 2003; 300: 486-9.

Kim JW, Chang TS, Lee JE, Huh SH, Yeon SW, Yang WS, Joe CO, Mook-Jung I, Tanzi RE, Kim TW, Choi EJ. Negative regulation of the SAPK/JNK signaling pathway by presenilin 1. J Cell Biol. 2001 Apr 30;153(3):457-63.

Klein WL, Krafft GA, Finch CE. Targeting small Abeta oligomers: the solution to an Alzheimer's disease conundrum? Trends Neurosci. 2001 Apr; 24(4): 219-24.

Klis FM, Mol P, Hellingwerf K, Brul S. Dynamics of cell wall structure in Saccharomyces cerevisiae. FEMS Microbiol Rev 2002; 26: 239-56.

Kraepelin E. Das senile und präsenile Irresein. In: Kraepelin E (ed) Psychiatrie. Ein Lehrbuch für Studierende und Ärzte. Verlag von Johann Ambrosius Barth, Leipzig, 1910, 593–632

Kumar A, Shapiro MB, Grady C, Haxby JV, Wagner E, Salerno JA, Friedland RP, Rapoport SI. High-resolution PET studies in Alzheimer's disease. Neuropsychopharmacology 1991; 4: 35-46.

Lalonde R, Dumont M, Fukuchi K, Strazielle C. Transgenic mice expressing the human C99 terminal fragment of betaAPP: effects on spatial learning, exploration, anxiety, and motor coordination. Exp Gerontol. 2002 Dec; 37(12): 1401-12.

Lambert JC, Heath S, Even G, Campion D, Sleegers K, Hiltunen M, Combarros O, Zelenika D, Bullido MJ, Tavernier B, Letenneur L, Bettens K, Berr C, Pasquier F, Fiévet N, Barberger-Gateau P, Engelborghs S, De Deyn P, Mateo I, Franck A, Helisalmi S, Porcellini E, Hanon O; European Alzheimer's Disease Initiative Investigators, de Pancorbo MM, Lendon C, Dufouil C, Jaillard C, Leveillard T, Alvarez V, Bosco P, Mancuso M, Panza F, Nacmias B, Bossù P, Piccardi P, Annoni G, Seripa D, Galimberti D, Hannequin D, Licastro F, Soininen H, Ritchie K, Blanché H, Dartigues JF, Tzourio C, Gut I, Van Broeckhoven C, Alpérovitch A, Lathrop M, Amouyel P. Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer’s disease. Nat Genet 2009; 41: 1094-9.

Lee CG, Da Silva CA, Lee JY, Hartl D, Elias JA.: Chitin regulation of immune responses: an old molecule with new roles. Curr Opin Immunol 2008; 20: 684-9.

Lemere CA, Masliah E. Can Alzheimer disease be prevented by amyloid-beta immunotherapy? Nat Rev Neurol. 2010 Feb; 6(2): 108-19.

Lemere CA, Spooner ET, Leverone JF, Mori C, Clements JD Intranasal immunotherapy for the treatment of Alzheimer's disease: Escherichia coli LT and LT(R192G) as mucosal adjuvants. Neurobiol Aging 2002; 23: 991-1000.

Lesné S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, Gallagher M, Ashe KH. A specific amyloid-β protein assembly in the brain impairs memory. Nature 2006; 440: 352-357.

Lewis J, Dickson DW, Lin WL, Chisholm L, Corral A, Jones G, Yen SH, Sahara N, Skipper L, Yager D, Eckman C, Hardy J, Hutton M, McGowan E. Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. Science. 2001 Aug 24; 293(5534): 1487-91.

Li S, Hong S, Shepardson NE, Walsh DM, Shankar GM, Selkoe D. Soluble oligomers of amyloid β protein facilitate hippocampal long-term depression by disrupting neuronal glutamate uptake. Neuron 2009; 62: 788-801.

Liu HP, Lin WY, Liu SH, Wang WF, Tsai CH, Wu BT, Wang CK, Tsai FJ. Genetic variation in N-methyl-D-aspartate receptor subunit NR3A but not NR3B influences susceptibility to Alzheimer's disease. Dement Geriatr Cogn Disord. 2009;28(6):521-7.

Liu IH, Uversky VN, Munishkina LA, Fink AL, Halfter W, Cole GJ. Agrin binds alpha-synuclein and modulates alpha-synuclein fibrillation. Glycobiology. 2005 Dec;15(12):1320-31.

Lleó A, Berezovska O, Herl L, Raju S, Deng A, Bacskai BJ, Frosch MP, Irizarry M, Hyman BT. Nonsteroidal anti-inflammatory drugs lower Abeta42 and change presenilin 1 conformation. Nat Med 2004; 10: 1065-6.

Love DC, Hanover JA. The hexosamine signaling pathway: deciphering the ‘‘O-GlcNAc code’’. Sci STKE 2005; 13: 654-62

Lue LF, Kuo YM, Roher AE, Brachova L, Shen Y, Sue L, Beach T, Kurth JH, Rydel RE, Rogers J. Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. Am J Pathol 1999; 155: 853-62.

Lustbader JW, Cirilli M, Lin C, Xu HW, Takuma K, Wang N, Caspersen C, Chen X, Pollak S, Chaney M, Trinchese F, Liu S, Gunn-Moore F, Lue LF, Walker DG, Kuppusamy P, Zewier ZL, Arancio O, Stern D, Yan SS, Wu H. ABAD directly links Abeta to mitochondrial toxicity in Alzheimer's disease. Science. 2004 Apr 16; 304(5669): 448-52.

Lynch MA: Long-term potentiation and memory. Physiol Rev 2004; 84: 87-136.

Malenka RC, Bear MF: LTP and LTD: an embarrassment of riches. Neuron 2004; 44: 5-21.

Masliah E, Mallory M, Alford M, DeTeresa R, Hansen LA, McKeel DW Jr, Morris JC. Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease. Neurology 2001; 56: 127-129.

Mattsson N, Zetterberg H, Hansson O, Andreasen N, Parnetti L, Jonsson M, Herukka SK, van der Flier WM, Blankenstein MA, Ewers M, Rich K, Kaiser E, Verbeek M, Tsolaki M, Mulugeta E, Rosén E, Aarsland D, Visser PJ, Schröder J, Marcusson J, de Leon M, Hampel H, Scheltens P, Pirttilä T, Wallin A, Jönhagen ME, Minthon L, Winblad B, Blennow K. CSF biomarkers and incipient. Alzheimer disease in patients with mild cognitive impairment. JAMA 2009; 302: 385-93.

McGeer EG, McGeer PL, Akiyama H, Harrop R. Cortical glutaminase, beta-glucuronidase and glucose utilization in Alzheimer's disease. Can J Neurol Sci 1989; 16: 511–5.

McGeer EG, McGeer PL. Innate immunity in Alzheimer's disease: a model for local inflammatory reactions. Mol Interv. 2001 Apr; 1(1): 22-9.

McGeer EG, Peppard RP, McGeer PL, Tuokko H, Crockett D, Parks R, Akiyama H, Calne DB, Beattie BL, Harrop R. 18Fluorodeoxyglucose positron emission

tomography studies in presumed Alzheimer cases, including 13 serial scans. Can J Neurol Sci 1990; 17: 1–11.

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology. 1984 Jul; 34(7): 939-44.

Meda L, Cassatella MA, Szendrei GI, Otvos L Jr, Baron P, Villalba M, Ferrari D, Rossi F. Activation of microglial cells by beta-amyloid protein and interferon-gamma. Nature. 1995 Apr 13; 374(6523): 647-50.

Meyer-Luehmann M, Coomaraswamy J, Bolmont T, Kaeser S, Schaefer C, Kilger E, Neuenschwander A, Abramowski D, Frey P, Jaton AL, Vigouret JM, Paganetti P, Walsh DM, Mathews PM, Ghiso J, Staufenbiel M, Walker LC, Jucker M. Exogenous induction of cerebral beta-amyloidogenesis is governed by agent and host. Science. 2006 Sep 22; 313(5794): 1781-4.

Mielke R, Herholz K, Grond M, Kessler J, Heiss WD. Clinical deterioration in probable Alzheimer's disease correlates with progressive metabolic impairment of association areas. Dementia. 1994 Jan-Feb; 5(1): 36-41.

Minoshima S, Frey KA, Koeppe RA, Foster NL, Kuhl DE. A diagnostic approach in Alzheimer’s disease using three-dimensional stereotactic surface projections of fluorine-18-FDG PET. J Nucl Med 1995; 36: 1238–48.

Mirra SS, Hart MN, Terry RD. Making the diagnosis of Alzheimer's disease. A primer for practicing pathologists. Arch Pathol Lab Med. 1993 Feb; 117(2): 132-44.

Morris RG. Long-term potentiation and memory. Philos Trans R Soc Lond B Biol Sci 2003; 358: 643-647.

Mosconi L, De Santi S, Rusinek H, Convit A, de Leon MJ. Magnetic resonance and PET studies in the early diagnosis of Alzheimer's disease. Expert Rev Neurother. 2004 Sep; 4(5): 831-49.

Mucke L, Masliah E, Yu GQ, Mallory M, Rockenstein EM, Tatsuno G, Hu K, Kholodenko D, Johnson-Wood K, McConlogue L. High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. J Neurosci. 2000 Jun 1;20(11):4050-8.

Muzzarelli RA. Human enzymatic activities related to the therapeutic administration of chitin derivatives. Cell Mol Life Sci. 1997 Feb; 53(2): 131-40.

Nishimura K, Nishimura S, Nishi N, Saiki I, Tokura S, Azuma I. Immunological activity of chitin and its derivatives. Vaccine 1984; 2: 93-99.

Nunomura A, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, Jones PK, Ghanbari H, Wataya T, Shimohama S, Chiba S, Atwood CS, Petersen RB, Smith MA. Oxidative damage is the erliest event in Alzheimer disease. J Neuropathol Exp Neurol; 60: 759-67.

Oakley H, Cole SL, Logan S, Maus E, Shao P, Craft J, Guillozet-Bongaarts A, Ohno M, Disterhoft J, Van Eldik L, Berry R, Vassar R. Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. J Neurosci. 2006 Oct 4; 26(40): 10129-40.

O'Callaghan P, Sandwall E, Li JP, Yu H, Ravid R, Guan ZZ, van Kuppevelt TH, Nilsson LN, Ingelsson M, Hyman BT, Kalimo H, Lindahl U, Lannfelt L, Zhang X. Heparan sulfate accumulation with Abeta deposits in Alzheimer's disease and Tg2576 mice is contributed by glial cells. Brain Pathol. 2008 Oct; 18(4): 548-61.

Oddo S, Caccamo A, Kitazawa M, Tseng BP, LaFerla FM. Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. Neurobiol Aging. 2003 Dec; 24(8): 1063-70.

Olson MI, Shawn CM. Presenile dementia and Alzheimer’s disease in mongolism. Brain 1969; (92): 147-156

Paquet D, Bhat R, Sydow A, Mandelkow EM, Berg S, Hellberg S, Fälting J, Distel M, Köster RW, Schmid B, Haass C. A zebrafish model of tauopathy allows

in vivo imaging of neuronal cell death and drug evaluation. J Clin Invest 2009; 119: 1382-1395.

Peat RA, Gécz J, Fallon JR, Tarpey PS, Smith R, Futreal A, Stratton MR, Lamandé SR, Yang N, North KN. Exclusion of biglycan mutations in a cohort of patients with neuromuscular disorders. Neuromuscul Disord. 2008 Aug;18(8):606-9.

Perl DP. Neuropathology of Alzheimer's disease and related disorders. Neurol Clin. 2000 Nov; 18(4): 847-64. Review.

Piert M, Koeppe RA, Giordani B, Berent S, Kuhl DE. Diminished glucose transport and phosphorylation in Alzheimer's disease determined by dynamic FDG-PET. J Nucl Med 1996; 37: 201-8.

Pietrini P, Azari NP, Grady CL, Salerno JA, Gonzales-Aviles A, Heston LL, Pettigrew KD, Horwitz B, Haxby JV, Schapiro MB. Pattern of cerebral metabolic interactions in a subject with isolated amnesia at risk for Alzheimer's disease: a longitudinal evaluation. Dementia. 1993 Mar-Apr; 4(2): 94-101.

Pietropaolo S, Delage P, Lebreton F, Crusio WE, Cho YH. Early development of social deficits in APP and APP-PS1 mice. Neurobiol Aging. 2011 Oct 18.

Puzzo D, Privitera L, Leznik E, Fà M, Staniszewski A, Palmeri A, Arancio O. Picomolar amyloid-beta positively modulates synaptic plasticity and memory in hippocampus. J Neurosci 2008; 28: 14537-14545.

Querfurth HW, La Ferla FM.: Alzheimer's Disease. N Engl J Med 2010; 362: 329-44.

Rapoport SI. Hydrogen magnetic resonance spectroscopy in Alzheimer's disease. Lancet Neurol. 2002 Jun; 1(2): 82.

Raschetti R, Albanese E, Vanacore N, Maggini M. Cholinesterase inhibitors in mild cognitive impairment: a systematic review of randomised trials. PLoS Med. 2007 Nov 27; 4 (11): e338.

Reiman EM, Chen K, Liu X, Bandy D, Yu M, Lee W, Ayutyanont N, Keppler J, Reeder SA, Langbaum JB, Alexander GE, Klunk WE, Mathis CA, Price JC, Aizenstein HJ, DeKosky ST, Caselli RJ. Fibrillar amyloid-beta burden in cognitively normal people at 3 levels of genetic risk for Alzheimer's disease. Proc Natl Acad Sci U S A 2009; 106: 6820-5.

Roberson ED, Scearce-Levie K, Palop JJ, Yan F, Cheng IH, Wu T, Gerstein H, Yu GQ, Mucke L. Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model. Science 2007; 316: 750–754.

Rogaeva E, Meng Y, Lee JH, Gu Y, Kawarai T, Zou F, Katayama T, Baldwin CT, Cheng R, Hasegawa H, Chen F, Shibata N, Lunetta KL, Pardossi-Piquard R, Bohm C, Wakutani Y, Cupples LA, Cuenco KT, Green RC, Pinessi L, Rainero I, Sorbi S, Bruni A, Duara R, Friedland RP, Inzelberg R, Hampe W, Bujo H, Song YQ, Andersen OM, Willnow TE, Graff-Radford N, Petersen RC, Dickson D, Der SD, Fraser PE, Schmitt-Ulms G, Younkin S, Mayeux R, Farrer LA, St George-Hyslop P . The neuronal sortilin-related receptor SORL1 is genetically associated with Alzheimer disease. Nat Genet 2007; 39: 168-77.

Roncero C. The genetic complexity of chitin synthesis in fungi. Curr Genet. 2002 Sep; 41(6): 367-78.

Rothman SM, Olney JW. Excitotoxicity and the NMDA receptor — still lethal after eight years. Trends Neurosci 1995; 18: 57-8.

Rottkamp CA, Raina AK, Zhu X, Gaier E, Bush AI, Atwood CS, Chevion M, Perry G, Smith MA. Redox-active iron mediates amyloid-beta toxicity. Free Radic Biol Med 2001; 30: 447-50.

Sager KL, Wuu J, Leurgans SE, Rees HD, Gearing M, Mufson EJ, Levey AI, Lah JJ. Neuronal LR11/sorLA expression is reduced in mild cognitive impairment. Ann Neurol 2007; 62: 640-7.

Scheff SW, Price DA, Schmitt FA, DeKosky ST, Mufson EJ. Synaptic alterations in CA1 in mild Alzheimer disease and mild cognitive impairment. Neurology 2007; 68: 1501-1508.

Scheff SW, Price DA, Schmitt FA, Mufson EJ. Hippocampal synaptic loss in early Alzheimer's disease and mild cognitive impairment. Neurobiol Aging 2006; 27: 1372-1384.

Schultzberg M, Lindberg C, Aronsson AF, Hjorth E, Spulber SD, Oprica M. Inflammation in the nervous system: physiological and pathophysiological aspects. Physiol Behav. 2007 Sep 10; 92(1-2): 121-8.

Selkoe DJ. Alzheimer's disease results from the cerebral accumulation and cytotoxicity of amyloid beta-protein. J Alzheimers Dis. 2001 Feb; 3(1): 75-80.

Selkoe DJ. Soluble oligomers of the amyloid beta-protein impair synaptic plasticity and behavior. Behav Brain Res 2008; 192: 106–113.

Selkoe DJ. Translating cell biology into therapeutic advances in Alzheimer's disease. Nature 1999; 399: A23-31.

Semino CE, Specht CA, Raimondi A, Robbins PW. Homologs of the Xenopus developmental gene DG42 are present in zebrafish and mouse and are involved in the synthesis of Nod-like chitin oligosaccharides during early embryogenesis. Proc Natl Acad Sci U S A 1996; 93: 4548-53.

Shankar GM, Bloodgood BL, Townsend M, Walsh DM, Selkoe DJ, Sabatini BL. Natural oligomers of the Alzheimer amyloid-β protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. J Neurosci 2007; 27: 2866-2875.

Shankar GM, Metha TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, Lemere CA, Regan CM, Walsh DM, Sabatini BL, Selkoe DJ. Amyloid-beta protein dimers isolated directly from Alzheimer’s brains impair synaptic plasticity and memory. Nat Med 2008; 14: 837-842.

Shibata Y, Foster LA, Bradfield JF, Myrvik QN. Oral administration of chitin down-regulates serum IgE levels and lung eosinophilia in the allergic mouse. J Immunol. 2000 Feb1; 164(3): 131421

Shibata Y, Honda I, Justice JP, Van Scott MR, Nakamura RM, Myrvik QN. Th1 adjuvant N-acetyl-D-glucosamine polymer up-regulates Th1 immunity but down-regulates Th2 immunity against a mycobacterial protein (MPB-59) in interleukin-10-knockout and wild-type mice. Infect Immun. 2001 Oct; 69(10): 6123-30.

Shibata Y, Metzger WJ, Myrvik QN. Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production. J Immunol. 1997 Sep 1; 159(5): 2462-7.

Simpson IA, Chundu KR, Davies-Hill T, Honer WG, Davies P. Decreased concentration of GLUT1 and GLUT3 glucose transporters in the brains of patients with Alzheimer's disease. Ann Neurol 1994; 35: 546-51.

Simpson IA, Davies P. Reduced glucose transporter concentrations in brains of patients with Alzheimer's disease. Ann Neurol 1994; 36: 800-1.

Sipe JD, Cohen AS. Review: history of the amyloid fibril. J Struct Biol. 2000 Jun; 130(2-3): 88-98.

Smith GS, de Leon MJ, George AE, Kluger A, Volkow ND, McRae T, Golomb J, Ferris SH, Reisberg B, Ciaravino J. Topography of cross-sectional and longitudinal glucose metabolic deficits in Alzheimer’s disease. Pathophysiologic implications. Arch Neurol 1992; 49: 1142–50.

Snow AD, Wight TN, Nochlin D, Koike Y, Kimata K, DeArmond SJ, Prusiner SB. Immunolocalization of heparan sulfate proteoglycans to the prion protein amyloid plaques of Gerstmann-Straussler syndrome, Creutzfeldt-Jakob disease and scrapie. Lab Invest. 1990 Nov;63(5):601-11.

Solomon B, Frenkel D. Generation and brain delivery of anti-aggregating antibodies against beta-amyloid plaques using phage display technology. J Neural Transm Suppl. 2002; (62): 321-5.

Sotgiu S, Barone R, Arru G, Fois ML, Pugliatti M, Sanna A, Rosati G, Musumeci S. Intrathecal chitotriosidase and the outcome of multiple sclerosis**.** Mult Scler 2006a; 12:551-557.

Sotgiu S, Barone R, Zanda B, Arru G, Fois ML, Arru A, Rosati G, Marchetti B, Musumeci S. Chitotriosidase in patients with acute ischemic stroke. Eur Neurol. 2005;54(3):149-53.

Sotgiu S, Musumeci S, Marconi S, Gini B, Bonetti B. Different content of chitin-like polysaccharides in multiple sclerosis and Alzheimer's disease brains. J Neuroimmunol 2008; 197: 70-3.

Sotgiu S, Piras MR, Barone R, Arru G, Fois ML, Rosati G, Musumeci S. Chitotriosidase and Alzheimer's disease. Curr Alzheimer Res 2007a; 4: 295-6.

Stahl SM. The new cholinesterase inhibitors for Alzheimer's disease, Part 1: their similarities are different. J Clin Psychiatry. 2000 Oct; 61(10): 710-1.

Stephenson DT, Rash K, Clemens JA. Amyloid precursor protein accumulates in regions of neurodegeneration following focal cerebral ischemia in the rat. Brain Res. 1992 Oct 9; 593(1): 128-35.

Strittmatter WJ, Roses AD. Apolipoprotein E and Alzheimer's disease: Annu Rev Neurosci 1996; 19: 53-77.

Strong P, Clark H, Reid K. Intranasal application of chitin microparticles down-regulates symptoms of allergic hypersensitivity to Dermatophagoides pteronyssinus and Aspergillus fumigatus in murine models of allergy. Clin Exp Allergy. 2002 Dec;32(12):1794-800.

Sur S, Lam J, Bouchard P, Sigounas A, Holbert D, Metzger WJ. Immunomodulatory effects of IL-12 on allergic lung inflammation depend on timing of doses. J Immunol. 1996 Nov 1;157(9):4173-80.

Szekely CA, Breitner JC, Fitzpatrick AL, Rea TD, Psaty BM, Kuller LH, Zandi PP. NSAID use and dementia risk in the Cardiovascular Health Study: role of APOE and NSAID type. Neurology 2008; 70: 17-24.

Tabert MH, Liu X, Doty RL, Serby M, Zamora D, Pelton GH, Marder K, Albers MW, Stern Y, Devanand DP. A 10-item smell identification scale related to risk for Alzheimer's disease. Ann Neurol. 2005 Jul; 58(1): 155-60.

Timmer NM, Herbert MK, Kleinovink JW, Kiliaan AJ, De Waal RM, Verbeek MM. Limited expression of heparan sulphate proteoglycans associated with Aβ deposits in the APPswe/PS1dE9 mouse model for Alzheimer's disease. Neuropathol Appl Neurobiol. 2010 Oct; 36(6): 478-86.

Timmer NM, Kuiperij HB, de Waal RM, Verbeek MM. Do amyloid β-associated factors co-deposit with Aβ in mouse models for Alzheimer's disease? J Alzheimers Dis. 2010; 22(2): 345-55.

Tomiyama T, Matsuyama S, Iso H, Umeda T, Takuma H, Ohnishi K, Ishibashi K, Teraoka R, Sakama N, Yamashita T, Nishitsuji K, Ito K, Shimada H, Lambert MP, Klein WL, Mori H. A model of amyloid β oligomers: their contribution to synaptic alteration, abnormal tau phosphorylation, glial activation, and neuronal loss in vivo. J Neurosci 2010; 30: 4845-4856.

Van Horssen J, Wesseling P, Van den Heuvel LP, de Waal RM, Verbeek MM. Heparan sulphate proteoglycans in Alzheimer's disease and amyloid-related disorders. Lancet Neurol. 2003 Aug; 2(8): 482-92.

Varki A. Does DG42 synthesize hyaluronan or chitin? A controversy about oligosaccharides in vertebrate development. Proc Natl Acad Sci U S A. 1996 May 14; 93(10): 4523-5.

Verdier Y, Penke B. Binding sites of amyloid beta-peptide in cell plasma membrane and implications for Alzheimer's disease. Curr Protein Pept Sci. 2004 Feb; 5(1): 19-31.

Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ. Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo. Nature 2002; 416: 535-539.

Walsh DM, Selkoe DJ. A β oligomers: a decade of discovery. J Neurochem 2007; 101: 1172-1184.

Wang HW, Nogales E. Nucleotide-dependent bending flexibility of tubulin regulates microtubule assembly. Nature. 2005 Jun 16;435(7044):911-5.

Wang J, Dickson DW, Trojanowski JQ, Lee VM. The levels of soluble versus insoluble brain Abeta distinguish Alzheimer's disease from normal and pathologic aging. Exp Neurol 1999; 158: 328-337.

Weggen S, Eriksen JL, Sagi SA, Pietrzik CU, Ozols V, Fauq A, Golde TE, Koo EH. Evidence that nonsteroidal anti-inflammatory drugs decrease amyloid beta42 production by direct modulation of gammasecretase activity. J Biol Chem 2003; 278: 31831-7.

Whitlock JR, Heynen AJ, Shuler MG, Bear MF. Learning induces long-term potentiation in the hippocampus. Science 2006; 313: 1093-1097.

Winkler J, Connor DJ, Frautschy SA, Behl C, Waite JJ, Cole GM, Thal LJ. Lack of long-term effects after beta-amyloid protein injections in rat brain. Neurobiol Aging. 1994 Sep-Oct; 15(5): 6017.

Winklhofer KF, Tatzelt J, Haass C. The two faces of protein misfolding: gain- and loss-of-function in neurodegenerative diseases. EMBO J. 2008 Jan 23; 27(2): 336-49.

Wisniewski HM, Frackowiak J. Commentary to: Differences between the pathogenesis of senile plaques and congophilic angiopathy in Alzheimer disease. J Neuropathol Exp Neurol 1997; 56:751-61) J Neuropathol Exp Neurol. 1998 Jan; 57(1): 96-8.

Wyss-Coray T, Mucke L. Inflammation in neurodegenerative disease-a double-edged sword. Neuron 2002a; 35: 419-32.

Wyss-Coray T, Yan F, Lin AH, Lambris JD, Alexander JJ, Quigg RJ, Masliah E Prominent neurodegeneration and increased plaque formation in complement-inhibited Alzheimer's mice. Proc Natl Acad Sci U S A. 2002 Aug 6; 99(16): 10837-42.

Xie Y, Yao Z, Chai H, Wong WM, Wu W. Potential roles of Alzheimer precursor protein A4 and beta-amyloid in survival and function of aged spinal motor neurons after axonal injury. J Neurosci Res. 2002 Aug 15; 73(4): 557-64.

Yoshida M, Itano N, Yamada Y, Kimata K. In vitro synthesis oh hyaluronan by a single protein derived from mouse HAS1 gene and characterization of amino acid residues essential for the activity. J Biol Chem 2002; 275: 497-506.