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TOPICAL REVIEW

Alzheimer's disease: biological aspects, therapeutic perspectives and diagnostic tools

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Abstract

Alzheimer's disease (AD) is the most common form of dementia among older people. Dementia is an irreversible brain disorder that seriously affects a person's ability to carry out daily activities. It is characterized by loss of cognitive functioning and behavioral abilities, to such an extent that it interferes with the daily life and activities of the affected patients.

Although it is still unknown how the disease process begins, it seems that brain damage starts a decade or more before problems become evident.

Scientific data seem to indicate that changes in the generation or the degradation of the amyloid-b peptide (Aβ) lead to the formation of aggregated structures that are the triggering molecular events in the pathogenic cascade of AD. This review summarizes some characteristic features of Aβ misfolding and aggregation and how cell damage and death mechanisms are induced by these supramolecular and toxic structures. Further, some interventions for the early diagnosis of AD are described and in the last part the potential therapeutic strategies adoptable to slow down, or better block, the progression of the pathology are reported.

(Some figures may appear in colour only in the online journal)

Contents

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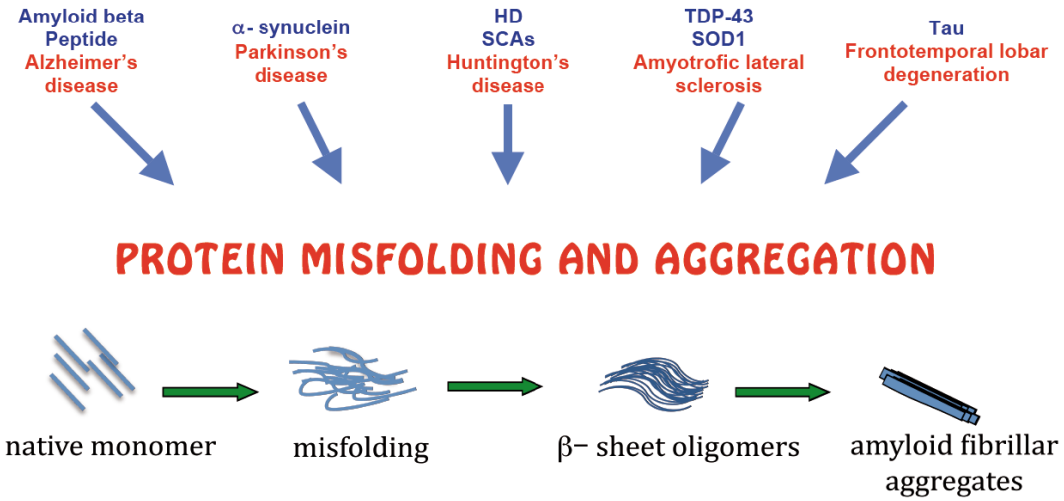

Figure 1. Some genetic mutations and environmental factors are responsible for many neurodegenerative diseases. The causative proteins are prone to misfolding and forming beta-sheet-rich oligomers and amyloid fibrillar aggregates, resulting in their accumulation in the affected neurons and eventually leading to degeneration in the brain.

1. Introduction

Protein aggregation is a very fascinating subject due to its implication in many human neurodegenerative diseases and its relevance in the food and pharmaceutical industries. In some cases, the aggregation of protein is a natural phenomenon occurring in living organisms. For instance, the reaction

$$n(\mathrm{G-Actin}) \rightarrow (\mathrm{F-Actin})_n$$

describes the assembly of the globular (G) monomeric actin form into its polymeric fibrillar (F) structure (Morris *et al* 2009). The case of polymerization of tropocollagen to obtain collagen fibrils is a process leading, in the case of type I collagen, to the formation of long fibrils with a wave pattern (Yadavalli *et al* 2010), structures that are fundamental for the robustness of tendons, ligaments and skin.

In general, a non-physiological aggregation, that is an aggregation process not naturally occurring, starts from 'activated' molecules having secondary and/or tertiary structures different from those corresponding to the 'native state' (Manno *et al* 2006, 2010, Morris *et al* 2009). In recent years, a better knowledge of the protein-folding process has led to the observation that several human diseases are caused by misfolding and dysmetabolism. In these pathologies, such as Alzheimer's, Parkinson's and Huntington's diseases, amyotrophic lateral sclerosis and frontotemporal lobar degeneration, the causative proteins lose their natural architecture and self-aggregate in toxic fibrillar structures denominated amyloid fibrils (figure 1).

The question of what triggers the transformation of a biologically active protein into an amyloidic structure with high self-assembly propensity is still unanswered. Some of the proposed explanations include the following:

(i) the propensity of some proteins to assume a pathological conformation which increases with aging (Uversky 2007, Saraiva 2001);

(ii) persistently high cellular or plasma concentrations (Singleton *et al* 2003, Farrer *et al* 2004);

(iii) an amino acid mutation or a genetic expansion of DNA sequences encoding proteins, as in the case of Huntington's disease (Cummings and Zoghbi 2000);

(iv) an abnormal post-translational modification of the causative protein (Goedert *et al* 1993);

(v) proteolitic cleavage of the precursor protein, as in the case of Aβ;

(vi) the influence of environmental factors.

Despite their common secondary structure conformation, it is well accepted that a correlation does not exist between amino acid sequence and tendency to amyloid structure formation, thus it is assumed that amyloid formation is a generic property of all polypeptides (Chiti and Dobson 2006). In particular, amyloid fibers share the following features (Xu 2007):

(i) all have a rope-like appearance;

(ii) they show a dominant beta-sheet structure;

(iii) their formation can be enhanced either by stirring or by the presence of seeds;

(iv) all aggregates start from spherical oligomers that in turn self-assemble linearly;

(v) all can incorporate special kinds of dye molecules such as Congo Red or Thioflavin *T*.

In the present review we focus on the recent insights into the Aβ amyloid peptide and the cellular damage caused by its aggregation. Further, current strategies to be used as therapies, addressing both the fibrillogenesis process and different Aβ biochemical pathways, are described. Finally, we present the state of the art in Alzheimer's disease (AD) diagnosis.

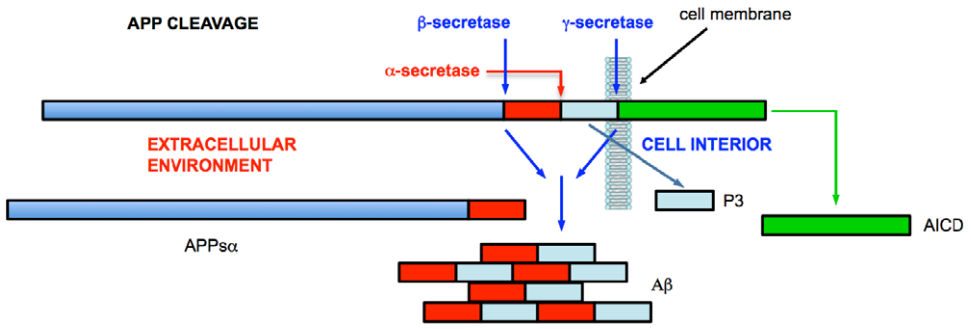

Figure 2. Mechanism of APP processing: β- and γ-secretases cleave in two sites of APP thus originating the Aβ fragment and the intracellular AICD fragment. The combined cleavage of α- and γ-secretases originates the APPsα, P3 and AICD fragments preventing Aβ generation.

2. The Aβ origin and fibril formation

Alzheimer's disease (AD) is the most common form of dementia in the elderly. It impairs higher brain functions such as memory, thinking and personality. The two forms of the disease are sporadic AD (late-onset), which can strike adults of either sex, and familial AD (early-onset), caused by a rare genetic mutation. Indeed, the only strongly confirmed genetic risk factor across many studies for early- and late-onset AD is the apolipoprotein E (APOE) gene on chromosome 19 (Corder *et al* 1993). Strong evidence suggests that the main mechanism by which APOE influences AD is via its effects on Aβ metabolism, mainly on Aβ aggregation and clearance (Kim *et al* 2009). However, the details of this process as well as the role played by APOE in non-Aβ mediated mechanisms remain to be fully clarified (Kim *et al* 2009). The human APOE is a 299 amino acid glycoprotein with variable post-translation modifications, expressed in several organs, with the highest expression in the liver, followed by the brain, mainly in astrocytes and microglia (Grehan *et al* 2001). The human APOE gene contains several single-nucleotide polymorphisms (SNPs) distributed across the gene (Nickerson *et al* 2000). The three most common SNPs lead to changes in the encoding sequence and result in the three common isoforms of APOE: APOE2 (cys112, cys158), APOE3 (cys112, arg158), and APOE4 (arg112, arg158). Although the three common isoforms differ by only one or two amino acids at residue 112 and/or 158, these differences profoundly alter APOE structure and function (Mahley *et al* 2006). However, the ε4 allele of the APOE gene was discovered to be the strongest genetic risk factor for AD (Bertram *et al* 2007).

AD is characterized by neuronal cell loss and increasing accumulation of neurofibrillary tangles (NTF) in neurons and amyloid fibers in neuritic plaques and in the walls of blood vessels (Wisniewski and Frackowiak 1997). Amyloid beta peptide (Aβ) (39–43 residues) is the main component of the amyloid plaques present in the brain of Alzheimer-affected patients. Accumulation of Aβ peptide is hypothesized to initiate by a pathogenic cascade that eventually leads to AD (Hardy and Selkoe 2002). This small peptide is the final product of a proteolitic cleavage of the larger transmembrane protein called amyloid precursor protein (APP) (Wilquet and De Strooper 2004), the functions of which are not yet known; recent data suggest that it is implicated in synapse formation (Priller *et al* 2006), neural plasticity (Turner *et al* 2003) and iron export (Duce *et al* 2010). The proteolitic cleavage of APP occurs through the action of the secretase family enzymes and Aβ is obtained by the sequential cleavage of β- and γ-secretase. A third secretase, α-secretase, cleaves within the Aβ sequence and is therefore usually considered as non-amyloidogenic. α-secretase, indeed, cleaves the ectodomain of APP, resulting in the formation of the APPsα fragment and precluding Aβ generation. Then, γ-secretase cleaves the transmembrane domain of the APP carboxy terminal fragments releasing the so-called P3 peptide and the APP intracellular domain (AICD). Alternatively, amyloidogenesis takes place when APP is first cleaved by β-secretase, producing APPsβ; successively, Aβ and AICD are generated upon cleavage by γ-secretase (Wilquet and De Strooper 2004) (figure 2).

The 42-residue beta peptide (Aβ42) is the predominant form found in plaques and under physiological conditions the ratio between Aβ42 and Aβ40 is about 1:10 (Iwatsubo *et al* 1994). Aβ42 has a neurotoxicity much greater than that of Aβ40 and its aggregation kinetics is faster than other beta-peptides (Davis and Van Nostrand 1996). The proteinaceous material is organized in structured linear aggregates (amyloid fibrils). A recent and now convincing belief is that small diffusible oligomers of Aβ, called ADDLs, are the determining pathogenic species causing synaptic dysfunction and eventually neuronal degeneration (Lambert *et al* 1998, Picone *et al* 2009a, 2009b).

Although the majority of AD researchers advocate the amyloid cascade hypothesis, some researchers have argued that tangles are central to AD pathogenesis. This has led to lively debates and the formation of a related but less clearly stated 'Tau and tangle' hypothesis. This hypothesis postulates that in AD the normal role of Tau in stabilizing microtubules within neurons is impaired, resulting in the replacement of microtubules with tangles (aggregated Tau), thus culminating in neuronal death. The process of Tau aggregation is still not well understood. However, it is clear that phosphorylation of Tau is a key factor, in fact aggregated Tau is highly phosphorylated and this chemical modification reduces its binding to microtubules (Mudher and Lovestone

2002). Other studies suggest that Tau phosphorylation occurs after its aggregation and that structural changes in Tau protein are associated with aggregation (Mena *et al* 1996). Attempts have been made to ascertain which pathological lesion (plaque or tangle) is the cause or consequence and no clear results are available. However, some experiments have demonstrated that Aβ could increase the activity of the Tau-phosphorylating kinases. Exposure of hippocampal neurons to fibrillar Aβ has been shown to activate MAPK and GSK3β pathways leading to hyperphosphorylation of Tau and dystrophic neurons suggesting that Tau phosphorylation is a consequence of Aβ production (Ferreira *et al* 1997, Hoshi *et al* 2003).

However, the cytotoxicity of Aβ is not only due to the ability to form fibrillar aggregates in the extracellular environment, but also to the presence of soluble Aβ oligomers in the intracellular environment (Lambert *et al* 1998). Both the forms, or their intermediates, produce severe damage to cell membrane and different organelles inducing alteration of physiological biochemical pathways and leading to oxidative stress, inflammation and, in the end, cell death *via* apoptosis (Chauhan and Chauhan 2006, Weiner and Frenkel 2006).

On a molecular lengthscale, Aβ can form aggregates of different shape originated *in vitro* under different conditions. These structures include amyloid fibrils (Ban *et al* 2004), small oligomers (Walsh *et al* 1999), spherical amyloid particle oligomers (Westlind-Danielsson and Arnerup 2001), annular pore-forming structures (Lashuel *et al* 2002), amyloid protofibrils (Harper *et al* 1997), beaded chain protofibrils (Huang *et al* 2000) and spherocylindrical micelles (Lomakin *et al* 1996, Yong *et al* 2002).

X-ray fiber diffraction showed that amyloid fibrils contain β-sheet structure lying orthogonally to the major fibril axis (Serpell 2000). In the 2000s, Tycko's group (Antzutkin *et al* 2000, 2002, Balbach *et al* 2002) obtained for the first time evidence of an extended parallel β-sheet organization for the Aβ40 fibrils using solid-state NMR. These authors showed that the methyl carbons of Ala-21 and Ala-30 must be placed in groups of at least four with internuclear distances of less than 5.5 Å. Although beta-sheets are the main constituent of the amyloid fibrils they are not the only structures present in the fibrils. Liquid-state NMR, FTIR and CD measurements have demonstrated in Aβ40 the existence of a turn formed by the amino acids at position 26–29. There is little information about the Aβ42 fibril structure and many mutant peptides have been synthetized to obtain an explanation of its secondary structure. The results have shown that the residues at positions 15–21 and 24–32 are involved in the beta-sheet formation and that the turn at positions 22 and 23 plays a crucial role in the aggregation of Aβ42.

The unbranched amyloid filaments have a diameter of 5–10 nm, in agreement with the thinnest fibrils seen by electron microscopy. Mature fibrils can be composed of more filaments and be up to 1 mm long (Antzutkin *et al* 2002). Actually, this model can be valid for all the fibrils, from a variety of amyloidosis, which yield a diffraction pattern remarkably similar to Aβ fibrils (Kirschner *et al* 1986). Different amyloid fibrils may be composed of protofilaments built from a different number of β-sheets and the filaments of a different number of protofilaments. Furthermore, a super-helical structure can be obtained by wrapping around more fibrillar filaments. *In vitro* studies have been and are still essential to obtain kinetic models of amyloid fibril formation. The understanding of all key steps of the process (preliminary conformational changes, oligomerization, nucleation, elongation and branching) can help to choose appropriate therapeutic strategies. No single experimental method can be considered exhaustive and reveal all aspects of the whole process. Furthermore, the structural properties of the Aβ peptide in physiological conditions are still uncertain, due to the low solubility of the molecule. However, the assembly of Aβ in supramolecular structures results to be toxic in both intra- and extracellular environments.

3. Oxidative stress

Oxidative stress is one of major cellular features in the pathophysiology of AD and it occurs when excessive generation of free radicals during normal metabolic processes in the cell is unbalanced by the antioxidative defense system. Free radicals, i.e. ROS (reactive oxygen species), are generated by oxygen- and nitrogen-based molecules with unpaired electrons and, for this reason, they are very unstable and highly reactive. Generally, oxidative damage to the cellular components results in alteration of the membrane properties such as fluidity, ion transport, enzyme activities, and protein cross-linking. Excessive oxidative damage eventually leads to neurodegeneration.

Under physiological conditions, free radicals are normally produced during the cellular metabolism, the main source being the mitochondrion and, in particular, the electron transport chain (ETC). The mitochondrial ETC is composed of five multimeric complexes (Enns 2003). Electron transport between complexes I and IV is coupled to extrusion of protons from complexes I, III and IV into inter-membrane space, creating an electrochemical gradient ($\Delta\psi$) across the inner mitochondrial membrane. This movement of electrons generates an alkaline matrix and an acidic inter-membrane space. Protons then flow through complex V (ATP synthase), which utilizes the energy to synthesize ATP from ADP (Han and Reynolds 2001). In addition, the Krebs cycle can, under appropriate conditions, produce free radicals (Brookes 2007, Starkov *et al* 2004).

The free radicals are toxic for the body and, if not removed or neutralized, can lead to cell death. In healthy condition, the brain is protected from such damage by an effective balance between pro-oxidant and antioxidant mechanisms, which include antioxidant enzymes, and free-radical-scavenging chemicals such as ascorbate, vitamin E and protein sulfhydryl. In AD, this balance appears to be disturbed, as demonstrated by pathological studies of biopsy and post-mortem cerebral tissue reporting excess DNA and protein oxidation (Mecocci *et al* 1994, Munch *et al* 1997), lipid peroxidation (Lovell *et al* 1995) and increased activity of the antioxidant enzyme superoxide dismutase (SOD). In

particular, lipid peroxidation may be particularly harmful in mitochondria, because of the presence of the lipid cardiolipin, the main constituent of the inner mitochondrial membrane (Capaldi 1982), required for the activity of cytochrome c oxidase (Robinson 1993) and other mitochondrial proteins. Oxidative stress causes a decrease of cardiolipin to a larger extent than other lipids, probably due to its high unsaturation level, and this, in turn, gives rise to the drastic reduction of cytochrome c oxidase action (Paradies *et al* 1997). In addition to the structural damage to membranes, lipid peroxidation causes the generation of secondary products (Montine *et al* 2002). The most abundant diffusible products derived from lipid peroxidation are reactive aldehydes such as 4-hydroxy-2-hexenal (HHE), and 4-hydroxy-2-nonenal (HNE), which, in a vicious circle, are highly reactive with proteins, nucleic acids and lipids (Esterbauer *et al* 1991, Kruman *et al* 1997). All these processes taking place in mitochondria cause a derangement in the functions of these organelles with consequences mainly in the cells requiring high-energy support, such as neurons and cardiac myocytes. Oxidative stress disrupts mitochondrial integrity through the opening of a large channel referred to as the permeability transition (PT) pore, thus producing mitochondrial depolarization, a key event to initiate the cell death cascade. The PT pore has been seen to be composed of the voltage-dependent anion channel (VDAC), adenine nucleotide translocator (ANT), cyclophilin D and hexokinase-II (HK-II) proteins, although perhaps other molecular constituents are present (Halestrap 2009). A link between intra-neuronal Aβ and mitochondria dysfunction has been more often evidenced. Aβ can interact with the mitochondrial matrix protein Aβ-binding alcohol dehydrogenase (ABAD) in human AD brain, believed to participate in mitochondrial dysfunction and oxidative stress (Lustbader *et al* 2004). Moreover, by using *in vivo* and *in vitro* approaches, it has been demonstrated that Aβ is transported into rat mitochondria via the translocase of the outer membrane (TOM) and localizes within the mitochondrial cristae (Hansson Petersen *et al* 2008). It is evident that by using antioxidant molecules or inhibiting /enhancing molecules, and/or signaling involved in oxidative stress and mitochondrial dysfunction a new AD therapeutic approach would be provided. Insulin signaling promotes cell survival and is able to confer mitochondrial protection (del Peso *et al* 1997). Recently, it has been demonstrated that insulin can reverse the damage caused by oxidative stress induced by Aβ in the LAN5 neuroblastoma cell line suggesting that insulin signaling could be a good target for new drug design (Picone *et al* 2011). Moreover, an important dramatic consequence of mitochondrial dysfunction involves cytochrome c. This fundamental protein is located in the inter-membrane space and shuttles electrons between mitochondrial complexes III and IV. In addition to this well known and essential function, cytochrome c plays a critical role in the activation of caspases during apoptosis (Chen *et al* 2003).

4. Two cellular defense mechanisms: apoptosis and autophagy

Apoptosis is a type of programmed cell death regulated in an orderly way by a series of signal cascades and committed by demolition enzymes called caspases. Apoptosis plays an essential role in regulating growth, development, immune response and eliminating excess or abnormal cells in organisms. Caspase signaling, together with several molecules with a regulation function such as the inhibitor of apoptosis protein, the Bcl-2 family proteins and calpain, is involved in the whole process. Caspases belong to a large protease family and, up to now, fourteen caspases have been identified, all sharing common features: they are aspartate-specific cysteine proteases and present the common amino acid sequence, glutamine, alanine, cysteine, X, glycine (QACXG) (where X is arginine, glutamine or glycine), in the active site. Two are the pathways involved in this programmed cell death: the extrinsic one, starting outside the cell, and the intrinsic one, initiating within the cell (Kerr 2002). Both these pathways are able to activate the executrix caspase-3 involved in the death process. Some studies have demonstrated the involvement of Aβ in activation of the apoptotic mechanism and there is evidence that soluble protofibrils and oligomers induce cell death by using this mechanism (Dickson 2004). Some experimental studies suggest that Aβ can activate caspases through the extrinsic pathway, by binding, directly or not, extracellular Aβ to receptors and activating caspase-8. Other studies suggest that the intrinsic pathway, in which the release of cytochrome c from mitochondria triggers the formation of the apoptosome composed of Apaf-1, pro-caspase-9, dATP, and cytochrome c, may be more relevant (Glabe 2001, Oddo *et al* 2003). Moreover, accumulation of unfolded proteins can induce endoplasmic reticulum (ER) stress and excessive and prolonged stresses lead cells to apoptosis *via* caspase-12 activation. The discovery that caspase-12 is processed downstream of Apaf-1 and the executive caspase-3 is very intriguing, suggesting that Apaf-1 and the mitochondrial pathway of apoptosis play a role in ER-stress induced apoptosis (Shiraishi *et al* 2006). Recently, utilizing a recombinant Aβ42 peptide, under oligomeric and fibrillar aggregate forms, and the LAN5 neuroblastoma cell line, it has been demonstrated that fibrillar aggregates are able to activate the extrinsic apoptotic pathway by inducing caspase-8, whereas oligomers are able to activate mainly the intrinsic apoptotic pathway by inducing caspase-9 (Picone *et al* 2009b); thus, a correlation between Aβ structures and different cellular responses has been demonstrated.

From this point of view, the use of natural antioxidants protecting against different types of cellular damage could be a promising therapy to defeat AD and probably other neurodegenerative diseases.

Autophagy is a process involved in the turnover of proteins and cell organelles and has an important role in the regulation of cell destiny after stress injury (Levine 2005, Shintani and Klionsky 2004). Autophagy works as a homeostatic non-lethal stress response mechanism for recycling proteins to protect cells from low supply of

nutrients and as a cell death mechanism. This process counts on the support of lysosome, an intracellular organelle completely devoted to the degradation or recycling of intra- and extracellular components. In the CNS of mammalians two autophagy processes are identified differing in the way by which cellular components are delivered to lysosomes for degradation: macroautophagy and chaperon mediated autophagy (CMA), although many others exist such as mitophagy (having the mitochondrion as target) (Kanki and Klionsky 2008), xenophagy (degradation of bacteria and viruses) (Levine 2005, Huang and Klionsky 2007) and microautophagy (incorporation of cytosol).

Macroautophagy is the degradation, not selective, of entire portions of the cytosol. In the first step of the process, the region involved is included in a limiting double membrane, called the 'autophagosome'. The origin of the material making up the membrane is still unknown, but recent data (Axe *et al* 2008) suggest its derivation from the endoplasmic reticulum. As soon as it is formed, the autophagosome has the same pH as the cytosol, and then gradually the pH becomes more acidic. The complete degradation of the autophagosome content occurs after its migration in the perinuclear region where there is a high lysosome content.

In the case of chaperon mediated autophagy (CMA) no autophagosome vesicles are involved. The protein, with a specific lysosomal motif (Dice 1990), is delivered to the surface membrane of the lysosome where it interacts with a receptor. Once recognized by a chaperon placed in the interior of the lysosome, the protein crosses the membrane to be degraded in the lumen of the lysosome. CMA activity is present in most tissue but increases in the case of stress conditions (Massey *et al* 2006).

The macroautophagy process is particularly active in the case of food deprivation as a source of amino acids for the synthesis of new proteins. If starvation persists, the more selective CMA process, having the proteins as specific target, takes place.

The CMA process is also useful in the case of protein damage because it allows the removal of altered proteins without modification of the healthy ones.

For rapid protein degradation, eukaryotic cells use the ubiquitin proteasome system (UPS) to remove anomalous proteins (Kraft *et al* 2010). This system requires the presence of the ubiquitin, a small protein present in all eukaryotic cells and highly conserved among all species: yeast and human share more than 95% of the amino acid primary sequence. Ubiquitin (Ub), first identified in 1975, is a small peptide of about 8.5 kDa. Its functions as a component of the ubiquitination pathway were elucidated in the 1980s by Aaron Ciechanover, Avram Hershko and Irwin Rose, winners, for this research, of the Nobel Prize in 2004 (for a review, see Ciechanover 2000). Misfolded proteins are recognized by the heat shock protein (HSP) family, which shield the hydrophobic surface exposed to the aqueous medium thus preventing its aggregation and facilitating its correct folding. Furthermore, HSPs interact with enzymes (E1, E2 and E3), which promote the binding of the defective proteins with several molecules of Ub (polyubiquitination) linked to lysine residues for degradation by the UPS (figure 3). The polyubiquitinated protein thus formed is directed into a structure called 'proteasome' for degradation. The proteolitic process, occurring in the interior of the proteasome, yields peptides of about 6–8 amino acids which can then be further degraded into amino acids and used in synthesizing new proteins (Thrower *et al* 2000).

Neuronal survival requires continuous lysosomal turnover of cellular constituents delivered by autophagy and endocytosis. Lysosomal dysfunction is well known to cause severe neurodegenerative phenotypes associated with accumulations of lysosomes and autophagic vacuoles (AVs). In AD endosomal–autophagic lysosomal dysfunction is particularly present and is driven by the genes that cause or promote this disease (Nixon *et al* 2008). Large AV accumulation within pathological neurites is a peculiarity in AD and it seems more consistent with a defect in their clearance rather than solely with an increased autophagy induction. However, the contribution of alterations in the autophagic system to the pathogenesis of Alzheimer's disease is controversial because even though *in vitro* evidence supports a negative effect of amyloid on proteasome activity (Oh *et al* 2005), other studies show no changes in proteasome activity in samples from patients with Alzheimer's disease (Blandini *et al* 2006). By contrast, the existence of changes in the lysosomal system and their contribution to the pathogenesis of the disorder are known and widely accepted now. Upregulation of the endocytic–lysosomal system is an early cellular event in Alzheimer's disease, evident even before amyloid deposits (Nixon *et al* 2008). Autophagic upregulation in these early stages is associated with lysosomal proliferation and increased expression of lysosomal enzymes (Cataldo *et al* 1995), necessary for the successful removal of aggregated and toxic proteins (Nixon *et al* 2008). As the disease progresses, the efficiency of the lysosomal system decreases, resulting in poor clearance of other intracellular components (Yu *et al* 2005). Most of these components are still sequestered in autophagic vacuoles, whose formation does not seem to be affected. Many of these vacuoles never receive the enzymes required for cargo degradation because they fail to fuse with lysosomes. Other vacuoles fuse with lysosomes but, for unknown reasons, their content is never degraded. Consequently, numerous autophagic vacuoles accumulate inside affected neurons (Nixon *et al* 2008). The series of events that leads to cell death at this stage is not clearly understood. The presence of these enlarged compartments may interfere with normal intracellular trafficking essential for specific neuronal functioning. Vacuoles may begin to leak after remaining in the cell for some time, and this release of enzymes and toxic undigested products is detrimental for cells. A proposed mechanism is that, in Alzheimer's disease, persistent autophagic vacuoles are eventually transformed into an internal source for Aβ because they contain the transmembrane protein and proteases that generate Aβ, thus contributing to amyloid deposition (Yu *et al* 2005). Increasing evidence supports a close association between autophagy and apoptosis (Pattingre and Levine 2006), thus raising

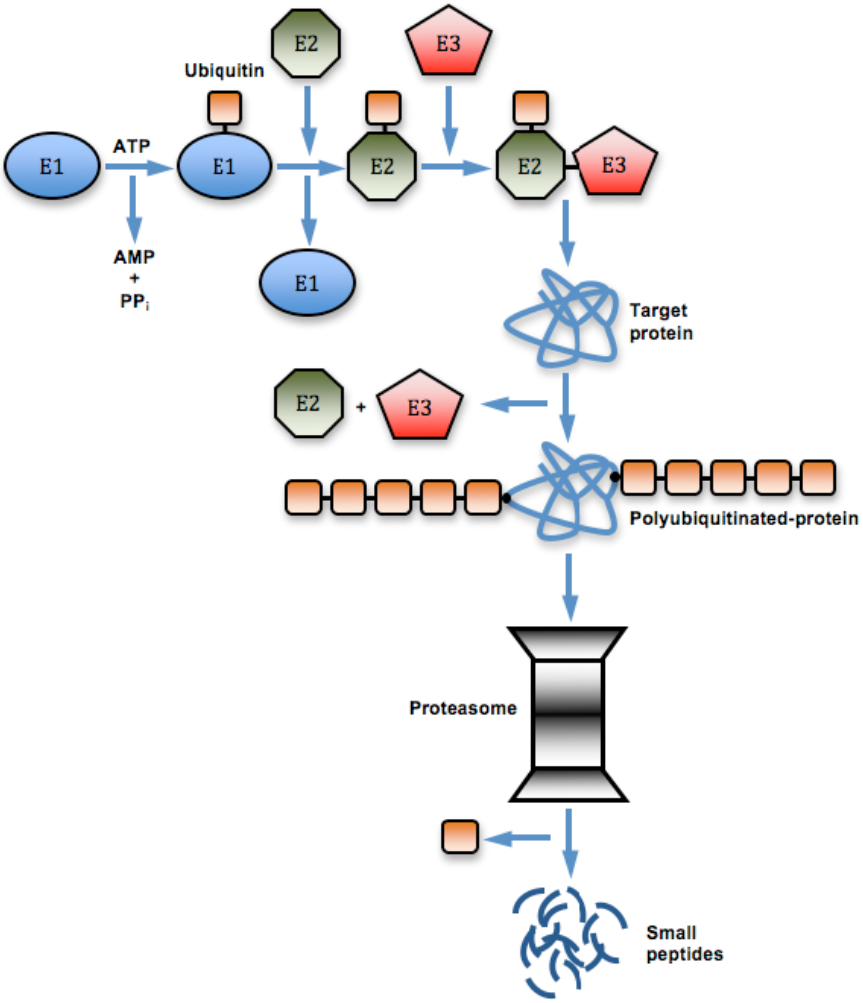

Figure 3. Ubiquitin is linked to the enzymes E1 in an ATP-dependent reaction. Then the protein is transferred to the enzyme E2 and the complex binds to E3. Ubiquitin binds the lysine residues of the protein to be degraded and the E2 and E3 are released. The polyubiquitinated protein enters into the proteasome where the proteolitic enzymes cleave the target protein in small peptides formed by 6–8 residues.

the possibility that alterations in autophagy could induce apoptotic cell death in the affected neurons. The reason for the failure of macroautophagy in Alzheimer's disease is still unknown, but aggravating events such as aging and oxidative stress may underlie defective autophagic functions. Future studies should focus on the mechanisms by which altered proteins affect clearance systems in order to develop therapeutic strategies to prevent their toxic effect. Recently, some evidence supporting the idea of a beneficial effect of activating macroautophagy on the clearance of altered proteins has opened a new window of therapeutic opportunity for these disorders. Activation of macroautophagy seems, indeed, to be the compensatory mechanism also elicited by the cells in response to the failure of the other proteolytic systems.

5. Development of therapeutic strategies

As the human population continues to age and the number of patients affected by AD increases, requirements for therapies preventing progression of the disease become more and more urgent. The effort of scientific research is focused on providing valuable information about how drug and non-drug approaches can improve day-to-day functioning and maximize quality of life. The drug (pharmacological) treatments currently available are used to manage the cognitive symptoms of Alzheimer's, such as changes in thinking, memory and perception. The treatment cannot stop the disease, but can slow down the progression of symptoms in some people, at least for a while. While drug therapy is important and beneficial, especially in the early stages, the management of Alzheimer's has evolved to include non-pharmacological therapies as integral aspects of care. These include various strategies aimed at managing problematic behavior, including involvement in therapeutic activities, home or 'environmental' modifications, and the use of appropriate communication techniques. Support and education for caregivers and family members is also crucial to the best care of people with Alzheimer's.

However, much of the research to find a therapy is based on the awareness that the progressive accumulation of Aβ aggregates is fundamental to the initial development and progression of AD, but at the same time Aβ toxicity

is a complex and multifaceted phenomenon. Aβ toxicity can be, indeed, induced by multiple assembly forms of Aβ that trigger a cascade of biochemical events such as neurotoxicity, oxidative damage, and inflammation. Thus, the strategies to use, for an efficient therapy, should target both the oligomerization process and the different activated biochemical pathways inducing toxicity and degeneration. At the moment many therapeutic efforts have been concentrated on reducing or modulating Aβ production, including secretase inhibition, increase of Aβ clearance with amyloid vaccines, or blocking of Aβ aggregation with different sources such as antibodies, breaker peptides, or small organic and natural molecules that selectively bind and inhibit Aβ aggregation and fibril formation (Walsh and Selkoe 2007).

Preventing the formation of cytotoxic oligomers should be an important goal for treating AD. While information is lacking regarding the range of Aβ assemblies present in the human brain, therapeutic intervention should target the earliest stages of oligomerization to remove all potential Aβ aggregation forms rather than a single Aβ assembly. Decrease of the production of soluble Aβ monomer is particularly attractive because it may be possible to titrate Aβ down to concentrations that will not permit oligomerization. However, different approaches have been investigated which target Aβ at different stages of the amyloid formation from its production from APP to its deposition. The inhibition of Aβ accumulation obtained by APP proteolysis should be the first level at which to act. The development of potent highly selective inhibitors of β- and γ-secretase that can readily enter the brain and lower Aβ production is being actively pursued. The inhibitor *NN*-[*N*-3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester, for example, has been shown to reduce brain Aβ level when administered orally to APPV717F transgenic mice (Dovey *et al* 2001).

Although not yet well developed, research on the employment of small chemical or natural molecules which bind and stabilize Aβ monomer preventing oligomerization and allowing the natural removal of the monomer by the brain has been undertaken. Many inhibitors of *in vitro* Aβ aggregation have been identified, although molecules capable of disrupting preformed oligomers have not yet reached clinical trials (Walsh *et al* 2005). Recent studies on animals using a small molecule inhibitor of *in vitro* fibrillogenesis, scyllo-cyclohexanehexol (AZD-103), are promising. Furthermore, natural molecules have been utilized to destabilize preformed Aβ fibrils. Ferulic acid (FA), a phenolic compound and a major constituent of fruit, inhibits Aβ fibril formation and also destabilizes preformed Aβ fibrils; this makes it a potential key molecule to be employed in AD therapy (Ono *et al* 2005). Moreover, FA has an antioxidant effect as demonstrated by experiments in which free FA or FA entrapped in solid lipid nanoparticles (SNL), used as a drug delivery system, reduces ROS production in the neuroblastoma cell line (Picone *et al* 2009a). It has been demonstrated by circular dichroism and fluorescence that Hypericin, a natural polycyclic pigment, can associate to precursors of the mature fibrils and perturb the aggregation process through intermolecular interactions with the Aβ peptides (Sgarbossa *et al* 2008).

Although a number of epidemiological studies have not found a clear link between antioxidant intake and reduced incidence of dementia and cognitive decline in elderly populations, some antioxidants such as Vitamins E and C (Ayasolla *et al* 2004), gossypin (Yoon *et al* 2004), melatonin (Zatta *et al* 2003), curcumin (Shishodia *et al* 2005, Lim *et al* 2001), Ginkgo biloba (Yao *et al* 2001) and ferulic acid (Picone *et al* 2009a) are reported to have protective effects against Aβ neurotoxicity. It is suggested that a combination of antioxidants might be of greater potential benefit for AD, especially if these agents work in different cellular compartments or have complementary activity.

Antioxidants react with ROS and reactive nitrogen species (RNS) and thus alleviate cellular damage by terminating the otherwise harmful free-radical chain reactions. A diet rich in antioxidants seems to offer hope in delaying the onset of many age-related disorders, such as atherosclerosis or neurodegenerative diseases. The most widely known natural antioxidants present in fruit and vegetables are derived from phytochemicals (plant-derived chemicals), which include polyphenols in the form of flavonoids. The flavonoids are broadly classified into anthocyanidins (e.g., cyanidin, delphinidin, malvidin), flavanols (e.g., catechin, epicatechin), flavonols (e.g., quercetin, fisetin), and flavones (e.g., luteolin) (figure 4). Some of the nonphenolic antioxidants include carotenoids (e.g., beta-carotene, lycopene), vitamin C (ascorbic acid), and vitamin E (tocopherols). These antioxidants have excellent free-radical quenching properties (Chen *et al* 2009).

When the vast antioxidant literature is explored, mixed results are reported concerning the benefits of antioxidant supplementation for treating age-related diseases. However, large clinical trials that used only selected antioxidants have not shown evidence for their benefit as therapeutic agents (Steinhubl 2008). Conversely, clinical trials involving relatively smaller groups of patients have shown beneficial effects for selected antioxidants, such as *N*-acetylcysteine, or when some antioxidants are used in combination with others (Steinhubl 2008). On the basis of these conflicting data, it becomes necessary to enroll ideal patients and use appropriate durations when undertaking this kind of clinical trial before we can make fair assessments of the therapeutic roles of these molecules.

Polyphenols, such as those found in aged red wine (Brouillard *et al* 1997), have particular importance for diabetes, cancer, aging, and neurodegenerative diseases because of their effect on certain enzymes that post-translationally modify the acetylation pattern of the histone proteins (Rahman *et al* 2006, Howitz *et al* 2003). France and Italy, the two major European wine producers demonstrate a paradoxical finding largely related to the diet in these countries. These same people are basically able to consume fat-rich and low-density lipid-saturated foods in greater quantities than most other groups, while at the same time remaining relatively unaffected in their cardiovascular health (Renaud and Gueguen 1998). The French diet, in particular,

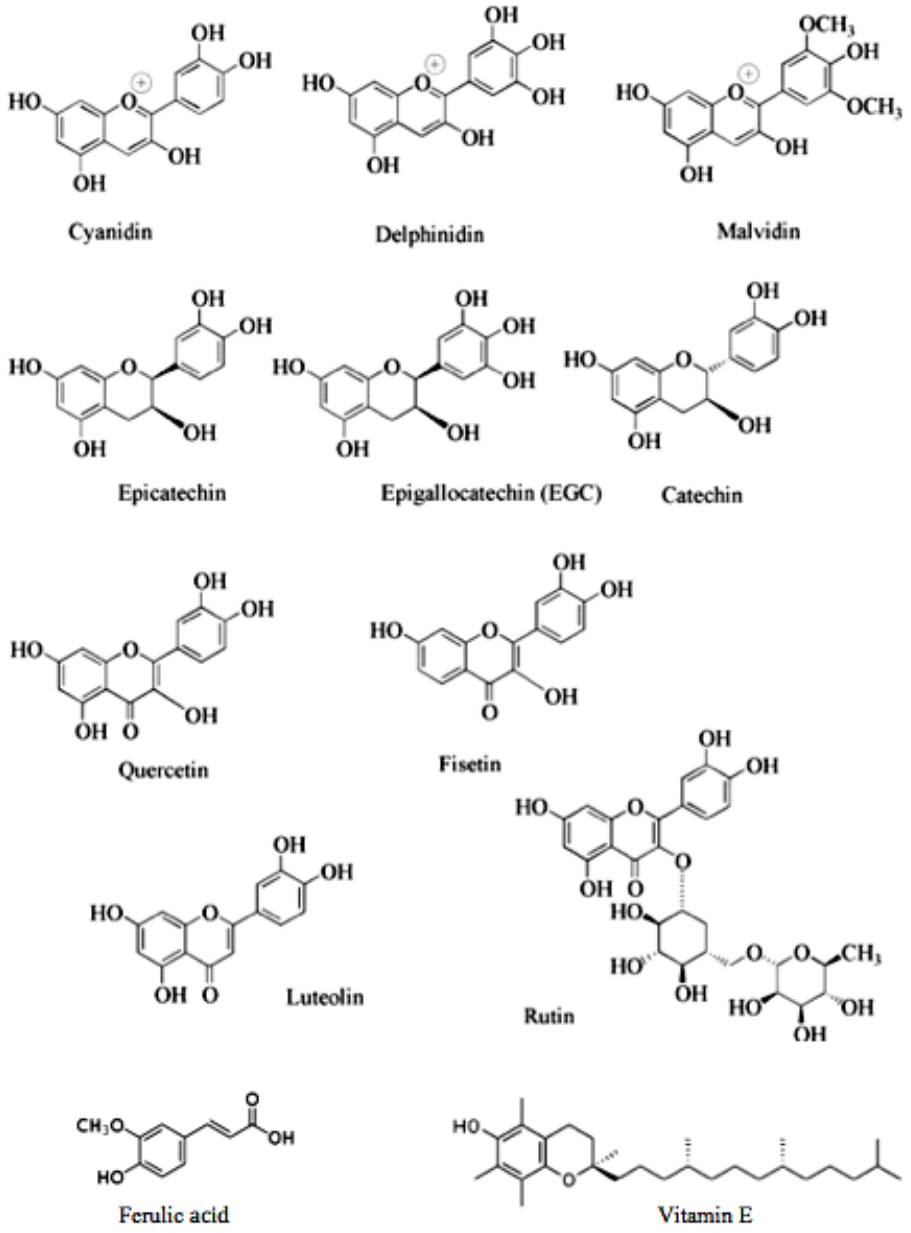

Figure 4. Chemical scheme of some antioxidant compounds.

is particularly lipid rich, and the French consume many fatty foods, yet cardiovascular pathologies are not prevalent. They frequently consume wine and have low morbidity and mortality through cardiovascular disease. This peculiarity has been commonly referred to as the 'French Paradox'.

Caloric restriction and antioxidants such as resveratrol protect cells from various cytotoxic insults and show useful effects in mouse models of aging as well as in models of AD and in limited clinical trials with human AD. It has been demonstrated that polyphenols can affect spatial memory, which is enhanced by physical activity. The relationship between neurodegeneration and heart disease, the so-called heart/brain connection, has been explored for some time (Obrenovich *et al* 2006). The French Paradox involves the cardiovasculature, which can be extrapolated to apply to the brain and cerebrovasculature as well.

Cytoprotective effects of catechin-rich flavanols and resveratrol include modulation of glutamatergic receptors, ion channels, and neuronal energy homeostasis. These compounds also appear to affect the cerebrovascular complications known to play a role in aging, vascular dementia, and AD, all of which have a particularly strong vascular component to the pathobiology of the disease (Aliev *et al* 2003), and appear to afford vascular protection (Schroeter *et al* 2006) in a manner similar to that of ischemic preconditioning. In Raval *et al* (2008), the authors demonstrated that resveratrol pretreatment confers neuroprotection against lethal ischemic insults in the brain and other organ systems via SIRT1 activation. The common factor

in all these diseases is their age-related nature. In that regard, age-related cognitive decline was reversed in mice whose diets were supplemented with blueberries, spinach or strawberries (Joseph *et al* 1999). Taken together the data suggest that an active lifestyle, combined with a polyphenol-rich diet, may prevent age-related cognitive disorders (Haque *et al* 2006) and neurodegenerative disease (van Praag *et al* 2007).

However, at present few commercial drugs have been developed to cure Alzheimer's disease. Present day therapies focus on treating associated symptoms like depression, agitation, sleep disorder, hallucinations and delusions. One of the principal targets is the basal forebrain cholinergic system, a region of the brain that is severely damaged in Alzheimer's disease. Several strategies have been developed to influence this cholinergic system. Many research groups and biotech companies are working on strategies to prevent and ameliorate Aβ deposition and/or to influence the role of inflammatory/immune mediated processes in the disease. Until now, only a few biotechnology products have reached the market. Only two compounds, cholinesterase inhibitor (tacrine) and aricept (donepexil), have been approved for the treatment of Alzheimer's disease. These two compounds prevent the breakdown of acetylcholine, an important neurotransmitter of the brain. In agreement with the putative key role of Aβ in the pathogenesis of Alzheimer's disease and on the basis of preclinical studies on animals, some biotech companies have developed immunotherapeutic strategies that aim to reduce levels of Aβ in the brain. Patients that had consented for long-term clinical follow-up, post-mortem neuropathological examination, or both, entered a phase I randomized, placebo-controlled trial of immunization with Aβ42 (AN-1792 vaccine) (Holmes *et al* 2008). Plaques were assessed in terms of the percentage area of the cortex with Aβ immunostaining (Aβ load) and in terms of characteristic histological features reflecting plaque removal. The data showed that immunization of patients with Alzheimer's disease with Aβ42 (AN-1792) was associated with a long-term reduction in Aβ load and a variable degree of plaque removal compared with unimmunized control individuals. However, no correlation between vaccine doses and removal of plaques was detected. Moreover, despite the evidence of disease modification, there was little evidence to suggest that there was any major effect on cognitive function and some cases of meningoencephalitis were described. However, the study program was continued by other pharmaceutical companies and the newer version of the vaccine, now in phase III, is known as bapinuezumab. Bapinuezumab is a monoclonal antibody produced in the laboratory (Salloway *et al* 2009, Rinne *et al* 2010). It strongly binds to and clears Aβ in the brain and in doing so hopefully facilitates memory and other cognitive processes.

Other immunotherapy approaches are currently under investigation. Solanezumab is a monoclonal antibody raised against Aβ_{13-28}, actually in phase II. It differs from bapineuzumab because it recognizes a distinct epitope in the central portion of the peptide. Solanezumab selectively binds to soluble Aβ with little to no affinity for the fibrillar form and presents fewer CNS adverse events than bapineuzumab (Siemers *et al* 2010). A phase III trial for solanezumab is now underway, with a planned completion date in 2012 (Delrieu *et al* 2012).

Another important target in AD is the NFT, composed primarily of hyperphosphorylated Tau proteins. Aβ immunotherapy results in a very limited indirect clearance of Tau aggregates in dystrophic neurites, showing the importance of developing a separate therapy that directly targets pathological Tau. In tangle mouse models, immunization with a phospho-Tau derivative reduces aggregated Tau in the brain and slows progression of NFTs (Sigurdsson 2008). Passive immunization with Tau antibodies can decrease Tau pathology and functional impairments (Boutajangout *et al* 2011); however, these results need to be confirmed in human studies.

6. Cellular therapies in AD

Neurodegenerative diseases create a tremendous burden on society, and, despite decades of research, as described above, effective treatments do not exist. Although many neurological disorders rely on complex genetic rodent models or chemical treatments that may not fully represent human neurodegenerative diseases, these cells afford options for disease modeling and provide novel sources for autologous cellular therapies. These kinds of therapies are attractive options, and the application of stem cell research to neurodegenerative diseases is rapidly expanding.

Stem cells have the ability to proliferate and differentiate into multiple cellular lineages. There are different classifications of stem cells that reflect the range of possible cell types they can produce and the ways in which the stem cells are derived. These include embryonic stem (ES) cells, progenitor cells, mesenchymal stem cells (MSCs), and induced pluripotent stem (iPS) cells. Each type possesses certain qualities and advantages, and the rationale for utilizing each depends on the desired applications and outcomes. ES cells are derived from the inner cell mass of a developing blastocyst and are pluripotent, possessing the capacity to give rise to all three germ layers. Progenitor cells, which are derived from more developed fetal or adult tissues, are multi-potent, meaning that they give rise to more restricted lineages than ES cells. These potential lineages are usually determined by the germ layer of origin. For example, neural progenitor cells (NPCs), or neural stem cells (NSCs), are capable of differentiating to cell types within a neural lineage (Gaspard and Vanderhaeghen 2010).

MSCs are an alternative source of multi-potent self-renewing cells and are derived from adult bone marrow. Naturally, they differentiate to produce osteoblasts, chondrocytes, and adipocytes; however, there is evidence that they can trans-differentiate to a neural lineage (Satija *et al* 2009). MSCs provide an accessible alternative to ES cells and potentially circumvent the need for immunosuppression in cellular therapies because they are derived from an autologous source. More recently, the development of iPS cells has provided an additional source of autologous stem cells for modeling and treating diseases. iPS cells are generated from

somatic tissue such as fibroblasts and are reprogrammed into ES-like cells by the addition of selected transcription factors as Oct 3/4, Klf, Sox2, and c-Myc (Yamanaka 2008). With the continued advancement of iPS technology, however, directed differentiation of patient iPS cells may be utilized to model human disease processes for mechanistic and therapeutic discovery. Selecting the appropriate stem cell type and understanding the desired mechanism of support is only the first step in developing and translating cellular therapies to patients. The course from bench to bedside is long and complex and it may take well over a decade of *in vitro*, *in vivo*, and large animal studies, and certain universal issues must be considered for a safe transition to patient therapies (Lo and Parham 2010).

The treatment objectives of stem cell therapies typically center on cellular replacement or providing environmental enrichment. Cellular replacement for neurodegenerative diseases involves the derivation of specific neuronal subtypes lost in disease and subsequent grafting into affected areas of the nervous system. The newly transplanted neurons may then integrate, synapse, and recapitulate a neural network similar to that lost in disease. Alternatively, stem cells may provide environmental enrichment to support host neurons by producing neurotrophic factors, scavenging toxic factors, or creating auxiliary neural networks around affected areas. Many strategies for environmental enrichment utilize stem cells to provide *de novo* synthesis and delivery of neuroprotective growth factors at the site of disease.

Current treatment options for AD are centered on regulating neurotransmitter activity. Enhancing cholinergic function improves AD behavioral and cognitive defects. Targeting the cholinergic system using stem cell therapies may provide environmental enrichment. Neurogenesis in the hippocampus decreases with aging and this phenomenon is exacerbated in AD (Drapeau and Nora Abrous 2008). Therefore, cellular therapies that enhance neurogenesis or replace lost neurons may also delay the progression of AD. Enhancement of brain-derived neurotrophic factor (BDNF) levels, which are decreased with age and in AD, promotes neurogenesis and protects neuronal function (Li *et al* 2009).

Rodent AD models receiving NPC grafts demonstrate increased hippocampal synaptic density and increased cognitive function associated with local production of BDNF (Blurton-Jones *et al* 2009). Similarly, BDNF upregulation along with NPC transplants also improves cell incorporation and functional outcomes in an AD rat model (Xuan *et al* 2008). Nerve growth factor (NGF) production is another mechanism of cellular therapy efficacy. Genetically engineered patient fibroblasts that produce NGF are currently being examined in a phase I trial for AD (Tuszynski *et al* 2005, Tuszynski 2007). Integration of NGF fibroblasts into a major cholinergic center of the basal forebrain provided some benefit to AD patients (Tuszynski 2007). Some companies are developing an NGF-releasing therapy using encapsulated epithelial cells. Combining engineered growth factor overexpression with the benefits of NPC integration into neural networks may provide an enhanced approach to treating AD. Furthermore, given the widespread neuronal loss involved in AD pathogenesis, targeting multiple systems simultaneously may be advantageous. As the understanding of the capacity of stem cell technologies increases, there is growing public hope that stem cell therapies will continue to progress into realistic and efficacious treatments for AD and other neurodegenerative diseases.

7. Biomarkers as diagnostic tools

At the moment definite AD diagnosis can occur only post-mortem, after verification of the presence of plaques and tangles. Early symptoms of the disease are, indeed, shared by a variety of neuropathological disorders. It is important to find one or more markers to diagnose and monitor the progression of the disease.

A biological marker, or biomarker (Frey *et al* 2005, Henley *et al* 2005, Sprott 2010, Ho *et al* 2010), is objectively measured and evaluated as an indicator of the normal or pathogenic processes and the pharmacological responses to a therapeutic intervention. A biomarker can serve as an indicator of health (i.e. biomarker of aging) and disease. The sensitivity, specificity and ease-of-use characteristics are the most important factors that ultimately define the diagnostic utility of a biomarker. Some biomarkers are more reasonably viewed as risk factors rather than true disease markers. Some criteria to define a good biomarker for the diagnosis of AD or other forms of dementia should be followed:

(i) it should reflect physiological aging processes and basic pathophysiological processes of the brain;
(ii) it should react upon pharmacological intervention and display high sensitivity and specificity for the disease as compared with related disorders;
(iii) it should allow measurements repeatedly over time and reproducibility in laboratories worldwide;
(iv) it should be measurable in non-invasive, easy-to-perform tests and not cause harm to the individuals being assessed.

Furthermore, tests should be inexpensive and rapid, and samples should be stable to allow easy and cheap transport. It is necessary to define good cut-off values to distinguish diseases. Experimental data should be published in peer-reviewed journals and reproduced by at least two independent laboratories.

8. AD biomarkers in cerebrospinal fluid (CSF)

The investigation of AD biomarkers in cerebrospinal fluid (CSF) could serve to detect and monitor the effects of drug candidates and their potential side effects on the disease process from the early stage of the pathology.

Neuroimaging techniques have been developed that provide evidence for Aβ deposition, Tau aggregation and neurodegeneration already at very early clinical disease stages. However, the current generation of biomarkers for AD is not yet solid against standard criteria that biomarkers in other branches of medicine satisfy.

CSF is a very useful fluid for AD diagnosis, because it reflects metabolic processes in the brain due to the direct contact between the brain and CSF. Unfortunately, its diagnostic use is limited because of the invasive collection by lumbar puncture.

Three biomarkers have been well established and validated internationally to diagnose AD in CSF with the ELISA method: Aβ42, total Tau and phospho-Tau-181. It is now accepted that only the combination of these three CSF biomarkers significantly increases the diagnostic validity for sporadic AD; it yields a combined sensitivity of >95% and a specificity of >85% (Blennow 2004, 2005, Blennow *et al* 2010, Marksteiner *et al* 2007).

Analysis of CSF Aβ42 shows a highly significant reduction in AD patients compared to controls, with a cut-off of <500 pg ml^{-1}. It has been suggested this reduced concentration is caused by decreased clearance of Aβ from the brain to the blood/CSF, as well as enhanced aggregation and plaque deposition in the brain. Changes in CSF Aβ levels differ based on the disease (Zetterberg *et al* 2010, Stefani *et al* 2005, Noguchi *et al* 2005). CSF levels of the shorter Aβ40 forms are unchanged or increased in AD. It has therefore been suggested that the Aβ42/Aβ40 ratio can improve AD diagnosis, but others have not found such changes (Sunderland *et al* 2004, Schoonenboom *et al* 2005). A novel approach based on the analysis of protein patterns has emerged that may provide a more effective means to diagnose diseases. The method is based on the use of surface-enhanced laser desorption/ionization (SELDI) time-of-flight mass spectrometry (TOF-MS) to detect differentially captured proteins from clinical samples, such as serum and plasma. This analysis results in the detection of 'proteomic' patterns that result to be an ideal method for the simultaneous detection and quantitation of a variety of Aβ peptide cleavage products (Xiao *et al* 2005).

The second hallmark of AD is intra-neuronal inclusion of the microtubule-associated protein Tau. In healthy controls, levels of total Tau in the CSF increase with age: <300 pg ml^{-1} (21–50 years), < 450 pg ml^{-1} (51–70 years), and <500 pg ml^{-1} (>70 years) (Sjögren 2001). Total Tau levels are significantly enhanced in AD patients as compared with age-matched control subjects, but this enhancement is common to other forms of neurodegenerative disease. Moreover, Tau is markedly hyperphoshorylated (39 possible sites) in AD, which results in a lack of function and axonal transport dysfunction The detection of Tau phosphorylated at position 181 is significantly enhanced in AD compared to controls, but also this form is not a prerogative of this pathology (Hampel *et al* 2010).

9. AD biomarkers in blood

The routine diagnosis of AD and mixed forms of dementia from CSF have several disadvantage: lumbar puncture and collection of CSF is an invasive treatment with potential side effects. Further, screening of patients is often difficult and follow-up analysis of the same patient over several years is problematic. Thus, there is a clear need to search for biomarkers in other body fluids to diagnose AD (Blennow 2004). Although saliva or urine can be easily collected, blood analysis is the best standard. It is still unknown how the concentration of analytes in the blood directly correlates with pathological changes in the brain, especially in AD. The search for blood biomarkers that correlate with AD should therefore begin with accepted CSF markers, such as Aβ and Tau-related biomarkers, and further include factors involved in inflammation, protein aging and cell death, and cerebrovascular dysfunctions.

Moreover, there are numerous other candidate biomarkers that reflect either elements of the primary pathogenic process in Alzheimer's disease or secondary events of the disease. Biomarkers mirroring the pathogenic process include, for example, Aβ oligomers, beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) activity and concentration, secreted isoforms of APP, and Aβ degradation products (Andreasson *et al* 2007). Secondary events include oxidative-stress responses, inflammation and gliosis (de Jong *et al* 2007). The diagnostic potential of these biomarkers is less well studied. However, some biomarkers such as BACE1 activity (Zhong *et al* 2007) and APP isoforms (Zetterberg *et al* 2008) may also give important information on desired biochemical effects of certain drug candidates, such as BACE1 inhibitors.

10. Imaging techniques

Four imaging modalities have been used as secondary end points in clinical trials on Alzheimer's disease: structural magnetic resonance imaging (MRI), functional MRI (FMRI), magnetic resonance spectroscopy (MRS) and positron emission tomography (PET). In structural MRI studies, there are correlations between MRI-based volume and neuron numbers in specific brain regions (Nagy *et al* 1996). The blood oxygen-dependent level (BODL) FMRI signal is primarily a measure of the input and processing of neuronal information within a brain region (Logothetis 2002). MRS represents changes in the biochemical composition of the brain tissue. PET using ^{18}-F-2-fluoro-2-deoxydglucose (FDG) is used for evidencing neuronal glucose consumption as the main determinant of neuronal metabolism (Reivich *et al* 1979). PET using tracers for Aβ is employed to detect accumulation of the amyloid plaques as a hallmark of Alzheimer's disease (Lockhart *et al* 2007). The validity of a biomarker with respect to a supposed neurobiological substrate will be relevant for the evaluation of disease modifying treatments. These imaging techniques provide information on the regional distribution of changes on a macroscopic (FMRI, PET, MRS) or a mesoscopic (MRI) scale. Such knowledge on the spatial distribution and temporal dynamic changes of the brain in AD results to be important because they may be an index of a correlation between the pathological changes and the stage of the disease.

10.1. Structural MRI

Prediction of the time to progress from mild cognitive impairment to clinical AD is of increasing importance

for therapeutic interventions for prevention or delay of dementia. Structural MRI provides visualization of the macroscopic tissue atrophy that results from the cellular changes underlying AD and, as such, offers one potential, non-invasive method for early detection and prediction of AD. In AD, structural MRI typically shows a pattern of decreased gray matter in the parahippocampal gyrus, the hippocampus, the amygdala, the posterior association cortex and the subcortical nuclei including the cholinergic basal forebrain. In longitudinal studies MRI can be used as a potential marker to discriminate between disease modifications and symptomatic treatment effects, by determining the rate of atrophy of brain regions. The reliability of volumetric measures obtained from repeated MRI scans is generally high, and this is an important prerequisite for its use as a disease progression marker. The most commonly MRI derived measurement is the hippocampus volume, which is measured by visual inspection or manual drawing on MRI slices. More recent automated whole brain approaches to identify mildly cognitively impaired individuals, at greatest risk for AD, have been employed (Whitwell *et al* 2008). Although manual region of interest methods offer several advantages, they have at the same time limited clinical use because they do not allow for the timely analysis of regions across the entire brain or in large datasets. In contrast, many whole brain approaches cannot evaluate the disease state in a single individual. More recent manual region of interest and whole brain studies have demonstrated that automated MRI-based computational measures can successfully discriminate those mildly cognitively impaired individuals who progress to AD from those mildly cognitively impaired individuals who do not progress (Bakkour *et al* 2009). Advances in image analysis algorithms have led to the development of structural MRI-based software tools that can automatically parcellate the entire brain into anatomic regions and quantify the tissue properties in these regions for a single individual (Desikan *et al* 2009). In a recent study, the feasibility of using automated MRI-based software tools as predictive markers for AD has been investigated (Desikan *et al* 2010). Using baseline MRI scans from 162 mildly cognitively impaired individuals, it is possible both to identify individuals in the earliest stages of the disease process and to predict the time to disease progression, and this can potentially serve as a predictive biomarker for AD.

10.2. Functional MRI

Studies of memory in patients with AD using FMRI discovered a pattern of altered activation in the medial temporal lobes and parietal lobes, which were consistent with structural MRI results. Based on initial single center studies, specific effects of treatment on regional brain activation could be detected in AD. Multicenter and longitudinal studies using FMRI in patients with AD have yet to be carried out; these will be a major step in the further development of FMRI-based biomarkers. Moreover, a variant of functional MRI has been used increasingly to map the modulatory effects of psychopharmacological agents on cognitive activation of large-scale networks in the human brain. Such pharmacological MRI (phMRI) studies can be informative about pharmacodynamics, specific neurotransmitter mechanisms that underlie the adaptivity of neurocognitive systems to variation in task difficulty and familiarity, and changes in neurophysiological drug effects associated with genetic variation, neuropsychiatric disorders and normal aging (Honey and Bullmore 2004). Application of this imaging technique has permitted the importance of individual differences in genotype and cognitive phenotype as conditioners of drug effects on brain activation to be evidenced. Moreover, phMRI has been successfully utilized to map the hemodynamic effects of psychotropic substances such as cocaine, which requires an understanding of their sites, mechanisms, and time courses of action in the mouse model of brain disorder (Perles-Barbacaru *et al* 2011).

10.3. Proton-MRS

Proton-MRS (^{1}H-MRS) provides quantitative biochemical measurements of the compounds in brain tissue. The best established ^{1}H-MRS marker is the amino acid *N*-acetyl aspartate (NAA), which reflects the functional status of neuronal mitochondria (Moffett *et al* 2007). A reduction of NAA levels independent of brain atrophy is a consistent finding in Alzheimer's disease. Preliminary single center studies have demonstrated that NAA levels are responsive to pharmacological treatment, and multicenter application of this technique has been demonstrated. In addition to NAA, several other metabolites such as choline containing compounds, creatine and phosophocreatine, myoinisitol, and gluatamate and glutamine are detectable with ^{1}H-MRS but their potential as biomarker candidates in Alzheimer's disease is controversial and requires more investigation (Kantarci 2007).

10.4. FDG-PET

Positron emission tomography (PET) is a nuclear medicine imaging technique that produces a three-dimensional image or picture of functional processes in the body. The system detects pairs of gamma rays emitted indirectly by a positron-emitting radionuclide (tracer), which is introduced into the body on a biologically active molecule. The glucose analog, 2-[fluorine-18]-fluoro-2-deoxy-D-glucose (FDG), is useful in neuroimaging because it permits measurements of local glucose metabolism as an index of neuronal activity at a resting state without the need for cognitive activation. In Alzheimer's disease, neuronal activity is impaired, as FDG uptake is reduced, mainly in temporo-parietal association areas. These changes are closely related to cognitive impairment as demonstrated in cross-sectional and longitudinal studies. The changes in FDG uptake can be measured objectively with smaller coefficients of variance than standard neuropsychological measures, thus increasing the power of these studies (Alexander *et al* 2002, Hirono *et al* 2004). Alterations in FDG uptake are usually attributed to pharmaco-dynamic drug effects but will also reflect disease

progression, especially when measured after several months of follow-up. This technique has been used as a secondary outcome parameter in some clinical trials (Stefanova *et al* 2006, Kadir *et al* 2008).

Moreover, some studies have demonstrated that Aβ plaques can be measured in patients with AD by the use of PET and the radiotracer carbon-11-labeled Pittsburgh compound B (^{11}C-PiB). PiB is a thioflavin analog that binds with low nanomolar affinity to aggregated fibrillar deposits of the amyloid-β peptide, enters the brain at concentrations detectable by PET, and clears rapidly from normal brain tissue (Price 2005). At the low nanomolar concentrations used in PET studies, PiB selectively binds to fibrillar Aβ deposits in post-mortem human brain (Fodero-Tavoletti *et al* 2007). This kind of scan has provided information regarding the Aβ plaque burden that is independent from structural change in brain anatomy. An important application of ^{11}C-PiB PET was for investigating whether bapineuzumab, the humanized anti-amyloid-β vaccine, described above, would reduce cortical fibrillar Aβ load in patients with Alzheimer's disease (Rinne *et al* 2010). Treatment with bapineuzumab for 78 weeks reduced cortical ^{11}C-PiB retention compared with both baseline and placebo. ^{11}C-PiB PET seems to be useful in assessing the effects of potential AD treatments on cortical fibrillar Aβ load *in vivo*.

Moreover, the ^{11}C-PiB PET technique has been utilized to investigate whether cognitively preserved monozygotic or dizygotic cotwins of persons with Alzheimer's disease exhibit increased brain amyloid accumulation (Scheinin *et al* 2011). By mapping of specific brain areas it was revealed that genetic factors appear to influence the development of Alzheimer's-like β-amyloid plaque pathology. The dissociation between cognitive impairment and brain β-amyloidosis in monozygotic twins implies that there may be important environmental/acquired factors that modulate the relationship between brain amyloidosis and neurodegeneration. AD may be detectable in high-risk individuals in its presymptomatic stage with ^{11}C-PiB PET, but clinical follow-up will be needed to confirm this.

11. Genetic test

AD is mainly considered a sporadic pathology and genetic screening cannot be utilized for its diagnosis. However, for the rare cases in which individuals have family members with early-onset familial AD, genetic tests are available.

These genetic screenings include testing for PSEN1, PSEN2, APP and APOE mutations; however, since APP or PSEN2 mutations are rare, screening for these mutations is not currently used. Although genetic testing of APOE and predictive testing in typical late-onset disease have been discouraged (Farrer *et al* 1995), the predictive value of APOE ε4 homozygosis is as high as for many other Mendelian diseases. Indeed, it seems that attitudes to APOE genotyping are changing (Green *et al* 2009), and it is likely that the predictive potential of genetic analysis will be considerably increased. This is in part due to a growing appreciation of the increase in predictive value given by genotyping the whole APOE locus.

A likely outcome of these efforts is that these predictive tests will classify individuals at risk of developing AD into three groups. The first group includes individuals who are APOE ε4 homozygotes at high risk of developing AD. This risk is modulated by other genes and the polymorphisms of the APOE promoter (about 3% of the population). The second group includes individuals who are APOE ε4 heterozygotes who are at modest risk of developing AD. For this group, the precise risk is modulated by other APOE alleles (ε2 being less risky than ε3) and by the polymorphisms of the APOE promoter, as well as by the other genome-wide association study hits. The third group includes individuals who do not carry an APOE ε4 allele and are therefore at low risk of developing AD. The interpretation of these data will need the development of a careful risk chart, and their application to the population will represent a challenge. Although still to be investigated, it is expected that the use of these genetic tests will help considerably in the identification of individuals with mild cognitive impairment who will later be diagnosed with Alzheimer's disease (Herukka *et al* 2007).

Scientific research is also providing valuable information about how drug and non-drug approaches to treatment can improve day-to-day functioning and maximize quality of life. The drug (pharmacological) treatments currently available are used to manage the cognitive symptoms of Alzheimer's, such as changes in thinking, memory and perception. They cannot stop the disease, but they can slow the progression of symptoms in some people, at least for a while. While drug therapy is important and beneficial, especially in early stages, the management of Alzheimer's has evolved to include non-pharmacological therapies as integral aspects of care. These include various strategies aimed at managing problematic behavior, including involvement in therapeutic activities, home or 'environmental' modifications, and the use of appropriate communication techniques. Support and education for caregivers and family members is also crucial to the best care of people with Alzheimer's.

12. Conclusions

The increased lifetime of the human population is coupled to the progression of neurodegenerative diseases. The effort of the scientific research in the field of neurodegeneration is focused on improving the quality of life of people affected by these diseases. New insights into basic neurodegeneration and cell death programs will offer new ways for future prevention and treatment strategies. Further, validation and development of new and good biomarkers will allow easy and cheap diagnosis. Discovery and improvement of efficacious therapies including chemical drugs, antioxidants, vaccines or cell treatments will be crucial to the best care of AD-affected patients.

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