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Neuroprotective strategies against neurodegeneration in cellular model systems

Presentata da: **ELEUTERI SIMONA CARMEN**

Coordinatore Dottorato **Relatore**

Dr. Michela Rugolo **Dr.Antonio Contestabile**

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ABSTRACT

In the present study we analyzed new neuroprotective therapeutical strategies in PD (Parkinson's disease) and AD (Alzheimer's disease). Current therapeutic strategies for treating PD and AD offer mainly transient symptomatic relief but it is still impossible to block the loss of neuron and then the progression of PD and AD. There is considerable consensus that the increased production and/or aggregation of α-synuclein (α-syn) and β-amyloid peptide (Aβ), plays a central role in the pathogenesis of PD, related synucleinopathies and AD. Therefore, we identified antiamyloidogenic compounds and we tested their effect as neuroprotective drug-like molecules against α-syn and β-amyloid cytotoxicity in PC12. Herein, we show that two nitro-catechol compounds (entacapone and tolcapone) and 5 cathecol-containing compounds (dopamine, pyrogallol, gallic acid, caffeic acid and quercetin) with antioxidant and anti-inflammatory properties, are potent inhibitors of α-syn and β-amyloid oligomerization and fibrillization. Subsequently, we show that the inhibition of α-syn and β-amyloid oligomerization and fibrillization is correlated with the neuroprotection of these compounds against the α-syn and β-amyloid-induced cytotoxicity in PC12.

Finally, we focused on the study of the neuroprotective role of microglia and on the possibility that the neuroprotection properties of these cells could be use as therapeutical strategy in PD and AD. Here, we have used an in vitro model to demonstrate neuroprotection of a 48 h-microglial conditioned medium (MCM) towards cerebellar granule neurons (CGNs) challenged with the neurotoxin 6-hydroxydopamine (6-OHDA), which induces a Parkinson-like neurodegeneration, with $A\beta_{42}$, which induces a Alzheimer-like neurodegeneration, and glutamate, involved in the major neurodegenerative diseases. We show that MCM nearly completely protects CGNs from 6-OHDA neurotoxicity, partially from glutamate excitotoxicity but not from Aβ42 toxin.

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INTRODUCTION

1.1 ALZHEIMER'S DISEASE

In November 1901, Alois Alzheimer admitted at Anatomic Laboratory at Royal Psychatric Clinic of the University of Munich a 51-year-old patient named Auguste D. with progressive memory loss, language disturbances, visuospatial deficits, imaired judgment, motivation and neuropsychiatric synptoms as depression, anxiety, sleep disturbance and focal simptoms. After the death of the patient the brain was analysed and discovered neuritic plaques and neuropathological fibrillary tangles using the silver staining methods. In the 1906 Alois Alzheimer presented the clinical and neurophatological characteristics of a disease that subsequently Emil Krapeln named Alzheimer's disease (AD).

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia among people age 65 and older. It has been estimated that 1997, the disease affected more than 2 million older people in the United States alone (Brookmeyer et al., 1998). In the 2003, the Alzheimer's Association estimed 4.5 million US cases in the year 2000 and predicted that the number will rise 3-fold to 13.2 million by 2050 (Hebert et al., 2000).

1.1.1 Clinical features and Neuropathology

AD characterized by early memory deficits, followed by progressive alteration of the major cognitive functions including deterioration of language as well as defects in visual and motor coordination, in judgement and emotions (Cummings et al., 2004).

Alzheimer disease pathology are characterized into three broad chapter : lesions realted to accumulation used to detect and constitute the basis of diagnosis ; losses of neurons and synapses and finally reactive process (i.e. inflammation and plasticity). The lesions related to accumulation are mainly constituted by extracellular plaques (Fig 1.1a) and intracellular aggregates of neurofibrillary tangles (NFTs) (Fig 1.1b).

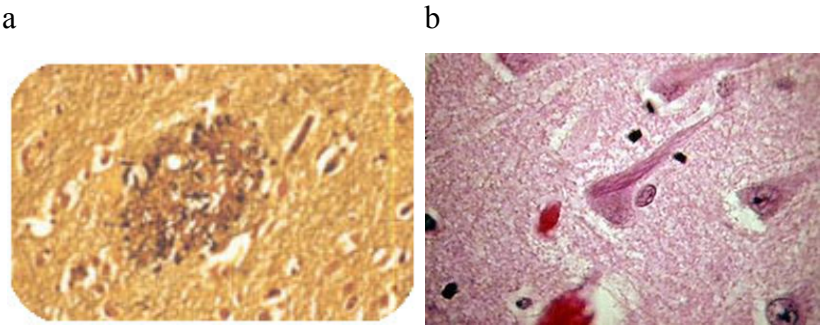


Figure 1.1 deposits in AD. Extracellular amyloid plaques (a) and intracellular aggregates of neurofibrillary tangles (b).

The AD brain is characterized by specific neuroanatomy alterations as neuronal cells and synapses loss at specific sites (Selkoe et al., 2002). The progressive loss of neurons results in athrophy of the affected regions of the brain, reported in Fig 1.2 and Fig 1.3.

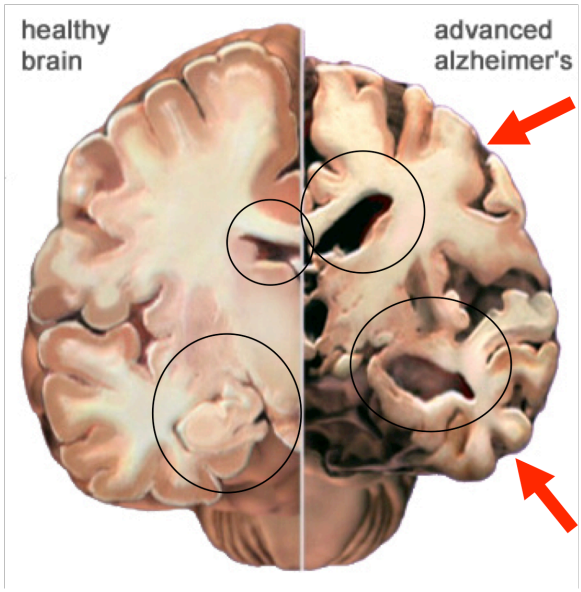


Figure 1.2 Athrophy of the parietal and temporal cortex, hyppocampus and amigdala in AD brain

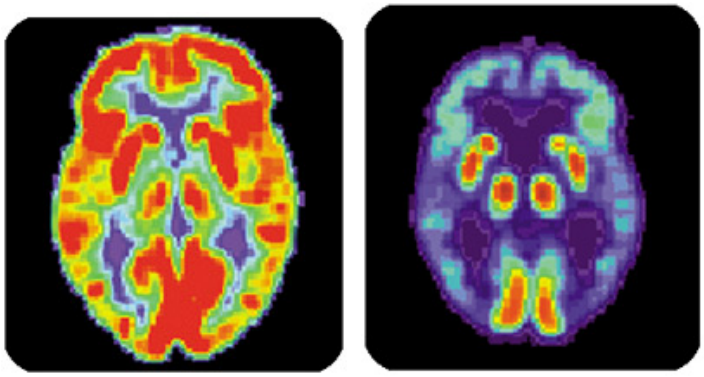


Figure 1.3 Pet scan of normal brain (a) and Pet scan of AD brain

The major neuropathological changes occur in the hippocampus, parietal and temporal cortex and subcortical structures including amygdala and nucleus basalis of Meynert (Arnold et al., 1991). Signs of preclinical AD are first noticed in the entorhinal cortex, them proceed to the hippocampus. The affected regions begin to shrink as nerve cells die and changes can begin 10-20 years before symptoms appear. The memory loss is the first sign of AD (fig 1.4 a and d). In the mild to moderate AD the cerebral cortex begins to shrink as more and more neurons stop working and die. Mild AD signs can include memory loss, confusion, trouble handing money, poor judgement, mood changes and increased anxiety. Moderate AD signs can include increased memory loss and confusion, problems recognizing people, difficulty with language and thoughts, restlessness, agitation, wandering, and repetitive statements (fig 1.4 b and e). In severe AD, extreme shrinkage occurs in the brain. Patients are completely dependent on others for care. Symptoms can include weight loss, seizures, skin infections, groaning, moaning, or grunting, increased sleeping, loss of bladder and bowel control. Death usually occurs from aspiration pneumonia or other infections. Caregivers can turn to a hospice for help and palliative care (fig 1.4 c and f).

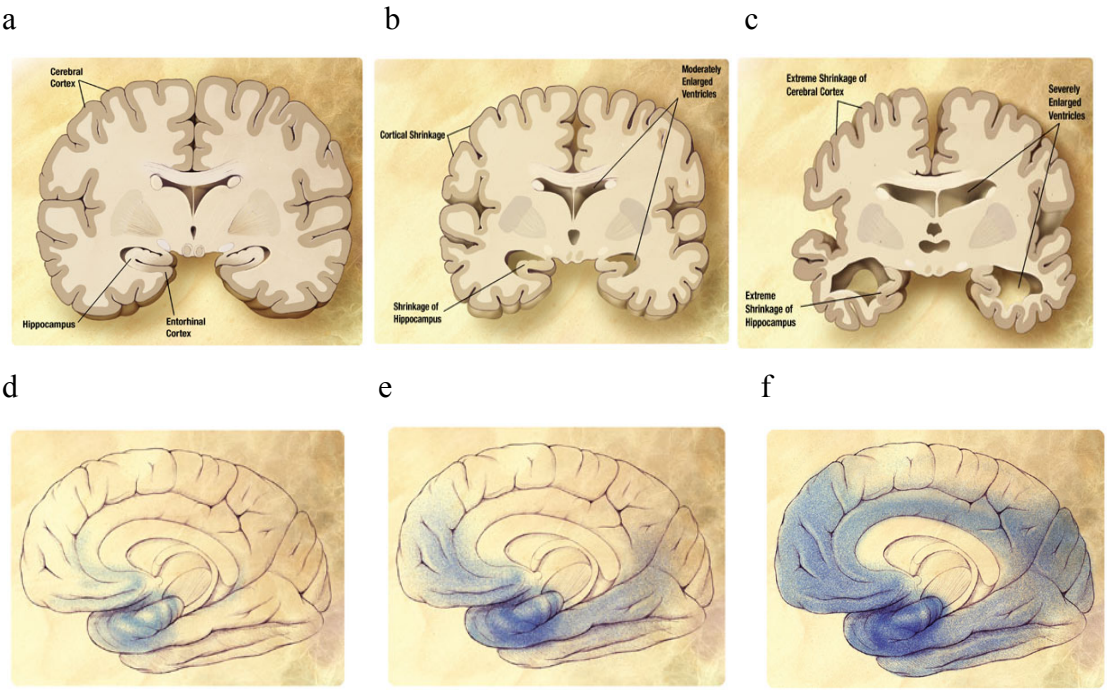


Figure 1.4 Preclinical AD (a and d) ; mild to moderate AD (b and c) ; severe AD (c and f). (National Institute on Aging, USA).

1.1.2 Amyloid plaques

The amyloid plaques are dense deposits of protein and cellular material accumulated outside and around nerve cells. The major constituent of plaques is a 40-42 amino acids peptide termed Aβ ($A\beta_{40}$ and $A\beta_{42}$) that is derived by proteolytic cleavage from the amyloid precursor protein (APP), the general mechanism is reported in Fig 1.5. box1 (Jurgen Gotz and Lars M Ittner, 2008).

The human APP gene was identified in 1987 localized on chromosome 21. More than 25 mutations in APP gene have been identified that are causative of the hereditary form of familial AD by the introduction of mutations within or flanking the Aβ domain. AD was found associated with Down syndrome patients in which the APP gene is triplicated (Lemere et al., 1996).

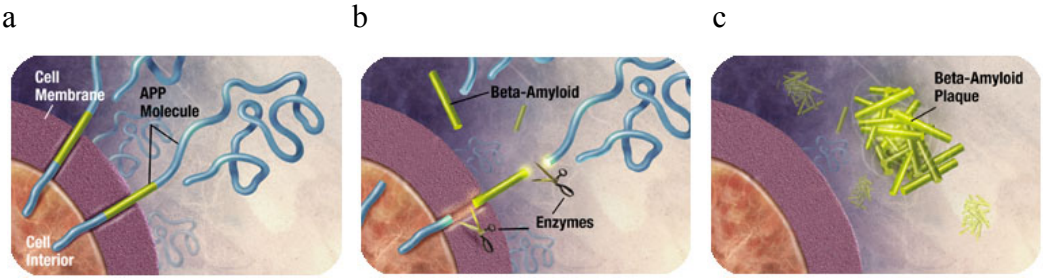


Figure 1.5 general mechanis of Aβ peptide formation from APP; APP sticks through the neuron membrane (a); enzymes cut the APP into fragments of protein, including beta-amyloid(b); beta-amyloid fragments come togheter in clumps to form plaques (c).

The APP is a transmembrane protein cleaved by different secretases. APP is first cleaved within the lumenal domain by β-secretase or γ-secretase resulting in the shedding of nearly the entire ectodomain and generation of memebrane-tethered β- or α-C-terminal fragments respectively. The β-fragment and γ-fragment are subsequently cleaved within the transmembrane domain by γ-secretase to release Aβ (4kDa) and p3 (3kDa) peptides into the extracellular milieu. In addition the γ-secretase generates a cytoplasmic polypeptide termed AICD, showed in fig 1.6 (Thinakaran et al., 2008). Several zinc metalloproteinases such as BACE2 can cleave APP at the α-secretase site and the major β-secretase is a transmembrane aspartyl protease termed BACE1. The proteolytic cleavage of APP by β-secretase generates the amino terminus of Aβ and γ-

secretase induces its length (40 or 42 amino acids). γ-secretase is formed of four subunits : presenilin-1 or presenilin-2, nicastrin, APH-1 and PEN-2. The deposits contain a mixture of various Aβ isoforms. The Aβ40 peptide is much more prevalent than the aggregation-prone and damaging Aβ42 that contains two supplementary amino acids at the C-terminus of the peptide. (Duyckaerts et al., 2009). An increasing ratio of the full-length, $A\beta_{42}$ peptide to te $A\beta_{40}$ form is associated with the disease (Kumar-Singh et al., 2006). The deposition of Aβ peptide is related to an imbalance between production and clearence of the amyloid protein (Glenner et al., 1984).

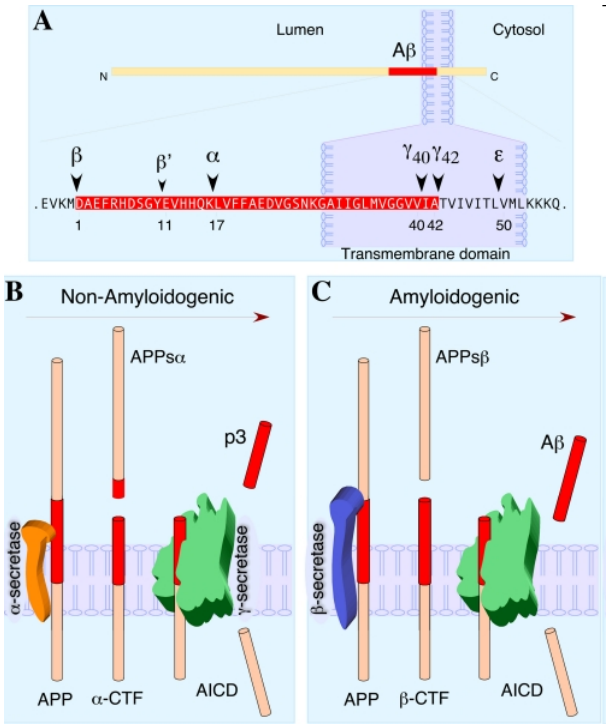


Figure 1.6 Proteolytic processing of APP. *A*, the schematic structure of APP is shown with the Aβ domain shaded in *red* and enlarged. The major sites of cleavage by α-, β-, and γ-secretases are indicated along with Aβ numbering from the N terminus of Aβ (Asp1). *B*, non-amyloidogenic processing of APP refers to sequential processing of APP by membrane-bound α- and γ-secretases. α-Secretase cleaves within the Aβ domain, thus precluding generation of intact Aβ peptide. The fates of N-terminally truncated Aβ (p3) and AICD are not fully resolved. *C*, amyloidogenic processing of APP is carried out by sequential action of membrane-bound β- and γ-secretases. *CTF*, C-terminal fragment. (J Biol Chem. 2008 October 31; 283(44): 29615–29619)

The function of Aβ peptide is still unknown, probably the peptide is physiologically produced at low concentration like a waste product. Recently studies showed that at picomolar concentration the Aβ peptide positively modulates synaptic plasticity and memory in hippocampus (Puzzo et al., 2008). The Aβ peptide is degradated by various emzymes as well as neprilysin and insulin degrading enzyme (IDE).

Neprilysin is a membrane-anchored zinc endopeptidase and the insulin-degrading enzyme is a thiol metalloendopeptidase that degrades small peptides such as insulin and monomeric Aβ. Oerexpression of neprilysin or insulin-degrading enzyme prevents plaque formation (Leissring et al., 2003).
The amyloid cascade hypothesis (selkoe lesson from Alzheimer's peptide)

1.1.3 Types of Aβ deposits in AD

The identification of extracellular deposits of Aβ peptide has been revealed by anti-Aβ antibodies or by Congo red and thioflavine S staining. The deposits could be parenchymal deposits or vascular deposits. The parenchymal deposits differ for their shape and may be distinguished in diffuse, focal or stellate Aβ deposits. (Delaere et al., 1990). The stellate deposits are probably related to astrocytes and rarely studied. The focal type of deposits may be amyloid (Congo red and thioflavine s positive). The diffuse Aβ deposits are usually large and ill-limited and in some regions of the brains the deposits are diffuse. The diffuse deposits have been found in the presubiculum, in the internal layers of the entorhinal cortex, in the striatum, in molecular layer of cerebellum and in the subpial region of the isocortex. The diffuse deposits have been found in subjects whose intellectual status had been evaluated normal to indicate that these lesions may not be directly toxic. (Duyckaerts et al., 2009). The focal deposits showed dense and spherical accumulations of Aβ peptide and may be amyloid or not. The parenchymal deposits are associated with various proteins, lipids and cells as well as ApoE produced by astrocytes and involved in cholesterol transport and the Apoε4 allele is considered a risk factor of AD ; (Namba et al., 1991). the deposits are associated with ApoJ, also called clusterin, (Martin-Rehrmann et al., 2005) with metals and components of the matrix like ICAM1 (Verbeek et al., 1994). thrombospondin (Buee et al., 1992), heparan sulfate proteoglycan. The Cathepsin D, a lysosomal enzyme, is localized around the the plaques and it could be related to the activation of the endosomal-lysosomal pathway (Cataldo et al., 1990).
Sometimes the Aβ deposits have been found in the vessel walls inducing lobar hemorrhages and small cortical infarcts. Mutation in APP gene has been found in hereditary cerebral hemorrhages (Lewy et al., 1990)

1.1.4 Tau accumulation

Neurofibrillary tangles, which are filamentous inclusion in neurons occur in AD and other neurodegenerative disorders termed taupathies. The major component of the tangles is an abnormally hyperphosphorylated and aggregated form of tau. Usually, tau is involved in the stability of the microtubules and vescicle transport. Hyperphosphorylated tau self-associates into paired helical filament structures (Fig 1.7).

Neurons have an internal support structure partly made up of microtubules. The protein tau helps stabilize microtubules. In AD tau changes, causing microtubules to collapse and tau proteins clump togheter to form neurofibrillary tangles.

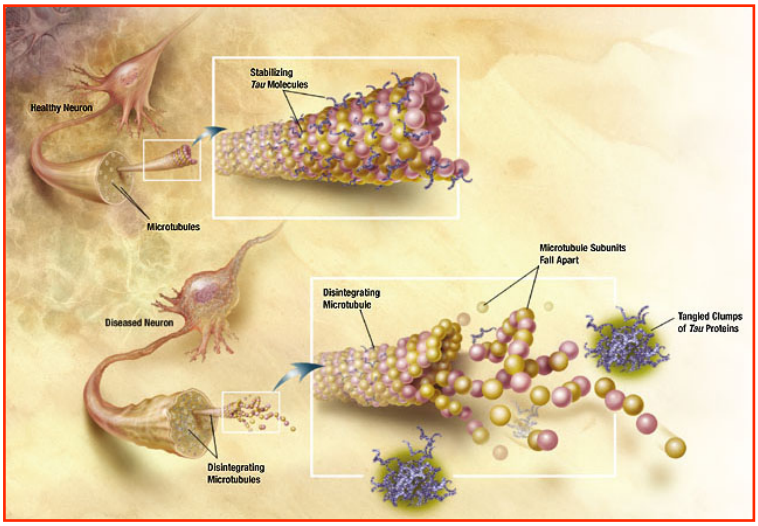


Figure 1.7 Tau structure and function

1.1.5 Genetics

The famlial AD (FAD) represent less than 1% of the total number of AD cases. Autosomal dominant mutations have been identified in three genes : APP, preselin1 (PSEN1) and preselin2 (PSEN2) which are proteolytic subunits of the γ-secretase complex generating the C-terminus of Aβ. The vast majority of FAD patients bear mutations in the genes encoding PS1 and PS2 ; more than 130 mutations in the PENS1 and PENS 2 have been identified. Pathogenic puntiform mutations have been identified in APP, and these mutation predominantly affect residues 22 and 23 including Dutch (E22Q), Italian (E22K), Iowa (D23N), Flemish (A21G) and arctic (E22G) (Nilsberth et al., 2001),V717I London mutation (Goate et al.,), These mutations in APP aggregate faster than wild type the peptide (Kirkitadze et al., 2001). In the sporadic AD (SAD) various susceptibility genes have been identified including apolipoprotein E (APOE) (Bertram & Tanzi, 2005).

The most prominent example of elevated Aβ concentration is the gene dosage effect in trisonomy 21 patients (Lemere et al., 1996).

1.2 PARKINSON'S DISEASE

1.2.1 Clinical features and Neuropathology

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease (AD). PD affects 1% of people beyond 65 years of age with high prevalence in men. Although approximately 10% of cases affect people under age 40. PD was first formally described in 1817 by a London physician named James Parkinson. The major symptoms involve: uncontrollable resting tremor, rigidity with increase muscle tone and increase resistance to movement, postural instability, bradykinesia and gait disturbance, which vary between the patients. Usually these symptoms could be accompanied with dementia, depression, dystonia.

Early pathological changes in PD include the selective loss of dopaminergic neurons of the *substantia nigra pars compacta* and other areas of the brain resulting in the degeneration of the nigro-striatal tract and loss of dopamine (Recchia rt al., 2004).

Neuropathologically, PD is characterized by the formation of intraneuronal Lewy bodies (LBs) and Lewy neuritis (LNs), reported in Fig 1.8. LBs and LNs consist primarily of fibrillar aggregated of α-synuclein (α-syn) (Spillantini et al., 1997) .

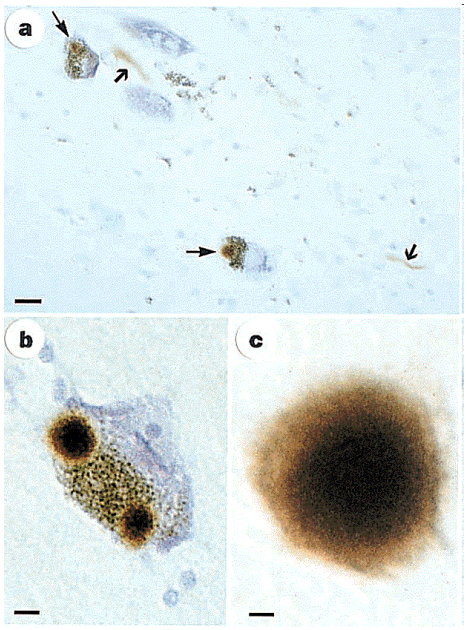


Figure 1.8 **a,** Two pigmented nerve cells, each containing an α-synuclein-positive Lewy body (thin arrows). Lewy neurites (thick arrows) are also immunopositive. Scale bar, 20m. **b,** A pigmented nerve cell with two α-synuclein-positive Lewy bodies. Scale bar, 8m. c, α-Synuclein-positive, extracellular Lewy body. Scale bar, 4m. (Spillantini, et al., 1997).

1.2.2 α-synuclein protein

The α-synuclein is a cytosolic protein of 14 kDa, 140 amino acids, "natively unfolded", present in extracellular amyloid plaques in some forms of Alzheimer's disease patients. (Recchia et al., 2004) .

The synuclein family include α-Syn, β-Syn and γ-Syn. α-Syn and β-Syn are predominantly expressed in brain at presynaptic terminals, particularly in the neocortex, hippocampus, striatum, thalamus, and cerebellum Figure 1.9 (Iwai et al., 1995). The α-syn is high expressed in the substantia nigra and has been found overexpressed in breast and ovarian tumors (Lavedan et al., 1998). The α-syn gene has been mapped to the human chromosome 4q21, the β-Syn to the chromosome 5q35 (Spillantini MG et al., 1995) and the γ-Syn to the chromosome 10q23 (Lavedan et al.,1998).

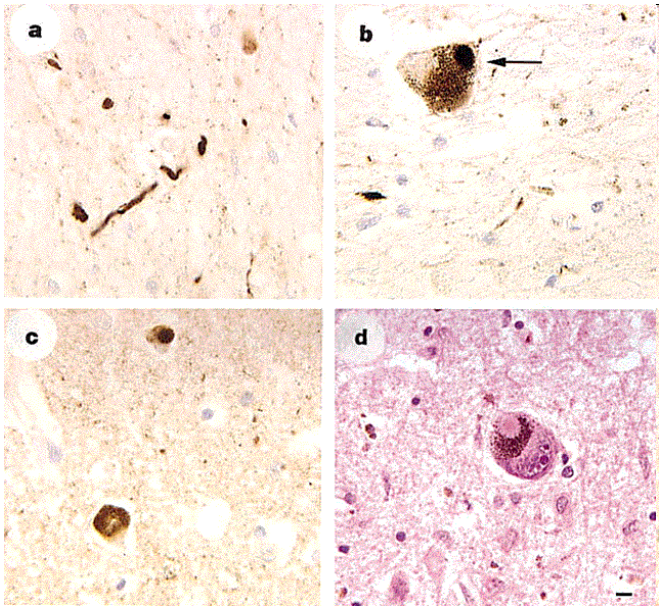


Figure 1.9 **a,** α-Synuclein-positive Lewy neurites in the substantia nigra. **b,** α -Synuclein-positive Lewy body (arrow) in pigmented nerve cell of the substantia nigra. **c,** Two α -synuclein-positive Lewy bodies in the cingulate cortex. **d,** Haematoxylin and eosin-stained section of substantia nigra with a pigmented nerve cell containing a Lewy body. Scale bar for **a-d,** 10 m. (Spillantini, et al., 1997).

The sequence of α-synuclein protein can be subdivided in three domains: N-terminal domain, NAC domain and C-terminal domain, reported in figure 1.10. The N-terminus domain is highly conserved (residues 1-65) include six copies of 11aa imperfect repeat that display variations of KTKEGV consensus sequence and can shift to an α-helical conformation that consist of two α-helixes. These amphipathic α-helixes can bind to negatively charged phospholipids suggesting that the protein could be associated with the membrane. In the alpha-helix forming domain have been found two independent missense mutations, A53T and A30P, associated with autosomal dominant heritable early-onset PD (Clayton et al., 1999). These mutations are linked to the disease inducing the protein structure destabilization and the subsequently protein aggregation (Rochet et al.).

The NAC region is a central hydrophobic domain (residues 66-95), involved in the aggregation of the protein for the shift of the random-coil to β-sheet structure (El-Agnaf et al.,2002). This domain has been found in the plaques in AD (Gisson et al, 2001).

The C-terminus domain (residues 96-140) is primarily composed of acidic amino acids and is not very conserved between species. In the C-terminus domain have been identified several phosphorylation sites: Tyr-125, Tyr-133, Tyr-136 and Ser-129. The

Ser 129 has been found phosphorilated in synucleinopathy lesions, including LBs (Fujiwara et al., 2002).

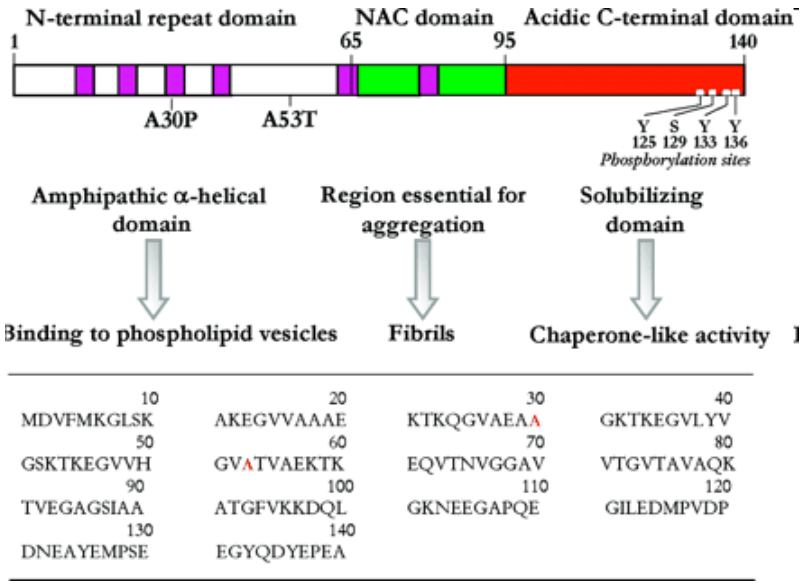


Figure 1.10. Human α-syn sequence and domains. The imperfect KTKEGV repeats are shown by violet columns. Missense mutations at residues 30 (A30P) and 53 (A53T) are shown in red. Recchia et al., 2004).

Accumulating evidence from genetics, animal models, biochemical and biophysical studies suggest that α-syn plays a central role in the initiation and/or progression of PD (Cookson et al., 2009) Mutations or increased expression of α-syn are associated with early-onset familial forms of PD (Chung et al.2003; Li et al., 2001; Zarranz et al., 2004).

Overexpression of wild type and disease-associated mutants enhances α-syn aggregation and toxicity in several animal and cellular models of synucleinopathies (Chen et al., 2005); Lo Bianco et al., 2008; Masliah et al., 2000). *In vitro* studies have consistently shown that disease-associated mutations accelerate and enhance α-syn oligomerization (A30P, A53T and E46K) and/or fibrillization (A53T and E46K) (El-Agnaf et al., 1998; Paleologou et al., 2008; Conway et al., 2000; Conway et al., 2000). Despite these advances in our understanding of the molecular mechanism of α-syn aggregation and toxicity *in vitro* and *in vivo*, very little is known about the 1) normal function of α-syn; 2) the relative contribution of α-syn aggregation to the

pathogenesis of PD; 3) identity of the toxic α-syn species; 4) the exact mechanism by which α-syn contributes to the loss of dopaminergic neurons and PD pathology also remains poorly understood (Horwich et al.,2002; Losic et al., 2006; Morgan et al.,2004).

1.3 AMYLOID PROTEIN, AGGREGATION AND ANTIAMYLOID APPROACH IN NEURODEGENERATIVE DISEASE

1.3.1 From misfolding proteins to protein deposition diseases

Different human neurodegenerative disorders share common pathogenic mechanisms involving aggregation and accumulation of misfolding proteins in the Central Nervous System (CNS). Proteins are the major components of the living cells, which play crucial role in the maintenance of life and the conversion of genetic informations into functional proteins is a central process in biology. The proteins to assolve their functions undergo folding process to assume their three-dimensional structures and then adopt the correct native state. The proteins are synthetized on the ribosome and the folding process can be a co-translational process or occurs in the cytoplasm or in specific compartements such as mitochondria or endoplasmic reticulum (ER) after trafficking and traslocation through membranes. The cells have developed specific strategies to avoid that incompletely folded proteins are exposed to the solvent and then to inappropriate interactions with other molecules and macromolecules crowding in intracellular environment in the tranport (Ellis et al., 2001). To counteract these dangeres, cells have developed a sophisticated system of protein quality control that trough degradation of aberrant or misfolded proteins contributes to a healthy intracellular environment. The degradation of proteins can be executed by different proteolytic system such as lysosomal degradation, chaperone-mediated autophagy and substrate-specific degradation by ubiquitin-proteosome system (UPS). The UPS is revelant in the neuronal development and plasticity other than in neurodegenerative disease where a direct linkage between UPS malfunction and disease pathogenesis was associated with an accumulation of ubiquitin conjugates and other UPS-related components in the neuropathological hallmarks of many neurodegenerative disease (van Tijin et al., 2008). The molecular chaperones are present in all type of cells and cellular compartments and they increase the efficency of the overall process by reducing the probability of competing reactions, particularly aggregation. Some chaperones interact with the nascent chains whereas others are involved in guiding later stages of the folding process (Dobson et al, 2003; Young et al., 2004).

Some proteins have the specific propensity to assume a pathogenic conformation which can become evident with the aging. In fact, a broad range of human diseases known as proetin misfolding disease arises from the failure of a specific peptide to adopt or remain in its native functional state. The misfolding diseases also known as protein deposition diseases, include neurodegenerative conditions, such as Alzheimer's disease (AD), Parkinson's disease (PD), the spongiform encephalopathies, Hunghtington's disease (HD), amyotrophic lateral sclerosis (ALS) and include systemic amyloidoses, such as light chain and lysozyme amyloidoses, familial amyloid polyneuropathy and dialysis-related amyloidosis, as well as localized amyloidoses, including type II diabetes and aortic medial amyloidosis, as summarized in table 1 and table 2 . Overall, more than 40 human protein deposition diseases have a distinct clinical profile and each associated with a single dominant amyloid protein or peptide that is prone to aggregate and form amyloid fibrils subsequently accumulate in deposits (Fig 1.11). Amyloids fibrils are derived from various amyloidogenic proteins that initially exist in either a « natively unfolded » or « naturally folded » state. α-synuclein protein accumulated in the Lewy bodies in PD, amiloyd polypeptide (IAPP) aggregates associated with diabetes mellitus type 2 and amyloid β-protein aggregates represent a group of polypeptides whose structures are predominantly random and this unfolded state allows the proteins to be readily self-assembled into the fibrillar suprastructures. On the contrary some amyloidogenic proteins retain their native structures before undergoing conformational transition and eventual fibril formation (Chiti et al., 2009). This group of proteins include prion, β2-microglobulin, lysozyme, transthyretin (TTR) and a variable region of immunoglobulin light chain (VL), which are responsible for mad cow disease, dialysis-related amyloidosis, hereditary systemic amyloidosis, systemic amyloidosis and light-chain amyloidosis (Bhak et al., 2009).

The first indication that protein misfolding and aggregation were involved in neurodegenerative diseases came from post-mortem neurophatological studies. In the post-mortem studies the misfolding proteins have been found accumulated in deposits (Fig 1.11). Support for a casual role of the protein misfolding in the diseases has come from genetic studies (Hardy et al., 1998). Mutations in the genes that encode for the misfolded proteins were associated with the manifestation of neurodegenerative diseases. In the 1991 a missense mutation in the amyloid precursor protein gene was associated with the familial AD (Goate et al., 1991). Subsequently, in the 1997, there

was the first association between the alpha-synuclein gene and familial PD (Polymeropoululos et al., 1997). In the same years genes encoding for misfolding proteins associated with HD (Huntington's Disease Collaborative Research Group (HDCRG) a novel gene containing a trinucleotide repeat that is unstable on Huntington's disease chromosomes Cell (1993) 72: 971-983) prion disease (Hsiao et al., 1989) and SLA (Rosen et al., 1993) were identified. Subsequently the generation of transgenic animal models with mutant forms of genes encoding the misfolding protein have provided evidence for the contribution of these proteins to disease pathogenesis (Soto et al., 2003). Transgenic mouse with overexpression of human amyloid protein precursor (APP) gene progressively developed cerebral amyloid deposits, neuritic dystrophy and behavioral alterations (Games et al., 1995). Similarly, transgenic mice overespressing human gene of α-synuclein showed several clinicophatological characteristics of PD as well as dopaminergic loss and inclusion body formation (Masliah et al., 2000). Transgenic mouse with human mutaded SOD1 gene developed motor neuron dysfunction and hyaline inclusion bodies in degenerating axons, muscle atrophy, astrocytic damage and loss of large myelinated axons of motor neurons (Gurney et al., 1994). In the same manner transgenic mice with human PrP gene overexpression resulted in spontaneous neurogical disease with spongiform degeneration (Hsiao et al., 1990).

The amyloidogenic protein constituent differs among the various neurodegenerative diseases and the aggregates accumulate in early in the lifetime of the individual, but only manifest clinical simptoms in middle or later life.

The deposition of these aggregated material can occur in a large variety of organs and tissues; as summarized in table 1.1 the protein deposition diseases can be also grouped into neurodegenerative conditions, in which aggregation occurs in the brain, non-neuropathic localized amyloidoses, in which aggregation occurs in a single type of tissue other than the brain, and nonneurophatic systemic amyloidoses in which aggregation occurs in multiple tissue, showed in table 1.2.

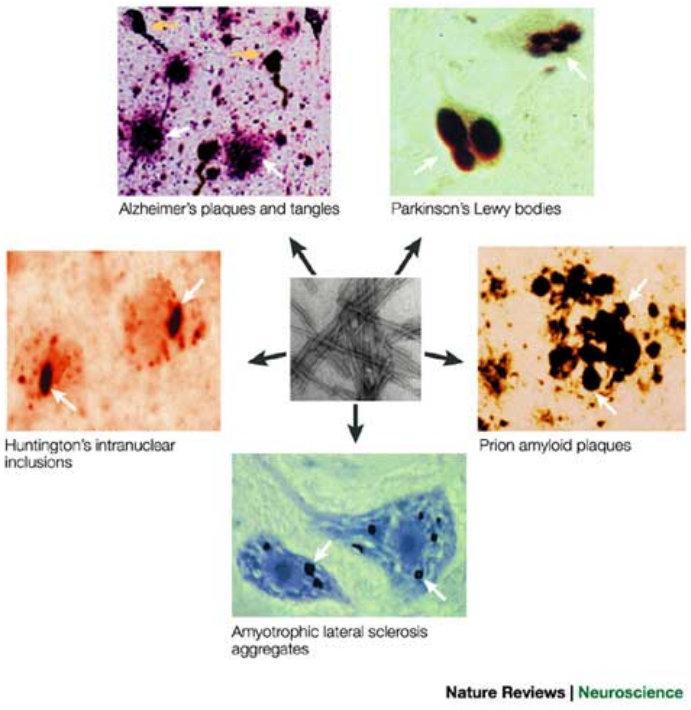


Figure 1.11 Cerebral aggregates in neurodegenerative diseases : Extracellular amyloid plaques (white arrows) and intracytoplasmic neurofibrillary tangles (yellow arrows) are the phatological signature of AD. Intracytoplasmic aggregates are tipically present in the neurons of people affected by PD and SLA. Intranuclear inclusions of huntingtin are observed in Huntington's disease patients and extracellular prion amyloid plaques that are located in different brain regions are present in some cases of transmissible spongiphorm encephalophathy. In spite of the different protein compositions, the ultrastructure of these deposits seems to be similar and composed mainly of a network of fibrillar polymers (centre)(Soto et al., 2003).

Some of these conditions, such as Alzheimer's and Parkinson's diseases are predominantly sporadic (labeled in [c] in Table1.1 and Table 1.2), but genetic forms are caused by mutations in the gene encoding the amyloidogenic protein (mutations in alpha-syn or other mutations). Other conditions, such as the lysozyme and fibrinogen amyloidoses, arise from specific mutations and are hereditary (labeled in [d] in Table1and Table 1.2). In addition to sporadic (85%) and hereditary (10%) forms, spongiform encephalopathies can also be transmissible (5%) in humans as well as in other mammals (Chiti et al., 2006 ; Chiti et al., 2009).

Table 1.1 **Human diseases associated with formation of extracellular amyloid deposits or intracellular inclusions with amyloid-like characteristics in the neurodegenerative diseases**

Disease	Aggregating protein or peptide	Number of residues [a]	Native structure of protein or peptide [b]
Alzheimer's disease [c]	Amyloid β peptide	40 or 42 [f]	Natively unfolded
Spongiform encephalopathies [e,g]	Prion protein or fragments thereof	253	Natively unfolded (residues 1–120) and α-helical (residues 121–230)
Parkinson's disease [c]	α-Synuclein	140	Natively unfolded
Dementia with Lewy bodies [c]	α-Synuclein	140	Natively unfolded
Frontotemporal dementia with Parkinsonism [c]	Tau	352–441 [f]	Natively unfolded
Amyotrophic lateral sclerosis [c]	Superoxide dismutase 1	153	All-β, Ig like
Huntington's disease [d]	Huntingtin with polyQ expansion	3144 [g]	Largely natively unfolded
Spinocerebellar ataxias [d]	Ataxins with polyQ expansion	816 [g,h]	All-β, AXH domain (residues 562–694); the rest are unknown
Spinocerebellar ataxia 17 [d]	TATA box-binding protein with polyQ expansion	339 [g]	α+β, TBP like (residues 159–339); unknown (residues 1–158)
Spinal and bulbar muscular atrophy [d]	Androgen receptor with polyQ expansion	919 [g]	All-α, nuclear receptor ligand-binding domain (residues 669–919); the rest are unknown
Hereditary dentatorubral-pallidoluysian atrophy [d]	Atrophin-1 with polyQ expansion	1185 [g]	Unknown
Familial British dementia [d]	ABri	23	Natively unfolded
Familial Danish dementia [d]	ADan	23	Natively unfolded

[a] Data refer to the number of residues of the processed polypeptide chains that deposit into aggregates,

not of the precursor proteins.[b] According to Structural Classification Of Proteins (SCOP), these are the structural class and fold of the native states of the processed peptides or proteins that deposit into aggregates prior to aggregation.[c] Predominantly sporadic, although in some cases hereditary forms associated with specific mutations are well documented.[d] Predominantly hereditary, although in some cases sporadic forms are documented.[e] Five percent of the cases are transmitted (e.g., iatrogenic).[f] Fragments of various lengths are generated and have been reported to be present in ex vivo fibrils.[g] Lengths shown refer to the normal sequences with nonpathogenic traits of polyQ.[h] Length shown is for ataxin-1.[i] The pathogenic mutation converts the stop codon into a Gly codon, extending the 77-residue protein by 21 additional residues.[j] Human insulin consists of two chains (A and B, with 21 and 30 residues, respectively) covalently linked by disulfide bridges.[k] Medin is the 245–294 fragment of

Table 1.2 **Human diseases associated with formation of extracellular amyloid deposits or intracellular inclusions with amyloid-like characteristics in non neuropathic systemic amyloidoses**

Nonneuropathic systemic amyloidoses			
AL amyloidosis [c]	Immunoglobulin light chains or fragments	90 [f]	All-β, Ig like
AA amyloidosis [c]	Fragments of serum amyloid A protein	76–104 [f]	All-α, unknown fold
Familial Mediterranean fever [c]	Fragments of serum amyloid A protein	76–104 [f]	All-α, unknown fold
Senile systemic amyloidosis [c]	Wild-type transthyretin	127	All-β, prealbumin like
Familial amyloidotic polyneuropathy [d]	Mutants of transthyretin	127	All-β, prealbumin like
Hemodialysis-related amyloidosis [c]	β2-microglobulin	99	All-β, Ig like
ApoAI amyloidosis [d]	N-terminal fragments of apolipoprotein AI	80–93 [f]	Natively unfolded
ApoAII amyloidosis [d]	N-terminal fragment of apolipoprotein AII	98 [i]	Unknown
ApoAIV amyloidosis [c]	N-terminal fragment of apolipoprotein AIV	70	Unknown
Finnish hereditary amyloidosis [d]	Fragments of gelsolin mutants	71	Natively unfolded
Lysozyme amyloidosis [d]	Mutants of lysozyme	130	α+β, lysozyme fold
Fibrinogen amyloidosis [d]	Variants of fibrinogen α-chain	27–81 [f]	Unknown
Icelandic hereditary cerebral amyloid angiopathy [d]	Mutant of cystatin C	120	α+β, cystatin like
Nonneuropathic localized diseases			
Type II diabetes [c]	Amylin, also called islet amyloid polypeptide (IAPP)	37	Natively unfolded
Medullary carcinoma of the thyroid [c]	Calcitonin	32	Natively unfolded
Atrial amyloidosis [c]	Atrial natriuretic factor	28	Natively unfolded
Hereditary cerebral haemorrhage with amyloidosis [d]	Mutants of amyloid β peptide	40 or 42 [f]	Natively unfolded
Pituitary prolactinoma	Prolactin	199	All-α, 4-helical cytokines
Injection-localized amyloidosis [c]	Insulin	21 + 30 [j]	All-α, insulin like

1.3.2 Pathological characterization of protein aggregated deposits

The neurodegenerative diseases are charaterized pathologically by the deposition of insoluble filamentous aggregates in extracellular plaques or intracellular inclusions in the CNS as reported in Figure 1.12. The advances of the human genetics, cellular biology and biochemistry allowed to identify the protein constituents of the deposits. The cytopathological hallmarks of Parkinson's disease are the Lewy bodies (LBs) and Lewy neurites (LNs), in Figure 1.12d. Missense mutations in the gene that encodes for the α-synuclein was identified in family with autosomal-dominant parkinsonism by linkage analysis and positional cloning studies (Polymeropoulos et al., 1997). Subsequently, an immunohistochemistry analysis of the substantia nigra and cingulate cortex extract from patients with Parkinson's disease, allowed the identification of the the α-synuclein protein as the major protein constituent of the LBs and LNs (Spillantini et al., 1997 ; Barba et al., 1998). In a similar manner trough discover of causative genes associated with the specific neurodegererative disorder was possible the identification of aggregation-prone proteins in disorders such as ALS (Rosen et al., 1993 ; Deng et al., 1993) and HD in Fig 1.12e (Hungtington's Disease Collaborative Research Group A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes., Cell 1993, 72 971-983). The pathological hallmarks of Alzheimer's disease are extracellular plaques of amyloid-β peptide (Aβ) and neurofibrillar tangle respectively reported in Figure 1.12 a-b. In the 1980s, the Aβ peptide was before isolated and purified from microvascular amyloid deposits from the meninges of AD brains (Glenner et al., 1984) and afterwards from amyloid plaques core of the post-mortem AD cortices (Masters et al.,1985). In the same years Tau protein phosphorilated was discovered to be the constituent of the neurofibrillar tangle (Haass et al., 2007). Many type of Aβ-deposits were identified using the immunohistochemistry as well as parenchyma deposits or vascular deposits.

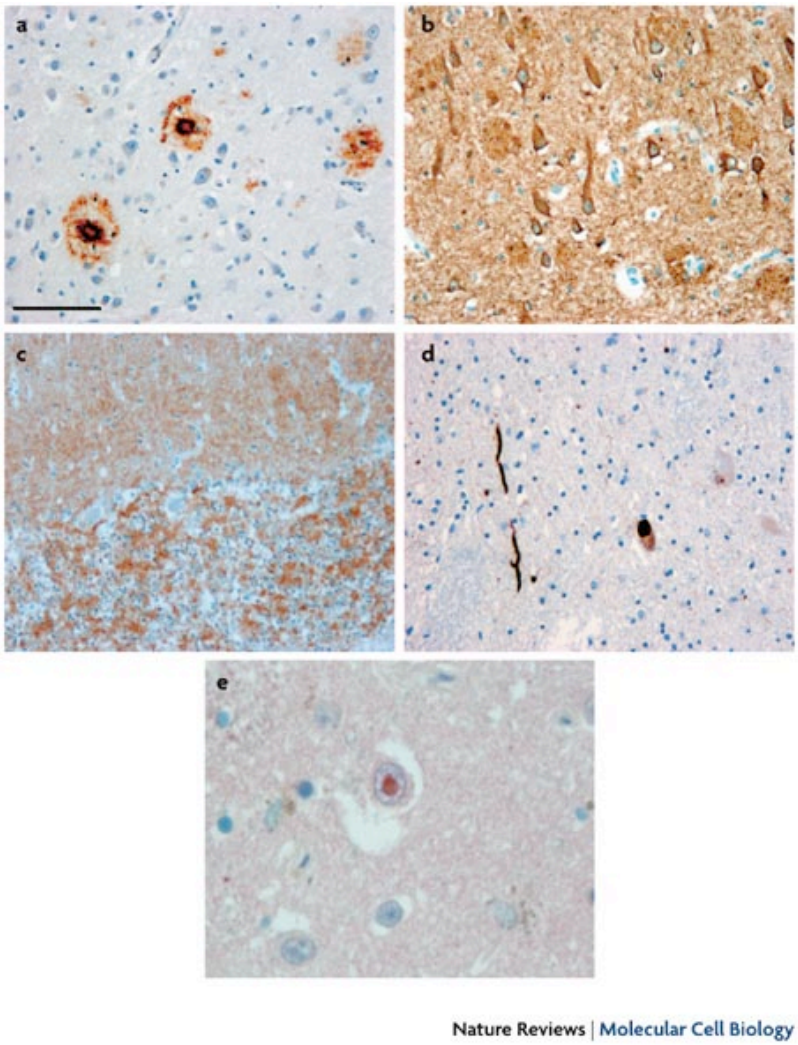


Figure 1.12 Panel **a** shows Alzheimer's disease with Aβ-positive senile plaques, and panel **b** shows tau-positive neurofibrillary tangles, neurophil threads and dystrophic neurites. Panel **c** shows PrP deposits in the cerebellum of a Creutzfeldt-Jacob case. Panel **d** shows substantia nigra of a Parkinson's disease case with an α-synuclein-positive Lewy body (right) and Lewy neurites (left). Panel **e** shows a ubiquitin-positive huntingtin inclusion in a case of Huntington's disease. The scale bar represents 100μm. (Haass et al., 2007)

The extracellular deposits found in patients suffering from any of the amyloid diseases have a major protein component, the misfolding proteins, that form the core and then additional associated species, including metal ions, glycosaminoglycans, the serum amyloid P component, apolipoprotein E, collagen, and many other (Hirschfield et al., 2005).

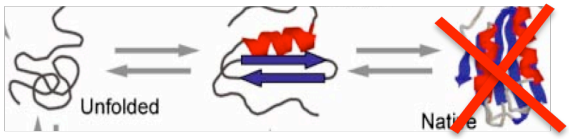
1.3.3 General properties of amyloid fibrils

Aminoacid composition and sequence analysis of the amyloid proteins revealed that each amyloid disorder was associated with a particular protein or peptide. In the neurodegenerative disorder a specific misfolding protein is unable to adopt or remain in its native state and it is converted from its soluble state into well-organised fibrillar aggregates with amyloid-like characteristics and resistant to degradation, reported in Fig 1.13. The characteristics of the soluble forms of proteins involved are very varied from globular proteins to largely unstructured peptide molecules with different primary sequences and with heterogeneity in secondary srtucture composition or chain length but they shared characteristics in commom and these characteristics confers the fibrillar, proteolytic resistant and insoluble properties to all form of amyloid.

Amyloid deposits have specific optical behaviour, they have the ability to bind specific fluorescent dyes such as Congo red (Puchteler et al., 1961 ; Puchtler et al., 1965;) and Thioflavine T (LeVine et al., 1993) that can also block the fibrillization process (Nilsson et al., 2004).

The amyloid fibrils also shared similar structural morphologies and characteristic cross-β X-ray fiber diffraction pattern (Sunde et al.,1997). In the 1971 lysosomal extracts was discovered to digeste immunoglobulin light chain precursor protein into amyloidogenic fragments suggesting that many amyloid forming peptides were produced by the proteolytic processing of a precursor protein (Glenner et al., 1971)GG, Terry W, Harada M, et al Amyloid fibril prpteins : proof of homology with immunoglobulin light chain by sequence analysis. Science 1971 ; 172 :1150-1).

a



b

Fig 13 The misfolding protein is unable to adopt and remain in its native state (a) and it is converted from its soluble state into well organised fibrillar aggregates with amyloid-like characteristics (b)

1.3.4 Structure of amyloid fibrils

The studies on the charcterization of amyloid fibrils were carried out in the 1960s. The term «amyloid » was coined initially by Schleiden and then by Virchow in the1954 to describe the apparently tinctorial strach-like properties of the deposits. (Virchow et al., 1954). Further staining with the dye Congo Red was observed to produce a characteristic green birefringence when examined between cross-polarized light microscope (in Fig 1.14) (Missmahl et al., 1953; Choen et al., 1965) suggesting that amyloids had fibrillar structure (Friedrich et al., 1859).

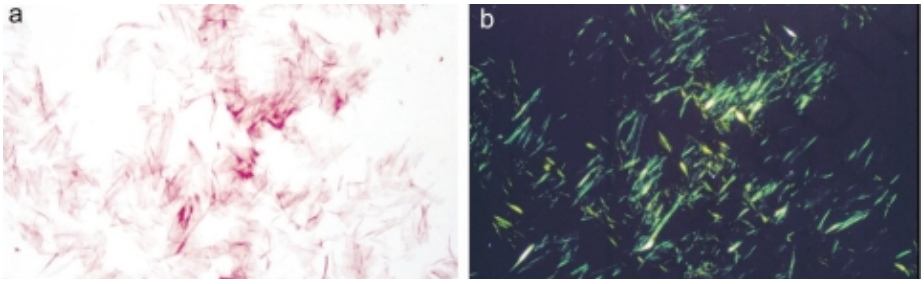


Fig 1.14 Isolated amyloid fibrils composed of Aα chain fragment of fibrinogen (a) stained with Congo Red and visualized by light microscopy and (b) between crossed polars, showing characteristicapple-green birefringence. Figure adapted from reference (amyloid fibrils Rambaran RN et al. Prion 2008)

Subsequently electron microscope studies in the 1959 of amyloid demonstrated that a variety of amyloids had a similar morphology and ultrastructure consisting of uniform fibrils about 100 A wide, reported in Figure 15A (Choen et al., 1959) and combined with the observation of birefringence with Congo Red suggested that the amyloid consist of linear structures arranged in an orderly and parallel fashion (Puchtler et al., 1962). Further progress in biochemical and biophysical tecniques allowed, in the 1968, the isolatation and purification of the amyloid fibrils from tissue, that stored the birefringent characteristic (Pras et al., 1968) and the fibrils subructure was confirmed using high-resolution electron microscopy (Choen et al., 1965 ; Glenner and Page, 1976). In the same years, the X-ray diffraction method, applied on isolated amyloid fibrils, produced diffraction patterns and showed that the fibrils were composed of polypeptide chains extended in cross-β-structure, reported in figure 15C (Eanes et al., 1969). The cross-β-structure were confirmed by NMR analysis (Lansbury et al., 1995). The initial work on the structural charatrization of amyloid fibrils established « ..that amyloid fibrils were defined by following overall structural features : (i) Straight, unbranched fibrils, about 100 A in diameter and indefinite lenght, which appear to be composed of two or more protofilaments, each 25-35 A in diameter. (ii) A molecular structure consisting of polypeptide chains in an extended β-conformation, hydrogen-bonded together into sheets running parallel to the axis of the fibrils, with their constituent β-strands arranged perpendicular to this axis. (iii) A molecular structure that, whatever the origin of the amyloid fibril, is able to bind Congo Red and interact with the planar bis-diazo dye in such a way that the bound molecules are spatially ordered with respect to the fibrils in similar physicochemical

environments and therefore generate the characteristic green birefringence» (Sunde and Blake, 1997).

The first complete X-ray diffraction analysis of fibrous protein with a cross-β structure was of the egg stalk of the lacewing Chrysopa by Geddes and co-workers in the 1968 (Geddes et al., 1968). The cross-β pattern of the amyloid fibrils has two characteristic signals, a sharp reflection at 4.7 Å along the same direction as the fibre and a more diffuse reflection at between 10 and 11 Å, related in Figure B, perpendicular to the fibre direction respectively arising from the hydrogen bonding distances between β-strands and side chain packing between the sheets, reported in Fig 1.15C (Makin and Serpell, 2005 ; Sunde et al., 1997; 273: 729-39 ; Makin et al., 2006).

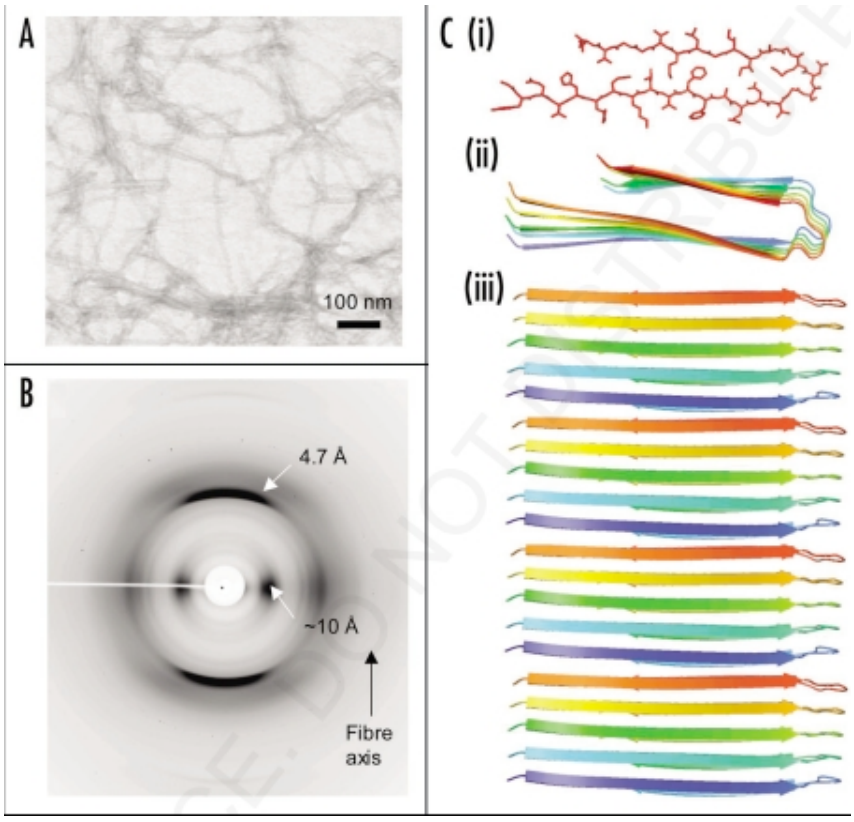


Figure 1.15. Synthetic amyloid fibrils made from Aβ peptide (A) electron micrograph showing long, straight, unbranching fibrils. (B) X-ray fiber diffraction pattern from partially aligned amyloid fibrils showing the characteristic "cross-β" diffraction pattern. (C) The structure of the Aβ amyloid fibril interpreted from ssNMR data,67 showing the top view of the fiber (i and ii) with side chains (i),showing the importance of side chain packing with in the fiber and as a cartoon (ii). The side view(iii) revealing the β-strands running perpendicular to the fiber axis. Figure adapted from reference (amyloid fibrils Rambaran RN et al. Prion 2008)

The pattern indicates that the fibrils have a particular core structure consisting of β-sheet conformation in which the hydrogen bonding direction runs parallel to the fibre axis and the β-strands are perpendicular (Fig 1.15C) EM shows that the fibrils are straight, unbranching, 70-120 Å in diameter (showed in figure 1.15A) and are made up of individual subunit named « protofilaments ». (Cohen et al., 1982 ; Goldsbury et al., 1999; Kistler et al., 1999; Serpell et al., 2000) Through cryo-electron microscopy and single particle processing of mature amyloid fibrils composed of SH3 domain of phosphotidylinositol-3`-kinase was discovered that a single fibril was comprised of 2–6 unbranched protofilaments each 2–5 nm in diameter and associated laterally or twisted together to form fibrils with 4–13 nm (Jimenez et al., 1999). Subsequently, a molecular model of a SH3 amyloid fibril has been generated showing that each protofilament was composed of β-sheet structure and this model was confirmed by studies on synthetic amyloid fibrils from insulin (Jimenez et al., 2002) lysozyme (Jimenez et al., 2001) and Aβ (1-40) (Sachse et al., 2006).

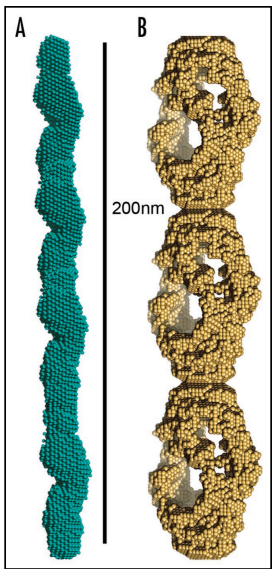


Figure 1.16 Models of mature protein fibrils based on Small-Angle X-rayscattering solution data. (A) Human alpha-synuclein fibrils and (B) humaninsulin fibrils.69 The results suggest that insulin fibrils (B) are formed of threeintertwining protofibrils, whereas α-synuclein fibril (A) consist of only oneprotofibril. Each protofibril is assumed to consist of two intertwining protofilaments.Four and three repeating units are shown for alpha-synuclein and insulin respectively. Figure adapted from reference (amyloid fibrils Rambaran RN et al. Prion 2008)

The formation of amyloid fibrils has been shown not only to be associated with pathology but also with functionality in organisms from bacteria to humans. An increasing number of proteins has been found to form fibrils in vitro with the same optical, morphologies and organizzation properties of the amyloid fibrils but without form protein deposits, reported in table 1.4. This finding has supported the idea that the ability to form the amyloid structure could be an inherent or generic property of polypeptide chains. This generic ability could be required from the cells in specific conditions and purposes and some organisms have been found to convert, during their normal physiological life cycle, one or more their proteins into amyloid fibrils to assolve specialized biological functions. (Stefani et al., 2003 ; Uversky et al., 1698).
Curlin, a protein produced by *Escherichia coli,* is used by the bacterium to colonize inert surfaces and to mediate the binding to host protein. This protein is able to form fibrils with the same amyloid structure. The fibrils are 6-12 diameter and with extensive β-sheet structure (Chapman et al., 2002). A class of protein called chaplins able to form amyloid fibrils were identified in the hyphae producted by the filamentous bacterium *Streptomyces coelicor* (Claessen et al., 2003).

Table 1.4 Proteins forming naturally nonpathological amyloid-like fibrils with specific functional roles (Chiti-Dobson et al., 2006).

Protein	Organism	Function of the resulting fibrils
Curlin	*E. coli* (bacterium)	To colonize inert surfaces and mediate binding to host protein
Chaplins	S. coelicolor (bacterium)	To lower the water surface tension and the development of hyphae
Hydrophobin[a] EAS	*Neurospora crassa* (fungus)	To lower the water surface tension and the development of hyphae
Protein of the chorion of eggshell[b]	*Bombyx mori* (silkworm)	To protect the oocyte and developing embryo
Spidroin	*Nephila edulis* (spider)	To form the silk fibers of the web
Intralumenal domain of Pmel17	*Homo sapiens*	To form, inside melanosomes, fibrous striations upon which melanine granules form
Ure2p (prion)	*S. cerevisiae* (yeast)	To promote the uptake of poor nitrogen sources
Sup35p (prion)	*S. cerevisiae* (yeast)	To confer new phenotypes (PSI+) by facilitating the readthrough of stop codons
Rnq1p (prion)	*S. cerevisiae* (yeast)	Not well understood
HET-s (prion)	*Podospora anserina* (fungus)	To trigger a programmed cell death phenomenon (heterokaryon incompatibility)
Neuron-specific isoform of CPEB (prion)	*Aplisia californica* (marine snail)	To promote long-term maintenance of synaptic changes associated with memory storage

[a] Other proteins form this class, collectively called hydrophobins, have been found to play similar roles in other species of filamentous fungi.

[b] Suggested to form amyloid-like fibrils in vivo, although amyloid formation has only been observed in vitro

1.3.5 Aggregation mechanism and fibrillogenesis of amyloid proteins in the neurodegenerative diseases

The aggregation and accumulation of amyloid proteins or peptides in the CNS is considered to be fundamental to the initial development of neurodegenerative pathologies. Biophysical and biochemical studies combined with the generation of several mice transgenic models allowed the identification of the relationship between amyloid protein and the pathology and the association between amyloid aggregation and the development of the disease. Preliminary studies in vitro were very important to better understand the amyloid aggregation pathway and to identify the formation of different intermediates such as oligomers and protofibrils. The model of fibrillization of amyloid proteins favored by most scientists is a nucleation-dependent polymerization mechanism, already studied for the process of crystallization of large and small molecules. This process is characterized by an initial lag-time phase (nucleation phase), followed by an exponential growth phase (polymerization phase) and a final plateau (equilibrium phase), reported in figure 1.17 (Harper et al., 1999; Stefani et al., 2003 ; Uversky et al., 2004).

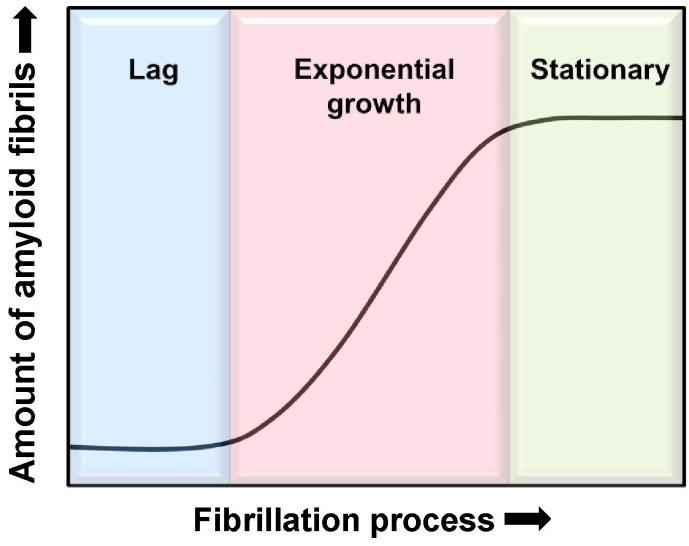


Figure 1.17 Kinetic of amyloidogenesis

The nucleation-dependent polymerization mechanism is characterized by (*a*) a slow nucleation phase, in which the protein undergoes a series of unfavorable association steps to form an ordered oligomeric nucleus, (*b*) a growth phase, in which the nucleus

rapidly grows to form larger polymers, and (*c*) a steady state phase, in which the ordered aggregate and the monomer appear to be at equilibrium (Figure 1.18).

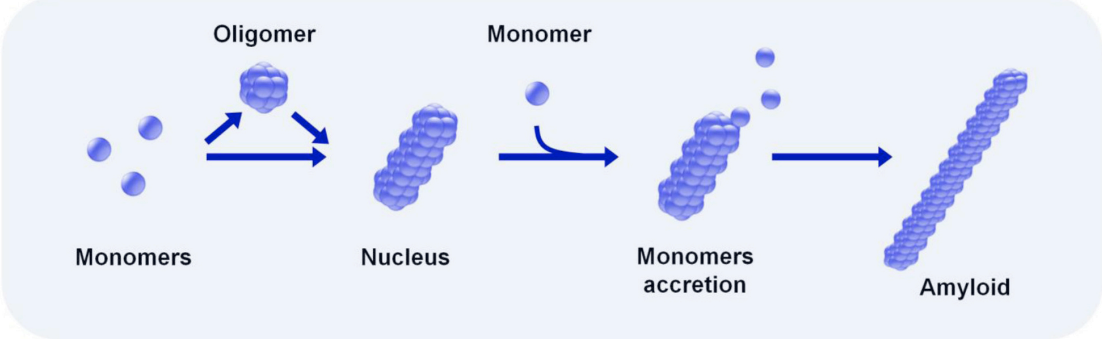


Fig. 1.18. Models of amyloidogenesis. (A) Nucleation-dependent fibrillation (Bhak et al. 2009)

The characteristic features of a simple nucleation-dependent polymerization are as follows: (*a*) No aggregation occurs at a protein concentration below the critical concentration (Fig 1.19). (*b*) At protein concentrations that exceed the critical concentration by a small amount, there is a lag time before polymerization occurs. (*c*) During the lag time, addition of a seed results in immediate polymerization (Jarrett et al., 1993; Andreu et al., 1986; Harper et al., 1997).

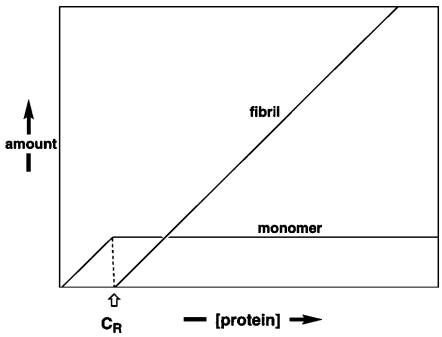


Figure 1.19 Fibril formation is concentration dependent. Below the critical concentration (C_R) no fibril formation will occur. Above that concentration all added protein will be incorporated into the fibrils such that the monomer concentration never exceeds C_R; this behavior is seen at equilibrum (Jarrett et al., 1993)

1.3.6 Aggregation toxicity in Alzheimer's disease model

A wide range of transgenic models allowed the capture of major features of neurological conditions and systemic amyloidoses. Many of these transgenic models successfully reproduce the Aβ plaques and the neurofibrillary tangles found in AD. Using transgenic mice models it is was possible to understand that there is an uncertain nature of the relationship between the deposits and the pathogenesis (Luheshi et al., 2009). Different studies showed that the Aβ plaques can be a poor indicator of cognitive decline in transgenic models (Oddo et al., 2003; Westerman et al., 2002), and this finding is consistent with neuropathological studies in humans (Lue et al., 1999; Reiman et al., 2009).Therefore, the number and size of plaques in a postmortem AD brains do not correlate with the severity of symptoms at the time of the death and amyloid plaques are found throughout the cortex of many cognitively normal 70-year-old. (Dickson et al., 1992; Lansbury et al., 2006). Morever, recent clinical trial results suggest that removing the existing plaques from the brain may not reverse the cognitive decline (Holmes et al., 2008). The best explanation for these findings is that the intermediate species, pre-fibrillar aggregates and oligomers, formed during the amyloid aggregation pathway are likely to be primarily responsible for cell damage, (Fig 1.20). (Hass et al., 2007) .

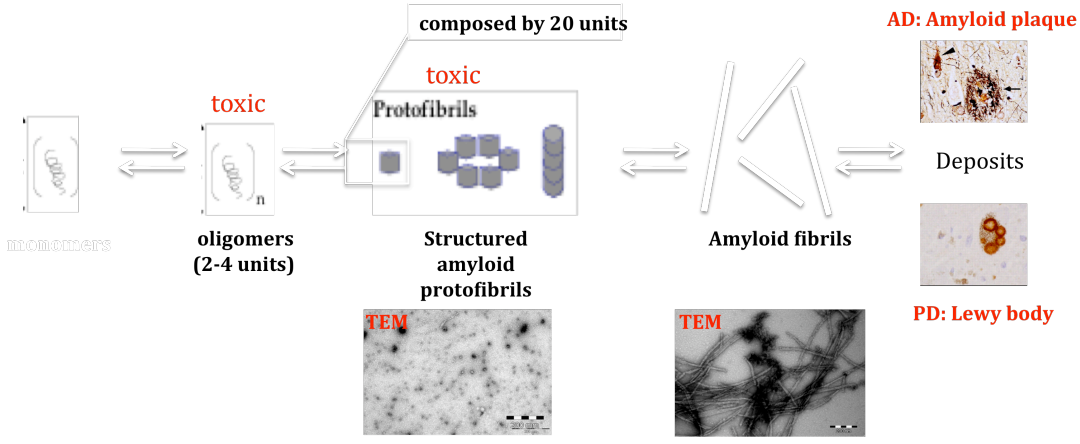


Figure 1.20 Amyloid aggregation mechanism; Aggregation of amyloid proteins, formation of intermediate toxic species and fibrils that were deposited in extracellular plaque such as in AD, or in intracellular inclusion such as PD.

Several studies have shown a correlation between soluble Aβ levels and the extent of synaptic loss and severity of cognitive impairment. The small oligomers can diffuse into synaptic clefts and may represent the better candidates to induce neuronal and synaptic dysfunctions. Several studies indicate that biochemically-measured levels of soluble Aβ, including soluble oligomers, correlate better than the presence of the plaques with the cognitive decline. (Lue et al., 1999; Wang et al., 1999).

The large plaques of fibrillary Aβ in AD brain show surrounding dystrophic neurites, and the aggregates seem to be correlate with the neuronal injury. Probably the large, insoluble protein aggregates could not be directly involved in neuronal injury, but the protein aggregates are likely surrounded by a number of small and diffusible oligomers that might be responsive for neronal death and synapses loss. (Haas et al., 2007) .

1.3.7 Intermediates in the pathway of Aβ fibrillization and different types of Aβ oligomers

Kinetic studies with different reporters and effect of different inhibitors of assembly suggest that a linear sequential process of fibrillar assembly is simplicistic. Inhibition of oligomeric formation but not fibril formation at low concentration of urea in vitro indicates that oligomers are not obligate in vitro precursor fibrils. (Levine et al., 2007).

Aβ assembly is a multistep process involving several transient intermediate species with different physical and biological properties, the intermediates species in the pathway of the Aβ aggregation are reported in Table 1.5.

Table 1.5 Intermediates in the pathway of Aβ fibrillization (Finder et al., 2007)

Aβ species	Characteristics
Monomers	soluble amphipathic molecule; generated from APP; potential α-helical, random coil or β-sheet conformation
Dimers	intracellular localization in vivo, in human brain extracts and in vitro; hydrophobic core; diameter of about 35nm
Trimers	observed in vivo in mouse models; potential key role as as subunit of toxic oligomers
Small Oligomers	observed in vivo in AD patients as well as in mouse models and in vitro; heteromorphous; comprising of 3-50 monomers; mostrly transient, unstable and toxic
Anular Oligomers	observed in cell culture and in vitro experiments; potential role as membrane-disrupting pores or ion channels
ADDLs	observed in murine and human brain extracts as well as in vitro; non fibrillar neurotoxic; 17-42 kDa; trimers to 24mers
Protofibrils	observed in vitro; short, flexible; rod-like structure; maximum size 8×200nm binding Congo red and thioflavine T; precursor of mature fibrils; toxic
Fibrils	observed in AD patients as well as in mouse moedels and in vitro; bind Congo red and thioflavine T; stable, filamentous Aβ aggregates composed of Aβ units perpendicolar to the fiber axis
Plaques	observed in vivo AD patients as well as in mouse models; large extracellular Aβ deposits; predominantly composed of fibrils; not toxic; surrounded by distrophic dendrites, axons, activated microglia and reactive astrocytes

Several intermediate aggregates have been described including protofibrils (PFs); The protofibrils were identified in the 1997 from Lansbury group and Teplow group; both groups reported that the $A\beta_{42}$ peptide is able to form protofibrils more rapidly that the Aβ40 (Harper et al., 1997; Hartley et al., 1999; Walsh et al., 1997; Walsh et al., 1999). Subsequently anular structures (Bitan et al.,2003; Lashuel et al., 2002), Aβ-derived diffusible ligands (ADDLs) (Lambert et al., 1998; Gong et al., 2003), amylospheroids (Hoshi et al., 2003), $A\beta_{56}$ (Lesne et al., 2006) and amyloid fibrils have been identified; the recognized Aβ oligomers are reported in table 1.6 (Haas et al., 2007).

Table 1.6 **Oligomeric assemblies of Aβ** (Haas et al., 2007)

Oligomeric assembly	**Characteristics**
Protofibril (PF)	Intermediates of synthetic Aβ fibrillization; up to 150nm in length 5 nm in width; β-sheet structure: bind Congo red and Thioflavin T
Anular assemblies	Doughnut-like structures of synthetic Aβ; outer diameter of 8-12nm Inner diameter of 2-2.5 nm
Aβ-derived diffusible Ligands (ADDLs)	Synthetic Aβ oligomers maller then annuli: might affect neuronal signal-tranductional pathways
Aβ56	Apparent dodecamer of endogenous brain Aβ; detected in the brains of an APP transgenic mouse line and might correlate with memory loss
Secreted soluble Aβ Dimers and trimers	Produced by cultured cells; resistant to SDS; resistant to resistant to the Aβ-degrading protease IDE; alter synaptic structure and function

1.3.8 Currently Theraphies in AD and PD

Currently avaiable treatments for AD are the cholinoesterase inhibitors donepezil, rivastigmine and galantamine cholinesterase inhibitors and N-methyl-aspartate (NMDA) antagonist memantine. These drugs offer primarily symptomatic benefits, providing temporary cognitive improvement and slowing disease progression.

The characterization of the pathophysiology of the AD allowed the identification of new potential targets in the treatment of this disorder. Molecular, genetic, animal and clinical studies suggested that the production and accumulation of the β-amyloid peptide is fondamental to the initial development of neurodegenerative patholoɡhy and to trigger a cascade of the events that contribute to the progression of AD (Hardy et al., 2002 ; McLean et al., 1999). The oligomeric form of the Aβ peptide triggers a variety of secondary events such : tau hyperphosphorylation, synaptic degeneration, oxidative stress, excitotoxicity, inflammation and neuronal apoptosis (Salloway et al., 2008).

Current therapeutic strategies for treating PD treatment offer mainly transient symptomatic relief and aim at restoring the loss of dopamine by “dopamine

replacement therapy". This is accomplished through the administration of levodopa (L-dopa), a direct precursor of DA and other drugs that increase the lifetime of DA by slowing its metabolism. Catechol-*O*-methyltransferase inhibitors (ICOMT), monoamine oxidase B inhibitors (IMAOB), dopamine receptor agonists and peripheral aromatic L-amino acid decarboxylase inhibitors (IADDC) are used as adjunctive medications to L-dopa used to slow DA degradation and increase the availability of brain DA (Sommer et al., 2008).

The therapeutic approaches under investigation for the treatment of AD and PD consist in antiamyloid and neuroprotective therapies.

1.3.9 Antiamyloid approaches in AD and PD

The antiamyloid approach has as targets the inhibition of the amyloid aggregation mechanism.

The targets of the antiamyloid approach in AD are the inhibition of the Aβ production, aggregation and accumulation in deposits and the clearance of the formed deposits. The antiamyloid treatments under active investigation in AD are reported in Fig 1.21.

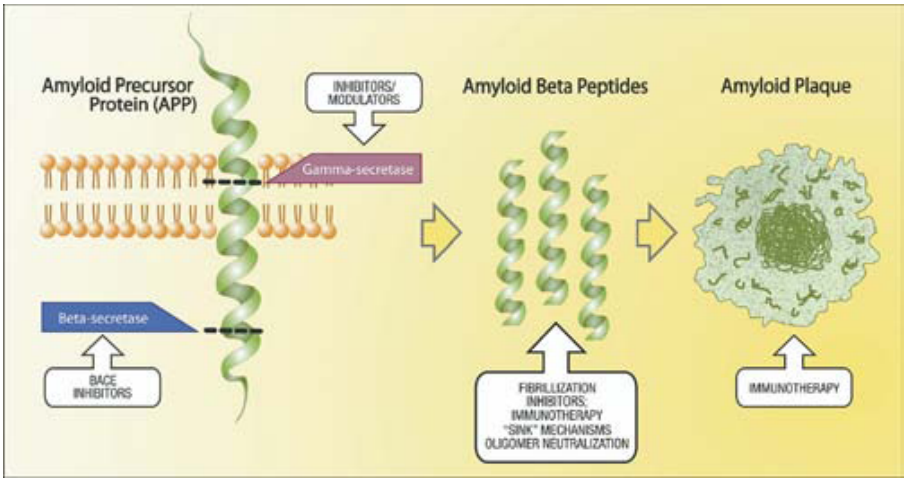


Figure 21 Amyloidogenic pathways in AD and sites for potential therapeutic intervention (Salloway et al. 2008)

Treatments currently under investigation are the vaccination and immunization therapies. Inoculation of mice with an Aβ-derived immunogen resulted in the reduction of amyloid plaques. Transgenic mice with human APP mutation showed an

attenuation of AD after immunization with Aβ peptide (Schenk et al., 1999). Clinical trials were performed with synthetic Aβ peptide AN1792 in combination with an adjuvant, QS-21, on patients with moderate AD. First analysis on a small subset of patient with AD showed formation of antibody against Aβ peptide and slow cognitive decline. (Orgozono et al., 2003). At the same time a study with Aβ peptide AN1792 was interrupted for the development of meningoencephalitis in a subset of immunized patients. (Hock et al., 2003). Currently several trials with passive immunization or vaccination with selective Aβ monoclonal antibodies are underway (Dodel et al., 2004).

A second antiamyloid strategy has as target the ihibition of secretases involved in the production of the Aβ peptide from APP, using β-secretase inhibitors, γ-secretase inhibitors, γ-secretase modulators. Several small molecules that inhibit the β-secretase and γ-secretase are under investigation. In vivo studies on APP transgenic mice the injection of a β-secretase inhibitor, KMI-429, into hippocampus reduced the Aβ production (Asai et al., 2006). Several inhibitors of γ-secretase as well as DAPT (El Mouedden et al., 2006) and LY450139 dihydrate (May et al., 2004) showed a reduction in Aβ levels in the brain, cerebrospinal fluid (CSF) and plasma. In a clinical trial with 70 patients with mild to moderate AD treated with LY450139 dihydrate for 6 week the plasma Aβ levels were reduced but the CSF levels showed no significant change (Siemers et al., 2006). The treatment with γ-secretase inhibitors was well-tollered and several studies are evaluating if high doses will have beneficial changes in Aβ levels without increase in toxicity.

A third strategy in AD and PD involves the inhibition of the Aβ and α-syn aggregation and fibrillogenesis . Interestingly, small molecules such as Congo red, EGCG and Lac have recently been reported to affect fibril formation of several amyloidogenic protein including α-syn and Aβ peptide and to interact with monomeric unstructured protein (Masuda et al., 2006). The compound 3-amino-1-propanesulfonic acid Tramiprasate, a sulfated glycosaminoglycan mimetic, is used to reduce of Aβ aggregation. This compounds reduces amyloid-plaque formation in mouse model and is well tolerated and can cross the blood-brain barrier (Gervais et al., 2006). Although many inhibitors of in vitro Aβ aggregation have been identified such as a small molecule inhibitor of in vitro fibrillogenesis, scyllo-cyclohexanehexol (AZD-103) that prevent and reverse Alzheimer phenotype in a mouse model (McLaurin et al., 2006).

Several factors could influence the balance between native amyloid protein, misfolded protein and the formation of the intermediates (oligomers, protofibrils, fibrils). Overproduction or inappropiate post-translational modifications, inhibition of the chaperone activity or of the protein degradation systems could induce the shift of the balance towards misfolded protein, oligomerization and fibril formation. Potential therapeuthical interventions on the amyloid aggregation pathway have been identified, reported in Fig 1.22 in blue. It is possible to block the formation of toxic species by upregulation of the chaperones activity or by increasing of misfolded proteins degradation. The inibition of the misfolded protein aggregation at different steps on the amyloidogenic pathway could represent an alternative way to avoid the formation of the toxic species and the fibrillogenesis (Skovronsky et al., 2006).

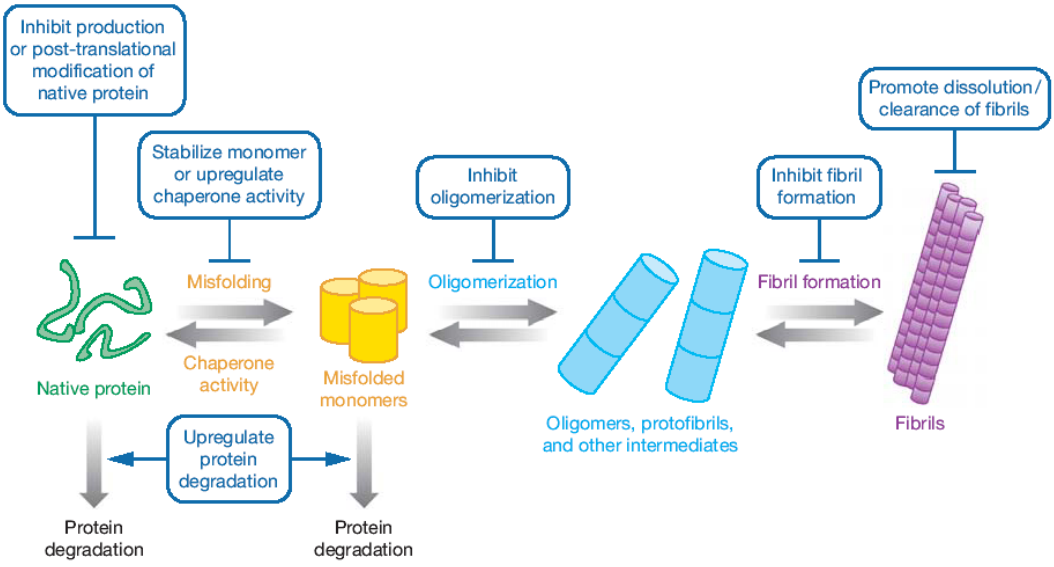


Figure 1.22 Amyloid aggregation pathway. Soluble native protein is misfolded and associates to form different intermediates

However, the ability to block protein aggregation using small molecules provides unique opportunity to address the link between amyloid aggregation and toxicity by allowing the unlinking of these two processes. The is especially important in PD where the majority of affected patients do not carry mutations in the α-syn gene, mutations in several other genes are known to cause rare cases of early onset familial

PD. Initial efforts aimed at blocking or reversing protein aggregation focused on preventing amyloid formation or disruption of preformed fibrils as a mean of promoting their clearance. The accumulating evidence demonstrating that early intermediates on the aggregation pathway are the primary cytotoxic species, emphasize the importance of targeting the initial misfolding and/or aggregation steps on the amyloid formation pathway. Current therapeutic approaches to treat PD, related synucleinopathies and AD are focused on slowing, blocking and/or reversing α-syn aggregation or Aβ aggregation and toxicity, include 1) reducing amyloid protein/peptide levels; 2) reducing and/or inhibiting amyloid protein/peptide oligomerization and fibrillogenesis; 3) promoting the clearance of amyloid protein/peptide aggregates; 4) preventing the formation of the toxic oligomers by diverting the aggregation pathway towards a non-toxic form of the protein or reducing their kinetic stability by accelerating/enhancing oligomer to fibril conversion.

Several small molecules and short peptides have been reported as inhibitors or modulators of α-syn and Aβ fibrillization. These anti-fibrillogenic compounds belong to different classes like polyphenols and catecholamines (Cappai et al., 2005; Li et al., 2005; Conway et al., 2001; Norris et al., 2005; Zhu et al., 2004). Dopamine (D) and other catechols. Dopamine agonists and IMAO B, currently used as anti-parkinsonian agents, have also shown to destabilize preformed fibrils (Li et al., 2007).

1.4 NEUROPROTECTIVE ROLE OF MICROGLIA AND POTENTIAL APPROACH APPROACH IN THE NEURODEGENERATIVE DISEASES

1.4.1 Microglia cells distribution in the CNS and origin

Microglia, the immune cells in the mammalian central nervous system (CNS), have been originally described by Rio-Hortega in the 1932.

Microglia form approximately 10% of the brain (Kennedy et al, 1997). These cells are present in large number in all major division of the brain and they are an heterogeneous population in distribution and morphology. The density differs from 5% in cortex and corpus callosum, to 12% in the substantia nigra. Particularly densely populated areas include the hippocampus, olfactory telencephalon, basal ganglia and substantia nigra (Lawson et al, 1990).

Their origin is still a field of intense controversial discussion. The most accepted view assigns to microglia an exogenous mesodermal origin from bone marrow precursor cells that invade the central nervous system (CNS). (Navascuès et al, 1998; Stoll et al, 1999; Chan et al, 2007). Their precursors of hemapoietic lineage enter in the nervous parenchyma from meninges, ventricular space and blood steam; the first brain colonization from microglial precursors occurs during fetal development and in early postnatal days. In these phases the microglial cells have properties of monocytes (Navascués et al.,, 2000; Hanisch et al., 2007).

1.4.2 Neuron- microglia cross-talk in the development

It has been demonstrated that the functioning of the CNS depends on a continuous inter-relationship between neuron and microglia (Schwab et al, 2004; Polazzi et al, 2002; Aihara et al, 2000; Saab et al, 2008).

These interactions consist in a "cross-talk" between the cells characterized by an exchange of molecular signals that starts in the development, continue in the adult brain and is enhanced in brain injury or in the microbe infections.

The CNS controls the migration and the proliferation of microglial precursors by specific factors release from developing neurons (Fig 1.23) (Navascues et al, 2000; Rock et al, 2004). Human fetal telencephalon can release signals like chemokine MCP-1, RANTES, IL-8 (Rezaie et al, 1997; Rezaie et al, 1999) and fractalkine (Harrison et al, 1998) or neuronal death that are able to recruit microglia in development (Rezaie et al, 2002). In the development, microglia interact with neurons at early stages of differentiation and secrete many neurotrophic factors implicated in all aspects of neuronal functions, neurogenesis and glial development (Hanischet et al, 2002; Butovsky et al, 2006; Monje et al, 2003). These cells are also implicated in induction of apoptosis and synaptogenesis (Bessis et al, 2007). One of primary function of the microglia during development is the removal of apoptotic neurons and the elimination of aberrant axons and entire denditric structures (Cullheim et al, 2007; Trapp et al, 2007; Rappert et al, 2004). In the retina has been demonstrated that nerve growth factor (NGF) of microglial source causes apoptotic elimination of retinal elemnets (Frade et al., 1998).

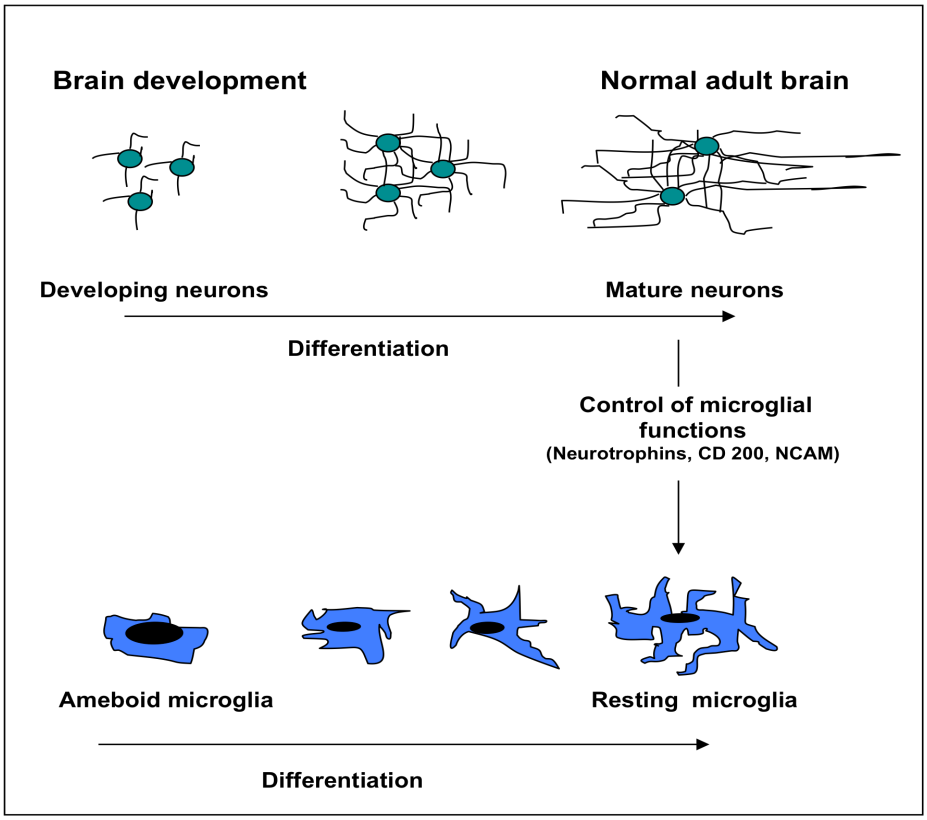


Figure 1.23 The maturation of neurons correspond to the acquisition of control functions on microglial cells.

Microglia cells migrate in a stereotypical manner suggesting that migration is controlled by specific factors. Factors released during cell death may influence microglia migration, as several studies have reported the microglia precursors are attracted towards regions of the nervous parenchyma where intense cell death occurs (Pearson et al., 1993)

1.4.3 Neuron-microglia cross-talk in the adult brain

Microglial cells have immune function; they are able to protect the CNS against microbe infections and injuries through phagocytosis, antigen presentation and cytokines secretion (Aloisi et al, 2001; Kreutzberg et al, 1996; Rock et al, 2004).
In adult healthy brain the microglial cells are in a resting state characterized by ramified morphology (Hanisch et al, 2007). The ramification of microglia is accompanied with changes in cellular functions in the motility and in the partial loss of macrophagic functions characteristic of ameboid microglial precursors. The CNS exercise an endogenous control on the microglial state, through the release of secreted factors like neurotrophins and cytokines/chemiokines (Neurrmann et al, 1998; Harrison et al, 1998; Streit et al, 2001).
Recently, it has been found that neurons produce several IgSF like CD200 (OX2 in rat), CD47 and CD22, chemokine like CX3CL1, neurotransmitters and neurotrophins like NGF, BDNF and NT-3 that keep microglia quiescent. It also has been found that the microglial cells express receptors that bound the respective factors (Biber et al, 2006; Hoek et al, 2000; Farber et al, 2005; Biber et al, 2007), In mice CD200-/- the microglia form activated cell clusters characterized by a loss of ramifications, shorter glail process, increase of CD11b and CD45 expression (Hoeck et al, 2000).
It has been demonstrated that neurons are able to block the production of inflammatory factors such as the release of nitric oxide and TNF-α by LPS simulation. The production of inflammatory factors after the LPS stimulation has been found high in mixed microglial cells and reduced in neuron-glia-coculture (Chang et al., 2000).
Microglia respond to changes in microenvironment to help brain, after traumatic CNS injuries and microbe infections, with migration at the sites of damage (Heppner et al, 1998) and activation. In the activated state, ramified microglia undergo a drastic change in cellular morphology, in cell size and they rapidly up-regulate the expression of a large number of cell surface molecules and secretory products like cytokines and growth factors. The microglia activation seem to have a double effect,neuroprotective and neurotoxic effect. It's possible discriminate between a physiological activation that occurs in adult brain afterwards a neuronal damage and an hyperactivated state that occurs in aging and neurodegenerative disease in response to different factors

released from damaged neurons and and to the neuronal damage intensity. Damaged neurons release factors, such as ATP, IL-1, IL-6, TNF-alpha, TGF-beta, LIF, MCP-1 CSFS, PAF, Glutamate and fractalkine that induce a physiological microglia activation. The physiological microglia activation induces the release of neuroprotective factors to repair the neuronal damage (Fig 1.26).

1.4.4 Microglial activation in aging brain and neurodegenerative diseases

In the adult brain the microglia is found in a ramified form that represent a quiescent condition of the microglia named “resting state” (Fig 1.24A). In the resting state the microglia have small bodies with several process and show a downregulation of the macrophagic characteristics. Microglia respond to changes in the microenvironment to help and maintain brain homeostasis. Following acute CNS injury microglia become activated and undergo phenotypical changes that include hypertrophy, an enhanced expression of immunological cell surface molecules, mitosis and changes in cytokines and growth factor production. Fully activated microglia retract their process and develop an enlarged cell body with the same shape of peripheral macrophages; the ameboid and reactive microglia have been found in pathological condition, with a rod-shape morphology and they are able to migrate to the site of injury (Fig 1.24B). In the activate state the microglia express on their surface molecules typical of the macrophage lineage, such as the major histocompatibility complex of class II (MCH II) (Streit et al 1999).

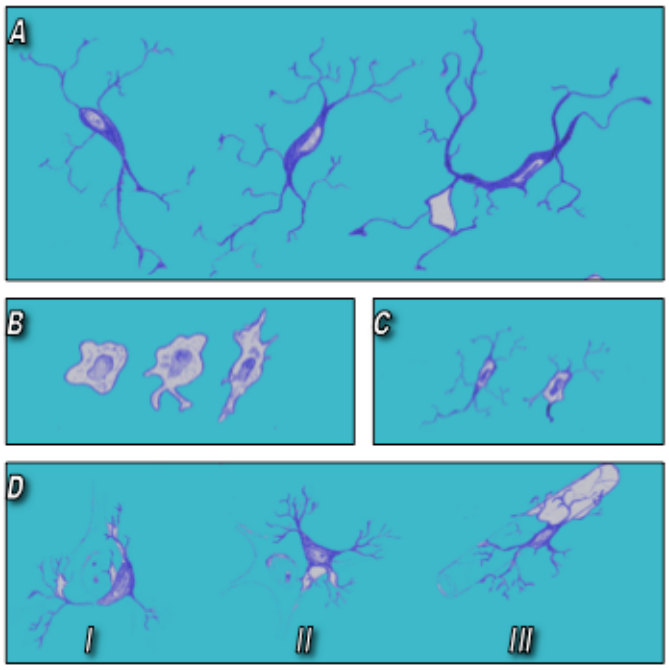


Figure 24 Morphology of microglial cells: A. Resting microglia. B. Ameboid microglia. C. Reactive microglia (intermediate stage). D. Specialize locations of microglia in the adult brain: I. Neuronal satellite microglia. II. Glial satellite microglia, around astrocytes or oligodendrocytes. III. Perivascular microglia (Rezaie and Male, 1999).

Microglial immunophenotype is highly dynamic and fluctuates with changing state of activation. Resting microglia express several surface molecules such as complement receptors (Graeber et al., 1988), Fc (Perry et al., 1985), macrophage specific antigen, CD4 (Perry et al., 1987) and leukocyte common antigen (LCA) (Akiyama et al., 1990). After 24 hours of microglial activation the cells express molecules important for the interaction between lymphocytes and antigen-presenting cells such as upregulation of CR3 (OX-42) expression (Graeber et al., 1988), an increase of IgG-immunoreactivity, thrombospondin and intercellular adhesion molecules (Kloss et al., 1999; Moller et al., 1996). Changes occur in the expression of P2 purinoreceptors following LPS-induced activation probably in response to extracellular ATP, and the expression of major histocompatibility complex type I and type II, MCH I and MCH II (Streit et al., 2008) .

Controversy remains concerning the effects of microglial activation on the CNS following brain injury. *In vitro* studies showed that highly reactive microglial cells produce neurotoxic factors which may be responsible for causing neurodegeneration. The microglia are able to release pro-inflammatory cytokines such as TNF-alpha, IL-1, IL-6 and cytotoxic factors such as oxygen radicals, nitric oxide, glutamate (Fig

1.25). (Boje et al., 1992; Giulian et al.,, 1996). IL-1 and TNF-α have been shown to be involved in the development of CNS inflammation through the disruption of BBB, the induction of adhesion molecules and chemiokines from astrocytes and endothelial cells, which facilitate the infliltration of leukocytes into the CNS (Oh JW, Schwiebert LM, Benveniste EN Cytokine regulation of CC and CXC chemokine expression by human astrocytes J neurovirol (1999) 5: 82-94). On the contrary, *in vivo* experiments demonstrated that the microglial activation could be a neuroprotective process. (Streit et al., 2006; Moran et al., 2004) . Activated microglia promote axonal regeneration in PNS (peripheral nervous system) and axonal regrowth in SNC (Lazarov-Spiegler et al, 1998). After six hours to transient middle cerebral artery occlusion (tMCAO) in the rats there was an increase of the TGF-β1 mRNA expression, an anti-inflammatory cytokines, released by microglia cells (Lehrmann et al, 1998). At the same time *in vitro* experiments showed that the microglia release anti-inflammatory cytokines TGF-β1, IL-10, IL-1 receptor antagonist (IL-1Ra) and neuroprotective factors such as fibroblast growth factor (bFGF), hepatocyte growth factor (HGF) and neurotrophins that could potentially rescue damaged neurons (Polazzi et al, 2002).

Cytokines release by activated microglia varies temporally and quantitatively in different types of insult. Many microglial-derived cytokines could have a positive or negative effects on CNS and their neuroprotective or neurotoxic effect depends on the degree of microglial activation and severity of neuronal damage. In the spinal cord damage microglia rapidly upregulate the production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, but in the regenerating facial nerve injury these cytokines are minimally elevated (Streit et al.,2008) .

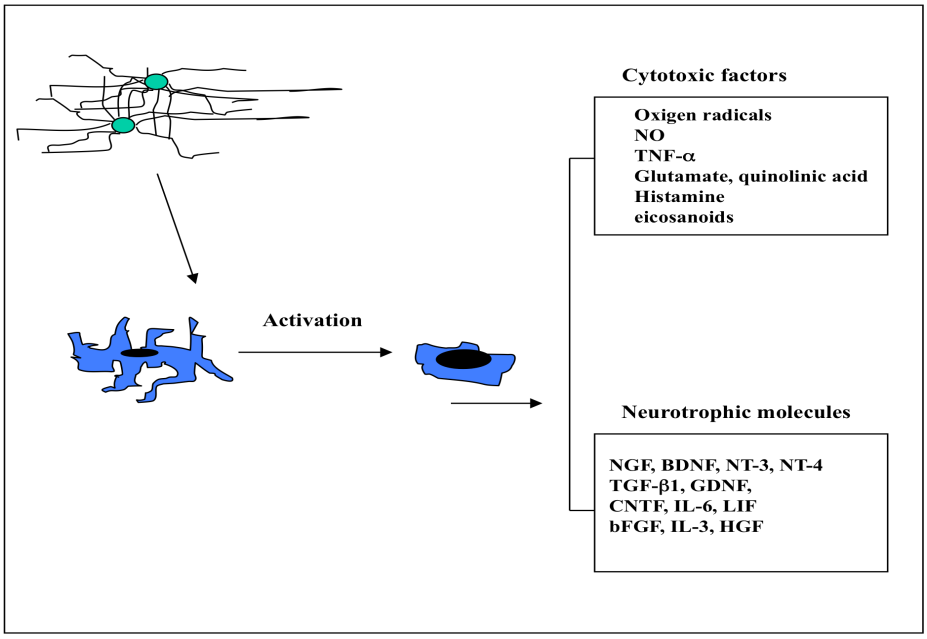


Figure 1.25 Microglia activation and release of cytotoxic factors or neurotrophic factors

Microglia undergo progressive activation in aged brain, suggesting that the control exerted by neuron is lost in parallel with aging-related impairment of neuronal function (Polazzi, 2002). The microglial age cause changes of the cytokines expression patterns and neuronal survival (Sawada ref63) and showed increased IL-1β immunoreactivity (Sheng et al., 1998). The treatment of neonatal mice with two microglial activators, 1-methyl-4phenyl1-1,2,3,6-tetrahydropyridine (MPTP) and lypopolysaccharide (LPS), showed upregulation of IL-1β and IL-6 and neuronal recovery following initial MPTP-induced cell loss. In aged mice treated with MPTP showed significant neuronal loss. A similar situation was observed in microglial cultures obtain from cerebral cortex of aging rats replicated in vitro some phenotypical features characteristic of the activation state observed in vivo (Rozovsky et al., 1998).

The lost of neuronal control on the microglial cells with the aging becomes much more dramatic in the presence of neurodegenerative diseases. The microglial cells were found activated in different neurodegenerative disease AD, PD, HIV and multiple sclerosis (MS). In AD an inflammatory response with the release of cytokines, protease, has been found associated with the presence of activated microglia at the level of the neuritic plaques (Cotter et al., 1999). In particular, has been proposed the neurotoxins released by activated microglia contribute to neurodegeneration in AD and this seems confirmed by the fact that the severity of

inflammatory response positively correlates with density of activated microglia in affected area (Carpenter et al., 1993).

Microglial activation is primarily intended to protect neurons and is a component of the regenerative process. Recently studies hypothesized that microglial activation following neuronal injury, primarily constitutes a brain protective mechanism to limit neurodegeneration. (Minghetti et al., 1999). However, in several neuropathologies, where chronic inflammation is present, the inflammatory products derived from activated microglia may secondarily promote neurodegeneration and contribute to neuronal loss (Gonza´lez-Scarano and Baltuch 1999; Polazzi and Contestabile 2002; Streit et al. 2008). The interaction between neuron-microglia, the physiological activation after neurons damage and the hyperactivation state of the microglia in aging and neurodegenerative diseases are summarized in Fig 1.26. In this setting, the possibility to shift the balance of microglial cells from pro inflammatory to anti-inflammatory states offers a good strategy to develop therapies for neurodegenerative diseases. The knowledge of the neuroprotective mechanisms of microglial cells, emerging from studies of in vitro neuron/microglia cross-talk, may help in identifying and targeting specific microglial-produced molecules that could account for neuronal survival.

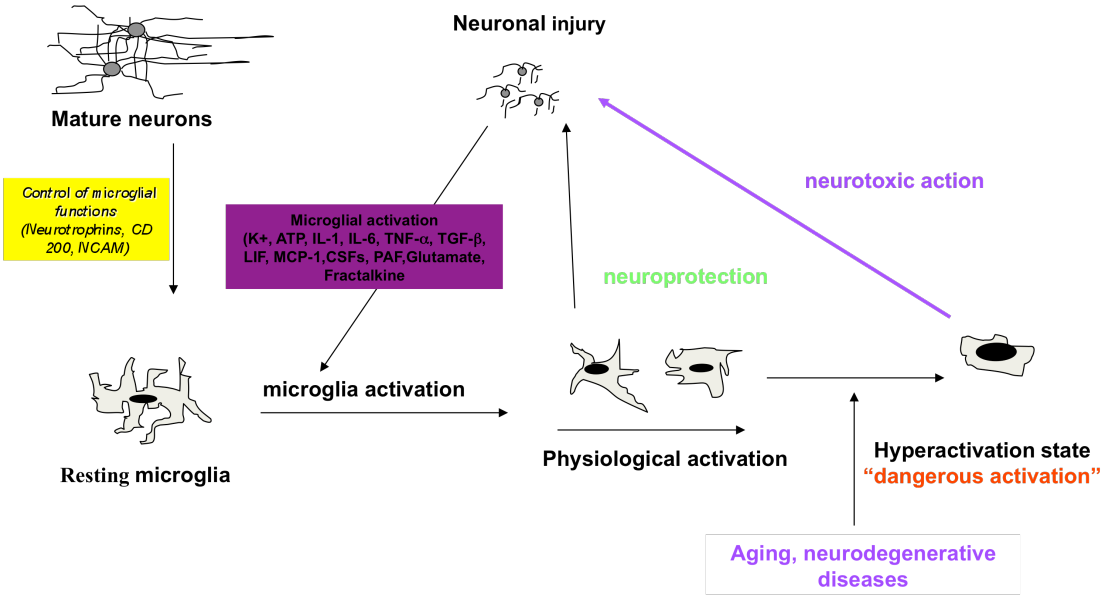


Figure 1.26 Interaction between neuron and microglia in adult brain, in aging and neurodegenerative disease.

1.5 AIM OF THE THE THESIS

The aim of the present thesis was the identification of new neuroprotective therapeutical strategies in the PD and AD. First, we analysed the correletaion between the inhibition of amyloid aggregation and the neuroprotection in PD and AD. We identified inhibitors (two iCOMT and four polyphenols) of alpha-syn and beta-amyloid aggregation, amyloid protein/peptide respectively involved in the PD and AD and afterwards we tested the neuroprotection of these amyloid inhibitors against alpha-syn and beta-amyloid-induced citotoxicity in PC12. Subsequently we focused on the study of the neuroprotective role of microglia and on the possibility that the neuroprotection properties of these cells could be use as therapeutical strategy in PD and AD. We tested the neuroprotective role of microglia on PD-like neurodegeneration model (using 6-OHDA), AD-like neurodegeneration model (using AB42) and in the excitotoxicity (using Glutamate), involved in the major neurodegenerative diseases.

CHAPTER 2

MATERIALS AND METHODS

EXPERIMENTAL PROCEDURES

Thioflavin T, gallic acid, quercetin and MTT were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) of microbiological quality,dopamine, caffeic acid and pyrogallol were from Fluka (Buchs, Switzerland). Entacapone and tolcapone were kindly donated by Prof. Alberto Gasco (Dipartimento della Scienza e tecnologia del Farmaco, University of Turin,Italy). DMEM, heat-inactivated horse serum,fetal bovine serum, gentamicin, insulin, NuPAGE 4-12% Bis- Tris gel, goat anti-mouseAlexaFluor680 and SilverXpress SilverStaining Kit were from Invitrogen. Betaamyloid Monoclonal Antibody 6E10 was from Covance and nitrocellulose membranes from Protran. All chemicals were of analytical grade and all solutions were prepared in autoclaved distillate water. Compounds solutions were prepared in 100 % DMSO (10 mM). Fresh solutions were prepared by diluting stock solutions in water to achieve a final amount of co-solvent less than 1%, in the reaction mixtures.

2.1 Expression and purification of α-synuclein-Human wild type (WT)

α-syn was expressed as previously described (Paleologou et al., 2008). Cells were harvested, resuspended in buffer and lysed. The supernatant was saved, concentrated and loaded onto a Superdex 200 size exclusion column. α-syn containing fractions were combined, lyophilized and stored at -20 °C until use.

2.2 Preparation and characterization of Aβ42 low molecular weight (LMW) and protofibrils (PF)

Aβ42 was synthesized and purified by Dr. James I. Elliot at Yale University (New Haven, CT). Monomeric Aβ42 stock solutions were prepared by dissolving the

peptide in 6 M Guanidine-HCl at concentration of 1 mg/ml and centrifuged at 8,600 rpm for 5 minutes. Low molecular weight (LMW) and protofibril (PF) Aβ42 stock solutions were prepared by dissolving the peptide in 5 % DMSO, 2 M Tris Base pH 7.6. The mixture was subjected to low speed centrifugation at 6000 rpm for 5 minutes. The supernatant of Aβ42 monomeric, LMW and PF stock solutions was loaded onto a Gel filtration column (Superdex 75 HR 10/30 Amersham) previously equilibrated with 10 mM Tris buffer pH 7.4 (Jan et al, 2008). The fractions were analyzed by SDS-PAGE and protein concentration was determined by UV absorbance at 280 nm in 10-mm path-length cuvettes using the theoretical molar extinction coefficient at 280 nm (1490 M-1 cm-1) (31). All Aβ42 stock solutions were diluted with Tris buffer pH 7.4 to a final peptide concentration of 10 μM. Samples of Aβ42 were incubated at 37° C in 1.5 mL polypropylene sterile tubes, with and without different concentrations of inhibitors, at molar ratios of Aβ42: inhibitor of 1:0.5 and 1:2.

2.3 Preparation of α-syn and Aβ42 seeds

The seeds were prepared by incubation of ‹-syn peptide solution (20 mM Tris buffer, 150 mM NaCl (pH 7.4)) and Aβ42 peptide solution (5% DMSO, 2 M Tris buffer (pH 7.6) at 37°C under agitation for three days. The fibrils were then mechanically fragmented to yield narrow distribution of smaller fibrillar structures (100-300 nm long) by ultrasonication on ice using SONICS Vibra Cell TM equipped with a fine tip (20 x 5 second pulses, amplitude 40, output watts 6). The sonicated fibrils were diluted in 10 mM Tris buffer pH 7.4. Seeds and monomeric α-syn or Aβ42 were incubated for 4 hours at 37° C with continuous shaking, with and without inhibitors, in polystyrene black 384 well plates (Nunc,USA).

2.4 Fibrilization Studies

Purified, lyophilized α-syn was dissolved in 20 mM Tris buffer, 150 mM NaCl. The samples of α-syn at a concentration of 100 μM (as estimated by spectroscopy) were incubated at 37° C in 1.5 mL sterile polypropylene tubes, with continuous shaking, in the absence and presence of inhibitors, at a molar ratio of α-syn: inhibitors of 1:1, 1:0.5, 1:0.1 and 1:0.01. Aliquots (10 μL) of the ‹-syn incubations (final protein concentration of 10 μM), previously incubated at 37° C with and without compounds,

were added to 80 µL of 50 µM Glycine-buffer (pH= 8.5) and 10 µL solution of 100 µM Aliquots of 80 µL of 10 µM Aβ42 solutions (LMW or PF), previously incubated at 37° C in absence and in presence of compounds were added to 10 µL of 100 µM ThT and 10 µL of 50 µM Glycine-buffer (pH=8.5). The time course of α-syn and Aβ42 fibrillization was measured by ThT fluorescence assay. Fluorescence measurements were carried out with a spectrofluorometer (Analyst™ AD 96-384, Bucher Biotec AG, Basel) at 25° C using polystyrene black 384 well plates. The excitation wavelength was set to 450 nm and emission was monitored at 485 nm. All measurements were done in triplicates by performing three identical experiments.

2.5 Seeding polymerization assay

Polymerization of soluble α-syn with or without α-syn fibrils added as seeds and Aβ42 with or without Aβ42 seeds was assayed as described elsewhere (32). Nunc 384 fluorescence plates were filled with monomeric and seed protein solutions. Compounds of interest (or Tris buffer containing DMSO 1% for the control) were finally added to the reaction mixture. The plate was incubated at 37°C for 3 hours under agitation. The kinetics and extent of fibrillization is monitored using the standard Thioflavin T (ThT) binding assay as described above. The assay was run in triplicate by processing three identical plates.

2.6 Quantification of soluble α-syn and Aβ42

SDS-page was performed to directly determine the amount of the monomeric α-syn and Aβ42 form after the incubation of the peptide (10µM) with the inhibitory compounds (20µM) at 37°C for 48h. Equal amount of protein samples were separated on NuPAGE 4-12% Bis-Tris gel and the electrophoresis was run at constant voltage of 130V. The gels were stained using the SilverXpress Staining Kit (Invitrogen), For western blotting analysis, the bands were transferred to a nitrocellulose membrane and blocked for 1h with the blocking solution (30% of Licor and 70% ofPBS). Subsequently the membranes were incubated with primary antibody, Betaamyloid Monoclonal Antibody 6E10 (1:5000), overnight at 4°C with agitation. The Nitrocellulose membranes were washed with PBS- Tween 0,1% and incubated with

the secondary Antibody, goat anti-mouse AlexaFluor680 (1:2000) for 1h at room temperature. Before to scan the membrane with Odyssey infared lector the membranes were washed with PBS-Tween 0,1% and PBS to remove the excess of antibody.

2.7 Electron microscopy analysis of fibril formation

Aβ and α-syn fibril formation was monitored by transmission electron microscopy (TEM). Samples for TEM analysis were prepared by placing 10 μL of the sample solution on formvar-carbon copper grid for 1 min before removing the excess solution. The grid was washed with two drops of distillated water and 1 drop of uranyl acetate before staining with 1 % of fresh uranyl acetate for 30 seconds. The grids were thoroughly examined to get an overall evaluation of the structures present in the sample. Specimens were inspected at 80 kV using a Philip CIME 12 electron microscope. Digitized photographs were recorded with a slow scan CCD camera (Gatan, Model 679).

2.8 NMR spectroscopy

NMR spectra were acquired on a Bruker Avance III 600 MHz NMR spectrometer at 15°C. Measurements were performed using 60 μM 15N-labeled αS in 20mM Tris, 150 mM NaCl pH 7.4. Two-dimensional 1H-15N Heteronuclear Single Quantum Coherence (HSQC) spectra were recorded using 256 × 1024 complex data points in the F1 and F2 dimensions, with a relaxation delay of 1.0 s (33). Sixty-four scans per increment were recorded for each spectrum. Spectral widths were 1612.9 and 7211.5 Hz in the 15N and 1H dimensions, respectively. Spectra were processed with Topspin 1.3 (Bruker Biospin) and NMRPipe (Goddand et al., 2003). Visualization and manipulation were performed using the public domain graphics program Sparky 3 [Goddard TD, Kneller DG (2004) SPARKY 3. University of California, San Francisco]. Resonance assignments had been previously obtained (Delaglio et al., 1995). The addition of 1% DMSO and a small change in temperature did not interfere with transfer of resonance assignment. 2D 1H-15N HSQC spectra were recorded to monitor chemical shift changes induced by the presence of three compounds (E,Q,T).

Compounds were freshly dissolved in DMSO to high concentration and added to the αS sample. The concentration of DMSO did not exceed 3% (v/v). Measurements were performed for molar ratios of 1:0.5, 1:2 and 1:10 αS:ligand. For each titration step, a reference spectrum was obtained by addition of the same amount of DMSO only. Both intensity and chemical shift differences were analyzed with respect to the DMSO reference spectrum. Mean weighted 1H-15N chemical shift differences were calculated according toΔav = {[(Δδ2HN + Δδ2N/25)]}/2 (Goddand et al., 2003; Delaglio et al., 1995; Grezesiek et al., 1996).

2.9 PC12 preparation and toxicity studies

The rat adrenal gland pheochrompcytoma cell line, PC12, were grown at 37°C in 5% of CO2 in DMEM supplemented with 6% heatinactivated horse serum, 6% of fetal bovine serum and Gentamicin 50μg/ml. Exponentially growing PC12 cells (5*104 cells per well) were plated in 96-well tissue culture plates (Falcon) in a media supplemented with insulin 2μM and gentamicin 50μg/ml; the cells were co-treated with Aβ42 crude preparation (40μM) and the compounds (40μM) for 24h at 37°C and in 5% of CO2; the peptide and the compounds were added directly in the medium DMEM supplement with insulin 2μM and gentamicin 50μg/ml. The viability of PC12 cells was evaluated by thiazolyl blue MTT (36) and luminescent cells assays. The MTT assay is based on conversion of tetrazolium salt to formazan (blue compound), a reaction that only occurs in viable cells since the chemical reaction is carried only by mithocondrial deidrogenases. MTT was added at final concentration of 0,5mg/ml for 2h at 37°C and the crystal formed were dissolved using 100μl of solubilizzation buffer containing 30% SDS, 70% of the Isopropanol in water. The optical density was determined at 570 nm using a microplate reader. The luminescent cells viability assay is a sensitive method of determining the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells. The assay procedure involves adding the single reagent (Promega CellTiter-Glo® Reagent) directly to cells cultured. The light emission, measured by Safire Instrument, is directly proportional to the ATP produced. All quantitative data are presented as means +/- SE. Statistical analysis between different treatments was calculated by using one way analysis of variance (ANOVA) followed by post-hoc comparison

through Bonferroni's test. A value of $p>0.05$ was considered statistically significant.

2.10 Microglial cell culture and microglial conditioned medium (MCM) preparation

Microglial cells were prepared from cerebral cortex of newborn Wistar rats as previously described (Levi et al., 1993). Briefly, brain tissue was cleaned from meninges, trypsinized for 15 min and, after mechanical dissociation cell suspension was washed and plated on poly-L-lysine (Sigma, 10μg/ml) coated flasks (75 cm^2). Mixed glial cells were cultured for 10-13 days in Basal Medium Eagle (BME, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 2 mM glutamine (Sigma) and 100 μM gentamicin sulphate (Sigma). Microglial cells were harvested from mixed glial cells cultures by mechanical shacking, resuspended in serum free BME, and plated on uncoated 40 mm dishes at a density of $1x10^6$ cells/ml medium. Cells were allowed to adhere for 30 min and then washed to remove non-adhering cells. After 48 hours microglial conditioned medium (MCM) was collected, filtered through 0.22 μM filters, aliquoted and stored at –20°C until used. To get insight into the nature and the range of molecular weight of the neuroprotective factor(s) present in MCM, some aliquots were boiled for 30 min before use. Other aliquots of MCM were treated with 1 μg/ml of proteinase k or peptidase (Sigma) for 1 hour at 37°C. Enzymatic activity was then killed heating the media at 95 °C for 10 minutes, a procedure that did not compromise the neuroprotective efficacy of MCM. Finally, other aliquots of MCM were filtered using Microcon-YM-10 filters (Millipore). In this way, we obtained two different fractions for each type of medium: the fraction containing low molecular weight (<10 kDa) substances and the one containing high molecular weight (>10 kDa) substances. The two fractions were separately resuspended in the original volume of medium to recreate a similar concentration of active factors, and used to test protection of neuronal cultures. In some experiments, to evaluate the neuroprotective effect of TGF β-2, we have added 10 ng/ml of human recombinant TGF β-2 (Sigma) to the fraction of MCM <10kDa that does not contain this cytokine. In another set of experiments we have also evaluated whether the neuroprotective effect of MCM was

inhibited when the signal transduction cascade of TGF-β2 was blocking by the compound SB 431542 (Inman et al., 2002).

2.11 Cerebellar granule cell cultures (CGC)

Primary cultures of CGC were prepared from 7 day-old rats of Wistar strain as previously described (Gallo et al., 1987). Briefly, cells were dissociated from cerebella and plated on 40 mm dishes or in 24 well plates coated with 10μg/ml poly-L-lysine at a density of $2x10^5$ cells cm^2 in BME supplemented with 10% heat-inactivated FBS, 2mM glutamine, 100μM gentamicin sulphate and 25mM K^+. After 16 hours, 10μM cytosine arabino-furanoside (Sigma) was added to avoid glial proliferation. After 7 days in vitro (7div) differentiated neurons were shifted to serum free BME medium containing 25mM K^+ and treated with different concentration of 6-hydroxydopamine (6-OHDA, Sigma) in the presence or in the absence of MCM to test its neuroprotective effect. We have also tested the neuroprotective effect of <10kDa and >10 kDa fractions of MCM, MCM previously heat-inactivated or MCM previously treated with proteinase K or peptidase (see above). All microglia conditioned media or their fractions, were added with KCl to reach a final 25mM K^+ concentration. After 24 hours neuronal survival was analyzed through MTT test or nuclei count after Hoechst staining. Experiments were authorized by a local bioethical committee and were performed in accordance with the Italian and European Community law on the use of animals for experimental purposes.

2.12 Western blot

To analyze protein expression of insulin growth factor–I (IGF-I), brain derived neurotrophic factor (BDNF) and transforming growth factor β -2 (TGF β-2) in their mature or precursor forms, on microglial cell cultures and on microglial conditioned medium, freshly detached microglial cells were plated in serum free BME at the same density used to obtain microglial conditioned medium. After 2, 24 or 48 hours, microglial cell were collected directly in Loading buffer 2X (0.5 M Tris-HCl pH.6.8; 4% SDS; 2% Glicerol; 0.2% Bromophenol Blue; 0.2M DTT). The cell from one well

(40 mm dish) were collected in 50 μl of Loading buffer 2X. Corresponding media were also collected and 1ml aliquots for every condition were lyophilized using Microcon-YM-3 (Millipore) and resuspended in 15 μl of Loading buffer 2X. Fifteen μl of each of the two samples were loaded per lane onto a 15% polyacrilamide gel. After electrophoresis and nitrocellulose membrane transfer, the membranes were blocked for 1 hour with 5% non-fat dried milk/0.1% Tween 20 in phosphate buffer, pH 7.4, and incubated overnight at 4°C with primary antibodies (rabbit polyclonal anti IGF-I, Santa Cruz Biotechnology, 1:1000; rabbit polyclonal anti BDNF, Santa Cruz Biotechnology, 1:1000, rabbit polyclonal anti TGF β 2, Santa Cruz Biotechnology, 1:1000). Subsequently, nitrocellulose membranes were incubated with a secondary antibody, a donkey anti-rabbit conjugated to horseradish peroxidase (1:2000, Santa Cruz Biotechnology), for 90 min at room temperature in 0.1% Tween 20 phosphate buffer, pH 7.4. The labeled bands were visualized by enhanced chemiluminescence method (ECL, GE Helthcare). The analysis of β-actin (Sigma) content, was performed to verify the amounts of proteins per lane.

2.13 MTT assay

The viability of CGC culture was evaluated by thiazolyl blue (MTT) assay (Hansen et al., 1989). This method is based on the conversion of the tetrazolium salt to a colored compound, a reaction that only occurs in viable cells since the chemical reaction is carried on by mitochondrial dehydrogenases. MTT was added to the culture-medium to reach a final concentration of 0.1 mg/ml. Following 15 min of incubation at 37°C the dark crystals formed were dissolved in 0.1 M Tris-HCl buffer containing 5% Triton X-100 and the absorbance was read at 570 nm in a Multiplate Spectophotometric Reader (Biorad).

2.14 Hoechst staining

To quantify neuronal cell death, normal and condensed nuclei were counted after Hoechst stain. CGC were fixed for 20 min with 4% paraformaldehyde in phosphate buffer, washed in PBS and incubated for 5 min at room temperature with 0.1μg/ml Hoechst 33258. Cultures were observed and photographed with a fluorescence

microscope using a 20x objective and count was performed in 3 randomly selected fields of each dish. Quantitative evaluation of cell death was determined by calculating the percent ratio: condensed nuclei/condensed + normal nuclei (Monti et al., 2001).

2.15 Fluoresceine diacetate (FDA) staining and propidium iodide staining

In experiments based on acute excitotoxic pulse of glutamate, living cells were stained with fluorescein diacetate (FDA) and nuclei of dead cells with propidium iodide (PI). Cells were washed with Locke's solution, stained for 3 min at room temperature with the same solution containing15µg/ml FDA and 5µg/ml PI, washed, observed and photographed with the fluorescence microscope.

2.16 Statistical analysis

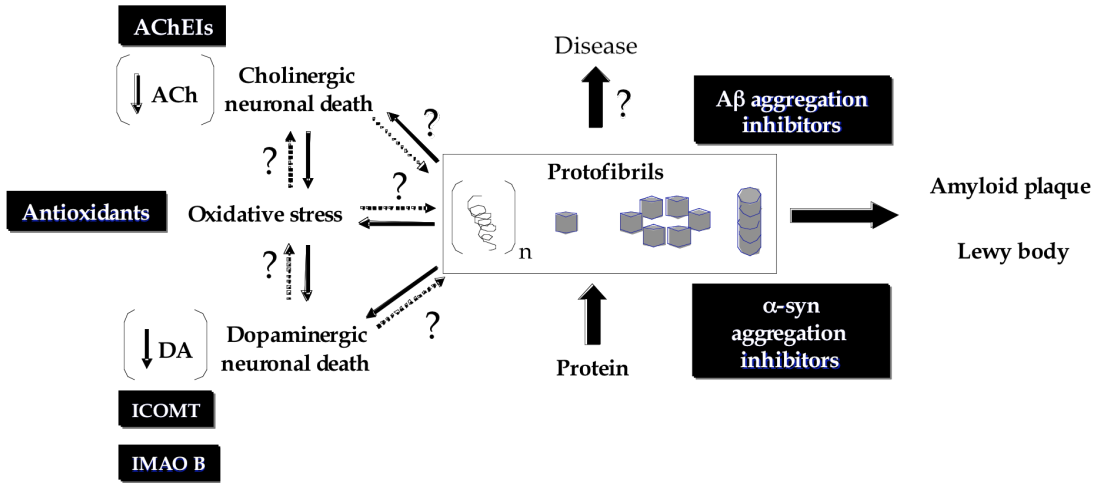
All quantitative data are presented as means ± SE from independent experiments. Statistical significance between different treatments, was calculated through one way analysis of variance (ANOVA) followed by post-hoc comparison through Bonferroni's test. A value of $p<0.05$ was considered statistically significant.

CHAPTER 3

RESULTS

3.1 PROTECTIVE EFFECT OF ANTIAMYLOIDOGENIC COMPOUNDS AGAINST α-SYN AND β-AMYLOID-induced CITOTOXICITY

We evaluated the inhibitory potency of anti-Parkinsonian drugs and catecholamines towards the oligomerization, fibrillogenesis and toxicity of monomeric and oligomeric α-syn and $A\beta_{42}$. These molecules include two nitrocatechols, i.e. Entacapone and Tolcapone (Bonifacio et al., 2007) approved as adjunct in the therapy of PD (scheme 3.1). These two coumpounds are Catechol-*O*-methyltransferase inhibitors, COMT inhibitors, used in the treatment of PD to block the oxidation of the levodopa, analogous of the dopamine.



Scheme 3.1 Working model illustrating that protein aggregation, i.e. α-syn in PD and Aβ in AD, has a central role in the generation of the cascade of events that result in neurodegeneration and disease.

Entacapone and Tolcapone have never been tested for their ability to modulate α-syn and $A\beta_{42}$ aggregation and toxicity. These molecules were also investigated for their ability to inhibit and/or reverse the growth of α-syn and $A\beta_{42}$ fibrils using a seeding polymerization assay. In addition, four natural polyphenols containing a catechols

moiety and which are known for their antioxidant properties, Quercetin, Caffeic acid, Gallic acid and an anti-inflammatory coumpound, Pyrogallol were tested as potential inhibitors of amyloid aggregation and inhibitors of the seeding polymerization. (Johnsonet al., 2005); Han et al, 2008; Dodo et al 2008; Armagan et al, 2008). The inhibitory potential of those compounds was compared to that of dopamine, a well-known inhibitor of α-syn and amyloid-β fibrillization. The structures of the nitrocatechols and polyphenols are reported in table 3.1. Finally, we investigated the protective effects of these anti-amylogenic compounds against α-syn and Aβ-induced cellular toxicity on PC12.

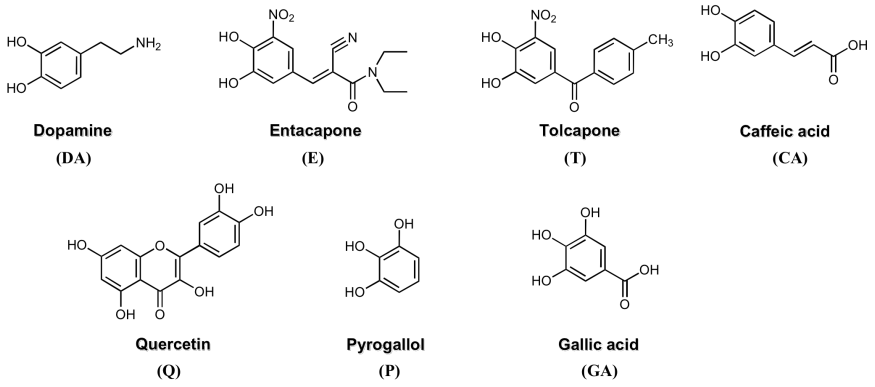


Table 3.1 Chemical structure of compounds examined as inhibitors of human WT α-syn and Aβ42 fibril formation.

3.1.1 Inhibition of α-syn fibrillization by Entacapone, Tolcapone and related catechols

α-syn fibril formation is a concentration dependent process and occurs readily *in vitro* only under conditions that combine high protein concentrations (100-200 μM) and mechanical agitation to accelerate the process (Fig. 3.1).

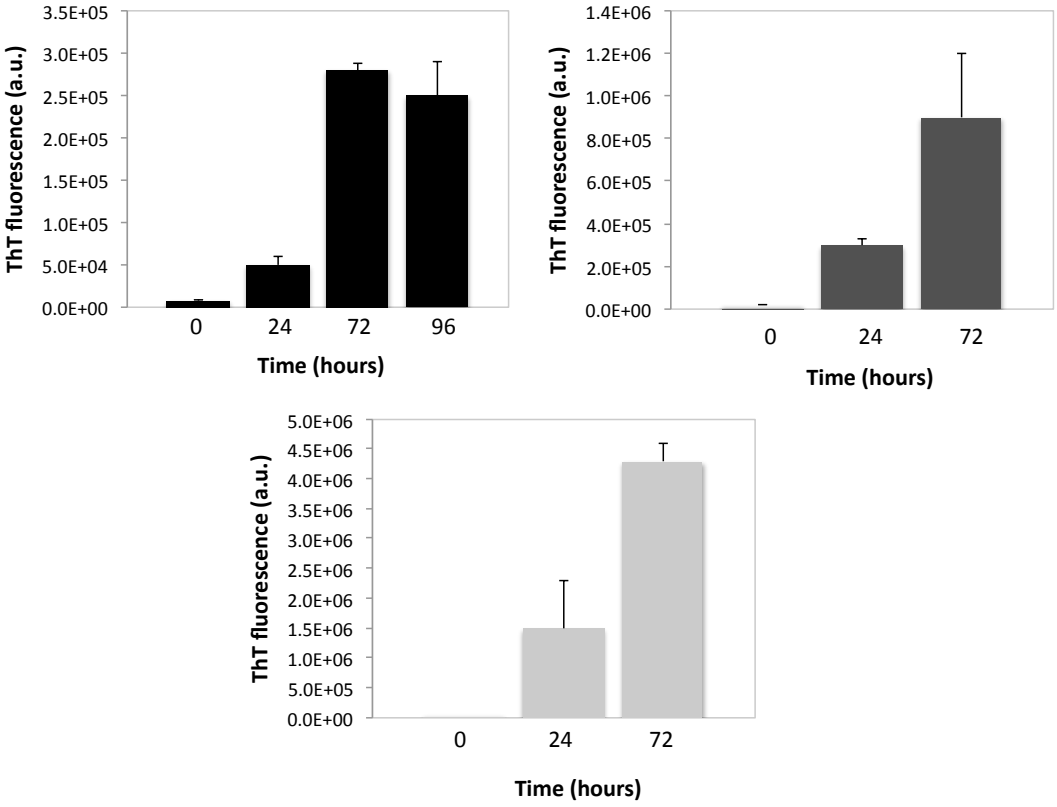
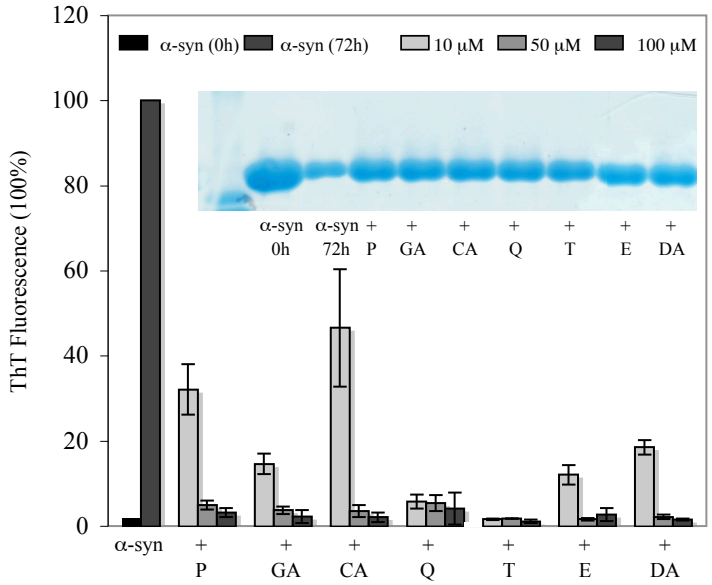


Fig. 3.1: α-syn fibril formation is a concentration-dependent process. Purified, lyohilized α-syn was dissolved in buffer 20 mM Tris, 150 mM NaCl pH 7.4. Samples α-syn were incubated at 37°C, with continuous shaking at 50 μM (a), 100 μM (b) and 200 μM (c). The time course of protein fibrillation was measured by ThT fluorescence assay after incubation for 48, 72 and 96h for the 50 μM sample and for 48 and 72 hours in the case of 100 μM and 200 μM samples.

We evaluated the fibrillization of human WT α-syn alone under agitation in the range of 50-200 μM and a 100 μM protein concentration was chosen as the optimal concentration to assess the inhibitory activity of the compounds (Fig. 3.2). Under these conditions, α-syn fibrillization was complete after 72-96 hr of incubation at 37 °C. To investigate the influence of the two nitrocatechols, entacapone (E) and Tolcapone (T), and the four natural compounds, quercetin (Q), caffeic acid (CA), gallic acid (GA) and pyrogallol (P) on α-syn aggregation, we monitored the

fibrillization of α-syn in the absence and presence of each compound in the concentration range of 1-100 μM using ThT fluorescence, SDS-PAGE analysis of soluble protein and TEM as a function of time. DA was chosen as a reference compound and was tested under the same working conditions. As shown in Fig. 3.2, after 72 hr of incubation, all compounds tested abolished α-syn fibril formation at concentrations of 50 and 100 μM. Compounds E, GA and Q exhibited strong inhibition of α-syn aggregation even at concentrations of 10 μM, with compound T demonstrating the greatest inhibition potency (Fig. 3.2A). More than 70-80% reduction of the ThT fluorescence signal was observed in all samples containing ≥ 10 μM of compounds. The ThT results were confirmed by TEM, which demonstrated the absence of significant amounts of amyloid fibrils in α-syn samples incubated with the compounds at 100 μM concentrations (Fig. 3.2B). After 72 h, WT α-syn alone formed extensive fibrillar structures with an average diameter of 35 nm. In the samples containing 100 μM of E, T, Q, CA, GA and P, the number of fibrils was significantly reduced, and spherical oligomers and short sheared fibrils were observed instead. Aggregates formed in the presence of the various compounds were morphologically distinct from those formed by WT α-syn. To further confirm the inhibitory effect observed by ThT and TEM assays, we quantified the amount of remaining soluble protein by SDS-PAGE analysis of the supernatant after removal of fibrils and insoluble materials by centrifugation. The sample containing α-syn alone showed a reduction in band intensity corresponding to > ~ 70-80% loss of soluble α-syn after 72 hr of incubation at 37 °C, suggesting that the majority of soluble α-syn has been converted into insoluble fibrils under these conditions. In contrast, all samples containing the compounds (100 μM) showed levels of soluble protein that correspond to the inhibitory effect reported by ThT and TEM. At 1:1 molar ratio, more than ~ 60% of the starting protein remained in solution after 72 hr of incubation (Fig. 3.2A).

A



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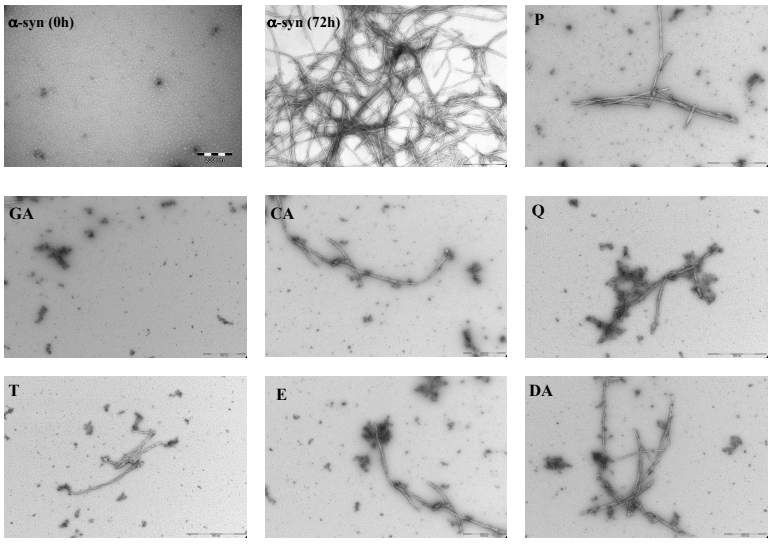


Fig. 3.2 The compounds abolish α-syn fibril formation and increase the soluble forms of α-syn after incubation for 72h. (A) Samples of 100 μM α-syn were incubated at 37 °C, with continuous shaking, with and without compounds, at molar ratios of α-syn: inhibitor of 1:0.1, 1:0.5 and 1:1. The time course of protein fibrillization was measured by ThT fluorescence assay after incubation for 72 hr. The bar graph represents the amount of fibril formation in absence and in presence of the compounds pyrogallol (P), gallic acid (GA), caffeic acid (CA), quercetin (Q), tolcapone (T), entacapone (E), and dopamine (DA). The figure shows means of three independent experiments ± SD (n=6). The inhibitory effect of the compounds on α-syn aggregation was evalueted by SDS-PAGE. Samples of α-syn (100 μM) were incubated with and without compounds, at molar ratios of α-syn:inhibitor of 1:1. The bands represent the amount of monomeric form of α-syn before incubation and after incubation for 72 hr in the absence and in the presence of the compounds. **(B)** Electron micrographs of negatively stained quaternary structures deposited from solutions of α-syn before incubation and after 72 hr incubation at 37°C in the absence and in the presence of 100 μM compound. *Scale bar* represents 200 nm.

3.1.2 Inhibition of the seeding capacity of fibrillar α-syn

The process of amyloid fibril formation follows a nucleation-dependent polymerization mechanism that is characterized by an initial lag-time phase (nucleation phase), followed by an exponential growth phase (polymerization phase) and a final plateau (equilibrium phase) (Harper et al., 1999) The spontaneous breakage of fibrils into smaller aggregates or their disassociation by small molecules or chaperones is believed to contribute to the spreading and acceleration of amyloid formation *in vivo* via a seeding mechanism (Grimminger-Marquardt et al., 2009).This is supported by *in vitro* and *in vivo* studies demonstrating that amyloid fibril formation is accelerated by the addition of preformed aggregates (Harper et al., Ann Rev Biochem 1997; Harper et al., Chem Biol 1997), which act as seeds that nucleate fibril formation and growth. In other words, the addition of seeds eliminates the lag phase associated with fibril formation. Therefore, blocking the seeding capacity of preformed fibrils is an attractive strategy for slowing amyloid formation and disease progression in PD and related disorders. For this purpose, the ability of the compounds to inhibit the seeding capacity of α-syn fibrils was investigated. To produce α-syn seeds, mature α-syn fibrils were mechanically disrupted by sonication to yield a narrow distribution of short fibrils. As expected, addition of small amounts of seeds abolished the lag phase and accelerated α-syn fibrillization (Fig. 3.3A). To determine the relative potency of the compounds towards blocking the seeding capacity of α-syn fibrils, 10 and 50 μM of each compound was added to a solution of freshly prepared monomeric α-syn. Fibrillar seeds at 2 μM final concentration were then added and the kinetics of fibrillization was monitored by ThT fluorescence. In the absence of compounds, fibrillization proceeds immediately to yield a dense network of amyloid fibrils. The fibrillization reaction was complete within 3 hr as opposed to 72-96 hr in the absence of the seeds. At 50 μM concentration of E, T, Q, P, and CA (full triangles in Fig. 3.3A) the seeding capacity of short α-syn fibrils was abolished by >90%. However, only Q and T exhibited a similar potency at lower concentrations (10 μM). The remaining compounds E, P, CA, GA, and DA still showed greater than 60-75% inhibition of seeded fibril growth at this concentration. These findings were confirmed by TEM, which demonstrated the presence of predominantly spherical structures and short isolated fibrillar assemblies, (Fig. 3.3B) in seeded samples containing 50 μM of the compounds compared to extensive fibril

formation in samples containing only α-syn.

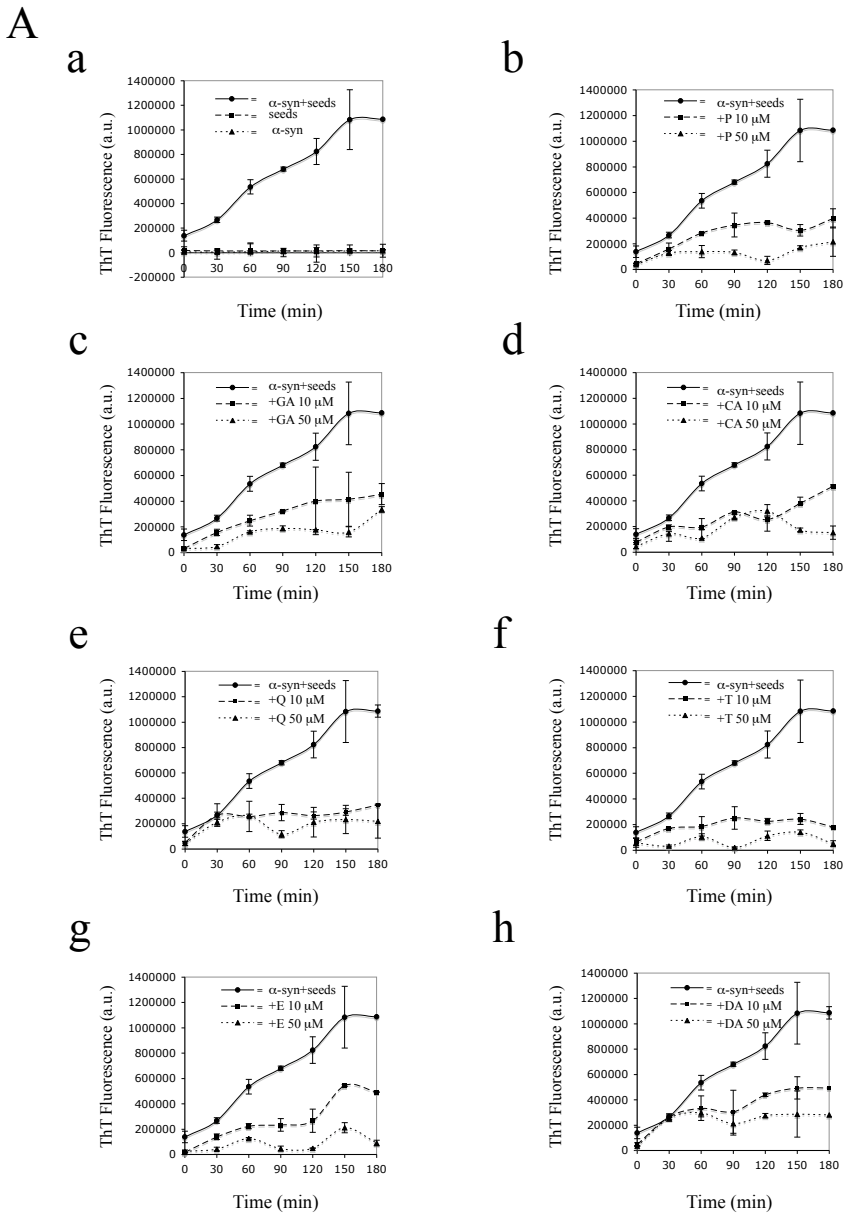
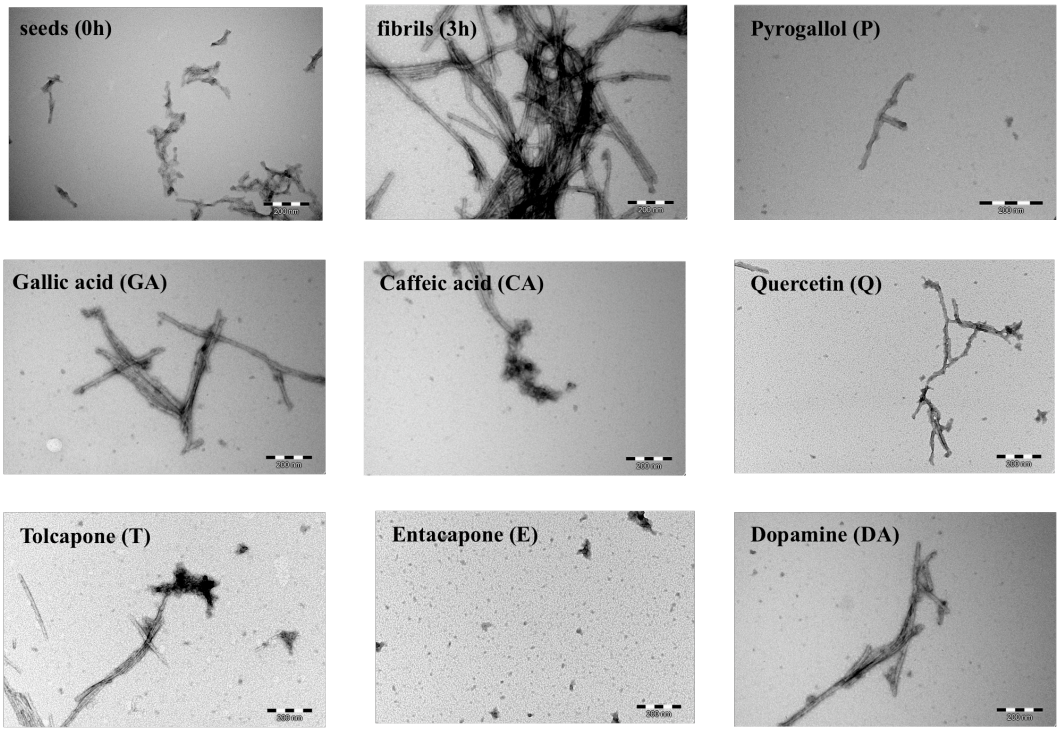


Fig. 3.3 The compounds have an inhibitory effect on the α-syn seeding polymerization. (A) Samples of monomeric α-syn (100 μM) were incubated with the seeds (2 μM) at 37°C, with continuous shaking, without (a) and with (b-h) 10 μM and 50 μM of compounds pyrogallol (P), gallic acid (GA), caffeic acid (CA), quercetin (Q), tolcapone (T), entacapone (E), and dopamine (DA). The time course of protein fibrillization was measured every 30 minutes by ThT fluorescence assay for 3 hr. **(B)** Electron micrographs of negatively stained quaternary structures deposited from solutions of seeds and α-syn+seeds after 3hr incubation in the absence and in the presence of the compounds (50 μM). *Scale bar* represents 200 nm.

B



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3.1.3 Entacapone (E), tolcapone (T) and related catechols do not bind to monomeric α-syn

Binding of E, T and Q to monomeric α- syn was probed using nuclear magnetic resonance (NMR) spectroscopy. NMR signals of backbone amides constitute excellent probes of complex formation providing maps of interaction interfaces (Craik et al, 1997). We monitored the position and intensity of the NMR signals of α -syn in the presence of E, T and Q for molar ratios up to 1:10 α-syn:compound. In the case of Q, only a 1:2 ratio was reached due to its lower solubility. No significant chemical shift changes were observed for any of the compounds, with the exception of very minor chemical shift changes for His50 and some N-terminal residues (Fig. 3.4A,B,C). As the very small chemical shift changes observed for His50 and the two to three N-terminal residues are most likely due to slight changes in pH, the NMR data suggest that there is no direct interaction of the compounds with the backbone of monomeric α-syn. Besides the position of NMR signals, their intensity is very sensitive to changes in the conformational properties of a protein as well as its chemical environment. For example, signal broadening indicates increased chemical exchange. When we compared the intensity of NMR signals in 2D 1H-15N HSQC spectra of α-syn in the free state and in the presence of the compounds E and T, residues in the C-terminal domain showed a different response than those of residues 20-105 (Fig. 3.4D, E, F). NMR signal intensities in the C-terminal domain in the presence of E and T were within 10-15% of the values in the free state. In contrast, a large number of residues in the N-terminal domain of α-syn, in particular in the NAC region showed an increase of up to 30% in NMR signal intensity in the presence of E and T when compared to the DMSO control spectrum. The increased NMR signal intensities point to an increase in the backbone flexibility of these residues or to a reduced amide proton exchange. Interestingly, a similar increase in NMR signal intensities was observed for residues 22-93 of α-syn in the presence of polyamines, which bind to the C-terminus of monomeric α-syn (Fernández et al., 2004).

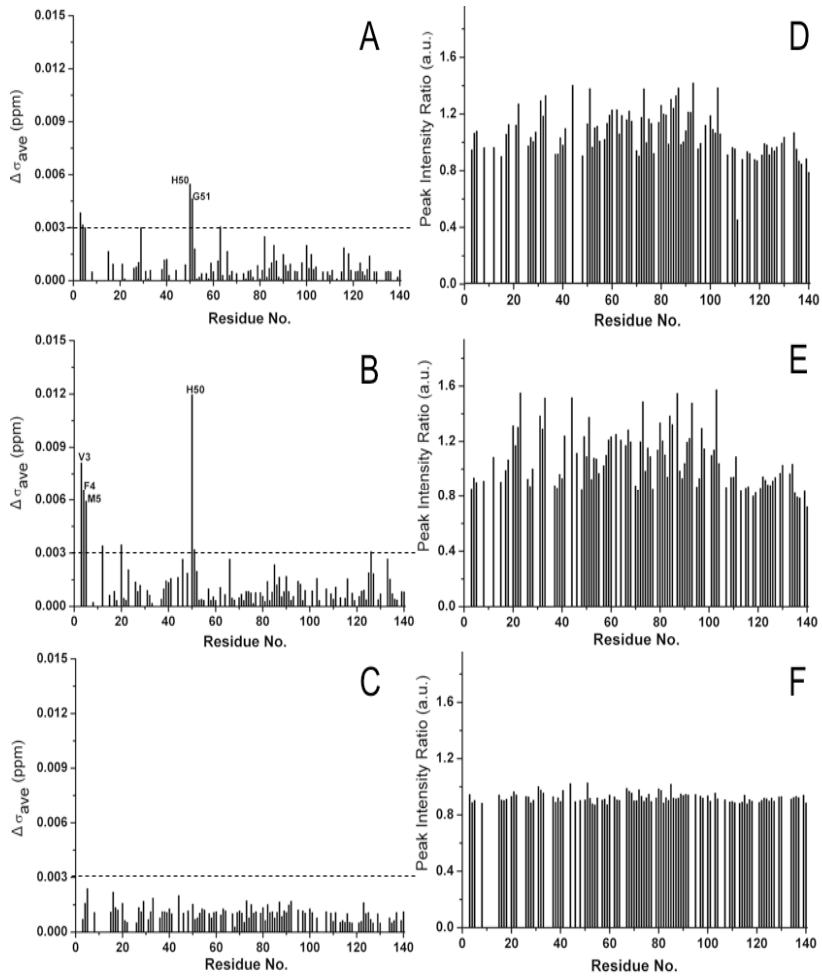


Fig. 3.4 Analysis of compound binding to monomeric α-syn by NMR spectroscopy. Changes in individual cross-peak positions (panels A, B, C) and intensities (panels D, E, F) of backbone 15N-1H resonances of α-syn (60 μM) in 2D 1H-15N HSQC spectra in the presence of compound E (panels A and D), T (panels B and E) and Q (panels C and F). For compounds E and F, molar ratios of 1:10 α-syn:compound were used. Compound Q is less soluble and only the 1:2 α-syn:compound ratio could be measured. Horizontal lines indicate the average variation of chemical shifts observed for α-syn from sample to sample due to slightly different buffer conditions.

3.1.4 All compounds protect PC12 against α-syn induced cell death

To investigate the effect of the E and T and related compounds in Fig.1 against α-syninduced extracellular toxicity on the PC12 cells, we used a preparation of α-syn which was incubated in the presence and/or absence of compounds for 72 °C at 37 °C under agitating conditions. The α-syn-compound mixtures were then added to the cell culture media and cell viability was evaluated using the MTT assay. The treatment with the preaggregated α-syn (40 μM) showed a reduction of cellular viability by ~ 40 % (Fig. 3.5). α-Syn samples which were incubated with inhibitors showed a significant increase of cell viability in the range of 10-30%. Interestingly, E was found to be the most active compound, with a protective effect close to 100%. These results suggest a direct correlation between the effect of these compounds on the fibrillization of α- syn and protection against α-syn-induced extracellular toxicity, which may be linked to the compounds ability to block the formation of the toxic entity or processes.

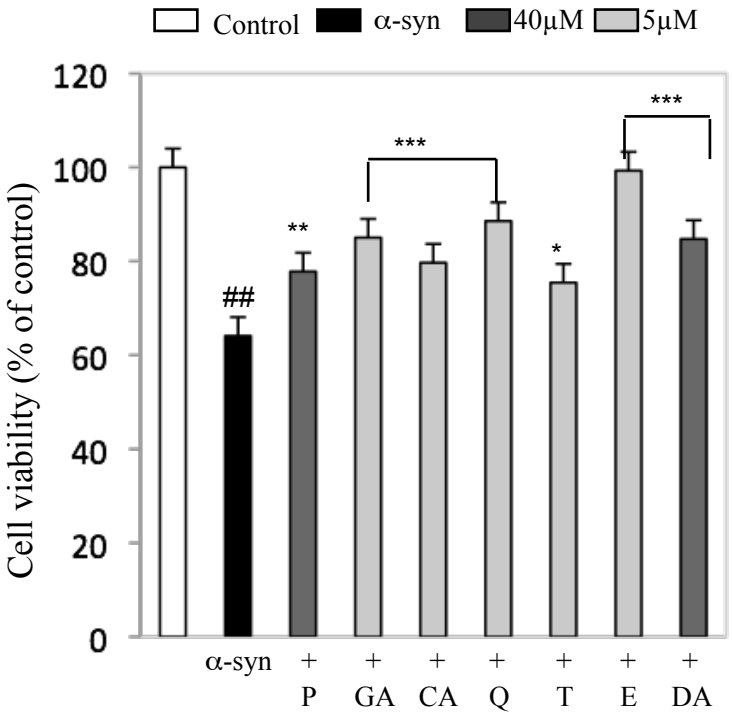
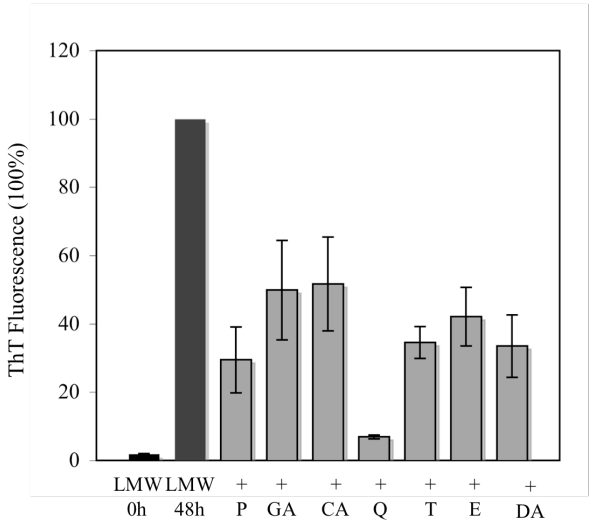


Fig. 3.5 Protective effect of the compounds against α-syn induced toxicity in PC12 cells. PC12 cells were treated with pre-incubated α-syn (40μM) alone or co-incubated with 5μM of gallic acid (GA), caffeic acid (CA), quercetin (Q), tolcapone (T) and entacapone (E) and 40μM of pyrogallol (P) and dopamine (D). The cellular viability was evaluated by MTT assay and the data were expressed as percentage of the control (non treated cells). The control treatment is set to 100%. Bars are means ± S.E. We used # to compare the data to the control and * respect to the treatments with α-syn. A:## $p < 0.001$, B:*** $p < 0.001$, C:** $p < 0.01$, D:* $p < 0.05$.

3.1.5 Entacapone (E), tolcapone (T) and related catechols inhibit the conversion of low molecular weight (LMW) Aβ42 into fibrils

In order to assess the inhibitory specificity of the compounds towards α-syn fibrillation, we investigated their capacity to inhibit the fibrillization of monomeric (LMW) and protofibrillar (PF) Aβ42, which were prepared freshly by size exclusion chromatography (SEC) as described previously (Jan et al, 2008). Fresh monomeric Aβ42 solutions were incubated (37°C), with and without compounds at molar ratios compound: Aβ42 of 0.5:1 and 2:1 for 24 hr and 48 hr. In the presence of 5 μM (data not shown) and 20 μM of compounds (Fig. 3.6A), Aβ42 fibrillization was decreased by ≥ 50% after 48 hr of incubation. Q was found to be the most potent compound, showing greater than 80% inhibition of Aβ42 fibrillization at 5- 20 μM, whereas the remaining compounds showed inhibition in the range of 60-70% at higher molar ratios (2:1, compound: Aβ42). These findings indicated that these compounds act by one of the following mechanisms: 1) by stabilizing monomeric Aβ; 2) by kinetic stabilization aggregation intermediates that precede mature fibril formation; or 3) by altering the aggregation properties of Aβ42 such that ThT negative large aggregates are formed. To determine their mode of action, the samples were analyzed by electron microscopy. TEM images of LMW Aβ42 following incubation for 48 hr in the presence of compounds indicate E and T stabilize distinct aggregate morphologies compared with the other compounds (Fig. 6B). Negatively stained TEM images of LMW alone revealed amyloid fibrils (diameter of 30nm) in the sample after 48 hr incubation (Fig. 3.6B). Aβ42 solutions containing 20 μM of E and T revealed predominantly large networks of PF-like tructures and the absence of mature fibrils. In comparison with E and T, the other compounds P, GA, CA and DA appeared to exert different effect on Aβ fibrillization. GA and CA were shown to stabilize smaller PF structures and result in the formation network of amorphous aggregates, after 48hr incubation at 37 °C. When Q was added to the sample containing Aβ42 LMW, we observed the formation of predominantly LMW and PF species.

A



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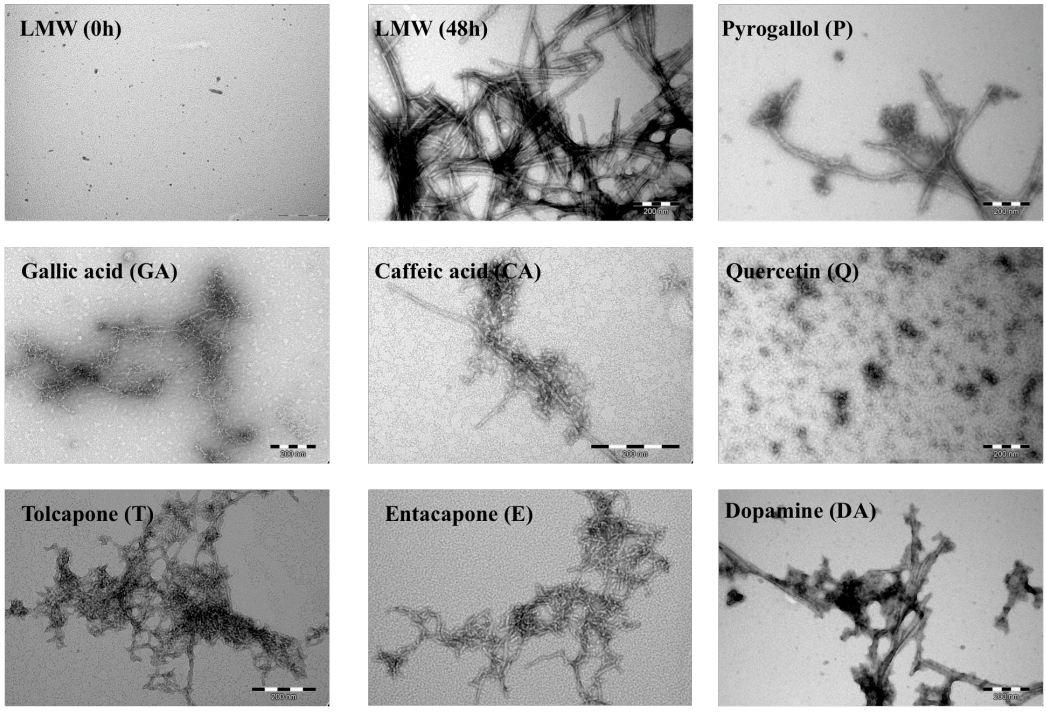
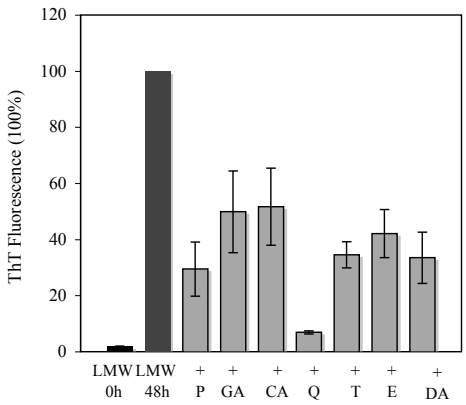


Fig. 3.6 The compounds inhibit low molecular weight (LMW) Aβ42 fibril formation. (A) Samples of LMW Aβ42 were incubated at 37°C with and without compounds, at molar ratios of Aβ42: inhibitor of 1:2. The time course of protein fibrillization was measured by ThT fluorescence assay. The bar graph represents the amount of fibril formation in absence and in presence of the compounds pyrogallol (P), gallic acid (GA), caffeic acid (CA), quercetin (Q), tolcapone (T), entacapone (E), and dopamine (DA). The samples containing the compounds showed a decrease of the ThT fluorescence signal after 48 hr incubation. The figure shows means of three independent experiments ± SD (n=6). **(B)** Electron micrographs of negatively stained quaternary structures deposited from solutions of LMW Aβ42 (10 μM) before and after 48 hr incubation at 37 °C in the absence and in the presence of 20 μM of the compounds listed. *Scale bar* represents 200 nm.

3.1.6 Entacapone (E), tolcapone (T) and related catechols inhibit the conversion of Aβ42 protofibrils (PF) into mature fibrils in a specific and concentration dependent manner

To test our hypothesis and determine if these compounds act by targeting intermediates on the amyloid pathway, we evaluated their capacity to block the conversion of PFs into mature amyloid fibrils. PFs are metastable oligomeric intermediates, which have been observed during the *in vitro* fibrillization of Aβ and almost all other amyloidogenic proteins (Lansbury et al., 2006). During the last decade, mounting evidence from *in vivo* and *in vitro* studies point toward early aggregation intermediates, including PFs, as the major cytotoxic species responsible for triggering neurodegeneration in AD, PD, prion diseases and other related diseases (Caughey et al., 2003). Freshly isolated PFs were co-incubated with 5 and 20 μM of compounds and the aggregation was monitored by ThT fluorescence and TEM after 24 hr (data not shown) and 48 hr at 37 °C. After 48 hr, the samples containing PFs alone showed an increase in ThT signal consistent with a conversion of the PFs into mature fibrils (Fig. 3.7A). When PFs were incubated with 20 μM GA and CA the increase in ThT fluorescence observed with PFs alone was reduced by approximately 50%. More than 70% reduction in the ThT signal was observed in the samples containing 20 μM of P, E and DA suggesting that these compounds are more effective at inhibiting the PF to fibril conversion. However, co-incubation of PF with 20 μM of Q or T resulted in > 80-90 % inhibition, with Q being the most potent inhibitor of PF growth and fibrillization in the series. As expected, TEM analysis of the samples containing PFs alone after 48 hr of aggregation showed dense networks of fibrils. In the presence of Q, T, E, DA, mainly PF-like structures, similar to those present at the starting conditions were observed, in addition to only some isolated short fibrils (Fig. 3.7B). Short fibrils were observed in the presence of GA and CA after 48 hr (Fig. 3.7B), which is consistent with the higher ThT signal in these samples (Fig. 3.7A).

A



B

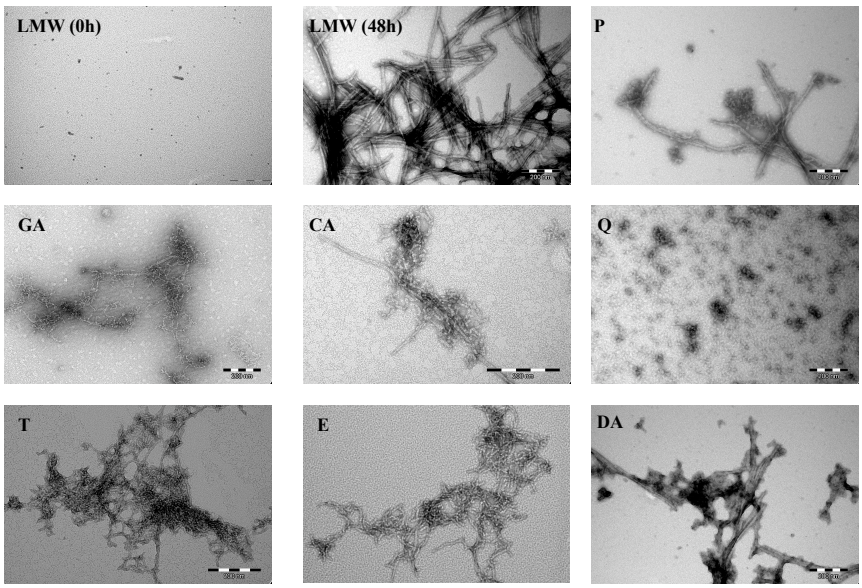


Fig. 3.7 The compounds prevent the conversion of Aβ42 protofibrils (PF) into mature fibrils in a specific and concentration-dependent manner. (A) Samples of PF Aβ42 stock solutions were prepared by dissolving the peptide in 5 % DMSO, 2 M Tris Base pH 7.6. Samples of Aβ42 were incubated at 37 °C with and without compounds, at molar ratios of Aβ42: inhibitor of 1:2. The time course of protein fibrillization was measured by ThT fluorescence assay. The bar graph represents the amount of fibril formation in absence and in presence of the compounds pyrogallol (P), gallic acid (GA), caffeic acid (CA), quercetin (Q), tolcapone (T), entacapone (E) and dopamine (DA). The samples containing the compounds showed a decrease of the ThT fluorescence signal after 48 hr incubation. The figure shows means of three independent experiments ± SD (n=6). **(B)** Electron micrographs of negatively stained quaternary structures deposited from solutions of PF Aβ42 (10 μM) before incubation and after 48 hr incubation at 37 °C in the absence and in the presence of 20 μM of the compounds listed. *Scale bar* represents 200 nm.

3.1.7 Only Entacapone (E), Tolcapone (T) are effective in inhibiting the seeding capacity of Aβ42

To determine if E, T and related compounds can block Aβ42 aggregation at a later stage of the fibrillization process, i.e. fibril growth, the capacity of the compounds to interfere with fibril elongation and the seeding capacity of Aβ42 fibrils was evaluated using the seeding polymerization assay described above. Fibrillar seeds of Aβ42 were prepared by fragmenting preformed and purified fibrils by sonication. The freshly prepared fibril seeds were added to a fresh monomeric solution of Aβ42 and co-incubated with each of the compounds (20 μM). The kinetics of fibrillization was followed over a period of 3 hr with ThT and the structures of the final aggregates were characterized by TEM. Among all the compounds, only E, T and Q showed significant reduction in fibril growth and inhibition of the seeding capacity by Aβ42 fragmented fibrils (Fig. 3.8). Analysis of these samples by TEM revealed predominantly short fibrillar structures resembling the fibrillar seeds, consistent with the ThT results (data not shown). These results further confirm that these E, T and Q interfere with the elongation and growth of Aβ42 fibrils possibly via direct interactions with either Aβ42 seeds or monomers or both. After 3 hr incubation, no inhibition was observed in the samples containing P and CA. Moreover, addition of GA and DA to the mixtures containing Aβ42 monomers and seeds did not affect the rate of fibrillization and resulted in only slight reduction of the ThT fluorescence signal (Fig. 3.8).

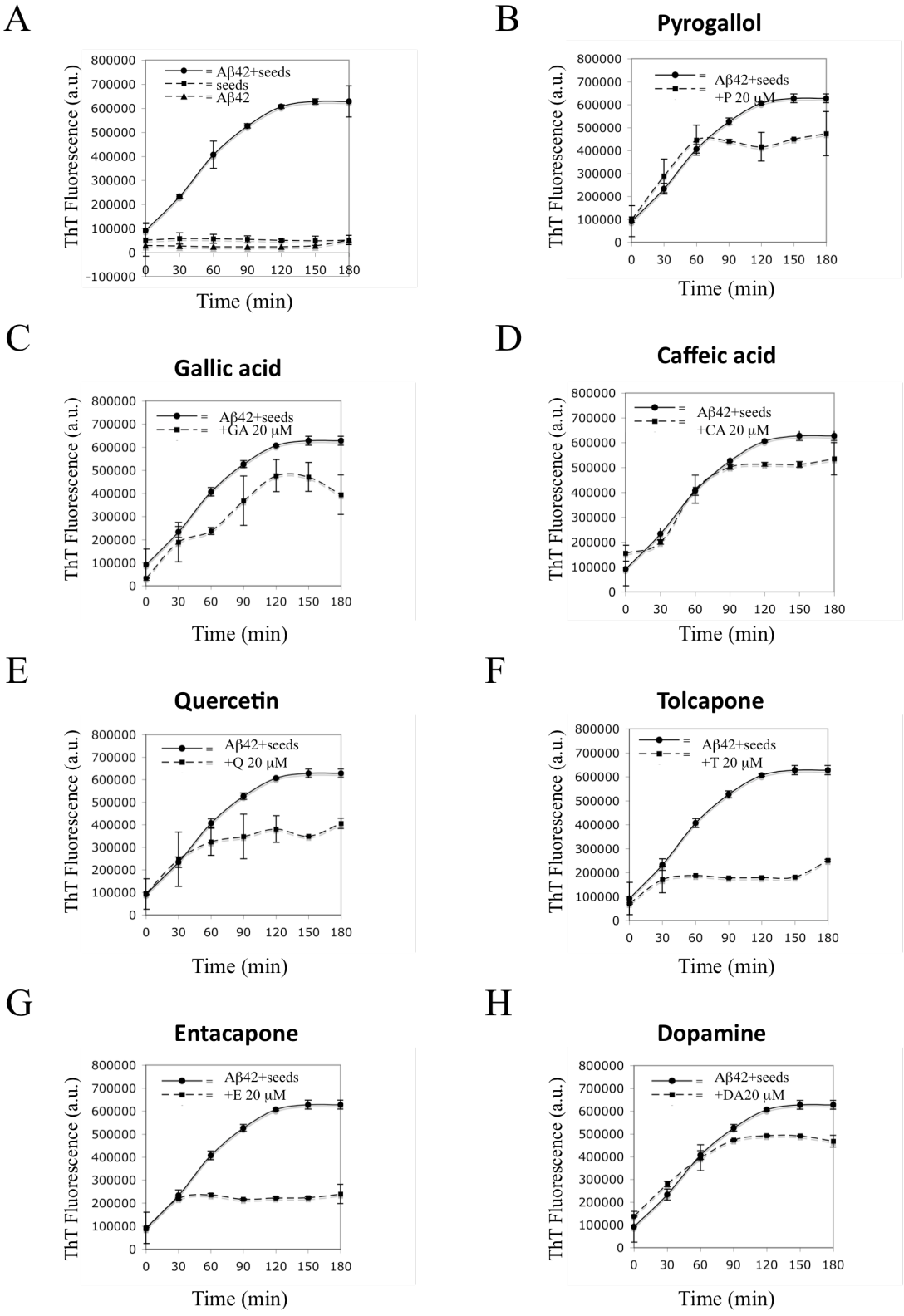


Fig. 3.8 The Aβ42 monomeric seeding polymerization assays revealed that Q, E and T have an inhibitory effect on the kinetic. Samples of monomeric Aβ42 (10 μM) were incubated with the seeds (2 μM) at 37 °C, with continuous shaking, without (A) and with (B-H) 20 μM of compounds pyrogallol (P), gallic acid (GA), caffeic acid (CA), quercetin (Q), tolcapone (T), entacapone (E), and dopamine (DA). The time course of protein fibrillization was measured every 30 minutes by ThT fluorescence assay for 3 hours.

3.1.8 Protection against Aβ42-induced toxicity in PC12 cells

Previous studies reported that natural polyphenols like Q, GA and CA are neuroprotective against Aβ toxicity. The effects of Q and GA have been studied against Aβ42 in primary cultures (Ansari et al., 2009; Ban et al, 2008; Bastianetto et al., 2006) and *in vivo* models of AD (Ehrnhoefer et al., 2008). Recently, the protective role of CA on Aβ-induced toxicity in PC12 cells was also described (Sul et al., 2009). We sought to determine if there is a correlation between the inhibitory potency of the polyphenolic compounds on Aβ aggregation and their effect on Aβ-induced toxicity in PC12 cells. The compounds were pre-incubated at concentrations in the range of 5 - 40 μM with crude preparation of Aβ42, i.e. preparations containing predominantly LMW and PF Aβ42. These conditions were chosen to mimic the pathological situation *in vivo* where both the LMW and PF species are populated in the diseased AD brain. After incubation with Aβ 42 for 20 - 30 min, the Aβ –compound mixtures were then added to PC12 in cell culture media. The cellular viability was assessed after 24 hr using MTT and luminescent assays (Jan et al, 2008). All the compounds tested did not increase the mortality of PC12 cells in the absence of Aβ42 even at the highest concentration of inhibitors (40 μM, see Fig.3.9), demonstrating that the compounds do not enhance cell viability on their own over a concentration range of 10 – 40 μM. The cells were then treated for 24 hr either with 40 μM of Aβ42 alone or in the presence of 5 μM, 10 μM, 20 μM and 40 μM of the compounds (Fig. 3.9).

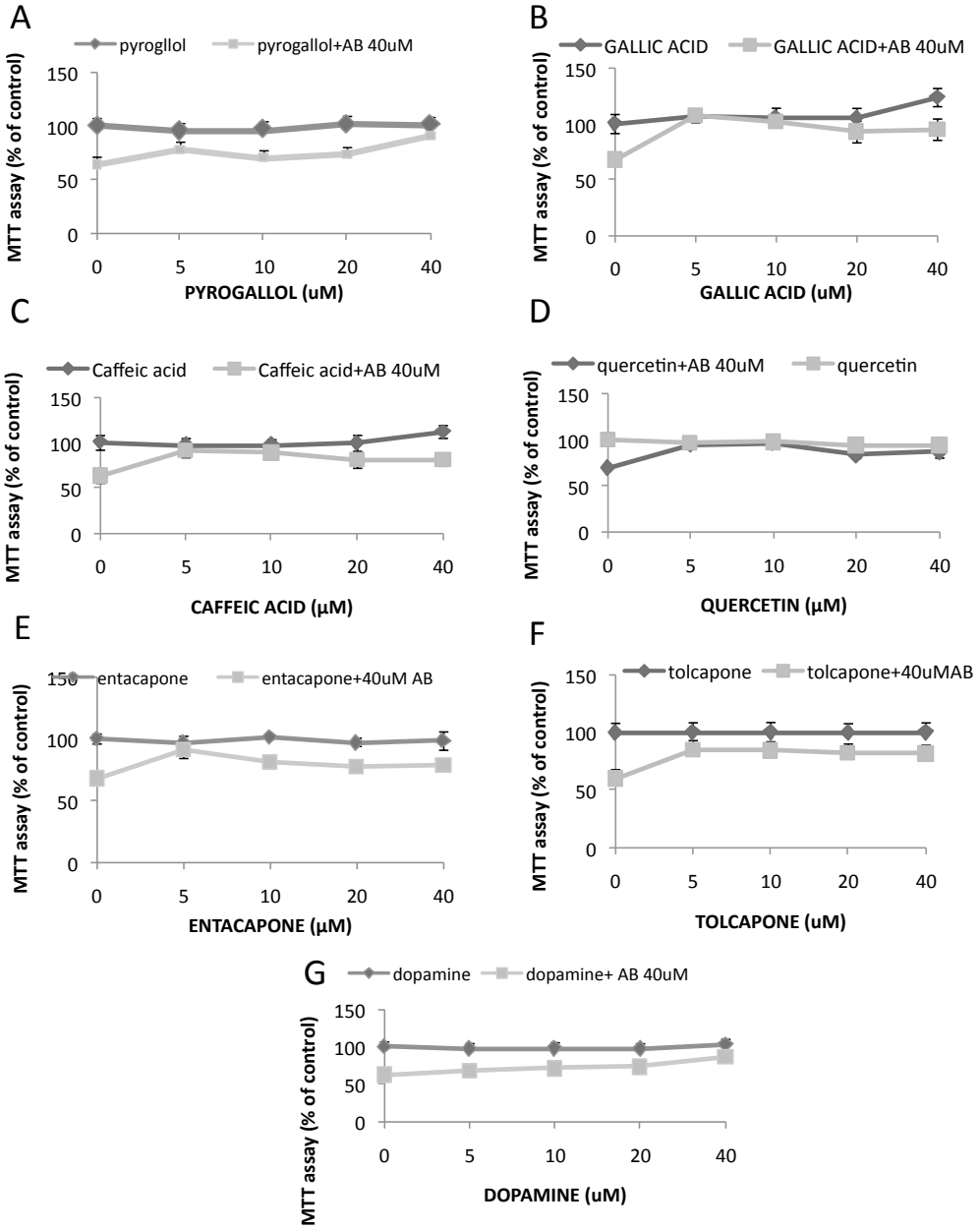


Figure 3.9 Concentration-dependent effects of the compounds on Aβ42-induced toxicity in PC12. The cells were treated with different concentration of compounds alone (5µM, 10µM, 20µM, 40µM) or co-treated with Ab42 (40µM) in presence of the compounds Pyrogallol (A), Gallic acid (B), Caffeic acid (C), Quercetin (D), Entacapone (E), Tolcapone (F) and Dopamine (G) for 24h at 37 °C. The cellular viability was evaluated by MTT assay and the data were expressed as percentage of control, the control treatment is set to 100%. Bars are means ± S.E.

Exposure of PC12 cells to 40µM of Aβ42 reduced the cellular viability by approximately 40% and 50% (Fig. 3.10 and 3.11) as determined by the MTT assay and ATP release, respectively (Fig. 3.11) with respect to the control (untreated cells). At higher concentrations of polyphenols (20 µM and 40 µM) were found to be protective against the Aβ42 toxicity but the attenuation of the toxicity was less

evident when compared to the effect observed at low concentration. However, P and DA showed a strong protective effect at a concentration of 40 μM. At low concentration (5 μM) E and T induced a significantly (~ 30%) protection against Aβ-induced toxicity whereas at higher concentration (10 μM, 20 μM and 40 μM) we found an increase of cellular viability and ATP release of approximately 20 %. These observations indicate that the protection in the co-treatment of the PC12 cells could be related to a dual activity of the compounds, i.e. their inhibitory activity against Aβ aggregation and protection against Aβ-induced cell toxicity.

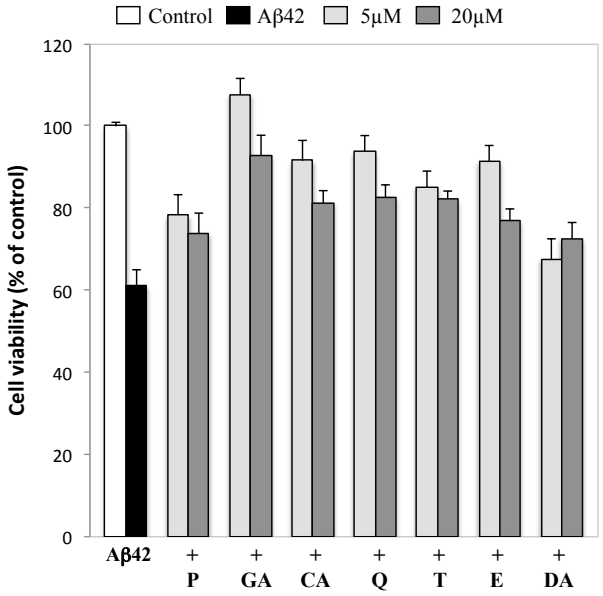


Fig. 3.10 Protective effect of the compounds against Aβ42–induced toxicity in PC12 cells. PC12 cells were treated with Aβ42 (40μM) or co-treated in presence of the compounds pyrogallol (P), gallic acid (GA), caffeic acid (CA), quercetin (Q), tolcapone (T), entacapone (E) and dopamine (D). The cells were treated with two different compound concentrations: 5 μM and 20 μM for 24 h. The cellular viability was evaluated by MTT assay and the data were expressed as percentage of control (non treated cells). The control treatment is set to 100%. Error bars are means ±S.E. We used # to compare the data to the control and * respect to the treatments with Aβ42 crude preparation. A: ### $p<0.001$, B: *** $p<0.001$, C: ** $p<0.01$, D: * $p<0.05$. The statistical value was ### for the treatment with Aβ42 crude preparation respect to the control; the statistical values for the compounds were *** $p<0.001$ for GA (5 μM and 20 μM), CA, Q, E (5 μM), and ** $p<0.01$ for P (5 μM and 20μM), T (5 μM and 20μM), CA, Q, E and D (20 μM).

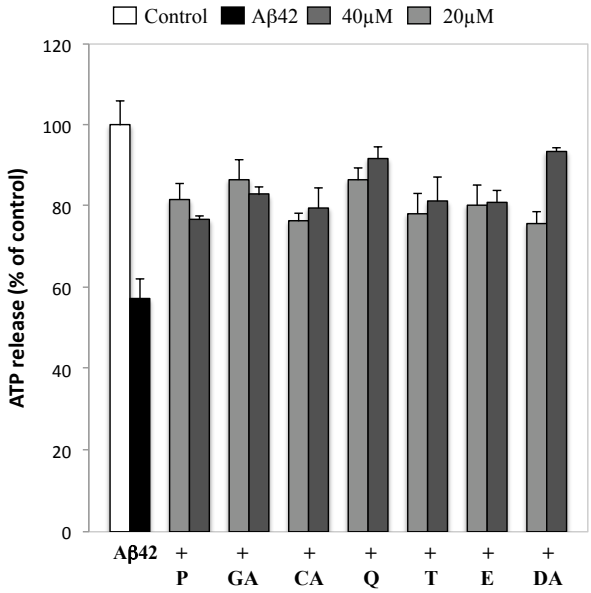


Fig. 3.11 Protective effect of the compounds against Aβ42–induced toxicity in PC12 cells. PC12 cells were treated with Aβ42 (40μM) or co-treated in presence of the compounds pyrogallol (P), gallic acid (GA), caffeic acid (CA), quercetin (Q), tolcapone (T), entacapone (E) and dopamine (D). The cells were treated with two different compound concentrations: 20 μM and 40 μM for 24 h. The cellular viability was evaluated by ATP luminescence assay and the data were expressed as percentage of control (non treated cells). The control treatment is set to 100%. Error bars are means ±S.E. We used # to compare the data to the control and * respect to the treatments with Aβ42 crude preparation. A: ### $p<0.001$, B: *** $p<0.001$, C: ** $p<0.01$, D: * $p<0.05$. The statistical value was ### for the treatment with Aβ42 crude preparation respect to the control; the statistical values for the compounds were *** $p<0.001$ for GA (5 μM and 20 μM), CA, Q, E (5 μM), and ** $p<0.01$ for P (5 μM and 20μM), T (5 μM and 20μM), CA, Q, E and D (20 μM).

NEUROPROTECTION OF MICROGLIA IN PD AND AD DEGENERATION MODELS

In recent years, our laboratory and others have performed in vitro studies based on co-culture of neurons and microglia or on the use of conditioned media to elucidate the neuroprotective functions of microglial cells (Toku et al, 1998; Zietlow et al, 1999; Park et al, 2001; Polazzi et al, 2001; Figueiredo et al, 2008; Lai and Todd, 2008). In particular, it has been demonstrated that microglia conditioned media prevent apoptosis of cerebellar granule neurons (CGNs) induced by shift to low potassium and that diffusible signals from apoptotic neurons increase microglial neuroprotective action (Polazzi et al., 2001). In this setting, the possibility to shift the balance of microglial cells from pro inflammatory to anti-inflammatory states offers a good strategy to develop therapies for neurodegenerative diseases. We investigated the neuroprotection of microglia *in vitro* models of Parkinson-like neurodegeneration, Alzheimer-like neurodegeneration and excitotoxicity induced by exogenous glutamate on cerebellar granule neurons (CGNs).

In the present study we have extended our in vitro 'microglial neuroprotective model' to an in vitro model of Parkinson-like neurodegeneration, i.e. 6-hydroxydopamine (6-OHDA)-induced death in CGNs, a widely used model to study mechanisms of neuronal survival/death (Contestabile et al, 2002). The neurotoxin 6-OHDA has been shown to induce CGNs death through production of free radicals, alteration of protein degradation mechanisms and accumulation of ubiquitinated proteins (Dodel et al., 1999; Lin et al., 2003; Chen et al., 2004; Ma et al., 2006; Monti et al., 2007), all typical features of the Parkinson's Disease (PD)-like death caused by the toxin on its specific target represented by dopaminergic neurons of the substantia nigra (reviewed by Blum et al., 2001; Bove´ et al., 2005; Olanow et al., 2007).

Subsequently, the neuroprotective effect of the microglia conditioned media was evaluated in a AD-like neurodegenerative models caused by Aβ42-induced cytotoxicity on CGNs.

We have used our in vitro 'microglial neuroprotective model' against apoptotic death of CGNs caused by staurosporine, and against a mild excitotoxic stimulus delivered through sub-chronic glutamate treatment and against a stronger and acute excitotoxic insult. In the excitotoxicity a non-physiologic increase of the glutamate in the

presynaptic region induces the iperstimulation of the glutamate receptors (metabotropic and ionotropic receptors). The iperactivation of both receptors induce an increase of intracytoplasmic calcium that is an apoptotic stimulus for the cell. The excitotoxicity represents a mechanism common to the major neurodegenerative diseases and the regulation of glutamate extracellular levels and glutamate-induced toxicity could be a potential therapeutical approach in many neurodegenerative disease such as: PD, AD, HD, amyotrophic lateral sclerosis (ASL).

Finally, we tried to identified the protective factor/s released as soluble factors present in the microglia conditioned medium in response to 6-OHDA toxin.

3.2.1 Neuroprotection of Microglial condition medium 48hours (MCM48h) against 6-OHDA in cultures of rat cerebellar granule neurons (CGNs)

In agreement with previous results (Dodel et al. 1999; Monti et al. 2007), cultures of fully differentiated CGNs at 7 days in vitro are dose-dependently sensible to the neurotoxic insult caused by 24-h exposure to 6-OHDA, based on the MTT assay and on nuclei count following Hoechst staining (Fig. 3.12). Exposure of CGNs to 6-OHDA in presence of medium previously conditioned for 48 h by cultured microglial cells (microglia conditioned medium, MCM) resulted in nearly complete preservation of cell viability, evaluated through MTT assay, even at the most toxic 6-OHDA concentrations (Fig. 3.10a). Any in vivo toxin hitting neurons would likely also affect microglia. In order to gain better insight into the functional significance of the neuroprotection granted by MCM, we verified whether the MCM neuroprotective effect was maintained following exposure of microglia to 6-OHDA. While a toxic effect towards microglia was exerted by 6-OHDA at high concentration (data not shown), the medium conditioned by microglia exposed to 20 lmol/L 6-OHDA for 48 hours protected CGNs, challenged with 6-OHDA themselves, similarly to the normal MCM (Fig. 3.12b). To obtain a more precise assessment of cell death caused by 6-OHDA and of the extent of the MCM neuroprotection, we counted the condensed and normal nuclei in randomly selected fields of cultures stained with Hoechst (Fig. 3.12c). The quantification of the condensed/total nuclei ratio confirmed that 20 lmol/L

6-OHDA was highly toxic, resulting in more than 80% CGNs death after 24 h of toxin exposure, and that MCM was able to completely revert this effect (Fig. 3.12d).

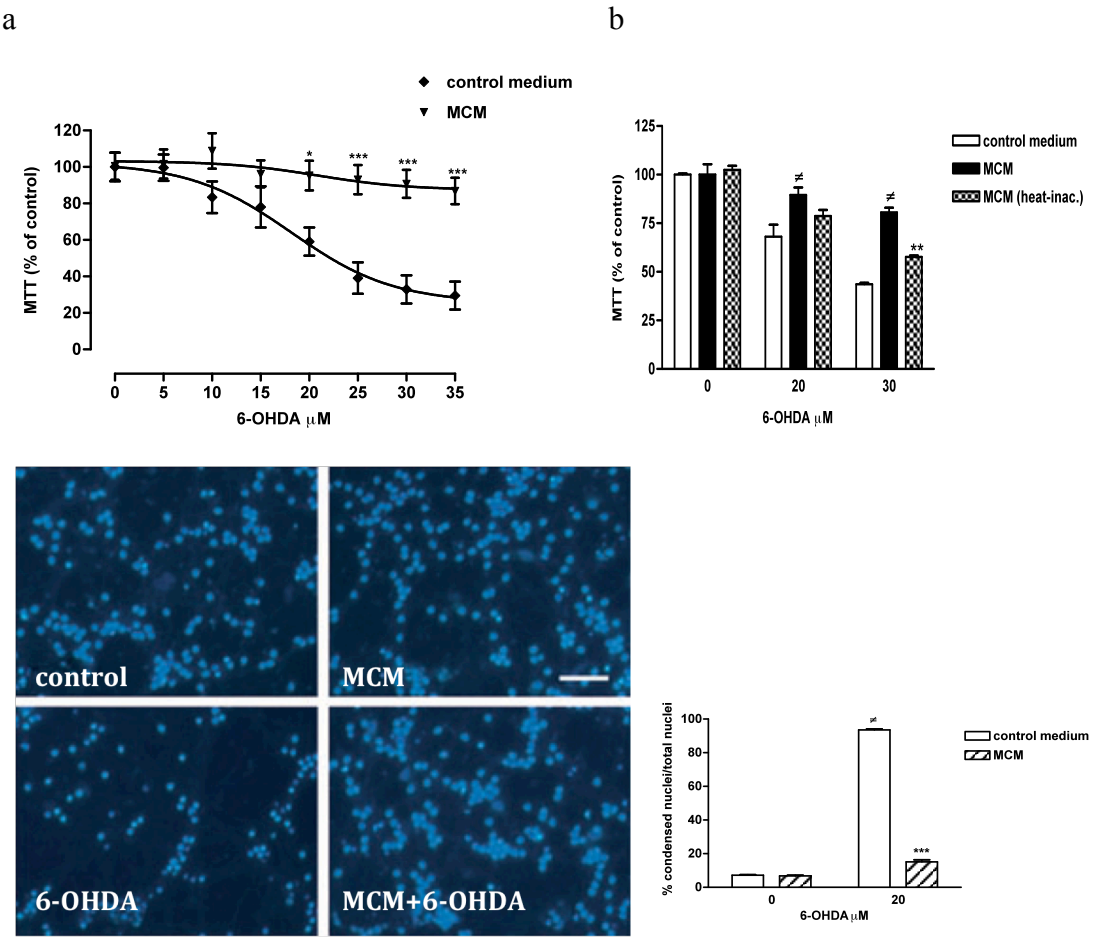
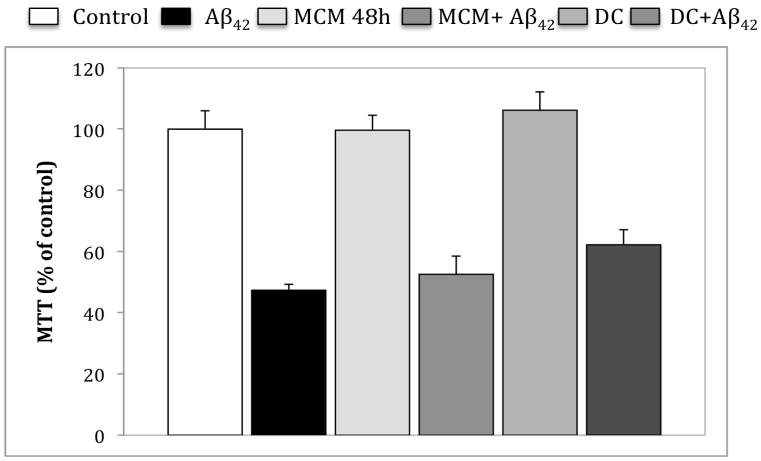


Fig. 3.12 Microglial conditioned medium (MCM) enhances neuronal viability and survival in CGNs exposed to 6-OHDA. (a) MTT assay of CGNs cultures treated for 24 h with increasing concentrations of 6-OHDA in the presence or absence of 48 h MCM to test its neuroprotection. Each point is the mean ± SE of six different experiments run in triplicate; *p < 0.05, ***p < 0.001 with respect to the conditions of treatment with 6-OHDA in non-conditioned medium. (b) MTT assay of CGNs treated for 24 h with 6-OHDA 20 lmol/L in the presence or absence of medium obtained by microglial cells exposed themselves to 6-OHDA 20 lM for 48 h to test its neuroprotection. Each point is the mean ± SE of three different experiments run in triplicate; „ p < 0.001 compared to controls; ***p < 0.001 with respect to the conditions of treatment with 6-OHDA in non-conditioned medium. (c) Representative Hoechst staining of apoptotic death. CGN cultures were treated for 24 h with 20 lmol/L 6-OHDA in the presence or absence of MCM. (d) Quantification based on count of condensed and normal nuclei following Hoechst staining. Bars are the mean ± SE of three different experiments run in duplicate; „ p < 0.001 respect to the control condition, ***p < 0.001 with respect to the condition of treatment with 6-OHDA in non-conditioned medium. Calibration bar 40 lm.

3.2.2 Neuroprotection of microglia conditioned media from apoptotic death induced by $A\beta_{42}$ in cultures of rat cerebellar granule cells

We tested the microglia neuroprotective model in AD-like neurodegeneration induced by $A\beta_{42}$ on CGNs. The CGNs were treated with a crude preparation of $A\beta_{42}$ (40μM) added directly in the medium serum free or in the presence of microglia conditioned media MCM48h or DC for 24h. Exposure of CGNs cells to 40μM of $A\beta_{42}$ reduced the cellular viability by approximately 50% (Fig. 3.13) as determined by the MTT with respect to the control (cells shifted in medium serum free, in MCM48 and DC).
The presence of the two conditioned media do not reverted the $A\beta_{42}$-induced damage on CGNs.



3.13 Effect of microglial-conditioned media against Aβ42–induced toxicity in CGNs. CGNs cells were treated with Aβ42 (40μM) added directly in the media serum free or added to the cells shifterd with two microglial-conditioned media: MCM48h or DC, for 24h. The cellular viability was evaluated by MTT assay and the data were expressed as percentage of control (non treated cells). The control treatment is set to 100%. Error bars are means ±S.E.

3.2.3 Neuroprotection of microglia conditioned media from apoptotic death induced by staurosporine in cultures of rat cerebellar granule neurons

We examined the neuroprotective role of microglia conditioned media on CGNs challenged with staurosporine-induced apoptosis.

Exposure to staurosporine resulted in apoptotic death of CGNs, as revealed by the appearance of condensed nuclei in Hoechst stained cultures (Fig. 3.14A). Through cell counting, it was determined that about 30% of cultured neurons were apoptotic by the end of the 24-h treatment and that MCM, and to a greater extent DCM, significantly protected CGNs from the neurotoxic insult (Fig. 3.14B). A similar degree of neuroprotection of the media conditioned by microglia was assessed by using the MTT assay to evaluate cell viability (Fig. 3.14C).

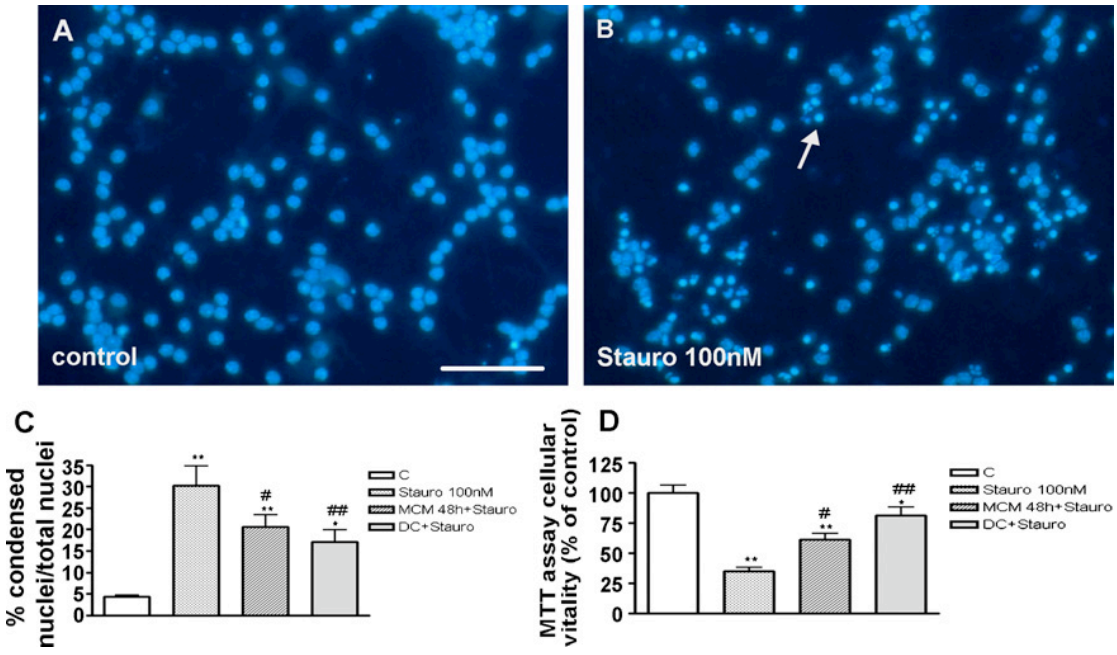


Fig. 3.14 Hoechst stain of control CGC at 8 DIV (A) or corresponding cultures treated for 24 h with staurosporine (B). Arrows point at condensed nuclei; calibration bar, 30_m. (C) Cell count of condensed and total nuclei after Hoechst stain. Data were expressed as percentage of condensed nuclei over total stained nuclei. Bars aremeans°æS.E. from four to eight cultures for each condition. $^{**}P < 0.001$, $^{*}P < 0.01$ compared to control; $\#P < 0.05$, $\#\#P < 0.01$ compared to staurosporine in non-conditioned medium. Newman–Keuls multiple comparison test after ANOVA. (D) MTT assay on corresponding CGC cultures. Bars aremeans°æS.E. from four to eight cultures for each condition. $^{*}P < 0.05$, $^{**}P < 0.001$ compared to control; $\#P < 0.05$, $\#\#P < 0.001$ compared to staurosporine in non-conditioned medium. Newman–Keuls multiple comparison test after ANOVA.

3.2.4 Neuroprotection of microglia conditioned media from apoptotic death induced by glutamate in cultures of rat cerebellar granule neurons

Sub-chronic glutamate excitotoxicity obtained through continuous exposure of the cultures to 100μM glutamate added to the serum-free medium, resulted after 24 h in the appearance of a sizeable number of condensed nuclei (Fig. 3.15A). Quantification through cell counting demonstrated around 20% of apoptotic neurons and also in this case MCM and DCM significantly protected from death neurons challenged with the excitotoxic insult (Fig. 3.15B). By assessing neurotoxicity through cell viability evaluated by MTT assay, we could confirm the significant neuroprotective action of media conditioned by microglia (Fig. 3.15C). Acute excitotoxicity, obtained through a short pulse of exposure of granule neuron cultures to glutamate in the absence of magnesium, resulted after 24h of subsequent incubation in the serum-free medium in massive neuronal death, as previously described (Ciani E et al., 1996). Under these conditions, neuronal death over the 24h period considered derives from a mix of apoptotic and necrotic mechanisms (Ankarcrona et al.,1995; Slagsvold et al., 2000) and cannot be reliably quantified through Hoechst stain. For this reason, we evaluated cell death from the ratio between PI positive cells (dead cells) and total cells present in the culture after 24 h from the initial excitotoxic insult (dead cells plus FDA-stained viable cells).

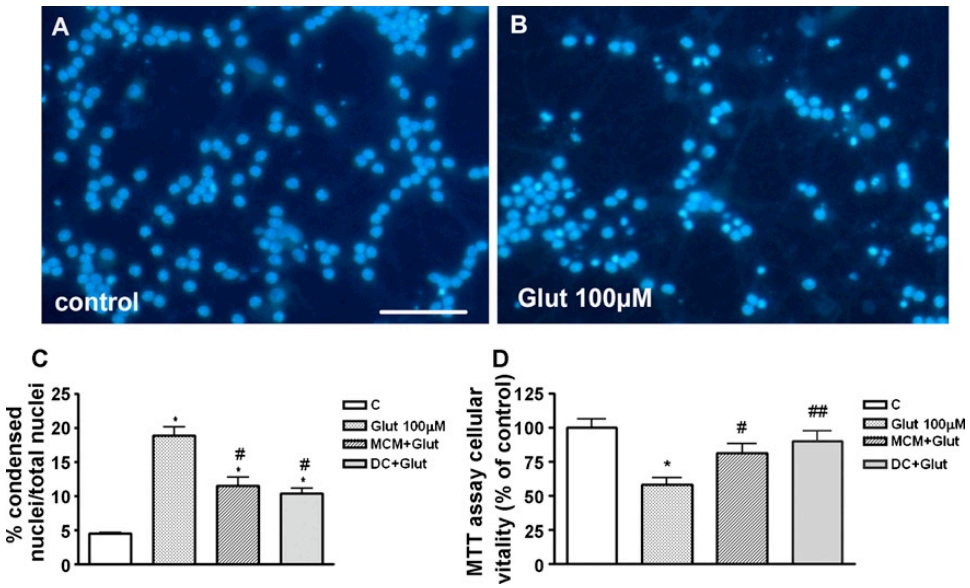


Fig. 3.15 Hoechst stain of control CGC at 8 DIV (A) or corresponding cultures treated for 24 h with glutamate (B). Arrows point at condensed nuclei; calibration bar, 30_m. (C) Cell count of condensed and total nuclei after Hoechst stain. Data were expressed as percentage of condensed nuclei over total stained nuclei. Bars are means°æS.E. from 8 to 20 cultures for each condition. **P* < 0.001 compared to control; #*P* < 0.001 compared to glutamate in non-conditioned medium. Newman–Keuls multiple comparison test after ANOVA. (D) MTT assay on corresponding CGC cultures. Bars are means°æS.E. from four to eight cultures. **P* < 0.001 compared to control; ##*P* < 0.01, #*P* < 0.05 compared to glutamate in non-conditioned medium. Newman–Keuls multiple comparison test after ANOVA.

As shown in Fig. 3.16A, the delivery of this excitotoxic insult to granule neuron cultures resulted in large decrease of FDA stained viable cells and corresponding increase of PI-stained dead cell nuclei. Cell counting (Fig. 3.16B) allowed to quantitatively estimate cell death at a value of at least 50% and to assess that, while MCM and to a greater extent DCM showed a tendency towards neuroprotection, this effect did not result statistically significant. In order to try to separate the effects of apoptosis from those of necrosis in this model of glutamate toxicity, we made experiments based on the measure of caspase 3 activity by determining it through the fluorogenic substrate Ac-DEVD-AMC (Calbiochem). While also in this case there was a tendency of MCM, and even more of DCM, to reduce caspase 3 activity in glutamate-treated cells, the results did not reach statistical significance (data not shown). Our present data substantially extend available information on the ability of microglia conditioned media to protect from death neurons challenged with neurotoxic stimuli (Polazzi et al., 2001; Toku et al., 1998).

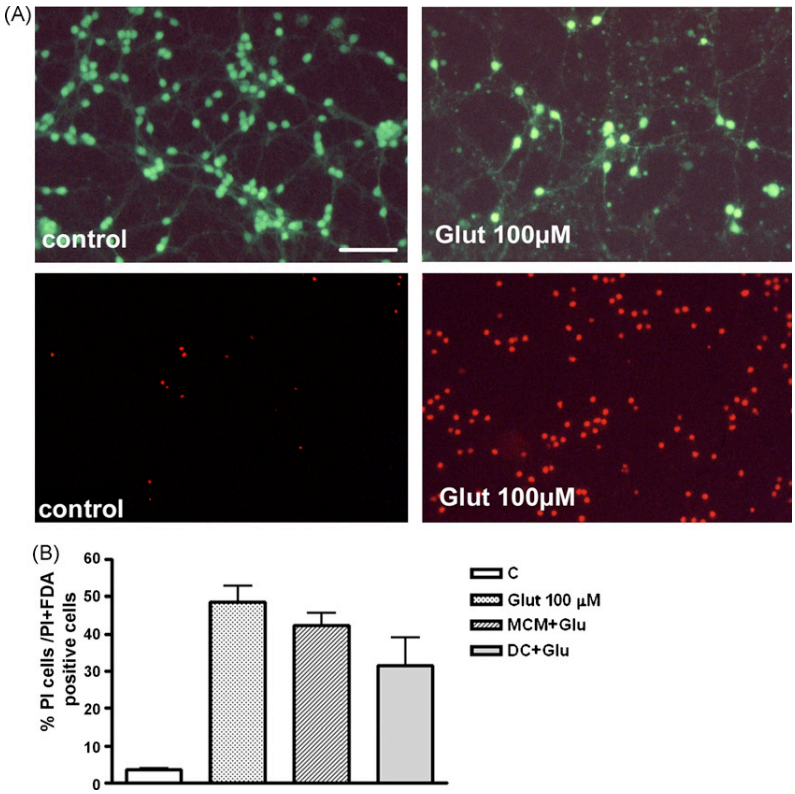


Fig. 3.16 (A) FDA (upper panels) and PI (lower panels) staining of CGC in control conditions and 24 h after an acute excitotoxic insult of glutamate. Calibration bar, 45_m. (B) Cell counting of FDA- and PI-stained cells in the various experimental conditions. Bars are means°æS.E. from six to eight cultures

3.2.5 Identification of putative neuroprotective factor(s) in MCM48h

In an effort to identify the nature of the putative neuroprotective factor(s) present in the conditioned medium, we exposed MCM to heath inactivation or to a pre-treatment with wide spectrum peptidases/proteases, before testing it for neuroprotection of CGNs exposed to 6-OHDA. As shown by Fig. 3.17 (a) and (b), both types of treatments resulted in a significant, but not complete abrogation of MCM neuroprotection, thus suggesting that peptidic factor(s) certainly contributed to, but not completely accounted for this MCM neuroprotective action.

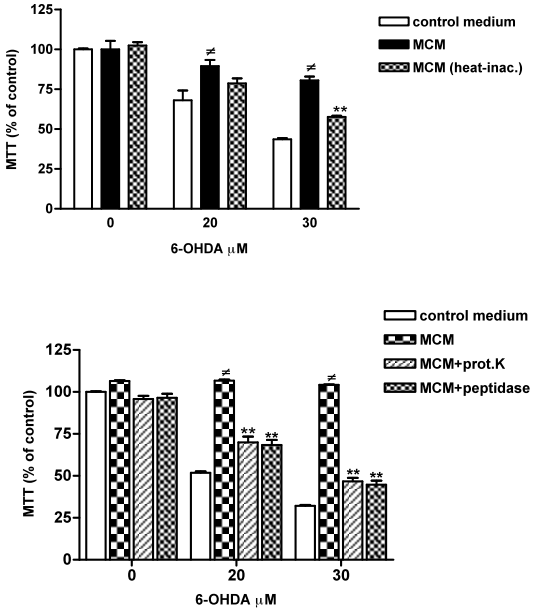


Fig. 3.17 MCM protects CGNs exposed to 6-OHDA through partial contribute of peptidic factors. CGN cultures were treated for 24 h with 30 lmol/L 6-OHDA in the presence or absence of MCM previously heat-inactivated (a) or previously enzimatically treated with proteinase K or peptidase (b). CGNs viability has been measured through MTT assay. (a) Bars are the mean ± SE of six different experiments run in triplicate; „ $p < 0.001$ with respect to the conditions of treatment with 6-OHDA in non-conditioned medium; **$p < 0.01$ with respect to the conditions of treatment with 6-OHDA in non-heat-inactivated MCM. (b) Bars are the mean ± SE of six different experiments run in triplicate; „ $p < 0.001$ with respect to the conditions of treatment with 6-OHDA in non conditioned medium. **$p < 0.01$ with respect to the conditions of treatment with 6-OHDA in non-enzymatically-treated MCM.

3.2.6 Identification of molecular weight of the neuroprotective factor(s)

To get more information on putative neuroprotective peptide(s), we performed a molecular weight based fractionation of MCM, by passing it through filters provided with a 10 kDa cut-off, before reconstituting the medium to the initial volume and testing it for its neurotoxicity/neuroprotection on 6-OHDA-treated CGNs. By itself, none of the two fractions obtained was able to replicate the neuroprotection, as done by the complete MCM (Fig. 3.18 a and b). Interestingly, even if the < 10 kDa fraction was neurotoxic towards CGNs per se, it significantly protected them from 6-OHDA neurotoxicity (Fig. 3.18).

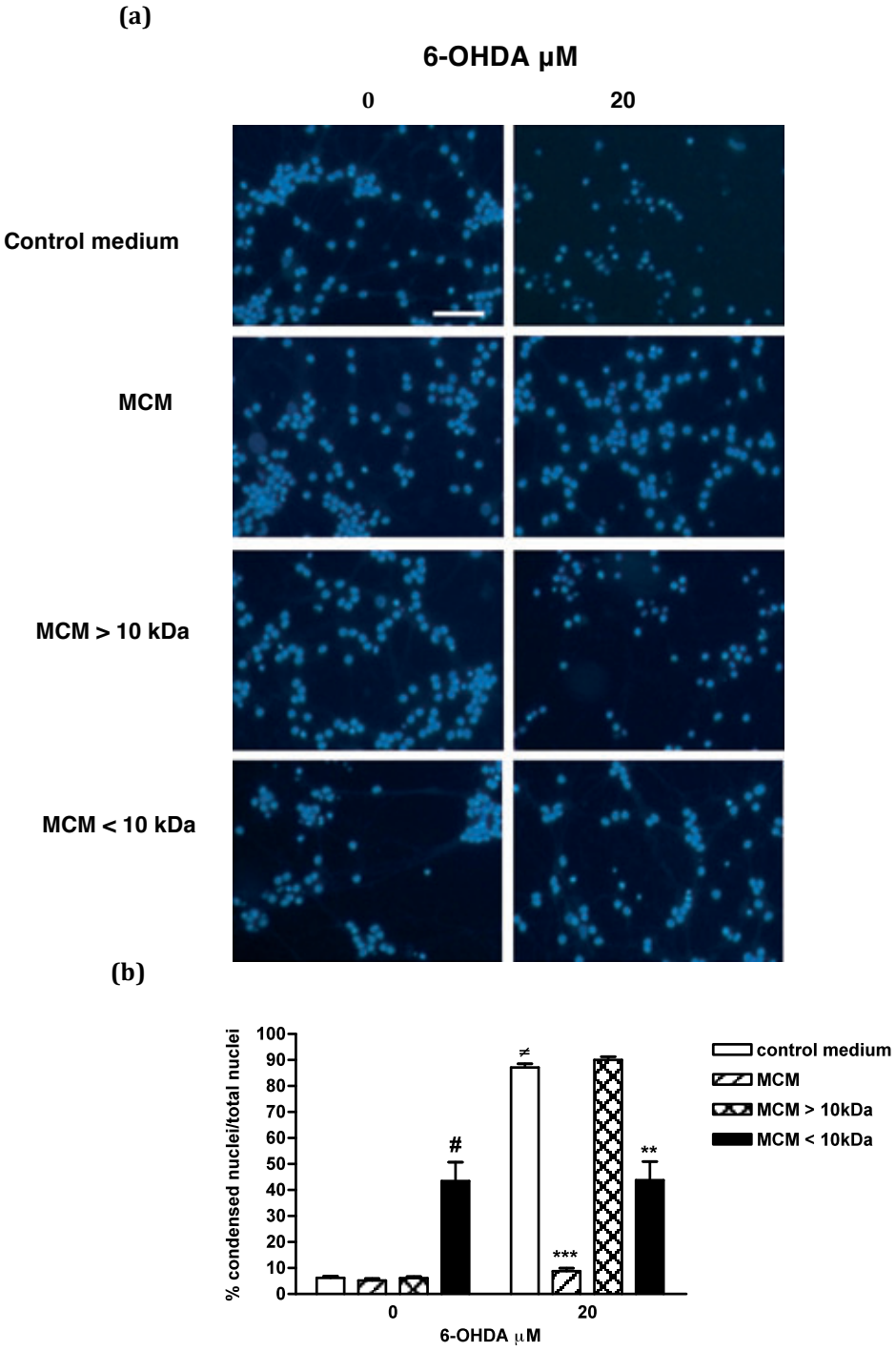


Fig. 3.18 Absence of neuroprotection by fractions of MCM obtained through filtration with cut-off at 10 kDa molecular weight. CGNs cultures were treated for 24 h with 20 lmol/L 6-OHDA in the presence or absence of MCM or its fractions containing high molecular weight (> 10 kDa) substances and low molecular weight (< 10 kDa) substances. Neuronal death has been measured by Hoechst staining (a), followed by nuclei count (b). Bars are the mean ± SE of three different experiments run in duplicate „ $p < 0.001$ with respect to the control medium, #$p < 0.001$ with respect to control medium and MCM; **$p < 0.01$, ***$p < 0.001$ with respect to the conditions of treatment with 20 lmol/L 6-OHDA with non-conditioned medium. Calibration bar 40 lm.

3.2.7 Identification of TGF-β_2 in the MCM48h

To more precisely target putative neuroprotective peptide(s), we tested MCM for the presence of proteins known to be neuroprotective. In particular, we performed western blot analysis of both cell lysates and MCM to verify whether some of these potentially neuroprotective factors were synthesized by microglial cells and accumulated in the medium. We decided to analyze the expression of BDNF, IGF-I and TGF-b proteins, as they are known to be potentially synthesized by microglia and to be neuroprotective towards CGNs (D'Mello et al., 1993; Lindholm et al., 1993; Nakajima et al., 2001; Elvers et al., 2005). As shown in Fig. 3.19, BDNF appeared to be synthesized, but not released in the medium by microglia, while IGF-I, TGF-b1 and TGF-b3 were both present in the cells and released into the medium conditioned for 48 h in their precursor high molecular weight forms. TGF-b2 precursor was constitutively synthesized by microglia and accumulated into the medium in its active form of estimated molecular weight of 12.5 kDa, reaching an apparently high level after 48 h of culture.

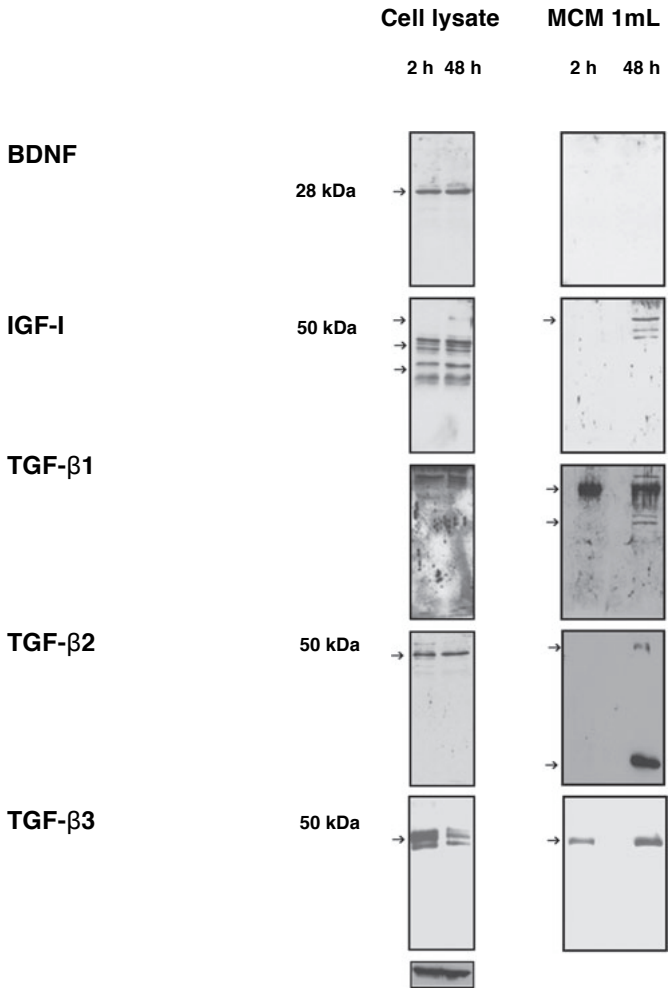


Fig. 3.19 Protein expression analysis of potential neuroprotective factors in microglial cells cultures and microglial conditioned medium. Microglial BDNF, IGF-I, TGF-b1, TGF-b2 and TGF-b3 expression and release into the conditioned medium have been detected by using western blot analysis on both microglial cell lysates (after 2 h and 48 h of culture) and concentrated aliquots of MCM (at 2 h and 48 h). b-Actin staining on microglial cell samples has been performed as a loading control.

In order to exclude that non-specific TGF-b-like ligands (see below) could interfere with the revealed immunoreactivity, we verified through real time PCR whether microglial cells actually synthetized TGF-b mRNAs. All the three TGF- b mRNAs were synthesized by microglial cells in culture both shortly after plating and after 48 h of culture, as shown by the presence of bands of the expected size in the gel and byreal time PCR quantification (Fig. 3.20).

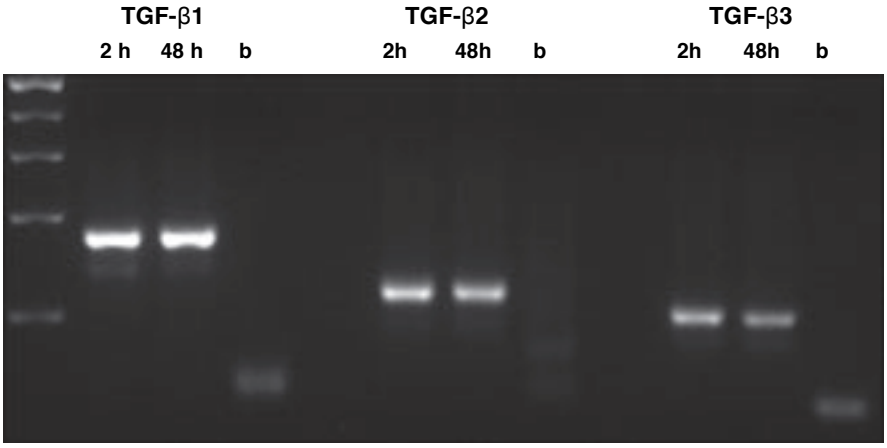
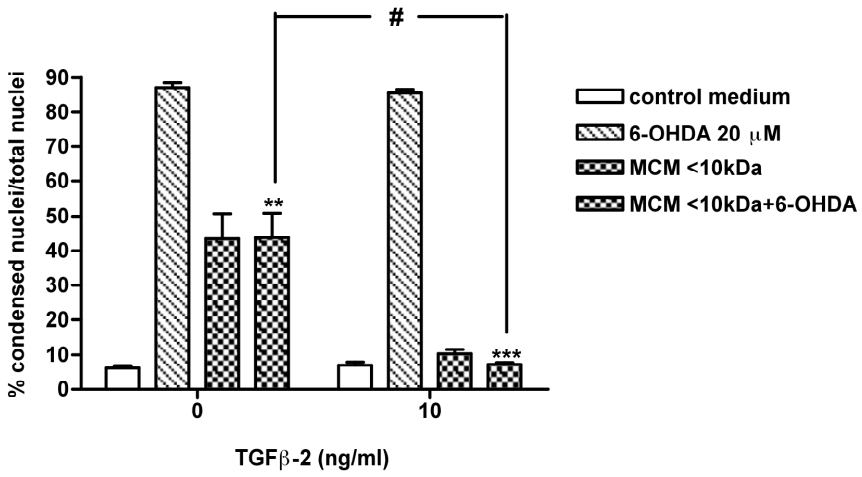


Fig. 3.20 PCR analysis and quantification of TGF-b1, TGF-b2 and TGFb3 expression in microglial cells cultures. Microglial TGF-b1, TGF-b2 and TGF-b3 RNA expression have been detected by using specific primer pairs through real-time PCR analysis in microglial cell at 2 h and 48 h. (a) Table of TGF-b1, TGF-b2 and TGF-b3 expression in Dct relative to actin (Dct = Ct of each gene-Ct of b-actin) at 2 h and the fold-expression of 48 h compared to 2 h. For the 2 h stage, three experiments in duplicate were run; for the 48 h, four experiments in duplicate; b is the negative control, without cDNA. (b) Representative gel of PCR analysis of microglial TGF-b1, TGF-b2 and TGF-b3 RNA expression at 2 and 48 h (b is the negative control).

Focusing on TGF-b2, which was the only tested peptide present in MCM in its mature form, by densitometric quantification of its content in western blots from MCM compared with western blots made from known amounts of recombinant TGF-b2, we estimated a peptide concentration of about 60 ng/mL in media conditioned for 48 h (data not shown). Therefore, TGF-b2 appeared to be a promising candidate as a possible neuroprotective agent, being released in MCM at high concentration in its active form and having previously shown a neuroprotective action towards CGNs and in PD-models (Elvers et al., 2005; Andrews et al., 2006). Attempts to abrogate MCM neuroprotection by blocking TGF-b2 with neutralizing antibodies (T4442 from Sigma- Aldrich or sc-90 Santa Cruz Biotechnology Inc.) were hampered by the fact that concentrations of antibodies still unable to remove the high amount of the peptide present in the medium, as verified through immunoprecipitation experiments, resulted toxic by themselves to CGNs (data not shown). We, therefore, tried to demonstrate

the involvement of TGF-b2 in MCM neuroprotection by using two different approaches. First, we tested whether addition of exogenous TGF-b2 to the MCM fraction, previously depleted of it through the fractionation procedure, was able to restore a neuroprotective action similar to the complete medium. This was actually the case, as supplementation of the < 10 kDa MCM fraction with 10 ng/mL, a concentration known to exert in vitro biological activity (Elvers et al., 2005) fully protected CGNs from exposure to 6-OHDA (Fig. 3.20a). Noticeably, the same addition of TGF-b2 to a control medium, not previously conditioned by exposure to microglia, was ineffective. This clearly demonstrated that the efficacy of TGF-b2 neuroprotection depended in our model on the cooperative action with other substance(s) present in the conditioned medium, in particular in its low molecular weight fraction. Furthermore, addition of exogenous TGF-b2 was also able to abrogate the intrinsic toxicity of < 10 kDa MCM fraction, thus recreating the effect of the complete conditioned medium (Fig. 3.20a). Second, we tested the role of TGF-b2 in the neuroprotection granted by MCM by adding to this medium inhibitors of the intracellular pathways mediating the receptorial effects of TGF-bs, i.e. the compounds SB 431542 and SB 525334 that act on ALK (Activin Receptor-like Kinase)-family kinases (Inman et al., 2002; Laping et al., 2002; Grygielko et al., 2005). The neuroprotective effect of MCM was significantly attenuated by the ALK antagonist SB 431542 (Fig. 3.20 b), thus supporting the role of TGF-b-activated pathway in MCM protection of CGNs. A similar effect was also exerted by another ALK antagonist, SB 525334 (data not shown). As both these inhibitors are not selective for ALK5, which is the specific downstream mediator of TGF- b2, we performed experiments able to exclude that other proteins of the activin family acting through ALK signaling (Schmierer and Hill, 2007) were involved in MCM-mediated neuroprotection.

a)



b)

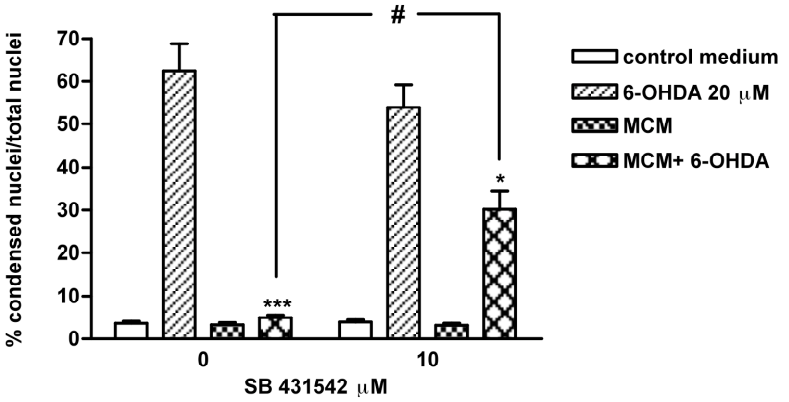


Fig. 3.20 Involvement of TGF-b2 in the neuroprotective action of MCM. (a) Human recombinant TGF-b2 (10 ng/mL) was added to the fraction of MCM containing low molecular weight (< 10 kDa) substances and its neuroprotective action was compared with that of complete medium on CGN cultures treated for 24 h with 20 lmol/L 6-OHDA. Nuclei count after Hoechst staining. Bars are the mean ± SE of three different experiments run in duplicate; **p < 0.01, ***p < 0.001 with respect to the conditions of treatment with 20 lmol/L 6-OHDA in non-conditioned medium; #p < 0.001 with respect to the condition of treatment with fraction of MCM containing low molecular weight (< 10 kDa) substances without the addiction of TGF-b2. (b) A specific inhibitor of TGF-bs signal transduction pathway, SB 431542 (10 lmol/L), was added to the cultures of CGNs treated for 24 h with 20 lmol/L 6-OHDA in the presence or absence of MCM. Nuclei count after Hoechst staining. Bars are the mean ± SE of two different experiments run in duplicate; *p < 0.05, ***p < 0.001 with respect to the conditions of treatment with 6-OHDA in non-conditioned medium; #p < 0.01 with respect to the condition in the absence SB 431542.

Western blots of four members of activin family showed that only precursor forms of these proteins, with estimated molecular weight > 30 kDa, were present in the medium conditioned by microglia (Fig. 3.21a). As mature forms of these proteins were not detected in MCM, their involvement in neuroprotection was unlike. To experimentally support this, we tested whether the neuroprotective effect of MCM was preserved after excluding all molecules with molecular weight > 30 kDa through selective filtration. Results showed that the conditioned medium deprived of > 30 kDa molecules retained its full neuroprotective activity (Fig. 3.21b). As a final experiment, we tested whether the putative neuroprotective factor(s) present in the < 10 kDa MCM fraction and required to obtain the full TGF-b2-mediated protection were also heat-labile molecules. As shown by Fig.3.21, this was not the case, as the neuroprotective effect of TGF-b2 was exactly the same when added to the native or to the heat-inactivated < 10 kDa fraction, suggesting a nonpeptidic nature of these low molecular weight factor(s).

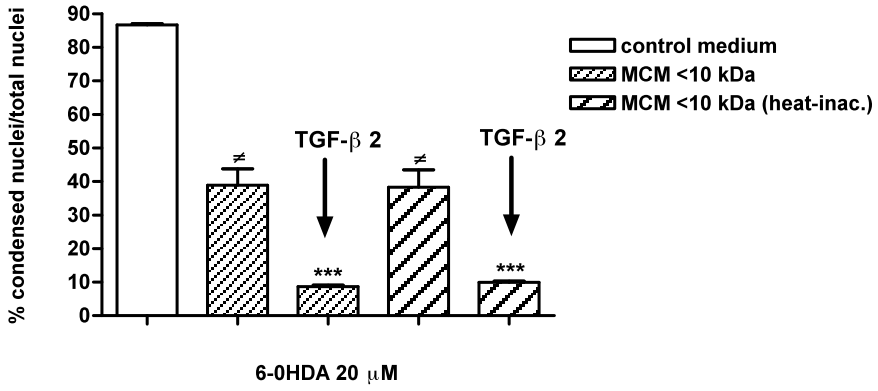


Fig. 3.21 Low molecular weight, heat-stable factors cooperate with TGFb2 in fulfill its neuroprotection. Human recombinant TGF-b2 (10 ng/ mL) was added to the fraction of MCM containing low molecular weight (< 10 kDa) substances, previously heat inactivated, then its neuroprotective action was compared with that of not heat-exposed medium on CGNs cultures treated for 24 h with 20 lmol/L 6-OHDA. Bars represent the mean ± SE of two different experiment run in duplicate „ $p < 0.001$ with respect to the conditions of treatment with 6-OHDA in non-conditioned medium; ***$p < 0.001$ with respect to the corresponding conditions in the absence of TGF-b2

CHAPTER 4
DISCUSSION

Converging evidence from various sources, including pathology, genetics, biochemistry, cell biology and animal models suggest that the aggregation of α-syn in PD and Aβ in AD play a critical role in the pathogenesis of these complex disorders. Hence, strategies aimed at inhibiting and/or reducing the aggregation and amyloid fibril formation of these proteins represent a viable therapeutic strategy to combat and/or prevent the progression of neurodegeneration in both diseases. Towards identifying potent drug-like aggregation inhibitors, E and T, two ICOMT currently approved as adjuncts in the therapy of PD, and five other catechol-containing small molecules (GA, CA, Q, P and DA) were selected and their effect on the oligomerization and fibrillization of α-syn and Aβ42 was investigated using ThT fluorescence, TEM and SDS-PAGE analysis. Our results demonstrate that all catechols containing compounds shown in Fig. 1 inhibited α-syn and Aβ fibrillization in vitro and showed protective effects against α-syn and Aβ42-induced toxicity in PC12 cells. At 1:1 protein to compound ratio, all compounds tested showed > 90% inhibition of α-syn fibrillization and blocked the growth and seeding capacity of α-syn fibrils. However, at lower protein to compound ratios (10:5 and 10:1) only Q, T, E showed the strongest inhibition. CA and P were the least effective at lower molar ratios and showed only 40-50% inhibition of α syn fibril formation. To probe the specificity of these compounds towards α-syn, we determined their capacity to inhibit the fibrillization of Aβ42 monomers, PFs and fibrils. All the compounds exhibited reduced (55-75%) inhibitory activity against the fibrillization of LMW and PF Aβ42, with the exception of Q, which resulted in >90% reduction in the fibrillization of Aβ42 fibril formation. When we examined the effect of these compounds on the fibrillization of Aβ42 PFs, we observed that T, E, and DA, showed greater inhibition (80-90%), with Q being the most effective compound. By TEM analysis, we did not observe significant differences in the structural properties of the aggregates and fibrils formed among the various compounds tested. There are several mechanisms by which these compounds could inhibit amyloid formation, including their ability to: 1) stabilize the native monomeric state of amyloid; 2) target different intermediates on

the amyloid pathway and block their conversion to fibrils; 3) alter the aggregation pathway in favour of non-amyloidogenic aggregates. In the case of α-syn, both SDS-PAGE analysis and TEM analysis demonstrate that the presence of these compounds enhances the solubility of α-syn (Fig. 3.2). On the other hand, none of the compounds tested were shown to stabilize monomeric Aβ42, instead the efficacy of these compounds appears to be linked to their ability to interfere with Aβ42 aggregation at different intermediate stages on the amyloid pathway (Fig. 3.6A and Fig. 3.7A). All compounds tested were shown to promote the formation of large non-fibrillar aggregates and/or protofibrillar species as discerned by TEM (Fig. 3.6B and Fig. 3.7B) and the reduced solubility of Aβ42 in presence of some compounds, despite the fact incubation with such compounds results in significant reduction in the ThT signals. Consistent were the TEM data, we failed to detect significant accumulation of monomeric Aβ42 after 96 hr of incubation in the presence and absence of compounds. Interestingly, each of the compounds appears to exert very specific effects and show preference for targeting different aggregation state on the amyloid pathway. For example, while T and E were equally effective in blocking the fibrillization of Aβ42 LMW and PF and showed strong inhibition of Aβ42 fibril growth and seeding capacity, DA and Q were most effective against the fibrillization of Aβ42 LMW and PF, but did not show significant inhibition of Aβ42 fibril growth and seeding capacity. Furthermore, incubation of each compound with Aβ42 resulted in the accumulation of Aβ42 aggregates of distinct size and morphological properties (Fig. 3.6B and Fig. 3.7B). Together, these results suggest that the presence of the catechol moiety in these compounds is sufficient to impart on them an anti-amyloidogenic activity against α-syn and Aβ42. However, the TEM and solubility studies demonstrate that the compounds interfere with the fibrillization of Aβ42 and α-syn via distinct mechanisms. This hypothesis is further supported by our findings that all the compounds showed strong inhibition of α-syn fibril growth and seeding capacity, whereas only T and E were effective in blocking the growth and seeding capacity of Aβ42 fibrils. The remaining (P, CA, GA, Q, and DA) compounds showed minimal effect even at 1:1 molar ratio, in contrast to their ability to block the seeding capacity of α-syn under similar conditions. To determine if the specificity and potency of these compounds is mediated by their interaction with specific sequences and/or structural motifs within these two proteins, we sought to determine which residues interact with the most potent compounds, Q, T and E using NMR. These studies did not show any

significant chemical shift changes for any of the compounds, with the exception of very minor chemical shift changes for His50 and some N terminal residues, suggesting that there is no direct binding of the compounds to the backbone of α-syn in its monomeric state. However, an increase in NMR signal intensity was observed in the presence of E and T for a large number of residues in the N-terminal domain of α-syn, in particular in the NAC region. This is in clear contrast, to the action of the polyphenols (-)-epigallocatechin gallate (EGCG), which binds to the backbone of monomeric α-syn and Aβ40, decreases the NMR signal intensity of monomeric α-syn and redirects both α-syn and Aβ40 into unstructured off-pathway oligomers (Ehrnhoefer et al, 2008). EGCG targets the polypeptide main chain that is identical in all proteins and easily accessible under unfolded conditions (Ehrnhoefer et al, 2008). In contrast, ThT fluorescence, SDS-PAGE and TEM measurements indicate that the COMT inhibitors appear to target a conformational feature of oligomers. Currently it is not known what this conformational feature is, but hydrophobic patches formed in oligomers in a rather unspecific manner could be a potential target. Thus, the COMT inhibitors or their scaffold are potentially more useful lead compounds than EGCG, as they preferentially bind to protein aggregates and not to unfolded polypeptide backbones.

Entacapone (E), tolcapone (T) and related catechols protect against extracellular a-syn and Aβ42-induced toxicity in PC12 cells

To determine if the ability of these compounds to block and/or alter the fibrillization pathway of Aβ42 and α-syn fibrillization could translate into protection against Aβ42 and α-syn cellular toxicity, we evaluated their protective effect in the cell culture using different assays. At concentrations that showed significant inhibition of Aβ42 and α-syn fibrillization, all the compounds were shown to protect PC12 cells from Aβ42 and α-syn-induced cytoxicity, by mechanisms that are directly linked to their ability to modulate the fibrillization of both proteins. These results are in agreement with previous studies. Several small polyphenols molecules were shown to exhibit strong antiamyloidogenic and neuroprotective properties in vitro and in vivo. Bastianetto et al. reported on the neuroprotective effects of GA and other green and black tea catechin gallate against Aβ40 in neuronal cell cultures (Bastianetto et al,

2006). Other groups have also demonstrated that cathechol and polyphenol compounds as potent antiamyloid agents and investigated the chemical and structural properties underlying their potency. However, to the best of our knowledge the anti-amyloidogenic properties of the two nitrocatechols E and T, already known for other biological activities, i.e. antioxidants and ICOMT, have never been described in literature. Although there is strong evidence in support of the protofibril hypothesis, the exact mechanisms by which protofibrils cause toxicity and the identity of the toxic species remain unknown. Protofibrils represent a heterogeneous mixture of aggregates of various size and morphologies, some of which are likely to contribute to toxicity. It is noteworthy, that all the fibrillization inhibitors reported in the literature, including those that protect against Aβ and α-syn toxicity exert their effect by acting at an intermediate step along the amyloid pathway, i.e. there are no known small molecule inhibitors that stabilize monomeric Aβ and α-syn. As shown in Figures 3.6 and 3.7, the different compounds we tested appear to stabilize or induce the formation of prefibrillar aggregates of distinct morphologies. Together, these results suggest that these molecules act by altering the structure of the aggregates and diverting toxic intermediates towards off-pathway non-toxic species. Studies from several groups have shown that small molecules, including inositol stereoisomers (McLaurin et al, 2000), (-)-epigallocatechin gallate (EGCC) (Ehrnhoefer et al, 2008) as well as Aβ42 derived peptides (Fradinger et al, 2008) were shown to alter the toxic properties of Aβ by stabilizing and/or inducing structural remodeling of protofibrillar and fibrillar aggregates. Resveratrol blocks Aβ toxicity without inhibiting oligomer formation (Feng et al, 2000). Moreover, recent studies from our group (Jan et al, 2008) and others (Wagulis et al, 2005) suggest that amyloid toxicity requires an on-going fibrillization process, i.e. the presence of protofibrils or fibril is not sufficient to cause toxicity unless these species are undergoing an on-going fibrillization process. Previous studies with DA and other catecholamines, e.g. apomorphine (Lashuel et al, 2000), linked their anti-amyloidogenic properties to their ability to undergo rapid autoxidation in aqueous solution, suggesting that one or more oxidation products is responsible for their inhibitor properties (Norris et al, 2001). However, the two major products of the catecholamine oxidation, i.e. quinines and aminochromes, are relatively unstable and difficult to isolate. In order to verify this hypothesis, the 3-methoxytyramine, the major metabolite of dopamine, was tested in the same working condition and in the presence of the two proteins, i.e. α-syn and Aβ42. As expected,

the results obtained confirmed that the methylated derivative of dopamine had no effect on the protein fibrillization process (data not shown). The mechanism by which catechol containing compounds block protein fibrillization remains controversial. Conway et al. reported that DA stabilise α-syn PFs by forming a DA-α-syn adduct (20). More recently, Norris et al. suggested a novel mechanism of action in which the dopaminochrome, the oxidized product of DA, inhibits α-syn fibrillization by interacting with the specific amino acid motif in the C terminus and NAC region of α-syn (Norris et al, 2005;Li et al, 2004; Jarret et al, 1993; Herrera et al, 2008; Yamin et al, 2005). Given the structural similarity amongst E, T and other known antiamyloidgenic catechol derivatives such as DA and Q, it is plausible to speculate that a shared mechanism may underlie the effectiveness of all these compounds. All seven molecules shown in Table 3.1 have in common the fact that each possesses at least one aromatic ring with catechol moiety.

Relevance to α-syn toxicity and Parkinson's disease

Although predominantly a cytosolic protein, several lines of evidence suggest a potential role of extracellular α-syn in mediating α-syn toxicity, Lewy body formation and the pathogenesis of PD and related synucleinopathies. These lines of evidence include: 1) recent studies from different laboratories which have shown that some monomeric and/or soluble aggregated forms of α-syn are secreted and can be detected in the blood plasma and cerebrospinal fluids of patients suffering from PD and related synucleinopathies (Borghi et al, 2000; Tokuda et al, 2006); 2) the fact that exogenous aggregated forms of α-syn have been shown to induce microglial activation, stimulate the production of reactive oxygen species (Zhang et al, 2005)), and pro-inflammatory factors (Klegeris et al, 2008) and are toxic to mammalian cells and primary neurons (El-Agnaf et al, 1998; Sung et al, 2008); 3) studies which have shown that the cellular uptake of extracellular α-syn occurs by passive diffusion (monomers) or via receptor mediated endocytic pathways (Ahn et al, 2006) (oligomers and protofibrils), depending on the aggregation state of α-syn (Lee et al, 2008) 4) the fact that small amounts of extracellular α-syn aggregates can efficiently catalyze the aggregation of intracellular α-syn inclusions. Specifically, Luk et al demonstrated that α-syn fibrils, prepared from recombinant full length or truncated α-syn, were uptaken by cells within cultures and act as seeds that catalyzed the aggregation and conversion of

soluble intracellular α-syn into LB-like inclusions (Luk et al, 2009). Finally, 5) Desplats and colleagues demonstrated neuron-to-neuron and neuron-to-glia transmission of monomeric and aggregated α-syn species in vivo and in cell cultures (Desplats et al 2009). These studies suggest that the release and uptake of monomeric and soluble aggregates of α-syn plays a central role in inclusion formation, neuronal cell death and spreading of α-syn pathology in PD. Therefore, targeting the aggregation of extracellular α-syn and/or promoting their clearance have emerged as viable therapeutic strategies for PD and related synucleinopathies. In this regard, the identification of small molecules that block or reverse the aggregation of extracellular α-syn is desirable. Herein, we demonstrated that entacapone, tolcapone and related catechols inhibit α-syn fibrillization in vitro and prevent the formation of toxic aggregates as discerned by their protection against α-syn induced extracellular toxicity in PC12 cells. Currently, the most effective treatment for PD continues to be the administration of L-DOPA together with a peripheral AADC inhibitor that is unable to enter the central nervous system. However, the amount of L-DOPA reaching the brain after oral administration is very low (about 5-10%). Furthermore, the subsequent metabolism of L-DOPA by catechol-O-methyltransferase (COMT) clearlylimits its availability in the brain. The two COMT inhibitors E and T, approved as adjuncts in the therapy of PD, increase the availability of L-DOPA for conversion to dopamine in the brain (Bonifacio et al, 2007) mainly by preventing the extensive metabolism of L-DOPA through O-methylation in the periphery (E and T) and partly in brain (T) (Lapish et al, 2009) However, the potential for the use of T as neuroprotective drug in AD or PD is limited due to the fact that it has been shown to cause severe hepatotoxicity resulting in its withdrawal from the market in many countries leaving entacapone as the only COMT inhibitor presently available in the clinic for the treatment of PD (Assal et al, 1998). However, recent studies demonstrate that tolcapone can be used with benefit when the liver function is actively monitored (Antonini et al, 2008; Truong et al, 2009). Although these two compounds share the same pharmacophore, their pharmacokinetic profiles are remarkably different (Navardi et al, 2006). Indeed, studies with rats have shown that tolcapone has a longer duration of action than entacapone and is both a central and peripheral COMT inhibitor, whereas entacapone is essentially a peripheral inhibitor (Di Stefano et al, 2009). However, the benefits of both entacapone and tolcapone in the L-DOPA treatment of patients suffering of Parkinson's disease were proved

(Haefeli et al, 2007; Damier et al, 2008).

In summary, our study showed that entacapone and tolcapone are potent inhibitors of α-syn and Aβ oligomerization and fibrillogensis and that the inhibition of the aggregation results in a protection against extracellular toxicity induced by the both proteins. Our results provide additional evidence for the potential of catechols as antiamyloidogenic agents and and demonstrate that entacapone and tolcapone belong to the classes of multifunctional drugs (Navardi et al, 2005) since they can inhibit COMT, act as good antioxidants and as effective inhibitors of protein aggregation (Bertolini et al, 2007). Whether the anti-amyloidogenic property of entacapone and tolcapone and the protection against α-syn extracellular toxicity contribute to their clinical benefits and enhanced symptomatic treatment of PD or not remains to be determined. Nonetheless, our findings suggest that the structure entacapone and tolcapone constitute molecular scaffolds that could guide the development of more potent inhibitors of amyloid formation and toxicity. Chemical modifications of entacapone and tolcapone can be envisaged in order to optimize its pharmacokinetic profile especially by avoiding hepatotoxicity (Boelsterli et al, 2006), modeling its peripherical metabolism and increasing its BBB permeation.

Protection of microglia conditioned media in PD-like neurodegeneration models induced by 6-OHDA toxin

Finally, we analyzed the neuroprotective effect of microglia conditioned media towards different insults. Our present data substantially extend available information on the ability of microglia conditioned media to protect from death neurons challenged with neurotoxic stimuli (Polazzi et al, 2001, Toku et al, 1998). It was previously demonstrated such a neuroprotective effect on a model of apoptotic neuronal death caused by the shift of mature CGC cultures from depolarizing to non depolarizing conditions (Polazzi et al, 2001) and we now show a strong and highly-specific neuroprotective action of microglia conditioned medium on an in vitro model of Parkinson-like neurodegeneration, the 6-OHDA-induced CGNs death. With the present report, we demonstrate a novel neuroprotective action of MCM on 6-OHDA neurotoxicity in CGNs, having previously described similar effects in different models of CGNs induced apoptosis (Polazzi et al. 2001; Eleuteri et al. 2008). 6-

OHDA is a neurotoxin with selectivity for dopaminergic neurons because of the presence of the dopamine transporter expressed in these cells, but CGNs are quite sensitive to 6-OHDA too (Dodel et al. 1999; Lin et al. 2003; Chen et al. 2004; Ma et al. 2006; Monti et al. 2007). CGNs have been used here as they are commonly considered a good model to study the cellular, biochemical and molecular mechanisms of neuronal survival/death and are among the most widely-used neuronal primary cultures (Contestabile 2002). In this model, 6-OHDA results in neurodegeneration presenting most of the characteristic features of Parkinson-like neuronal death, i.e. an apoptotic process with oxidative stress, proteasome impairment and protein aggregation (Dodel et al. 1999; Lin et al. 2003; Chen et al. 2004; Ma et al. 2006; Monti et al. 2007). Here, we have found that the pro-survival action exerted by MCM completely reversed 6-OHDA neurotoxic effect, not with standing its high toxicity, thus suggesting a high specificity of MCM secreted survival factors for this model of cell death. Indeed, in different models of CGNs death, MCM only partially counteracted neurotoxic insults (Polazzi et al. 2001; Eleuteri et al. 2008).

We extended the neuroprotective analysis of the conditioned media (MCM48 and DC) against the $A\beta_{42}$-induced toxicity in CGNs. Using this *in vitro* models of AD-like neurodegeneration we did not find protection against the $A\beta_{42}$-induced toxicity already seen for the inhibitors of the α-syn and β-amyloid aggregation. We also tested the neuroprotective effect of microglia conditioned media against excitotoxicity and staurosporine-induced apoptosis.

Staurosporine, a broad spectrum protein kinase inhibitor, is a wellknown inducer of apoptosis in various types of cells, including CGCs (Franco-Cea et al, 2004; Melchiorri et al, 2002). As indicated above, glutamate may induce neuronal death through both apoptotic and necrotic mechanisms (Ankarcrona et al, 1995; Slagsvold et al, 2000), apoptosis being the prevalent modality of cell death when the excitotoxic stimulus is mild and long-lasting (sub-chronic treatment) as previously described (Leng et al, 2006), and necrosis prevailing for short pulses of glutamate given in the absence of magnesium to maximize the involvement of NMDA receptors in the excitotoxic insult (acute treatment). In the present experiments, having delivered the excitotoxic stimulus in both ways, we were able to demonstrate a significant neuroprotection of media conditioned by microglia in the case of the sub-chronic mild treatment but not in the case of the acute excitotoxic treatment. The similarity of the glutamate mild excitotoxic stimulus with the neuroprotective effect noticed for

staurosporine treatment, a recognized apoptosis inducer suggests that microglia conditioned media preferentially protect neurons challenged with apoptotic death stimuli. While one should be obviously cautious in extending findings obtained in culture to the balance apoptosis/necrosis in vivo, this may reflect an in vivo differential involvement of microglia in chronic vs. acute insults. Our present data also demonstrate that the neuroprotection granted by a medium conditioned for 48 h by microglia is replicated, and indeed some what enhanced, by a medium conditioned by microglia for only 24 h but previously exposed for 24 h to apoptotic neurons. As previously discussed (Polazzi et al, 2001), this result strongly suggests that apoptotic neurons release in the medium diffusible factors that are in turn able to elicit an increased production of neuroprotective substances from microglial cells. This suggestion has recently received an important confirm from a study demonstrating that media exposed to neurons challenged with insults of moderate level, preferentially elicit the production of neurotrophins from microglia (Lai et al, 2008). The use of this experimental approach, or of other similar approaches, is of interest as it is potentially able to overcome some of the limitations that are inherent to the use of separate microglia and neuronal cultures to study the relationships between the two players. Indeed, they allow to re-create a form of reciprocal cross-talk of functional significance, in conditions of physical separation of the two cell populations. This situation may be advantageous, with respect to co-culture conditions (Neumann et al, 2006; Polazzi et al, 2001) in order to identify specific factors released in the media and responsible for the reciprocal functional influence and the neuroprotective action of microglia. Indeed, while the use of co-cultures has the advantage of creating conditions more similar to the in vivo situation, it renders more complicated to attribute the production of specific molecules to neurons or to microglia, as they are obviously mixed in the medium.

This neuroprotection is exerted through the release into the medium of peptidic molecules, which cooperate with low molecular weight, heat-resistant factor(s). Moreover, based on multiple evidence, we have identified TGF-b2 as one of these neuroprotective agents. As outlined in the introductory section, microglia can secrete inflammatory cytokines and other neurotoxic molecules, but also neuroprotective factors. A robust inflammatory reaction is observed in PD and other neurodegenerative pathologies and reactive microglia are prominent in affected brain areas of these patients (recently reviewed by Klegeris et al. 2007; McGeer and

McGeer 2008; Smith 2008; Villoslada et al. 2008; Whitton 2007). Therefore, blocking inflammation or shifting the balance from pro-inflammatory to anti-inflammatory states of microglia offers a promising strategy for therapy. In this setting, the knowledge of the neuroprotective mechanisms of microglia, coming out from in vitro studies of neuron/microglia cross-talk, may help to identify and target specific microglial molecules accounting for neuronal survival. Moreover, we have demonstrated here that MCM exerts its neuroprotective effect even if microglia are exposed themselves to 6-OHDA, thus suggesting that this neurotoxin does not induce microglial pro-inflammatory activation. We further provide evidence that TGF-b2 accumulated in the medium conditioned by microglial cells is an essential, even if not unique, factor mediating the MCM neuroprotective effect. Indeed, addition of the exogenous peptide to the conditioned medium fraction, previously depleted of endogenous TGF-b2 through molecular weight-based fractionation, was able to restore the neuroprotective action of the complete medium and also to efficiently counteract the intrinsic toxicity of this < 10 kDa MCM fraction towards CGNs. On the other hand, TGF b2 requires the presence of at least one factor released by microglia in this low molecular weight range to ensure its neuroprotective action. This is demonstrated by the fact that the > 10 kDa MCM fraction, in which TGF-b2 is present in large amount, lacks neuroprotection by itself and that the addition of the exogenous peptide to an unconditioned medium is ineffective too. The finding that TGF-b2 needs to cooperate with other factors to explicate its biological activity on CGNs is in agreement with previous data (Kane et al., 1996), demonstrating that this cytokine differentially regulates cerebellar granule cell neurogenesis depending on the presence of serum factors. Regarding the possible cooperation of other factors with TGF-b2, it should be considered that the conditioned medium here used derives from unstimulated cultured microglia. While evidence exists for functional interactions modulating TGF-b activity in response to pro-inflammatory cytokines, such as interleukin 6 (Samanta et al., 2008), no production of these cytokines does occur from primary microglia not stimulated with LPS (lipopolysaccharide) or other activating factors (Horvath et al., 2008; Vollmar et al., 2008).We also provide pharmacological evidence for the neuroprotective role of TGF-b2 contained in MCM, by significantly decreasing its efficacy through an antagonist of its main receptor/transduction system, which acts through kinases of ALK family (Laping et al. 2002; Grygielko et al. 2005). Since this pharmacological tool could not unequivocally identify TGF-b2 as the

neuroprotective factor, because of the possible interference with other proteins acting through ALK pathway, we verified that our cultured microglial cells produced the specific mRNA for TGF-b2, as well as for TGF-b1 and TGF-b3. Notwithstanding some authors could not detect TGF-b2 production in microglia in early studies (Morgan et al. 1993; Constam et al. 1992), more recently other groups demonstrated the microglial expression of TGFb2 RNA and protein both in vitro and in vivo (De Groot et al. 1999; Ma and Streilein 1999; Yuan and Neufeld 2001). Our PCR analysis revealed a continuous production of TGF-b2 mRNA from microglial cells throughout the culture period that could well account for the accumulation of the mature form of the protein in the medium after 48 h. Furthermore, we demonstrated that other proteins of the TGF-bs family, which could be involved in microglial neuroprotective action, including activins, nodal and vg-1 (Schmierer and Hill, 2007), were released in the conditioned medium at molecular weight > 30 kDa and thus could not contribute to neuroprotection as MCM deprived of its > 30 kDa fraction retained its full neuroprotective capacity. These experiments, together with our demonstration that addition of exogenous TGF-b2 to the fraction of MCM previously deprived of it through selective filtration efficiently granted neuroprotection, strongly support our identification of TGF-b2 as a neuroprotective factor constitutively released by microglia in its medium. Microglia-conditioned medium inactivation shows that neuroprotection granted against 6-OHDA toxicity is in part related to heat-resistant molecule(s), as the effect does not completely disappear after heat inactivation or peptide enzymatic destruction of the medium. This is in agreement with previous results, showing that the neuroprotective action of MCM on neurons in different neurodegenerative conditions was not completely abolished by heat inactivation (Toku et al., 1998; Zietlow et al., 1999; Park et al., 2001; Polazzi et al., 2001). Present experiments of heat inactivation on the < 10 kDa MCM fraction demonstrate the presence in this fraction of heat-resistant neuroprotective molecule(s), which are required to ensure the full protection of the complete medium or of the exogenous TGF-b2 added to this same fraction. Hypotheses on the possible nature of heat resistant neuroprotective molecule(s) are at present speculative, as they obviously require to be subjected to specific experimental analysis. On the basis of literature data, potential candidates may belong to prostaglandins and other lipids or sugars, as low molecular weight molecules of this kind are known to be released by microglia and to result neuroprotective under some conditions (Hicks et al. 1998; Bachis et al.

2002; Carrasco et al. 2008; Zhang et al. 2008). The family of TGF-bs comprises multifunctional growth factors abundantly expressed in the nervous system and promoting neuronal survival in a variety of physiological and neuropathological conditions (Flanders et al. 1998; Krieglstein et al. 2002). TGF-b2, in particular, protects neurons from excitotoxic insults, promotes differentiation and survival of nigral dopaminergic neurons, modulates CREB (cAMP-response element binding protein) activity in hippocampal neurons, thus promoting synaptic plasticity, and negatively regulates the inflammatory response of the central nervous system (Bruno et al. 1998; Andrews et al. 2006; Roussa et al. 2006; Fukushima et al. 2007; Siglienti et al. 2007). Cerebellar granule neurons have been demonstrated to be a target of TGF-b2 during development. In particular, TGF-b2 has been shown to take part in the neurogenesis of this neuronal population through regulation of precursor proliferation, apoptotic elimination and maturation (Constam et al. 1994; Kane et al. 1996; de Luca et al. 1996; Kaltschmidt and Kaltschmidt 2001; Elvers et al. 2005). Our results indicate that TGF-b2, by acting together with other microglia-derived molecules, represents a survival factor for these neurons when they are exposed to a neurotoxic insult, such as the one represented by 6-OHDA. The interest of this observation is increased by the fact that 6-OHDA is a known neurotoxin for dopaminergic nigral neurons found to massively degenerate in Parkinson's disease and by the fact that TGF-b2 protects these neurons both in vitro and in vivo (Macauley et al. 2004; Roussa et al. 2006). Furthermore, transgenic mice expressing low levels of TGF-b2 showed an age-related nigro-striatal dopaminergic deficit (Andrews et al. 2006) and a recent population study preliminarily demonstrated a trend towards association of the 5¢ region of TGF-b2 gene and susceptibility to PD (Goris et al. 2007; Klegeris et al. 2007). Thus, our present study should stimulate novel researches on the role played by microglia in the beginning and progression of PD, with the aim of identifying new potential targets for therapy. Another member of the same protein family, TGFb1, has been reported to be potentially neuroprotective under various experimental conditions (Flanders et al. 1998; Zhu et al. 2002, 2004; Boche et al. 2003). Our microglial cells expressed both mRNA and precursor of the protein, but no accumulation of mature TGF-b1 was detected in MCM even after 48 h of culture, thus suggesting that this factor does not play a role in MCM neuroprotection. The relevance of our present findings should be discussed with respect to how and to what extent the artificial situation of in vitro isolation of microglial cells reflects the situation occurring in

vivo. In vivo ‘resting microglia’ is actually continuously active (Raivich 2005) and ‘activated microglia’ is present in the brain not only with harmful phenotypes, but also with beneficial phenotypes that actively contribute to

imit neuronal damage and to promote neuron recovery (Schwartz et al. 2006). However, this ‘neuroprotective phenotype’ is still not fully characterized. Accordingly, in vivo immunosuppressant treatment inhibits the neurodegenerative role of microglia in a PD rat model, without completely blocking microglial activation itself (Wright et al. 2008). The various microglial states are not autonomously determined by microglial cells but are, instead, the result of a continuous and reciprocal exchange of molecular messages (Kreutzberg 1996; Neumann 2001; Polazzi and Contestabile 2002, 2006; Schwartz et al. 2006). The impossibility to re-create such complex interrelationships in in vitro systems is an obvious limitation of studies based on interactions in co-cultures or on the use of conditioned media to reciprocally test the responses of the different players. On the other hand, as considered in the introduction, this approach makes easier to isolate and identify specific factors involved in microglia-neuron cross-talk, as it is the case for TGF-b2 in the present study. In the case of experiments here presented, for instance, results suggest a continuous production of neuron survival factors from microglia and, therefore, they are consistent with the idea that microglia constitutively serve a functionally supportive and neuroprotective role (Streit et al. 2008). While obtained in separate cultures, our result, demonstrating that 6-OHDA toxicity towards microglia does not disrupt the neuroprotective action of the conditioned medium, suggests that the occurrence of neurotoxic insults affecting both neurons and microglia in vivo may not compromise microglial neuroprotection. Furthermore, in line with the idea that microglia in culture may reflect a functional state of activation of these cells, in some way similar to a state found in chronic neurodegenerative diseases (Streit et al. 1999), these experimental evidences in culture represent an important conceptual advancement in understanding microglial role in normal and pathological brain and in targeting modulation of microglia activation for therapeutic purposes in neurodegenerative diseases.

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