DOI: 10.1002/anie.200802808

Medicinal Chemistry

Alzheimer's Disease: From Pathology to Therapeutic Approaches

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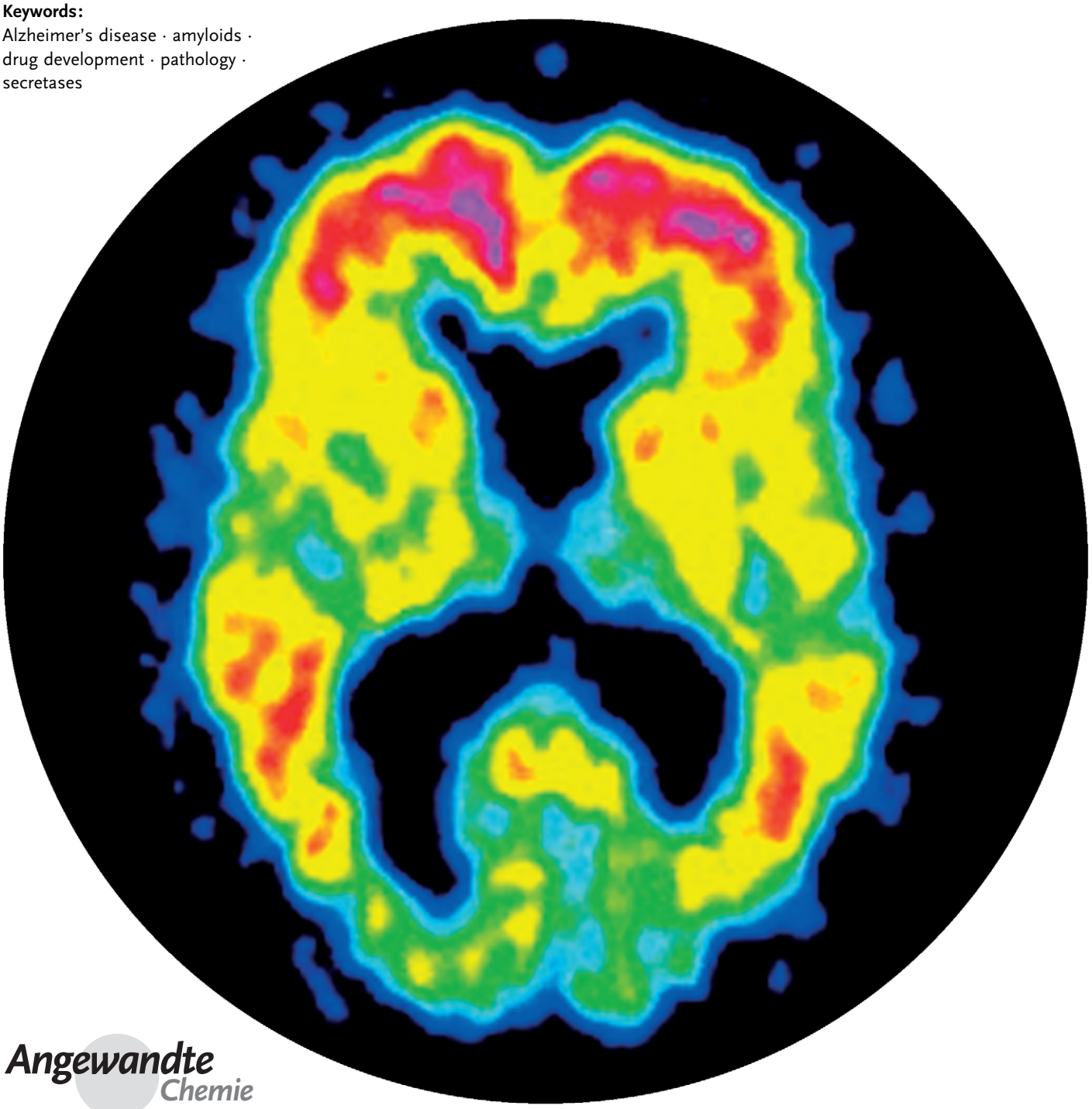
Keywords:
Alzheimer's disease · amyloids · drug development · pathology · secretases



Research on senile dementia and Alzheimer's disease covers an extremely broad range of scientific activities. At the recent international meeting of the Alzheimer's Association (ICAD 2008, Chicago) more than 2200 individual scientific contributions were presented. The aim of this Review is to give an overview of the field and to outline its main areas, starting from behavioral abnormalities and visible pathological findings and then focusing on the molecular details of the pathology. The "amyloid hypothesis" of Alzheimer's disease is given particular attention, since the majority of the ongoing therapeutic approaches are based on its theoretical framework.

From the Contents

> "*The fact is, I think we almost always underestimate the complexity of life and of nature*"
> Craig C. Mello, Nobel lecture 2006[1]

1. Introduction

When Auguste died, 56 years old, she had gone through years of life suffering. Her drama started when she began to distrust her husband, when she felt she had to hide things in her own home and when she thought that there were people out trying to kill her—all signs of profound personality changes. Within a short time a rapid loss of memory made her disoriented in her own home and she became afraid and desperate. The medical doctors and the nursing staff at the psychiatric clinic of Frankfurt am Main took care of her. She complained that she failed to understand anything, that everything appeared strange to her. Sometimes she seemed to suffer from auditory hallucinations, called for her husband or daughter and often she screamed for many hours on. When objects were shown to her, she could name them correctly, but she forgot the event immediately. Although she did not remember the use of some objects, she could use her hands and she could walk normally. Her ability to read, to write, and to speak became more and more impaired. Her mental deficiencies widened with time. After four and half years in the hospital she developed bedsores in spite of all nursing care and died probably as consequence of a subsequent sepsis.

We write the year 1906. Histological examination of Auguste's brain at the psychiatric hospital in Munich revealed the presence of intraneuronal fibrils, the deposition of a "pathological metabolic substance", changes in glia cells and atrophy but almost no sign of atheriosclerosis. "*Considering everything, it seems we are dealing with a special illness*", wrote the psychiatrist Alois Alzheimer, who had followed the progression of Auguste's disease and who had done the histological examinations.[2] Today, 100 years later, the presence of neurofibrillary tangles and amyloid deposits are still the disease-defining parameters. Neurofibrillary tangles and neuropil threads are aggregates of paired helical filaments of an abnormally phosphorylated and abnormally β-folded protein called tau. Amyloid deposits are formed by the Aβ peptide. Auguste would be classified now as an early onset AD patient (EOAD), a rare form of Alzheimer's disease (AD). With the high life expectancy today the majority of patients show onset of the disease at a higher age and are categorized as late-onset AD patients (LOAD). The estimated number of patients is 7–8 million in Europe, 4–5 million in the USA, and 24 million worldwide. The number is expected to increase to 42 million in the year 2020 because of demographic changes.[3] Alzheimer's disease is the most common neurodegenerative disorder and a major health concern to societies worldwide.

2. Pathobiological changes in Alzheimer's Disease

2.1. Episodic Memory Impairment

Often the beginning of dementia is recognized in everyday situations. While the mother is talking in great detail about events in the distant past, she is wondering where you are, even though you just told her that you would go to the garden. New information is not taken up, encoded, and stored properly long term and consequently cannot be retrieved, thereby resulting in what is called episodic memory impairment. In the initial phases of the disease the semantic memory—the meaning of a word—and procedural memory—how to dress or how to ride a bicycle—are relatively spared. Memory is made up of electrical impulses along the neuronal connections in the brain, formed by the opening and closing of synapses and facilitating for a specific time frame a specific circuit that encodes specific information. Learning involves the formation of new synaptic connections and this requires gene expression in the cell nucleus and the synthesis of protein at synapses. The formation of synaptic information is thus linked to events in the cell body.[4,5]

The formation of episodic memory requires, in particular, neuronal connections of small areas of the entorhinal cortex and of the hippocampus in the medial temporal lobe (hippocampus and the parahippocampal gyrus). The huge amount of

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information acquired by seeing, hearing, and feeling is processed in the neocortex and funneled by projections from almost all neocortical areas to the entorhinal region (Figure 1). This kind of associated data is then transmitted to

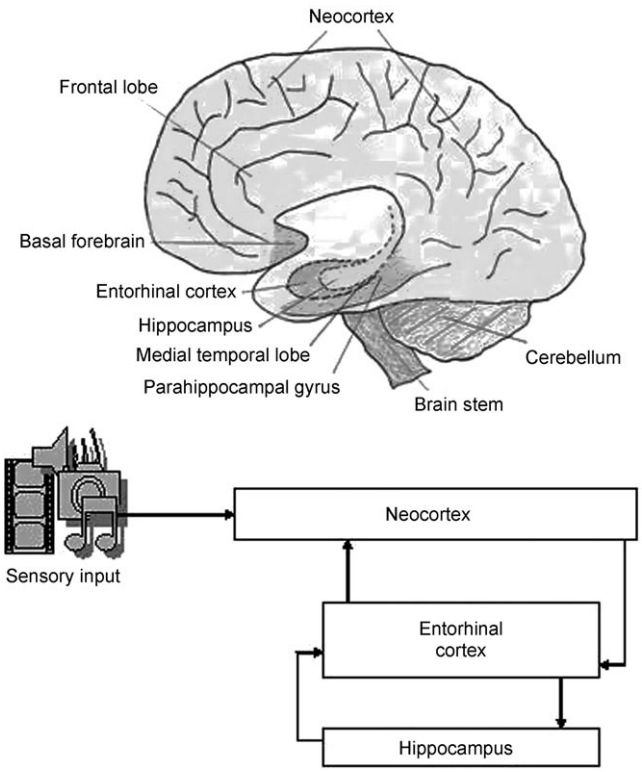

Figure 1. Schematic illustration of key brain areas and circuits involved in memory processes which are affected in Alzheimer's disease.

the hippocampus from where it is fed back again into a deep entorhinal layer, and from there information is distributed again back to the neocortex. Although the entorhinal cortex and the medial temporal lobe are only a tiny fraction of the whole cerebral cortex, they are a key interface and the functioning of their connections is essential for the formation of long-term memory. Loss of these connections directly causes clinical symptoms of episodic memory impairment. This was accidentally found in 1954 after surgical bilateral removal of the medial temporal lobes of patient H. M. and later in other patients. This removal led to an anterograde amnesia, where all events subsequent to the operation could not be remembered. Although the patient H. M. could, for example, read a newspaper he forgot the content after a few minutes.[6–8] The deficit in episodic memory is the hallmark of AD at the beginning of the disease, despite a certain heterogeneity in the clinical symptoms even at that stage.

2.2. Imaging Structural and Fuctional Changes in an AD-Damaged Brain

At the time of the first symptoms of episodic memory problems severe pathological changes are already present in the brains of AD patients. These changes develop gradually over years in the key areas for memory formation. The severity of the symptoms is paralleled by the development of characteristic brain changes. Early longitudinal imaging studies based on computed tomogram techniques followed the reduction of the thickness of the medial temporal lobe in normal aging and in AD patients. There is a tiny reduction with normal aging. However, in AD patients, at a distinct point in time that almost indicates the beginning of the disease process, the thickness of the medial temporal lobe declines steeply, with parts of it shrinking at a rate of 15% per year.[9,10] Modern magnetic resonance imaging (MRI) techniques not only confirmed the volume reduction in the gray matter (neurons) but demonstrated a correlation between the entorhinal cortex atrophy and episodic memory impairment in the AD patients.[11–13] In addition to the structural MRI techniques, positron-emission tomography (PET) studies measuring Aβ deposition with ^{11}C-PIB or other PET ligands and functional techniques such as glucose utilization and functional MRI (fMRI) are used today to follow the progress of the disease and to potentially allow early diagnosis (Figures 2 and 3).[14–17] In particular, the measurement of the magnetization transfer ratio seems to detect the very early changes in the brains of patients with mild cognitive impairment (MCI), a clinical condition of increased risk for the development of dementia.[18–20] The atrophy in the affected Alzheimer brain areas is caused by loss of dendrites and axons, myelin reduction, shrinkage, and finally the death of neurons.[21] The 1.5 mL sized cornu ammonis of a healthy

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Roland Jakob-Roetne was born in Kronach, studied chemistry at the Julius-Maximilian University of Würzburg, and received his PhD in 1983 working with Prof. Helmut Quast. After a year as a post-doctoral fellow at the ETH Zürich with Prof. Dieter Seebach, he joined the medicinal chemistry group of Hoffmann–La Roche in Basel in 1984. He has led several projects in the CNS area, and is particularly involved in research on Alzheimer's disease.

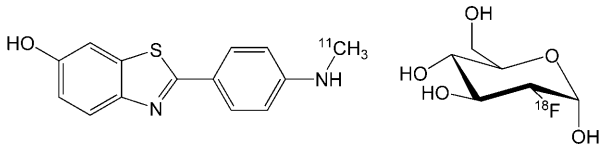

Figure 2. Structure of the thioflavine-T derivative [^{11}C]-PiB (Pittsburgh compound B) used in PET imaging of Aβ amyloid and of 2-deoxy-2[^{18}F]-fluoro-D-glucose (FDG) used in PET studies of glucose metabolism.

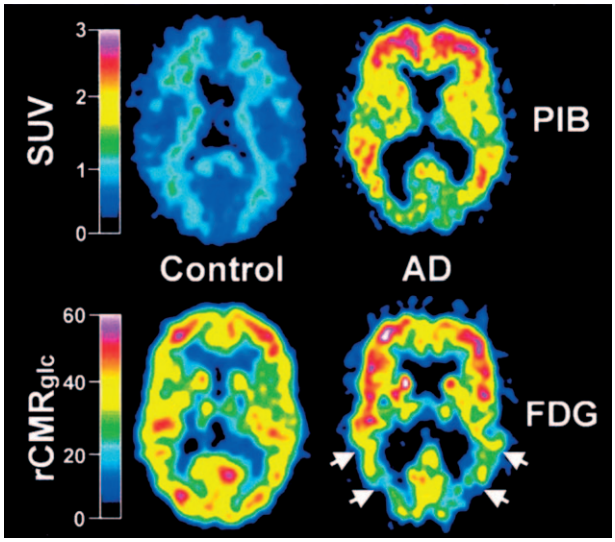

Figure 3. PIB standardized uptake value (SUV) images demonstrate a marked difference between PIB retention in Alzheimer's disease (AD) patients and healthy control (HC) subjects. The PET images of a 67-year-old HC subject (left) and a 79-year-old AD patient are shown (MMSE = 21; right). Top: SUV PIB images summed over 40 to 60 min; bottom: ^{18}FDG $rCMR_{glc}$ images (regional cerebral glucose metabolic rate, μmol min^{-1}/100 mL). The left column shows lack of PIB retention in the entire gray matter of the HC subject (top left) and normal ^{18}FDG uptake (bottom left). Nonspecific PIB retention is seen in the white matter (top left). The right column shows high PIB retention in the frontal and temporoparietal cortices of the AD patient (top right) and a typical pattern of ^{18}FDG hypometabolism present in the temporoparietal cortex (arrows; bottom right) along with preserved metabolic rate in the frontal cortex. The PIB and ^{18}FDG scans were obtained within 3 days of each other (reprinted with permission from W. E. Klunk et al., *Ann. Neurol.* **2004**, *55*, 306–319).

hippocampus contains about 9 million neurons. In the end stages of AD more than 80% of these neurons have disappeared and the volume is reduced to less than half.[22,23]

2.3. Stages of the Disease

Pathology distinguishes six "Braak stages" to describe the severity of the cortical destruction which are based on the development of neurofibrillary tangles in cell bodies and of neuropil threads in dendrites.[24,25] The presence and distribution of tangles and threads correlate with the degree of cognitive decline. These neurofibrillary changes follow a similar sequence in AD patients, with only minor variations between individuals. Braak stage I refers to a few tangles in the transentorhinal region only. In stage II there are more tangles and threads in this region and also occasionally some in the hippocampus, but still without any apparent clinical symptoms. Stages III and IV are characterized by mild to modest cognitive impairment and personality changes, and involve the superficial entorhinal layer to a large extent. In stage IV the changes expand to the deep entorhinal area, from where information is projected back to the neocortex. The neurofibrillary changes are still present mainly in the small entorhinal region. In the late stages V and VI the fibrillary changes spread fully to the hippocampus and the connected neocortical areas, and are associated with full dementia and additional symptoms such as speech disturbances and motor dysfunction (Figure 4). Extracellular Aβ-amyloid deposits in the medial temporal lobe develop concomitantly to the initiation of the neurofibrillary lesions, and neurofibrillary tangles appear in the absence of adjacent Aβ-amyloid deposits and vice versa.[26]

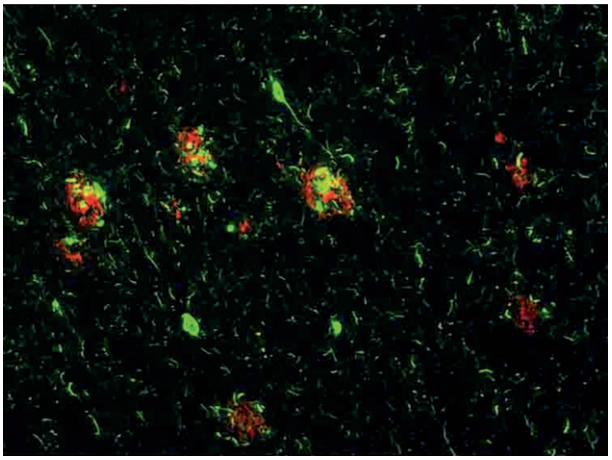

Figure 4. Immunohistochemistry on postmortem human brain frontal cortex tissue of an Alzheimer patient at the Braak VI stage. Double immunofluorescence staining of neurofibrillary tangles in green (stained with an antibody specific for phosphorylated tau protein) and dense amyloid Aβ plaques in red (stained with an antibody specific for Aβ) has been used. The numerous small green elongated strands indicate neuropil threads. The black background corresponds to areas of normal cells which do not show the fluorescence signals specific to the pathological lesions (reprinted courtesy of Dr. Berd Bohrmann).

Pathologists distinguish a sequence of five phases of Aβ deposition in the whole brain. The first Aβ deposits always develop in the neocortex only (phase 1) followed by deposition in the entorhinal and medial temporal lobe area (phase 2). Then structures in the middle of the brain close to the hippocampus (putamen, caudate nucleus) and parts of the basal forebrain (diencephalic nuclei, substantia innominata with nucleus basalis of Meynert) are affected (phase 3). Finally Aβ deposits occur in the brainstem (phase 4) and in the cerebellum (phase 5).[27] However, a general correlation between the degree of amyloid β-peptide deposition and the severity of the dementia still does not seem to exist. Deposition of Aβ has also been described in nondemented individuals, and atrophy and neuronal loss was described in

the cerebellum without the deposition of amyloids and fibrils.[28–31] The Aβ deposition spreads from regions with early on existing Aβ deposits into regions which receive their neuronal input from there, starting with Aβ deposits in the neocortex.

Axonal swellings occur long before detectable Aβ deposition, thus pointing to disturbances in the axonal transport, which may play a role in Aβ deposition.[32] Three classes of axonal defects in AD can be distinguished.[33,34] The first class relates to dystrophic axons in proximity to amyloid. The second class is associated with neurofibrillary tangles. The third class is neither spatially associated with amyloid nor with tangles, shows focal axonal swelling as a result of abnormal accumulation of axonally transported cargos, and is regarded as preceding the formation of amyloid and tangles.

2.4. Disturbance of Intracellular Transport

2.4.1. Tau, Paired Helical Filaments, and Neurofibrils

Continuous anterograde (from cell body to synapse) and retrograde, fast (100–400 mm day^{-1}) and slow (ca. 0.3–3 mm day^{-1}), axonal transport is an absolute requirement for the functioning of neurons. Motor proteins such as kinesins and dyneins carry their cargo over long distances through microtubules.[35] The microtubules, which are part of the cytoskeleton, are polymers of tubulin and they bind microtubule-associated proteins (MAPs), which regulate their stability. The most prominent MAP in the axon is the protein tau (MAPT). The six isoforms of human tau in neurons are between 352 and 441 amino acids long, and are expressed in a constant ratio. They contain in their C-terminal region three or four repeating units with a length of 31 amino acids each. These are the microtubule-binding domains and also the domains responsible for aggregation into the paired helical filaments. Tau is a very hydrophilic, water-soluble, and mostly unfolded protein which contains along its sequence at least 25 potential phosphorylation sites. The sites outside the repeat domains are mainly phosporylated by the kinases CDK5 (cyclin-dependent kinase 5), GSK3β (glycogen synthase kinase 3β), and ERK2 (extracellular signal-regulated kinase 2). Phosphorylation inside the repeat domains by MARKs (microtubule-affinity regulating kinases) can detach tau from microtubules. Specific phosphorylation sites have been correlated with the severity of the neuronal damage in AD.[36] Correctly phosphorylated tau at a certain concentration stabilizes microtubules, keeping a constant dynamic equilibrium between microtubule-bound tau and free tau, thereby maintaining the morphology of the neuron and ensuring axonal transport.

The key principle of the current "tau hypothesis" of AD is abnormal hyperphosphorylation of tau as a result of an imbalance in the kinase and phosphatase activities. Total tau levels in brains of AD patients are about eightfold higher than in controls, and the increased tau protein is in an abnormally hyperphosphorylated form.[37] The reason for this is not well understood. It has been speculated that chronically increased regenerative activity may be the origin, with the neuron trying to counteract cell-damaging events of various origins. High levels of neuroplasticity have been associated with increased phosphorylation and expression of tau, since tau can promote neurite outgrowth.[38] What is initially a normal element of neuronal plasticity may result in cytotoxicity after exceeding a certain threshold of duration and intensity. Too much tau protein bound to the microtubules affects the binding of the motor proteins to the microtubules. In particular, the anterograde kinesin-dependent transport can become impaired, thereby resulting in a net flow of vesicles to the cell body.[39,40] Importantly, hyperphosphorylated tau from AD-damaged brain can sequester normal tau and other microtubule-associated proteins. This results in destabilization and depolymerization of the microtubules, impairment of the axonal transport, and decreased neurotransmission, and has an immediate impact on cognitive functions. In addition, the increased phosphorylation stimulates the self-assembly of tau. One model for this self-assembly assumes an intramolecular binding of a flanking region to microtubule binding domains in normally phosphorylated tau (Figure 5). Excess phosphorylation disrupts this binding, and the freed microtubule binding domains assemble intermolecularly and form small deposits (pretangles) which finally adopt β-sheet structures in paired helical filaments. The paired helical filaments finally assemble into the large neurofibrillary tangles in which part of the protein has undergone a series of additional modifications, such as truncations, glycations, or cross-linking by transglutaminases, which confer an additional toxic lesion inside the neuron.

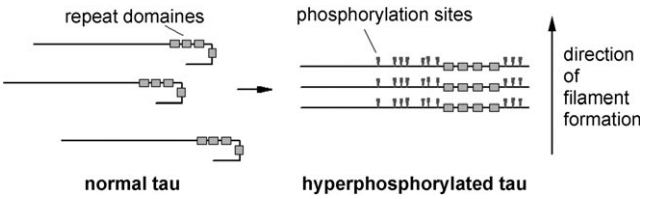

Figure 5. Model of tau self-assembly. Hyperphosphorylation changes the conformation of tau and allows contact between the repeat domains through which the protein assembles.

Disturbance of the delicate equilibria of tau function, without any additional pathology, can be sufficient to produce neurodegeneration. Several other neurodegenerative diseases, such as amyotrophic lateral sclerosis, Pick's disease, progressive supranuclear palsy, and FTDP17 (frontotemporal dementia with Parkinsonism-17) are also tauopathies.[41]

2.4.2. Changes in Axonally Transported Proteins

The cargo carried by the motor proteins along the microtubules consist of organelles such as mitochondria and peroxisomes and of vesicles filled with various proteins which fulfill various important functions at their destination site. In the pathological condition of AD, anterogradely transported material cannot arrive at the terminal processes and synapses which leads to starvation at these distal sites. In particular, the lack of mitochondria will lead to energy deprivation through lack of ATP (Figure 6). On the other hand, material that has

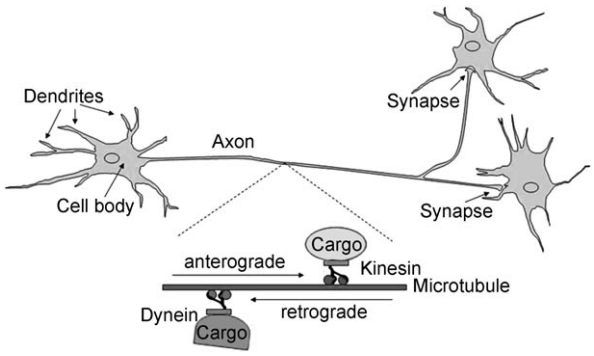

Figure 6. Schematic illustration of the motor proteins kinesin and dynein involved in the axonal transport.

to be removed from the distal sites for recycling or clearance may accumulate at the distal sites or may be released into the extracellular space. Thus, the distal sites with the synapses seem more vulnerable than the cell bodies, and it is not surprising that synapse loss correlates well with cognitive deficits.[42] Biopsies from the temporal and frontal cortex of AD patients revealed a 15 to 35 % reduction in the number of synapses per cortical neuron.[43] The level of the presynaptic marker synaptophysin also correlates with the degree of cognitive decline in patients with very mild AD. It can be reduced by up to 25 %.

Cholinergic and glutamatergic synapses are at the focus of these events since they are intimately involved in the learning and memory-forming processes. Interestingly, the cholinergic and glutamatergic presynaptic bouton density is upregulated in certain brain areas in persons with MCI, likely as a compensatory mechanism to an ongoing damaging process. Imaging studies in MCI patients demonstrated more activation in the medial temporal lobe and more active synapses than in subjects without cognitive impairment.[44] However, the disease eventually progresses to a continuous depletion. Studies in the 1980s of AD-damaged brains already revealed that the numbers of cholinergic neurons are decreased in the nucleus basalis of Meynert; this region is the major compartment of cholinergic cells in the basal forebrain which plays an important modulatory function in cognitive processes from where their axons innervate the whole cortex.[45] These axons and their terminals normally contain most of the choline acetyltransferase (ChAT) of the cerebral cortex. Strikingly, there is a more than 300 % increase in ChAT in the cell body per cholinergic neuron of the nucleus basalis of Meynert in Alzheimer's patients.[46] A similar increase in the amount of acetylcholine esterase (AChE) per nucleus basalis neuron was observed. ChAT is moved by slow axonal transport, and AChE by fast axonal transport. The accumulation of both enzymes in the cell bodies points towards impaired slow and fast axonal transport to the axonal terminals.

One of the most prominent fast anterograde compounds that is transported along the neuronal axon is the β-amyloid precursor protein (APP). Experiments in mice indicated that APP binds to the kinesin-1 light chain and mediates the axonal transport of β-secretase and presenilin-1 in membrane compartments in which Aβ can be formed.[47–49] APP is not just an innocent passenger on a ride to the synaptic environment, it may play an important role in axonal transport itself. APP can carry additional cargoes and mediate their transport through kinesin-1. A nice illustration of this function is the way the herpes simplex virus travels along the axons. Each single virus coats itself with a huge amount of APP molecules (between 1000 and 1 000 000 molecules per virus). This occurs probably during its passage through the Golgi apparatus. In this way the virus can hook up to the kinesin motor to form a huge vesicle and manages to travel at a fourfold faster rate than a mitochondrion.[50]

Another "victim" of disturbed axonal trafficking is the neurotrophin NGF (nerve growth factor). Defective retrograde transport of NGF from the cerebral cortex and hippocampus to the nucleus basalis in the basal forebrain has been described.[51] The cell bodies of the neurons in the nucleus basalis are dependent on NGF, and a decrease in the available NGF contributes to their atrophy and death. NGF is released by cortical neurons, dependent on the neuronal activity, and is then taken up into the axonal projections of the cholinergic neurons of the basal forebrain by binding to its receptors. These cholinergic neurons express two types of NGF receptors called p75NTR (pan-neurotrophin receptor, low affinity nerve growth factor receptor, binds all neurotophins) and TrkA (tropomyosin receptor kinase, p140trkA, high affinity for NGF). Both receptors can form a complex with high NGF affinity and selectivity for TrkA. The receptors are synthesized in the cell bodies of the nucleus basalis neurons and are anterogradely transported along the axons to the terminals. NGF released by the postsynaptic cells binds to the receptors and is then retrogradely transported to the cholinergic cell bodies. Only the binding of NGF to the TrkA/p75NTR complex results in survival of the neurons.[52,53] The expression of the TrkA receptor is positively regulated by NGF. Less NGF in the cell bodies is, therefore, expected to result in the synthesis of less TrkA. Reduced levels of TrkA per basal forebrain neuron are early events in the disease progression and are already present in MCI patients. A highly significant association between the decrease of TrkA and global cognitive score or minimental state examination has been found.[54] In contrast, levels of the p75NTR receptor in the cholinergic basal forebrain remain unchanged during the progression of the disease. It is reported that, in vitro, the Aβ protein can specifically bind to p75NTR with an affinity similar to NGF, but not to TrkA, and that this binding initiates intracellular apoptotic signaling. Given the imbalance between p75NTR and TrkA, and the increased formation of Aβ in AD, the direct activation of p75NTR by Aβ may contribute to neuronal death.[52]

2.4.3. *Induction and Impairment of Autophagy*

The neurons respond to stress factors such as axonal transport impairment by initiating macroautophagy as a means of rescue. The autophagy pathway is a common response of a cell to nutrient or growth-factor deprivation. Macroautophagy is a highly regulated process for the degradation of intracellular proteins, in particular for long-lived, folded or aggregated proteins and even whole organ-

elles that cannot be cleared by the proteasome pathway.[55] The process starts by the formation of a membrane that sequesters cytoplasm and its containing proteins. This then closes to form a double-membrane autophagosome (Figure 7). This autophagosome is transported intracellularly

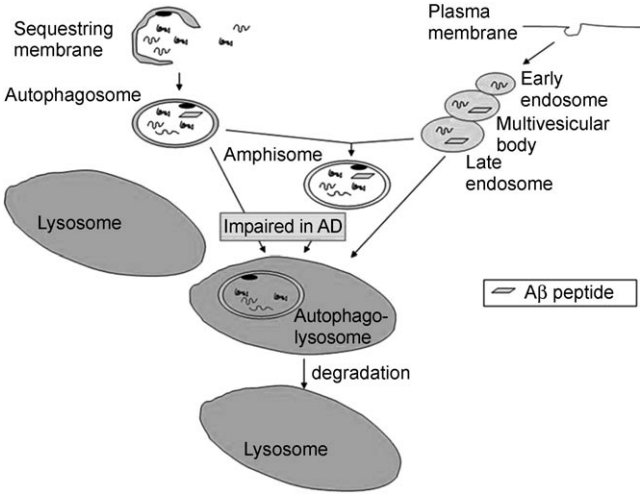

Figure 7. Autophagic and endosomal pathways for the degradation of intracellular proteins.

to finally meet and fuse with a lysosome to form a single-membrane autophagolysosome, in which the sequestered proteins are then degraded to fragments that can be reused in the metabolism of the cell. The collective term for all vesicles of the autophagic pathway is autophagic vacuoles. When the digestion of the various substrates in the autophagolysosome is completed, the organelle regains the appearance of a lysosome.

Neurons of normal adult brain are almost devoid of such autophagic vacuoles. In an AD-damaged brain, however, autophagic vacuoles are very frequent, occuring mostly in the dendrites and axons as well as the synapses of neocortical and hippocampal neurons. Autophagic vacuoles are already seen early in the disease, when these neurites are not yet dystrophic. With disease progression, the autophagic vacuoles accumulate in these neurites to such an extent that the cytoplasmic contents are almost replaced, thereby resulting in neurites swollen to sizes many times larger than normal.[13,56,57] The majority of these autophagic vacuoles are autophagosomes that are clearly unable to reach and fuse with the lysosomes. Thus, while macroautophagy is induced in AD neurons, its function is impaired.

Furthermore, the autophagic vacuoles in the AD neurons are immunoreactive for PS1 (presenilin 1), a key component of the γ-secretase complex. Experiments with isolated vacuoles and in cell cultures using a model fluorogenic substrate demonstrated that the autophagic vacuoles had the highest γ-secretase activity of all the cell organelles tested and account for about 20% of the total γ-secretase activity of the cell. The cleavage product of the model substrate corresponded to the 42 site of Aβ. This cleavage product was not cleaved further and accumulated in the autophagosomes, whereas it was further degraded in autophagolysosomes. This is evidence that Aβ may be generated in autophagic vacuoles before their maturation into autophagolysosomes. Given the accumulation of the autophagosomes and the lack of degradation by fusion with lysosomes, the autophagic Aβ may substantially contribute to the amyloid burden of the disease. The reason for the accumulation of the autophagosomes is currently not known, and one can only speculate if, for example, increased formation of Aβ in these vacuoles might hinder their maturation into autophagolysosomes, or if simply local breakdown of intracellular transport because of a lack of ATP stops them from reaching a lysosome, or if the lysosomes carry a defect that impairs fusion with the autophagosome. Ingestion of autophagocytosed mitochondrial material may overload the lysosomes with reactive oxygen species and damage the sensitive machinery of the lysosomal membrane necessary for fusion with the autophagosome. Of all the cellular membranes, nature has protected the lysosomal membrane with the highest content of the antioxidant vitamin E (30 times higher than in microsomal membranes).[58]

Equally important as the autophagic pathway in the pathology of AD is the endocytic pathway (Figure 7). In the endocytic pathway, extracellular material at the plasma membrane is internalized into early endosomes, which mature into multivesicular bodies and into late endosomes. The endosomes acquire proteolytic enzymes and a proton pump which leads to an internal pH value of 3.5–6.0. Endosomes may fuse with autophagosomes, autophagolysosomes, or lysosomes. In sporadic AD, an increased delivery of proteases to early endosomes and an increase in the endocytic activity is observed. Endosomes become abnormally enlarged because of the increased availability of endosome-fusion effectors. Many endosomes contain Aβ, and they have the substrate, the enzymes, and the right pH value for its production. The increased endocytic activity is one of the earliest pathophysiologies of AD, and is not observed in normal aging or other neurodegenerative diseases (apart from Down Syndrome, where such endosomal changes are present even before birth, and Niemann-Pick type C; both diseases share certain pathologies with AD).[59]

2.5. Mitochondrial Abnormalities

Impairment of autophagy increases the half-life of components that should be degraded by this pathway. Aged mitochondria are normally degraded by autophagy. The vulnerable and most affected neurons in the hippocampus and the cortex in AD show, compared to controls on a per neuron base, an accumulation of mitochondrial degradation products in the cytoplasm (mitochondrial DNA, cytochrome oxidase) and in autophagic vacuoles (mitochondrial DNA, lipoic acid).[60] Specifically, lipoic acid, the cofactor of several mitochondrial enzymes, was used as a marker for the fate of mitochondrial components in AD, and was found to be present in autophagic vacuoles of AD patients but not in the brains of a control group.[61] Morphometric analysis of these vulnerable neurons from biopsy samples from AD patients

showed a reduction of about 25% in the number of intact mitochondria. Purified mitochondrial fractions from brains of AD patients were demonstrated to have about 50% less COX activity (cytochrome oxidase, the enzyme responsible for reducing molecular oxygen) compared to controls. This finding is probably due to a catalytic defect in the enzyme, although other studies with different techniques gave contradictory results.[62–64] Decreased activity was also reported for the pyruvate dehydrogenase complex and the α-ketoglutarate dehydrogenase complex.[65] Another feature of the disease is that the control region of the mitochondrial DNA of AD patients carries many more mutations associated with dysfunction than the corresponding DNA from healthy controls.[66]

Several cell-culture studies, including experiments mimicking physiological conditions, relevant for sporadic AD have clearly demonstrated that the Aβ amyloid protein is an important contributor to the diminished mitochondrial function, even at a sub-nanomolar concentration.[67] One example of an Aβ binding protein identified in the mitochondria of neurons from AD patients is ABAD (Aβ-binding alcohol dehydrogenase, previously called ERAB, endoplasmic reticulum associated amyloid-β-peptide binding protein, although it is a mitochondrial protein). ABAD is identical to SCHAD (human brain short chain L-3-hydroxyacetyl-CoA dehydrogenase type II, E.C.1.1.1.35), which is encoded by the gene HADH II (also called HSD10, 17β-hydroxysteroid dehydrogenase).[68,69] The ABAD–Aβ complex has been detected in the brains of AD patients and localized to mitochondria.[70] ABAD is upregulated in AD brains (hippocampus) and is also present in activated astrocytes in the amyloid plaque corona.[71,72] A crystal structure of human ABAD in the presence of Aβ at a resolution of 2.3 Å has been elucidated, although no electron density could be obtained for the bound Aβ. Experiments in cultured neurons demonstrate that the ABAD–Aβ interaction results in oxidative stress and finally apoptosis. Transgenic mice overexpressing ABAD and mutant APP fail to learn efficiently, and ESR spectra of their frozen brains show abnormally high concentrations of free radicals.[70] The binding to ABAD occurs at Aβ concentrations of 40–70 nM (half-maximally occupied binding sites). However, Aβ inhibits the activity of the ABAD enzyme only at nonphysiological concentrations of 2–3 μM. The initiation of cytotoxicity is, therefore, expected not to result from direct inhibition of the enzyme activity, but from changes in its localization within the mitochondrion, where its capacity to dehydrogenate a broad range of alcohols may contribute to the increased formation of unwanted reactive aldehydes such as malondialdehyde or *trans*-4-hydroxy-2-nonenal.[72] Since mitochondrial abnormalities are found in neurons that lack pathologic neurofibrils, it is likely that these abnormalities occur at very early stages of the disease.

The consequences of compromised mitochondrial function are manifold: ATP-dependent processes will be impaired, including the ATP-dependent functioning of the axonal motor proteins dynein and kinesin. Furthermore, mitochondria also play an important role in intracellular calcium homeostasis and can store large quantities of calcium. Increased basal calcium concentrations were measured in the mitochondrial cybrid model, in which mitochondria of a human neuroblastoma cell line were replaced by mitochondria from patients with AD. A delayed recovery of the resulting calcium peak to the baseline level was found after stimulus with the cholinergic agonist carbachol.[73] Under such conditions, any additional stimulation, for example, activation of *N*-methylaspartate (NMDA) receptors by glutamate may become problematic for the cell, as has been shown in rats for the cholinergic neurons of the nucleus basalis after compromising mitochondrial activity.[74] Ca^{2+}-activated enzymes are affected, for example, tissue transglutaminase, which was shown in vitro to cross-link tau, and which is elevated in the brains of AD patients.[75]

The increase in the intracellular calcium level is considered to be the reason for the threefold activation of calpain I (μCANP, calcium-activated neutral proteinase) in the prefrontal cortex of AD patients. This results in an increase in the proteolysis of enzymes involved in signal transduction, namely, calcium-dependent protein kinases and phosphatases, membrane skeleton proteins, and the cytoplasmic domains of transmembrane proteins affecting membrane fusion.[76] The level of the endogenous calpain inhibitor calpastatin was found to be lower in vulnerable cortical layers of AD-damaged brain, but not in the cerebellum where the amyloid deposition and the neuronal loss occurs only at the late stages of the disease.[77] Soluble Aβ oligomers induced an increase in the level of intracellular calcium, and the subsequent calpain activation was reported to result in the degradation of dynamin 1 in hippocampal neuron cultures.[78] Dynamin 1 is a protein responsible for the disconnection of vesicles from their parent membrane and is depleted in the brain of AD patients. In the absence of dynamin 1, synapses cannot release neurotransmitters because the vesicles for the neurotransmitters are absent at the synapse and accumulate instead in the lumen. The source of the Ca^{2+} ions in the hippocampal neuron-culture experiment was clearly extracellular. Its influx was mediated by NMDA receptors and could be blocked by the NMDA receptor antagonists MK801 and memantine, which suggests direct or indirect contact of the Aβ oligomers with the receptor. These findings are relevant to the pathology of AD, and may explain the abnormalities of the endosomal-lysosomal system that are seen early in the affected neurons.

2.6. Oxidative Stress and Metallobiology of Aβ

Oxidative stress refers to the generation of abnormally high concentrations of otherwise physiologically important reactive oxygen species (ROS) such as O_2^- (superoxide anion), $HO^{\bullet}$ (hydroxyl radical), NO, and H_2O_2. When oxygen is reduced in the mitochondria, O_2^- is a normal side product of about 1%. The brain corresponds to only 2–3% of the body mass, but it utilizes about 20% of the oxygen taken up by the body and is, with its nonregenerating neurons, at higher risk of oxidative damage. However, despite the high load of ROS in the mitochondria, they normally do not show particularly high oxidative damage since detoxifying mechanisms are in place—such as SOD2 (manganese superoxide

dismutase) that converts O_2^- into oxygen and H_2O_2, or catalase that converts H_2O_2 into oxygen and water. Abnormal mitochondria, however, which produce much more O_2^-, can release H_2O_2 into the cytoplasm, and in cases where there is an irregular iron homeostasis very reactive and toxic HO· may form through the Fenton reaction ($Fe^{2+} + H_20_2 \rightarrow Fe^{3+} + HO\cdot + HO^-$). In normal brains, the levels of metal ions are tightly regulated by binding to metalloproteins. In AD-damaged brains, IRP-1 and IRP-2 (iron regulatory proteins 1 and 2) and their interaction with IRE (iron responsive element, the RNA binding site for IRPs) is disturbed and the ferritin concentration is decreased, thereby leading to increased concentrations of free iron.[79] The neurons protect themselves through increased formation of reductants such as NADPH, glutathione, and other SH compounds, or Cu/Zn superoxide dismutase.

H_2O_2 is, however, not only a source for HO· generation but is also a signaling molecule to stimulate protein cascade pathways that influence the cell cycle or that induce the expression of inflammatory genes such as SAPK (stress-activated protein kinase). In neuronal cells and in mouse experiments, Aβ has been consistently shown to induce such kinases which principally can contribute to the phosphorylation of tau.[79] Degenerating neurons in AD brain show signs of aberrant entry in the cell cycle, a condition where phosphorylation of microtubule-associated proteins (such as tau) is increased.[80] Free radicals are also strong inducers of the heat-shock proteins HSP27 and HSP70 in AD. These molecular chaperones are involved in the stabilization of tau and Aβ, they prevent aggregation, but stabilize damaged proteins, and prolong potential toxic effects by keeping them in solution.[81,82]

Imbalance in the sensitive redox system may be induced in various ways—disease related or as part of normal aging. Although initial changes may be local and invisible, with time these changes may reinforce each other and build up a vicious cycle. The free ROS radicals oxidize lipids and damage membranes in the brains of AD patients.[83] Lipid peroxidation products, for example, from the oxidation of polyunsaturated fatty acids, are aldehydes, which have much longer half-lives than the radicals and can diffuse to other sites within the cell and react there. Acrolein is such a reactive and toxic product, as is *trans*-4-hydroxy-2-nonenal, both found at increased levels in AD-damaged brain. They are Michael acceptors and bind cysteine, lysine, and histidine residues and thereby impair the function of proteins such as neuronal glucose transporter type-3 and Na^+/K^+ ATPases. This results in membrane depolarization, decrease in the ATP concentration, and increase in the level of neuronal intracellular free Ca^{2+} ions, with the consequences mentioned above.[84] In particular *trans*-4-hydroxy-2-nonenal forms adducts with tau and inhibits its dephosphorylation. Oxidative cholesterol metabolites, such as 3β-hydroxy-5-oxo-5,6-secocholestan-6-al, can covalently modify Aβ and lower the critical concentration for its aggregation into the nanomolar range.[85]

Free ROS radicals can oxidize proteins to form protein carbonyl species, which are increased in several brain regions in AD patients. In consequence, the functioning of these proteins is reduced or lost, as has been described in AD, for example, for creatine kinase (involved in energy transfer), β-actin (cytoskeleton), glutamine synthetase (degrades the neurotransmitter glutamate) and the glutamate transporter Glt-1 (clearance of the neurotransmitter glutamate), and pMSR (peptide methionine reductase).[83] Free ROS radicals can also oxidize DNA and RNA. The oxidation products 8-OHdG (8-hydroxy-2′-deoxyguanosine) and 8-OHG (8-hydroxyguanosine) were found to be elevated in vulnerable neurons in the brain of AD patients. In particular, the ribosomal RNA, which is highly abundant in neurons, contains 8-OHG, and was suggested to be a key binding partner for redox-active iron in the cytoplasm. This would have an impact on ribosomal functioning and reduce protein synthesis.[86] Interestingly, when Aβ deposition increases in the diffuse amyloid plaques in the cortex, the neuronal levels of 8-OHG decrease as if aggregation might prevent oxidative damage.

In addition to iron homeostasis, disturbed zinc and copper homeostasis also plays an important role in AD pathology.[87] Both metal ions bind readily to Aβ and can promote the formation of Aβ oligomers. The highest binding affinity of Zn^{2+} to Aβ42 and Aβ40 was determined at 100 nM.[88] Unlike copper, zinc is redox silent and does not contribute directly to the formation of reactive oxygen species. It plays an essential role at the glutamatergic synapses in the neocortex, the region where the first Aβ deposits develop. The neurotransmitter glutamate is released together with Zn^{2+} (probably as a counterion), and very high zinc concentrations of about 300 μM (compared to < 0.5 μM basal concentration) are transiently reached in the synaptic cleft. About 10% of the Zn^{2+} in the brain is in neuronal synaptic vesicles, the rest is protein-bound. The cortex and hippocampus achieve the highest concentrations of free, synaptic Zn^{2+} ions. Reuptake clears the high Zn^{2+} concentrations rapidly, but this is an energy-dependent process and may be delayed if the synapse does not receive sufficient energy. Glutamatergic neurons express the zinc transporter ZnT3, which loads the zinc ions into vesicles for synaptic release. The high zinc concentration may contribute to Aβ deposition, as was shown for mice deficient in ZnT3 and crossed with TG2576 mice (a transgenic mouse model for AD). The AD mice deficient in the zinc transporter developed 50% less Aβ amyloid plaque than the TG2576 mice with the transporter present.[89] The synaptic Zn^{2+} concentrations can promote aggregation and deposition if soluble Aβ is present.

Copper ions are stored in postsynaptic vesicles of the NMDA receptor. They are released at postsynaptic stimulation and reach transient concentrations of about 15 μM in the synaptic cleft. Extracellular copper levels in plaque-free brain areas in AD patients were found to be fourfold higher than in healthy brain.[90] However, the intracellular copper concentrations have been regarded as being too low, since the activity of several cuproenzymes is reduced, for example, the cytochrome oxidase activity in mitochondria, although the level of the COX-1 protein was found to be increased three to fourfold in mitochondria of vulnerable neurons.[60] Similarly, the antioxidant enzyme SOD1 (Cu/Zn superoxide dismutase) is expressed to a higher degree in brains of AD patients, but its activity is decreased. The SOD1 protein was also found to

be elevated but its activity decreased in APP23 transgenic mice. In this case the enzyme activity could be restored with dietary Cu^{2+}.[91] The recovery of the activity argues against potential inactivation as a result of oxidation of the histidine 118 residue in the active site (to 2-oxohistidine) by abnormally high H_2O_2 concentrations.[92] The deficiency in the intracellular Cu^{2+} concentrations have been interpreted as the result of the presence of other competing binding partners, such as APP and elevated Aβ concentrations, which have an impact on the metal homeostasis in the disease. The possibility that Aβ directs Cu^{2+} into the bloodstream was even considered, since the Cu^{2+} levels in serum is markedly elevated in AD patients.[93]

Human Aβ is a metalloprotein with an attomolar affinity for copper. The lowest K_D value for the binding of Cu^{2+} to Aβ42 is about 10^{-17} M and for binding to Aβ40 about 10^{-11} M at pH 7.4.[94] The current data suggest that monomeric Aβ binds Cu^{2+} at three histidine residues and a tyrosine residue.[95] Rat and mouse Aβ42 differ in two copper-binding amino acids (Tyr10→Phe, His13→Arg), and the resulting lower affinity to copper has been associated with less formation of H_2O_2 and, in consequence, less peroxidation products in these species, which do not develop Aβ amyloid.[96] High-resolution mass spectrometry and cyclic voltametry experiments in vitro have demonstrated that Cu^{2+} binds to Aβ42 in a 1:1 ratio. On its own, this Cu^{II}–Aβ42 complex is stable—the copper ion stays in the oxidation state +2 and the Tyr10 and Met35 residues are not oxidized. The redox potential of the complex for reduction to Cu^{I}–Aβ42 excludes tyrosine and methionine as reductants; however, it is high enough for the reduction to be effected by a variety of common redox-active molecules such as ascorbic acid, pyruvate/lactate, glutathione, vitamin B_{12}, or NAD^+/NADH as electron sources. This reduced Cu^{II}–Aβ42 complex is then able to reduce oxygen and to generate H_2O_2.[97] These results are in line with an indirect antioxidative and neuroprotective effect of only monomeric (not oligomeric) Aβ40 and Aβ42 in cultured neurons based on the sequestration of metal ions.[98] The situation is definitely more complex in vivo in the brain of AD patients, where Met35, for example, was found to be oxidized in autopsy samples, and where Aβ is to a large extent oligomeric and aggregated. These oligomers have not only lost their neuroprotective properties, but are clearly toxic.

Copper and zinc can coordinate to electron pairs on N or O atoms of the Aβ peptide not only intramolecularly but also intermolecularly, to form multimers of different length depending on the environment and the pH value. At pH 7.4, redox-silent Zn^{2+} is the dominant complex builder, and equivalent amounts of Zn^{2+} and Cu^{2+} are bound to Aβ in vitro. In contrast, more Cu^{2+} is incorporated at mildly acidic pH values of 6.6–7.0, and only Cu^{2+} is bound in vitro at pH 6.6, thus allowing more and more redox chemistry and toxicity.[94,99] In cell cultures, Zn^{2+} ions were shown to inhibit the Cu^{2+}-induced toxicity of Aβ42, which correlated with the suppression of H_2O_2 formation.[100] Such multimers can adopt ordered structures and can easily penetrate negatively charged membranes. Modeling studies suggest that the complexed Aβ42 peptide forms a hexameric helix, but, depending on the composition of the lipid bilayer, β-sheet structures have also been proposed.[95] The insertion of these Aβ multimers changes the conductance of the membrane, thereby leading to an increase in intracellular Ca^{2+} and cytotoxicity. This led to the theory that the Aβ multimers may assemble under appropriate structural circumstances into a conducting ion channel.[101] The evidence is, however, limited to in vitro systems. In these cases, the channel conductance could also be blocked by high micromolar concentrations of Cu^{2+} or Zn^{2+} ions and by specially designed peptides. Other proposals are that the Aβ multimers and also oligomers of other amyloidogenic proteins, such as prion protein or islet amyloid, increase the membrane conductance in general for cations as well as for anions by spreading the lipid head groups apart, thinning the lipid bilayer, and allowing increased partition of water into the membrane.[102–104]

2.7. Senile Plaques

2.7.1. Role of Astrocytes and Microglia

The neuronal cell membrane bound nonfibrillar form of Aβ42 is the predominant form found consistently in all diffuse plaques (nonfibrillar Aβ deposits, non-birefringent if stained with Congo red; previously also called preamyloid deposits) of AD patients, and is the major form in the cerebellum. This neuronal membrane associated Aβ42 is regarded as an initial form of Aβ42 deposition.[105] In pyramidal neurons (glutamatergic neurons) of the entorinal cortex and the hippocampus of AD patients, Aβ42 was described to first selectively accumulate intracellularly as discrete granules—potential lysosomes or lysosome-derived structures—and occupy a range of 20–83 % of the total cytoplasmic volume. There is evidence of lysis of these neurons, leading to a local dispersion of their cytoplasmic contents and leaving a nuclear residue.[106] There is also evidence that Aβ peptides may be released in association with exosomes (secreted vesicles), since exosomal proteins were found accumulated in plaques of AD patients.[107] The death of neurons in AD seems to follow AD-specific routes which do not correlate well with the terms apoptosis or necrosis.[108] Diffuse Aβ amyloid plaques are mostly associated with reactive astrocytes, but only less than 50 % are associated with microglia, and these are in their resting state. This is in sharp contrast to the dense fibrillar neuritic plaques (fibrillar Aβ amyloid deposits, birefringent if stained with Congo red), where more than 80 % are associated with reactive microglia.[109] One astrocyte is estimated to be in contact with more than 100 000 synapses and to protect these synapses and neuronal processes by shielding them (also named "walling-off") from amyloid. However, it is likely that the astrocytes at the diffuse plaques also prevent the ability of microglia cells to remove amyloid material, as observed in vitro,[110,111] since astrocytes in cell cultures were shown to suppress normal microglial clearance of senile plaque material by releasing diffusible proteoglycans that bind to Aβ. Astrocytes in contact with diffuse amyloid in the brain of AD patients were shown to accumulate substantial amounts of Aβ42 as well as debris from degenerated synapses and dendrites.[112] It is claimed that Aβ42 (not Aβ40) binds with high affinity to α7nAChRs (α7-nicotinic

acetylcholine receptors) on neuronal surfaces and is internalized by astrocytes mostly as an Aβ42/α7nAChR complex after rupture from the neuronal membrane.[113] Aβ42-overburdened astrocytes can undergo lysis similar to that observed with neurons. This lysis leads to a smaller type of amyloid plaque.

To the best of our knowledge, there is no ultrastructural documentation in the brains of AD patients of the internalization and phagocytosis of Aβ by microglia within plaques.[114,115] If it occurs, it is very inefficient and does not prevent accumulation of the Aβ peptide. The activation of microglia in the brain of AD patients seems to result in two functionally different states. One is the "good" phagocytic phenotype, which is able to degrade Aβ peptide, but which is not prominent in the brain of AD patients. The second abundant "bad" phenotype without phagocytic activity may lead to the release of H_2O_2 and pro-inflammatory cytokines as well as chronic microgliosis, which contributes to the progress of the disease through alterations to the vascular processes at the blood–brain barrier. Importantly, TNFα (tumor necrosis factor α) is increased in the microglia of brains from AD patients.[116] TNFα is not only a pro-inflammatory cytokine, but is a gliotransmitter which affects the synaptic strength, expression of BACE (β-amyloid cleaving enzyme), and clearance of Aβ.[117] In hippocampal slices, neutralizing TNFα prevented Aβ-induced inhibition of LTP (long-term potentiation, a neurophysiological correlator of learning and memory).[118] The phenotype of the microglia is at least partly determined by the local environment. Blockade of microglial cell-surface receptors, such as CD40 (TNF receptor superfamily member 5), or lack of activation, for example, of their Fc receptors (binding the Fc end of an antibody), may play a role. The interaction of CD40 with its ligand CD40L, expressed in the central nervous system (CNS) by astrocytes, retarded microglial phagocytosis and clearance of Aβ42 in vitro.[119] A clear induction of the phagocytic phenotype with Aβ present within microglia was induced in brains of AD patients by immunization with Aβ42.[120]

The activation of microglia in brains of AD patients parallels the maturation of the diffuse plaques to classic fibrillary neuritic plaques. The contact of diffuse Aβ with microglia eventually becomes the turning point for the formation of fibrillar plaques. It has been proposed that microglia cells attract and concentrate Aβ into narrow channels at their surface, where fibrillization starts.[112] In membrane fractions prepared from the cortices of brains from AD patients, a unique complex of Aβ with ganglioside GM1 was identified which is considered as the seed for the Aβ fibrillogenesis.[121] GM1 forms clusters in cholesterol-rich parts of membranes, and these clusters avidly bind Aβ. A higher cholesterol content of membranes, such as in the presence of apoE4 (a risk factor for developing AD) or with aging, may facilitate formation of the GM1–Aβ complex. GM1 facilitates the insertion of Aβ into the membrane, its fibrillization, and the disruption of the membrane.[122] Unlike in vitro conditions, fibrillar Aβ is always complexed with other Aβ-associated proteins in the brains of AD patients. The clusters of activated microglia are exclusively found in plaques, in which the C1q (complement 1q) and SAP (serum amyloid P component) could also be immunolabeled.[123] Fibrillar Aβ complexed with C1q and SAP increased the production of pro-inflammatory cytokines by microglia in cultures. C1q alone can bind Aβ42, and was shown to block its uptake and phagocytosis by cultured rat microglia.[124] The presence of activated microglia and their ability to potentially release neurotoxic substances has been interpreted as harmful chronic neuroinflammation, with the term "neuroinflammation" used to describe internal CNS events and not CNS inflammations such as, for example, multiple sclerosis.[125–127] Senile plaques containing the fibrillar Aβ amyloid (not diffuse plaques) show full activation of the complement cascade. Fibrillar Aβ, neurofibrillary tangles, and SAP can all activate the complement pathway. Clinicopathological studies found that the amount of Aβ/microglia complex increased in early stages (Braak IV) of the disease, and PET studies with PK11195 (a marker for activated microglia) demonstrated the presence of activated microglia before brain atrophy. The majority of the microglia are thought to originate from blood monocytes and to migrate into the brain parenchyma from cerebral capillaries.

2.7.2. *Vascular Amyloid*

About 90% of human amyloid plaques are in direct contact with capillaries.[112] The vascular basement membranes of the capillaries are another pool of Aβ, mainly of Aβ40.[128] If fibrillar amyloid is formed there, it is also associated with the presence of microglia cells. Focal disruption of the blood–brain barrier has been demonstrated in brain biopsy samples from AD patients. This effect arises as a result of Aβ deposition (different from vascular, multi-infarct dementia, the second most common dementia after AD, where disturbed blood circulation is the primary cause) and leads to a thickening of the basement membrane, degeneration of endothelial cells, and necrosis of cellular components in the vascular wall.[129] Such cerebral amyloid angiopathy in the arteries and capillaries is a prominent feature of AD. In a study of 117 brains of patients with autopsy-confirmed AD, 83% showed evidence of cerebral amyloid angiopathy.[130]

Aβ amyloid fibrils isolated from brain tissue or formed from recombinant material (used in 3D structure determination by H/D-exchange NMR or atomic force microscopy)[131–133] are unbranched, about 10 nm in diameter, and are composed of 2–4 nm thick twisting filaments.[134] However, observation of the Aβ filaments directly in the plaque tissue shows that the Aβ protein is associated with other components, such as with HSPG (heparin sulfate proteoglycan), CSPG (chondroitin sulfate proteoglycan), and SAP. In amyloid angiopathy, HSPG is decreased in the vascular basement membrane, probably because newly synthesized HSPG cannot be incorporated and might then be the source for the HSPG bound to Aβ. One research group has suggested basotubules incorporated into the wall of the microvasculature. According to these authors, the core of these basotubules consists of a long, tubelike assembly of pentameric SAP molecules which are wrapped up helically with a band of CSPG. A diameter of about 3–5 nm was reported for this complex. It is covered by a thicker surface layer of HSPG, on

top of which the Aβ protein binds.[135,136] These findings require further confirmation. In fact, SAP is present in all types of amyloid, irrespective of the underlying protein. SAP is a plasma protein (8–55 mg L^{-1}) that originates in the liver, although many cells including neurons are able to synthesize small amounts.[137] It belongs to the family of pentraxins and circulates in the blood as a pentamer consisting of 5 identical SAP units of 204 amino acids each. It was shown in vitro to bind to fibrillar Aβ (not to monomers), and the coating of amyloid fibrils by SAP protects them from digestion by proteases.[138,139] SAP is unusually enriched in the basement membranes in brains of AD patients. Not all the SAP leaving the basement membrane may incorporate into amyloid, and it is possible that parts penetrate deep into the cortical tissue.[140] There it can not only stabilize amyloid fibers or activate the complement, but may even cause direct damage if taken up by neurons. Marked toxicity of SAP to neurons was reported in vivo as well as in cell cultures.[141,142]

2.7.3. Deposition of Proteins and Metal Ions

Several additional proteins have been identified in the senile plaques, among them cytokines, immunoglobulins, apolipoprotein E, α1-antichymotrypsin, ubiquitin, cathepsins, and choline esterases.[143,144] Ferritin is present in neuritic processes within the plaque, where it complexes the Fe^{3+} found at very high millimolar concentrations.[145] Fe^{3+} does not seem to interact directly with the Aβ in the plaque and it also does not co-purify with Aβ extracted from plaques. Cu^{2+} and Zn^{2+} are highly concentrated within the plaque deposits and reach concentrations of 400 μM and 1 mM, respectively.[90] More Aβ can be extracted from plaque material in the presence of chelators for Cu^{2+} and Zn^{2+}, thus these ions seem to be involved in the deposition of Aβ. As with many long-lived proteins, posttranslational modifications of Aβ and other plaque components have been described. Most prominent are tyrosine cross-linked dimeric and trimeric Aβ species as well as racemized and pyroglutamated forms.

About 50% of the Aβ present in plaques is truncated at the N terminus, starting at position 3 with pyroglutamate instead of glutamate [AβN3(pE)].[146,147] The soluble Aβ fraction extracted from brains of patients with sporadic AD was found to be composed mainly of the truncated 3-pyroglutamate form (about 50% AβN3(pE)3-42, 35% Aβ1-40/Aβ1-42, and 15% AβN11(pE)11-42). In contrast, the soluble Aβ fraction extracted from brains of cognitively normal elderly people with amyloid plaques present consisted predominantly of full-length forms (about 50% Aβ1-40/Aβ1-42, 30% AßN3(pE)3-42, 20% AβN11(pE)11-42). Thus, the soluble Aβ forms in AD patients differ from those found in normal aged people, with the truncated 3-pyroglutamated derivative regarded as potentially the most pathogenic.[148] Clearly, the amyloid β peptide is highly increased in the brains of AD patients compared to controls. The total Aβ40 and Aβ42/43 levels in normal brains were determined to be 2 and 2 pmol g^{-1} wet tissue compared to 661 and 2100 pmol g^{-1} wet tissue based on an average from the brains of 23 AD patients.[149]

3. The Amyloid Hypothesis

Since its first introduction in the early 1990s, the amyloid hypothesis has shaped to a large extent the current thinking about Alzheimer's disease. In its basic form, it states that the accumulation of amyloid β peptides in the CNS is the primary cause which initiates and drives a pathogenic cascade that eventually results in the complex, multilayered pathology and the clinical manifestation of the disease (Figure 8).[150,151] Inhibition and reversion of the accumulation of amyloid β peptide are thus appropriate choices for rationale approaches to therapy, and consequently many of the currently ongoing attempts in the pharmaceutical industry are based on this hypothesis. However, it should be stressed that the amyloid hypothesis is far from being universally accepted, and alternative explanations and ideas have repeatedly been proposed.[152–154] Its final test will be the clinical evaluation of new medicines which directly interfere with the formation and deposition of the Aβ peptide. Such tests are currently undergoing clinical trials.

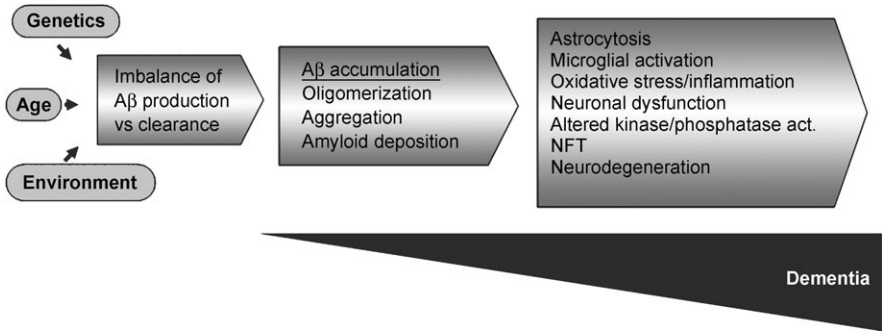

Figure 8. The amyloid cascade hypothesis of Alzheimer's disease.

The amyloid hypothesis is founded on several lines of evidence, the most important being: A) the disease pathology itself, B) the genetics of Alzheimer's disease, C) the cell biology of the amyloid β peptides, and D) more recently, the introduction of transgenic animal models which recapitulate some crucial aspects of the disease.

In terms of point A: Amyloid deposition in the CNS is invariably part of the AD pathology. Importantly, it affects primarily those regions of the brain which also show impairment in the cognitive and behavioral tests developed for diagnosing the disease and monitoring its progression. The concordance between clinical diagnosis of AD and its postmortem confirmation by amyloid pathology reaches 80% and more, thus underscoring the tight correlation between the presence of amyloid and disease.[155,156] The remaining 10 to 20% are usually misdiagnosed dementias of different origins. However, even when accepting the tight correlation between amyloid pathology and disease, a major criticism of the opponents of the amyloid hypothesis is the

lack of a quantitative correlation between amyloid deposits (measured as plaque load) in the CNS and disease severity.[157] There are documented cases where postmortem examination revealed severe amyloid pathology, while these individuals showed no signs of dementia at the time of their death.[158] New neuroimaging techniques which allow a non-invasive detection of amyloid deposition in living subjects have also revealed cases of significant amyloid deposits in cognitive normal elderly individuals.[14,159] Critics of the amyloid hypothesis interpret this as evidence that amyloid deposition in the CNS is an epiphenomenon or even a protective response of the organism, which would be weakened by any anti-amyloid therapy currently in development. For the proponents of the amyloid hypothesis, these individuals represent prodromal cases of AD and provide supportive evidence that amyloid accumulation is indeed the early and first initiator of the disease. Amyloid then drives the secondary pathologies, which may be more closely related to the graded severity of the clinical symptoms, such as the tau pathology in the Braak staging.[24] This interpretation is also supported by recent studies which showed, especially for the very early phases of the disease, a quantitative correlation between the total amounts of Aβ40 and Aβ42 in several cortical regions and the clinical dementia ratings (CDR), a measure for cognitive impairments.[160,161]

The amyloid hypothesis has been modified recently such that the macroscopic plaques by themselves are no longer considered to be the only or major mediators of disease symptoms and progression. This role is increasingly attributed to smaller, soluble aggregates (oligomers) of the Aβ peptide.[162,163] In a widely used transgenic mouse model of AD it was recently shown that the occurrence of Aβ-peptide oligomers correlates closely with cognitive impairments in behavioral tests.[164,165]

In terms of point B: A second major line of arguments supporting the amyloid hypothesis of AD is based on genetics. AD cases are classified as either "early onset AD" (EOAD) or "late onset AD" (LOAD), with the dividing line between these two categories being drawn at an age of onset of 65 years.[166,167] EOAD almost always has a strong genetic component. It occurs in affected families with an approximately 50% chance in every generation, which is typical for an autosomal-dominant inheritance with high penetrance, that is, a single copy of the corresponding gene carrying the risk mutation is enough to predispose the carrier for disease, and the likelihood that the carrier will indeed be affected by the disease is close to 100%. Although these EOAD cases occur very rarely (<2% of all AD cases), they have greatly contributed to our understanding of AD and strongly support the amyloid hypothesis, since all the genes which carry the EOAD mutations lead to an enhanced production of the Aβ peptide Aβ42 and thus to early amyloidosis of the brain. These genes code for the β-amyloid precursor protein (APP), which gives rise to the Aβ peptides after its proteolytic processing and the presenilin 1 and 2 genes (PSEN1 and PSEN2) which are part of the unique proteolytic machinery which processes APP.[166,168–171] LOAD, commonly called sporadic AD, occurs at a later age, and its pattern of inheritance is less pronounced. While it is clear that there are genetic factors which predispose for LOAD, the corresponding gene variants only carry an enhanced risk for disease—whether the carrier actually develops AD depends on additional genetic and environmental factors, which are much less understood. The best documented risk gene for LOAD is the ε4 variant of the ApoE gene, which is found in approximately 15% of the general population but close to 50% of all LOAD cases. There is evidence that the ApoE4 gene also enhances amyloid deposition, but its effect is less direct than that of the EOAD genes, and additional properties of ApoE4 likely contribute to the risk.[172–174] Whereas mutant APP and presenilins clearly cause enhanced and early deposition of amyloid through increased production of Aβ peptide, this is not the case for the ε4 allele of ApoE. Amyloid accumulation at a late age seems to be instead a consequence of decreased clearance of the Aβ peptide from the CNS, and thus a "downstream effect" of one or several unknown primary pathogenic disturbance(s).[175]

However, although EOAD and LOAD may differ profoundly in their primary pathogenic pathway and pathology,[176] they are largely similar in their final pathology and their clinical symptoms, and they are thus considered to be highly similar diseases. This similarity also argues strongly for a crucial pathogenic role of Aβ peptide in LOAD.[177]

In terms of point C: Despite these compelling arguments from pathology and genetics for a central role of the Aβ peptide in AD pathogenesis, the exact mechanism of how it exerts its toxic effects are not completely understood. As mentioned above, the amyloid plaques, which define the pathology of the disease, are most likely not the direct mediators of toxicity, and indeed are considered by some investigators to be "dumping sites" where the organism rids itself of a toxic agent. The lack of correlation between the amyloid plaque load and the disease severity also argues against a direct effect. The pathway from the original monomeric Aβ peptide to the macroscopic plaque proceeds through a number of intermediate steps where nonfibrillary and fibrillary aggregates of different sizes are formed. This process is largely driven by the long Aβ42 peptide, which is only a minor constituent of the total Aβ peptides produced (5–10%) but which has a much higher propensity for the formation of aggregates—both with itself and also with other proteins. Importantly, all the mutations in APP and presenilins that have been linked to EOAD either raise the amount of Aβ peptide produced above the normal level or increase the amount of Aβ42, which thus assumes a central role in AD pathology. Soluble Aβ-peptide oligomers are currently seen as the most important mediators of amyloid toxicity.[163]

In terms of point D: Research into AD has always suffered from the lack of suitable, naturally occurring animal models. Amyloid deposits have been observed in, for example, old dogs and monkeys. However, these animals are not ideal for research purposes, and the link between brain pathology and cognitive impairments such as learning and memory deficits is not straightforward. About 15 years ago transgenic mice were introduced which overexpress human genes linked to EOAD (APP and presenilin 1 or 2) and which reproduce within their short lifespan some central elements of AD pathology such as extensive amyloid plaque

deposition, toxic oligomer formation, and in some cases further pathologies such as neuroinflammation and formation of intracellular tau fibrillary tangles.[178,179] The mice also show age- and amyloid-dependent defects in learning and memory tests. However, for unknown reasons, they show little or no neuronal loss, and thus lack important aspects of the human disease.

The amyloid hypothesis incorporates central elements of AD pathology, genetics, and biochemistry. It certainly has gaps, and amyloid-independent, perhaps only age-related, changes in the CNS may contribute to the initiation and progression of the disease. However, no other current hypothesis provides a similar comprehensive framework to explain the various aspects of the disease and to guide new experiments, and it has thus remained the central theoretical framework in current AD research. Its final proof (or rejection) will also depend on the outcome of clinical trials with therapeutics which block the amyloid pathway or reverse brain amyloidosis.

3.1. APP and Aβ Peptides

The β-amyloid precursor protein (APP) is at the root of the pathway leading to amyloidosis (Figure 9).[180,181] APP is a type-1 membrane protein which is expressed in various tissues

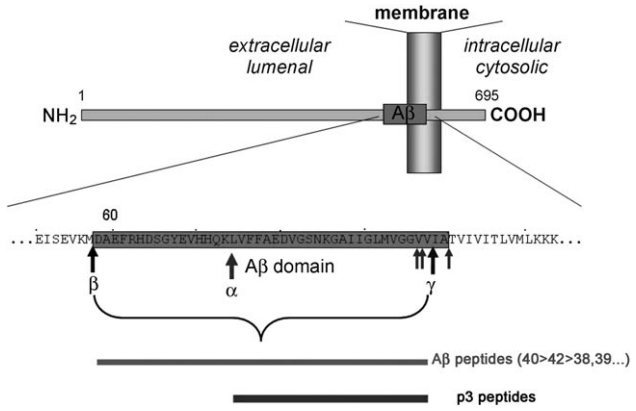

Figure 9. Proteolytic processing of the β-amyloid precursor protein (APP).

of the organism, both in the periphery and the CNS, but its expression levels are highest in the CNS compartment. APP occurs in splice variants of different length, but all can give rise to the Aβ peptide. The major part of the protein forms an extracellular domain followed by a transmembrane domain (TMD) and a cytoplasmic domain of approximately 50 amino acids length (AICD). Its true physiological function is still not completely understood; the extracellular domain has been proposed to serve as a neurotrophic factor, whereas the AICD may have a role as a regulator of gene transcription.[182–184] APP can be proteolytically processed by three proteases, namely α-, β-, and γ-secretase.[185] Cleavage by either α- or β-secretase occurs at its extracellular part close to the TMD and results in a shedding of the extracellular domain into the extracellular space. They both generate a short C-terminal fragment of 83 (CTFα) or 99 (CTFβ) amino acids length, which remains anchored in the membrane with its TMD. Both CTFs can be further processed by γ-secretase and this proteolytical cleavage results, in the case of CTFβ, in the formation of the Aβ peptides, while CTFα affords the shorter p3 peptides. The latter are rapidly degraded and play no pathological role.[186,187]

Since γ-secretase cuts its substrates at several neighboring positions, the so-called Aβ peptide is in reality a group of peptides which differ in length at their C terminus. The dominant species is the Aβ40 peptide, which normally constitutes 80 to 90% of all Aβ peptides. The second major species is the Aβ42 (ca. 5 to 10%); the rest is mostly made of shorter species including Aβ37 and Aβ38. As mentioned before, Aβ42 readily aggregates and thus forms the seed for larger oligomers and fibrils and finally for the macroscopic amyloid plaques.[188–192] The formation of aggregates is structurally accompanied by a transition from an α-helical conformation to β-sheet structures. Early aggregates and plaques may be formed exclusively of Aβ42, with Aβ40 adding in later when the concentration of amyloid aggregates reaches a threshold value. The shorter Aβ species, for example, Aβ38, are not prone to aggregation, and may actually interfere with this process.

Aβ-peptide monomers are not toxic at physiological concentration. They are produced constitutively by the organism as a by-product of APP proteolysis or, according to some authors, may even serve a physiological function. Different toxic aggregates can form under in vitro and in vivo conditions, but it is still far from clear which of these various aggregates are relevant for the disease and how the toxicity is brought about. Aβ oligomers have been shown to bind to cells, and their binding may show some specificity for certain neuronal cells and, more specifically, to some of their receptors, for example, the α-7-nicotinic receptor (discussed above), the p75 neurotrophin receptor, and the FPRL receptor.[52,193–195] Cells exposed to oligomers show activation of certain protein kinases such as c-jun N-terminal kinase, p38 MAP kinase, and cyclin-dependent kinase 5. As mentioned above, Aβ aggregates may form pores in cell membranes and thus disturb the ionic homoeostasis. Aβ peptides interact with the cell in a number of subtle ways, and toxicity may finally be the result of a number of such interactions. Nevertheless, understanding the really crucial interactions between Aβ and its cellular targets could provide valuable information for therapeutic intervention.

A potentially important new development was the recent demonstration that Aβ oligomers derived from cells over-expressing the human APP can disturb the induction of long-term potentiation (LTP) in cultured brain slices. This electrophysiological parameter of neuron activity is widely considered an in vitro analogue to the synaptic activation that occurs during real-life learning and memory formation (long-term potentiation is a measure of the connectivity of two neurons at their synapses). This connectivity, that is, the strength by which a signal is transmitted from the presynaptic neuron to the postsynaptic neuron, can be modulated by electric stimuli, thereby resulting in a long-lasting enhancement of the signal

strength. It is assumed that LTP reflects the changes in a neuronal circuit which are the basis for learning and the formation of short-term memory.[191,192,196]

Injection of such Aβ oligomers directly into rat brains impaired these animals in behavioral tests for learning. The appearance of a specific Aβ oligomeric species in the brain of transgenic mice which overexpress in their brain an EOAD mutant form of the human APP coincided with the onset of learning impairments, well in advance of the formation of larger amyloid deposits.[164] The amount of soluble, non-fibrillar Aβ peptides corresponded better to the severity of dementia than the total amount of β peptides in the brains of human AD patients.[160,163] Taken together, these observations support a role for soluble Aβ peptide in the learning and memory deficits that are typical for AD patients. The exact structure and composition of toxic Aβ species in vivo remain to be elucidated, as does the pathway leading to their generation and their relation to the amyloid plaques which form the "traditional" hallmark of the amyloid pathology. This relates also to the questions of whether plaques are indeed pathologically inert "dumping sides" for Aβ peptides which can not be cleared by other pathways or whether they exist in a dynamic equilibrium with soluble Aβ species and are thus a reservoir for the formation of toxic oligomers. This question clearly has important implications for therapeutic strategies. A very recent publication reported that Aβ-peptide dimers, which were isolated directly from the brains of AD patients, could inhibit LTP in slices from mouse brains and disrupted the memory of learned behavior when injected into the brains of rats.[197] Such toxic dimers could also be obtained from amyloid plaques.

An important, but yet unanswered question, is the true biological role of the APP protein. It is expressed at high levels in the CNS, but also occurs in other tissues. Its genetic ablation in mice leads to subtle changes in behavior, but is without gross consequences for viability and reproduction. This finding suggests that it has either a redundant function which can be supplied by other proteins or a repair/protection function which is not essential for the normal life of a laboratory mouse. The extracellular domain reportedly has a neurotrophic activity, yet how it exerts such a role is again unknown. The expression level can dramatically increase after traumatic brain injuries, thus making such injuries a significant risk factor for the later development of AD. While constitutive high expression of APP predisposes for early AD (shown for patients with Down's Syndrome who carry a third copy of the APP gene), it is not yet known if lower than normal expression can protect humans from the risk of Alzheimer's disease. Nevertheless, there is one compound in clinical development which reportedly reduces the expression level of APP and thus lowers the production of Aβ peptides.[198,199]

3.2. The β-Secretase

After a long search, β-secretase (BACE1, also named Asp2 or memapsin2) was finally identified almost simultaneously in 1999 by five research groups from industry and academia.[200–204] BACE1 belongs to the group of aspartyl proteases and it is, like APP, a type-1 membrane protein with a large extracellular domain that contains the catalytic aspartic acids, a transmembrane domain, and a short intracellular domain. (Type-1 membrane proteins are inserted into the cell membrane with one "transmembrane domain", a stretch of ca. 20–30 mostly hydrophobic amino acids which anchor the protein in the membrane. Type-1 membrane proteins are oriented such that their N terminus is in the extracellular space (including the lumen of intracellular vesicles) and their C terminus is located in the cytoplasma. Type-2 membrane proteins have the opposite polarity.) The highest expression has been documented in the CNS, but lower levels are also found in peripheral tissues. A related protease named BACE2 has also been described which is expressed mainly in the periphery and is not linked to AD.

It was shown that the preferred cleavage site of BACE1 within APP corresponds exactly to the N terminus of Aβ peptides (NH_2-D(672)AEF…). A mutated APP, which was found earlier in a Swedish family with an aggressive form of EOAD, has two amino acid exchanges at the BACE1 cleavage site (K670 > N; M671 > L) which turns this APPsw into a highly efficient BACE1 substrate. This leads to a much increased level of CTFβ and subsequently to higher levels of the Aβ peptide in the carriers of this mutation. Its acidic pH-optimum suggests that BACE is active primarily in the endosomal vesicles.

Genetic ablation of the BACE1 gene in mice completely abolishes production of the Aβ peptide, and thus proves that BACE1 has a unique proteolytic activity and is indispensable for the amyloidogenic processing of APP. These observations taken together—that is, belonging to a class of proteins that has been used successfully in the past for drug development (for example, for HIV-1 protease inhibitors) and having a unique activity—have made BACE1 a highly promising drug target for AD.[205]

BACE1 has, in addition to its signal peptide, a pro-domain (residues 22–45) which is cleaved off during maturation by a furin-type protease. It also has secondary modifications such as several intramolecular disulfide bridges and N-linked sugars, and can be phosphorylated at several sites.

There are other BACE1 substrates besides APP which could potentially limit the use of therapeutic BACE inhibitors, including the sialyl transferase St6Gal I, the P-selectin glycoprotein ligand, the APP-related proteins APLP1 and 2, and the growth-factor neuregulin 1.[100,206–208] Inhibition of neuregulin processing by inactivation of the BACE1 gene leads to a severe hypomyelination of peripheral and central nerves in a developing mouse, and it will be important to see if pharmacological inhibition of BACE1 in the adult organism can cause similar effects.[209]

Although inhibitors of aspartic acid proteases have been discovered and developed successfully in the past, for example, a number of inhibitors of the HIV-1 protease are in clinical use, the hunt for BACE1 inhibitors with suitable druglike properties has been slower than anticipated, and so far only one compound has entered early clinical trials (http://www.athenagen.com/index.php?/athenagen/press_releases/49/).

3.3. γ-Secretase

The CTFβ fragment of APP, which is generated by the cleavage reaction of BACE1, becomes a substrate for γ-secretase and is further cleaved into the Aβ peptides. These in turn are liberated into the extracellular space and the cytoplasmic domain AICD, which is released from its membrane anchor into the cytoplasma. γ-Secretase has a number of unusual features which set it clearly apart from other proteases: 1) it cleaves (hydrolyzes) its substrates within the lipophilic environment of the TMD, 2) it is itself firmly integrated into the cell membrane through multiple TMDs, and 3) it is a high-molecular-weight complex made of at least four different proteins, namely, PSEN1 or PSEN2, nicastrin (NCSTN), aph-1 (APH1 A, APH1B; anterior pharynx defective—a name derived from the phenotype of a C. elegans mutant), and pen-2 (PSENEN, presenilin enhancer 2) in a 1:1:1:1 ratio.[210–213] PSEN1/PSEN2 are believed to form the catalytic subunit of the γ-secretase complex. They are polytopic membrane proteins with eight or nine TMDs, and in their active state they are themselves proteolytically cleaved into an N- and C-terminal fragment, probably through autoproteolysis. The transmembrane domains 6 and 7 each contain an aspartic acid residue which very likely form the catalytic center of its proteolytic activity (D257 and D385 in the case of human PSEN1). The seventh TMD aspartate occurs within a conserved GXGD motif which is also found in a group of related proteases, the signal peptide peptidases, and which forms the active-site signature for this class of proteases.[214,215] Replacement of any of the aspartyl residues by another amino acid leads to complete loss of γ-secretase activity and also loss of autoproteolysis.[216]

The role of presenilin as the catalytic center of the complex is also supported by photoaffinity labeling with structurally different γ-secretase inhibitors which always targeted specifically the presenilin protein within the complex. The nicastrin protein may be important for the binding of substrates, since they recognize specifically truncated type-1 membrane proteins after shedding of their extracellular domain.[217] The role of the remaining two members is less clear; however, they are indispensable for the formation of the active enzyme complex.

Additional proteins, for example, TMP21, may be associated with the complex; however, reconstitution experiments in yeast have shown unequivocally that the above mentioned four partners are sufficient for the formation of the active complex.[218,219] How the hydrolytic cleavage of the peptide bonds can take place in the hydrophobic environment of the TMD is unclear, and it also remains to be shown if the two aspartyl residues serve a function similar to that in the other "typical" aspartyl proteases, namely, activation of a water molecule, which then leads to the nucleophilic attack at the peptide bond. The structures were recently elucidated of two different proteases which also cleave their respective substrates within their TMD, namely, a rhomboid protease (serine-type) and a site-2 protease (Zn-metallo type).[220,221] A hydrophilic cavity within the hydrophobic membrane environment existed in both structures which allowed water molecules to access the site of substrate cleavage.

As mentioned earlier, a number of mutations have been identified in PSEN1 and PSEN2 which cause the affected carriers to appear with EOAD, sometimes as early as in the third or fourth decade of life.[222] All these mutations increase the amount of Aβ42 at the expense of the otherwise predominant Aβ40 peptide, thereby raising its amount from the usual 5 to 10% of the total Aβ to up to 50%.[223] It is assumed that this shift occurs through subtle structural changes in the protein. However, in the absence of firm structural information on the complex, our understanding of these changes is still limited. In the case of PSEN1, more than 100 different EOAD mutations have been found which occur over almost the complete length of the protein, all resulting in the same change, namely, an increase in Aβ42 production. In this sense, they all represent "gain-of-function" mutations. A partial loss of overall proteolytic activity has been reported for at least some of these mutants. Whether this "loss-of-function" phenotype also contributes to their pathogenic role is currently not clear, but it may be relevant, for example, for the Notch receptors, which signal through a γ-secretase-dependent mechanism.[224,225]

It should be noted that, in addition to their proteolytic function in the γ-secretase complex, presenilins may have additional roles. It has been reported that PSEN1 interacts with β-catenin; it also regulates the wnt-signaling pathway and has an important role in intracellular calcium homeostasis. FAD PSEN1 reportedly increases the release of Ca^{2+} from endoplasmatic reticulum stores which could make neurons more vulnerable to excitatory stress.[226]

The cleavages which liberate the Aβ peptides take place in the middle of the APP transmembrane domain, and they occur with a rather low sequence specificity and produce a wide spectrum of Aβ peptides. Additionally, these γ cleavages are preceded by (or concommitent with) a proteolytic cut at the border between the TMD and the cytoplasm which releases the cytoplasmic domain of the APP. This prior cleavage is called ε cleavage; however, it is important to stress that ε cleavage and all γ cleavages are carried out by γ-secretase itself, which thus shows it has a remarkably "fuzzy" activity pattern. A similar cleavage pattern was shown for other γ-secretase substrates such as Notch 1 and CD44, and appears to be a general feature of γ-secretase proteolysis.[213,227] This picture is further complicated by studies with certain inhibitors of γ-secretase which showed that in the presence of an inhibitor some Aβ species were suppressed while other longer species, such as an Ab46, appeared.[228,229] Some inhibitors may in fact be "modulators" of γ-secretase specificity and this concept of modulation has been used in a second generation of inhibitors which are currently in development.[230]

While BACE1 preferentially localizes in the CNS and its range of substrates besides APP is still rather limited, the γ-secretase complex is found in most tissues of the organism, and the number of potential substrates is steadily increasing (Table 1). It is mandatory for all these substrates that their extracellular domain is cleaved off before they can be further processed by γ-secretase.

The best characterized of these substrates are the Notch receptors. Like APP, they are type-1 membrane proteins,

Table 1: Some substrates of the γ-secretase.

Protein	Function
βAPP	receptor (?), neurotrophin (?)
APLP 1 and 2	receptor (?)
Notch 1–4	receptor
Jagged, Delta	Notch ligands
p75 (NTR)	neurotrophin receptor
ErbB4	receptor tyrosine kinase
CD44	receptor
E- and N-cadherins	intercellular adhesion molecules
nectin 1α	intercellular adhesion molecule
LDL-receptor-related protein	cargo receptor

which shed their ectodomain upon binding of their ligands Jagged (JAG) or Delta (DLL) and thus become a γ-secretase substrate. Cleavage by the γ-secretase releases the Notch intracellular domain which travels to the nucleus and acts as a regulator of transcription. The γ-secretase-mediated signaling of Notch receptors plays an important role in, for example, maintenance of skin and gut epithelia, B- and T-cell development and other tissues which undergo continuous proliferation and differentiation during embryonal development, and in the adult organism. Reducing Notch signaling through inhibition of γ-secretase activity can severely affect the homeostasis of such tissues, and thus limits the suitability of γ-secretase as a drug target for AD.

3.4. APP Transport and α-Secretase Processing

APP is expressed in many tissues in addition to the CNS, but it is only in the CNS where a deposition of Aβ peptide in the form of plaques occurs. One reason is likely the co-occurrence of BACE1, which is also expressed highest in the CNS. In other tissues the cleavage of the APP extracellular domain is predominantly due to a third proteolytic activity called α-secretase. Cleavage by α-secretase occurs closer to the APP transmembrane domain and results in the previously described APP CTFα, a C-terminal APP fragment of 83 amino acids length. When CTFα is subsequently cleaved by γ-secretase it yields a peptide corresponding to the Aβ peptide, but which is 17 amino acids shorter at its N terminus. This so-called p3 peptide is pathologically innocent, it neither aggregates nor accumulates. It was shown that the α-secretase activity is actually provided by a group of ADAM proteases including the TNF-converting enzyme TACE.[231–234]

Like other membrane proteins, APP is synthesized at the endoplasmatic reticulum and then transferred to the Golgi apparatus and onward to the cell surface through the trans-Golgi network (TGN). From the cell surface, it can be re-internalized by endosomes. These endosomes can fuse with lysosomes, the cell organelles which carry out the terminal degradation of "used" cell material. Alternatively, the endosomes may eventually fuse with vesicles of the TGN and reenter the flow of traffic leading to the cell surface. The sorting of APP into these cellular transport systems is highly regulated, and seems to depend on the interaction of its cytoplasmic tail with different adaptor proteins. The re-internalization by endosomes is essential for its cleavage by β-secretase, since only in these organelles is the pH value sufficiently acidic to allow efficient activation of BACE (BACE has a pH-optimum of 4–4.5; in vitro it shows little activity at the (close to neutral) pH value found at the cell surface). Since γ-secretase is also found in endosomes, these organelles may be the principal compartment where Aβ petides are generated.[235]

The proportion of APP undergoing cleavage by α- or β-secretase is variable and probably depends first of all on the BACE activity in a given cell. However, it has also been shown that activation of certain GPCR-type receptors such as the M1 acetylcholin receptor or the 5-HT4 serotonin receptor can shift the APP processing to the α-secretase pathway, and thus reduce the BACE1-dependent production of Aβ petides. This may occur by modulation of proteinkinase C, since activators of PKC have similar effects. The activation of muscarinic receptors M1, in particular, has been actively pursued as a therapeutic approach to AD.[233,236,237]

3.5. Clearance of Aβ Peptide and Transport across the Blood–Brain Barrier

The CNS contains a continuously high steady-state level of Aβ peptide. However, this high level of Aβ peptide does not lead to the formation of amyloid aggregates or amyloid plaques in healthy individuals. Instead, freshly produced Aβ peptides are rapidly removed, and their half-life in a healthy brain is on the order of a few hours.[238,239] Thus, effective clearance mechanisms must exist.

The first line of clearance are brain proteases, which degrade the peptide. The list of such candidate proteases which can cleave Aβ peptide is long, but two of them are particularly strong candidates: neprilysin (MME, membrane metallo-endopeptidase or -enkephalinase) and the insulin-degrading enzyme (IDE, insulysin). As their name indicates, neprilysin reportedly plays a role in the degradation of enkephalins and other regulatory peptides in the CNS and the periphery, while IDE has a high activity for degrading insulin. Their importance for the degradation of Aβ peptide has been indicated by genetic experiments: the targeted inactivation of either gene in mice leads to a significant increase in the Aβ-peptide level. On the other hand, their transgenic over-expression in mice which also express a human APP (and develop a pronounced amyloidosis with age) reduces the Aβ-peptide level and greatly diminishes the age-dependent deposition of amyloid. The intracerebral infusion of a neprilyin inhibitor also leads to increased levels of the Aβ peptide. Additionally, the human IDE gene is contained in a larger locus on chromosome 10, which in genetic linkage studies was repeatedly shown to be associated with an increased risk for LOAD.

It is not yet clear if both proteases target the same species of Aβ peptides. Both prefer the monomeric species over aggregated forms. While IDE is preferentially found in the interior of the cell, the neprilysin is mainly located on its outside. Thus, their activities may be complementary and they

may target Aβ-peptide pools in different compartments.[175,240–242]

Proteolytic degradation is only one means by which the CNS rids itself of the harmful presence of Aβ peptides. A second major pathway seems to be the transporter-mediated efflux across the blood–brain barrier into the periphery. Of major importance for this pathway is the protein LRP1 (low-density lipoprotein receptor related protein). LRP1 is a so-called scavenger receptor which binds and transports a variety of proteins, among them the aforementioned apolipoprotein E (ApoE) and the α2-macroglobulin (A2M). Both of the latter proteins are known for binding Aβ peptides, and it is assumed that Aβ is primarily transported in this protein-bound state. However, more recent evidence suggests that Aβ can also directly bind to LRP without a mediator. Again, it is interesting to note that the importance of all the partners involved in this efflux system is also supported by genetic studies. ApoE4 is the best established genetic risk for LOAD, and the chromosomal loci for both LRP and A2M have been implicated in studies for LOAD risk genes.[243–245]

An additional efflux pathway for Aβ peptides may be provided by the ATP-binding transporter glycoprotein P (ABCB1). This plays a well-established role as a transporter of xenobiotics, including many known drugs, across the blood–brain barrier.[246]

The transport of Aβ peptides across the blood–brain barrier is not necessarily unidirectional from the CNS to the periphery. There is evidence that Aβ in the vascular system can be transferred across the blood–brain barrier into the CNS, and this transport is mediated by the RAGE receptors (receptor for advanced glycation end products).[245] RAGE is a cell-surface receptor which binds a multitude of ligands and is expressed on endothelial cells lining the blood vessels and also on neurons and microglia cells. Its binding affinity for soluble Aβ peptides is in the nanomolar range. Since its expression is increased by ligands, the elevation of Aβ-peptide levels in blood vessels of the brain could form a feedback loop which leads to an increased influx into the CNS. It remains, however, to be proven if or how much Aβ of peripheral origin contributes to the CNS amyloidosis: The expression of both BACE and βAPP is higher in CNS compared to peripheral tissues, and also the levels of Aβ peptides are much higher in the cerebrospinal fluid (CSF) than in plasma. Data from transgenic mice show that the expression of the APP transgene in the CNS is sufficient to cause early and severe amyloidosis. Nevertheless, inhibitors of the RAGE/Aβ-peptide interaction have entered clinical development.

4. The Discovery of Drugs for Alzheimer's Disease

The First Generation: Symptomatic Treatment

The hunt for drugs which ameliorate the symptoms of the disease or delay its progression (not even to mention a true cure) has been started, but is certainly not yet a success story. The first drugs which entered the market were the nootropics piracetam and aniracetam. Their development was based on behavior pharmacology, and they showed statistical improvement in cognitive tasks.[247] The drugs which entered the clinics in the 1990s are based on the early noted deficits in the acetylcholine neurotransmitter system and the loss of the cholinergic neurons. They are inhibitors of acetylcholine esterase and provide symptomatic treatment, addressing the cognitive impairments of the patients, but do not target the underlying pathology. Their therapeutic efficacy can be significant, but is mostly moderate and wears off after a certain treatment time. Their use is indicated for patients with mildly to moderately severe AD. Three compounds are currently on the market: donepezil, rivastigmine, and galantamine (for a review article see Ref. [248]).

Another approach was followed with memantine, which is a noncompetitive antagonist of the NMDA receptor and supposedly targets the neuron-damaging excitotoxic activities in the brain of AD patients. Whether it also provides lasting benefits or even amelioration of disease progression is still a matter of debate. In contrast to acetylcholine esterase inhibitors, memantine is approved for patients with moderate to severe disease.[249]

The Second Generation: Disease-Modifying Treatment

The second generation of substances currently in clinical trials is to a large extent based on the amyloid hypothesis and targets different steps in the amyloidogenic pathway which lead from the β-amyloid precursor protein to the disease-defining amyloid plaques in the brain. The drugs in development are based on various immunotherapeutic approaches: inhibitors and modulators of secretases, inhibitors of amyloid aggregation, and compounds which seek to increase the clearance of the Aβ peptides. In contrast to symptomatic treatment, which provides only transient improvement, it is hoped that disease-modifying treatments will significantly retard disease progression.

4.1. Immunotherapy for Alzheimer's Disease

The preclinical concept for the treatment of AD by immunotherapy originated from a study in APP transgenic mice which was published in 1999 by scientists at ELAN Pharmaceuticals.[250] When they immunized their PDAPP mice subcutaneously with human fibrillar Aβ42 in Freund's adjuvant, the animals developed high titers of anti-Aβ antibodies. Analysis of brain amyloidosis in these mice several months after vaccination revealed that the plaque load was dramatically reduced compared to PDAPP mice which were not vaccinated. This effect was shown both in young mice receiving vaccination before onset of amyloidosis but, more importantly, also in older animals which were first vaccinated after significant brain amyloid had already developed. This latter finding suggested that the vaccination of human AD patients already with existing amyloidosis would also lead to a therapeutically beneficial reduction of Aβ peptide in the brain. In addition to the reduction of the amyloid load, the vaccinated animals also showed less neuroinflam-

mation and less signs of synaptic loss. The results were reproduced in several other mouse models of AD with different forms of Aβ vaccine (for example, C-terminally truncated peptides) and different adjuvant or route of vaccination (for example, intranasal administration of the vaccine; for review articles see Refs. [251,252]).

Some of the transgenic mouse AD models showed significant impairments in learning and memory capacity, which developed in parallel to the formation and deposition of Aβ aggregates. Vaccination-mediated reduction of the Aβ deposits also led to improved performance in different learning tasks, which again suggests that that vaccination could be a promising avenue for AD therapy (and also gave additional support to the amyloid hypothesis, which holds that Aβ peptides are the ultimate culprits of the disease).[253,254]

Similar effects have been shown when, instead of active vaccination, the AD mice received passive vaccination with monoclonal antibodies specific for Aβ peptides. The antibodies were injected directly into the CNS, but were also active when applied to the periphery, despite the fact that only a very small amount of the total number of antibodies crosses the blood–brain barrier to the CNS. Similar to active vaccination, the anti-Aβ antibodies not only reduced the amount of amyloid in the brain, but also improved markers for synaptic loss and ameliorated the known impairments in spatial learning tasks.[255,256] Interestingly, the improvement of cognition could occur very rapidly after application of the antibody and before a significant reduction in the total amount of amyloid in the brain was observed. A possible explanation for this somewhat unexpected immediate effect is a rapid inactivation of soluble, synapto-toxic Aβ aggregates by binding to the antibody.[257]

Several mechanisms have been proposed that could account for the effects of anti-Aβ antibodies on the amyloid level in the brain, synaptic markers, and performance in behavioral tasks: 1) The binding of the antibody to Aβ peptides prevents their aggregation to oligomers and fibrils, and thus blocks the formation of neurotoxic species. The antibodies can disassemble preformed fibrills and the resulting monomeric peptides can then become subject to the normal clearance pathways. Under these conditions antibodies act like other direct aggregation inhibitors. 2) Antibody-bound Aβ becomes a target for Fc-receptor-mediated uptake into microglial cells, where it is subsequently degraded by lysosomal proteolysis. It was shown that antibodies applied to the periphery cross-over into the brain, bind to plaques, and attract microglia. In plaque-containing brain slices cultivated in vitro, anti-Aβ antibodies attracted macrophages which phagocytose the fibrillar amyloid. 3) While the previous mechanisms require uptake of the antibody into the CNS, an alternative "peripheral sink" hypothesis proposes that Aβ-binding antibodies in the periphery can effectively reduce the Aβ-peptide level in the CNS. This hypothesis is again based on experiments in transgenic APP mice with the monoclonal antibody m266, which binds with very high affinity (picomolar) to the middle domain of the Aβ peptide. Its intravenous injection leads to a rapid increase of the total plasma level of the Aβ peptide (up to 1000 times) in the absence of decreases in plaque-bound Aβ peptide in the CNS. The sequestering of the Aβ peptide by antibodies creates a disequilibrium, and subsequently a net efflux of the brain Aβ to the periphery. However, such an increase in the peripheral Aβ was not noted with other antibodies, and may be a mechanism specific to m266.[188,258]

In this context it is interesting to note that natural anti-Aβ antibodies have been found repeatedly in AD patients. However, it is unknown what triggers their formation and if they have any effect on the course of the disease.

The effectiveness of both active and passive vaccination against Aβ peptide in the mouse AD models has initiated several clinical trials in AD patients. ELAN Pharmaceuticals led the field with an active vaccination trial with aggregated Aβ42 peptide in mild-to-moderate AD patients. In a multi-dose phase I study in 80 patients, significant anti-Aβ antibody titers were induced in more than 50% of the subjects after 1 to 3 inoculations. No treatment-related adverse effects were noted. However, a phase II study had to be stopped shortly after its initiation as 6% of those receiving the Aβ vaccine developed an aseptic meningoencephalitis. Although clearly treatment-related, it did not correlate with the final anti-Aβ antibody titer as the most immediate read-out for vaccination efficacy.

The study participants have been followed even after discontinuation of the study because of the long duration of the immune response. These follow-ups include some post-mortem examinations of individuals who died from unrelated causes. Histological examination of relevant brain regions showed in all cases the absence or reduction of Aβ-positive plaques, fewer dystrophic neurites (compared to "historical" control examinations of brains from untreated patients), and activated microglia cells associated with amyloid deposits. There was no clear decrease in the number of neurons with tau-fibrillar tangles. An infiltration of T cells was noted in those cases of patients who had experienced meningoencephalitis. MRI scans after the first year of follow-up of patients with significant antibody titers showed an unexpected decrease in the brain volume, which exceeded the decreases seen in the patients that did not show an immune reaction, but which, however, stabilized later to normal decline. The relevance of this observation remains open to speculation. One clinical center reported a significant slower decline in cognitive factors for antibody responders, but this was not seen at other study sites.[259] There was a hint of a positive effect in responders in the Neuropsychological Test Battery (NTB) which was not seen in other measures of cognition (ADAS-coq, MMSE; for a review see Refs. [120,251,260]).

Although the risks for adverse effects by this active vaccination approach are clear, and the therapeutic efficacy is not yet proven, an antibody-based therapy for AD remains a promising avenue. Several clinical trials are currently in progress in regard to passive vaccination with human or humanized anti-Aβ antibodies directed against different epitopes of the Aβ peptide. Passive vaccination offers the benefit that the treatment can be stopped in the case of adverse effects and the titer of active antibody will wane off within a couple of weeks. Furthermore, antibodies with the desired specificity, affinity, and mechanism of action (for example, binding to the Fc receptor) can be obtained by

repeated cycles of optimization. One study with an anti-Aβ antibody has entered phase III of a clinical trial (Bapineuzumab of ELAN), and several are in earlier clinical phases (Gantemerumab of Roche).

4.2. Inhibitors of Secretases

While anti-Aβ antibodies target the amyloid cascade at the level of Aβ-peptide aggregation and accumulation, the inhibitors of secretases approach the amyloid cascade at its root, namely, production of Aβ peptides. This could be the "cleanest" approach which would remove all the monomeric Aβ peptides and subsequent oligomers and aggregates. The potential problem with secretase inhibitors is the physiological function of secretases. Both cleave a number of additional substrates and the inhibition of these other cleavages may limit the clinical utility of inhibitors. This limitation is quite apparent for γ-secretase inhibitors, but is also a potential threat for BACE inhibitors. An additional issue is the Aβ peptides themselves, which according to some authors are not just by-products of the complicated γ-secretase cleavage activity but serve some physiological role.[261]

4.2.1. Inhibition of γ-Secretase

Despite its structural complexity and the absence of any real structural information, γ-secretase has in the past been the more attractive target for inhibitor discovery, and consequently their clinical development is more advanced than that of BACE inhibitors. It is likely that most lead structures were discovered historically in cellular "black-box" screens for Aβ-peptide-lowering compounds, and that their inhibition of γ-secretase was elucidated in secondary assays which looked for inhibition of CTFβ processing, or in cell-free assays based on partially purified membrane preparations and recombinant CTFβ substrate. Nevertheless, a number of structurally diverse, highly potent γ-secretase inhibitors have been identified and described in the scientific literature and patents. Some compounds have entered clinical trials for AD. Only the more advanced compounds with clear in vivo activity will be discussed here; more information has been described in some recent extensive reviews.[262,263]

The first clear proof for in vivo efficacy of a γ-secretase inhibitor was obtained with the Elan/Eli Lilly compound DAPT (Figure 10) in the transgenic mouse PDAPP.[264] Oral application of the compound led to a rapid decrease of the Aβ level in brains, which became evident as early as one hour posttreatment. This study also proved for the first time that soluble, preaggregate Aβ peptides in the CNS are subject to a rapid turnover. Reduction in the Aβ level in the brain correlated with the drug level in the brain, and an EC_{50} value of about 100 mg kg^{-1} was determined. The decrease correlated with the relative accumulation of the reaction substrate APP-CTFs. The in vivo activity of DAPT was subsequently confirmed in another transgenic mouse model, the Tg2576 mouse.[265] This study also showed that changes in the level of Aβ in the plasma and CSF mirrored the changes in the Aβ level in the brain, but only in young, preamyloid mice. The immediate changes in the Aβ levels in the plasma and CSF were still observed in older animals which already had insoluble Aβ aggregates in the brain, but the total level of Aβ in the brain stayed nearly constant because of the presence of an excess of slow-turnover Aβ aggregates.

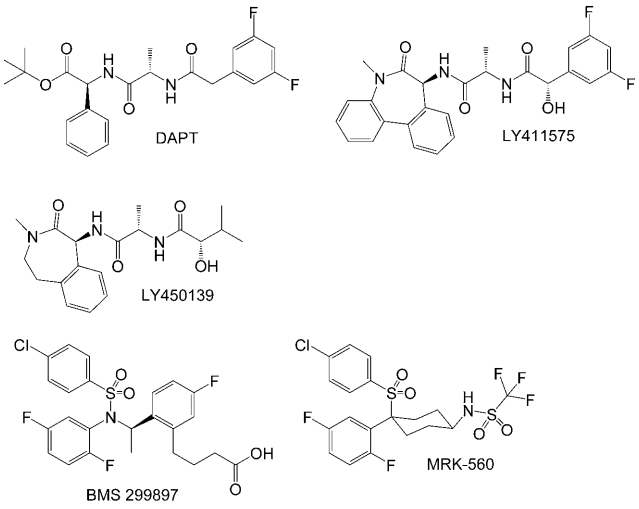

Figure 10. Selected γ-secretase inhibitors.

A compound later described by Eli Lilly, LY411575 (Figure 10), has subsequently been widely used for in vivo studies on γ-secretase inhibition. This compound is active in the low- to sub-nanomolar range in cellular assays, and in transgenic mice it reduced the Aβ level in the brain at doses as low as 0.3 mg kg^{-1}. The reduction of Aβ in the brain was again observed after a single application, but became greater after subchronic treatment for several days. As in the case of DAPT, the reduction of Aβ peptides was greatest in plasma, followed by CSF and brain. There was a clear trend towards recovery of the Aβ concentration in all the compartments measured within 24 h of treatment. The immediate and transient changes in the plasma and CSF Aβ levels suggested their use as biomarkers for assessing the efficacy of compounds in clinical trials. In addition to its activity in the transgenic mouse model, LY411575 also showed activity in a nontransgenic rat model.[266] This study also demonstrated a robust correlation between changes of Aβ40 levels in the CSF and brain.

Eli Lilly has finished a phase I and phase II study with LY-450139 (Figure 10), a compound related to LY411575 but almost two orders of magnitude less potent in cellular assays. In a study with nontransgenic guinea pigs, the compound showed a dose-dependent, transient decrease in the level of Aβ peptide in the plasma, CSF, and brain.[267] A transient increase in the plasma Aβ level was observed at low concentrations. This latter effect was also reported in clinical studies, where a transient increase in the Aβ levels in plasma above the baseline level was observed.[268–270] The start of a phase III study with this compound has recently been announced.

An active in vivo compound from a different structural class has been reported by a group from Bristol–Myers Squibb. BMS-299897 (Figure 10) also showed a time- and

dose-dependent decrease of Aβ in the plasma, CSF, and brain in young Tg2576 mice, with a good correlation between the changes in the CSF and brain. As noted before for LY411575, the total amount of Aβ in the brain did not change in older mice with amyloid plaques after acute treatment.[271] In contrast to the aforementioned compounds, BMS-299897 reportedly showed a 15-fold preference for an APP substrate over a Notch substrate in cellular assays. No Notch-related adverse effects were observed in a two-week study at doses sufficient to reduce the amount of Aβ in the brain. This was an important observation, since previous animal studies with compound LY411575 had shown that such adverse effects are a serious and potential dose-limiting problem with γ-secretase inhibitors.

Mice treated for two weeks with LY411575 at 10 mg kg^{-1} per day showed profound thymic atrophy and inhibition of splenic Marginal Zone B cells. Both effects were previously noted after genetic inhibition of the Notch signaling pathway.[272–274] Also noted was a hyperplasia of the goblet cells in the intestinal epithelia at the expense of enterocytes, an effect also reproduced in rats after subchronic treatment.[275] Similar findings were reported by other authors with different γ-secretase inhibitors.[276]

A potent and selective γ-secretase inhibitor (MRK-560) was recently reported by a group from Merck (Figure 10).[277–279] Treatment of Tg2576 for three months reduced the Aβ peptide and amyloid burden in the brain without any signs of Notch inhibition in the gut, spleen, or thymus. The selectivity of BMS-299897 and MRK-560, as well as the reported in vivo absence of Notch effects, for LY450139 are intriguing, but their mechanistic basis remains to be elucidated.

4.2.2. *Modulation of γ-Secretase*

As an alternative to the "classic" inhibitor approach, modulators of γ-secretase have received wide-spread attention in the last few years. This concept originated from the chance observation that certain nonsteroidal anti-inflammatory drugs (NSAIDs; ibuprofen, flurbiprofen, indomethacin, sulindac sulfide) altered the pattern of Aβ peptides produced in cellular assays: the level of Aβ42 decreased, Aβ40 remained unchanged, while a shorter species, Aβ38, increased. The cleavage at the ε site, which releases the intracellular domain, was not affected. This is evidently an almost ideal outcome, since Aβ42 is the "bad guy" among the Aβ peptides which drives the formation of cell-toxic aggregates and deposits, whereas shorter forms such as Aβ38 are considered to be "innocent" in this context. Sparing the inhibition of the ε site allows continued activity of, for example, the Notch pathway, and thus avoids complete inhibition of γ-secretase.

It has meanwhile become accepted that the NSAIDs indeed have a direct effect on the γ-secretase which is unrelated to their inhibition of COX-1 and -2 enzymes. The R form of flurbiprofen, which lacks any COX-inhibitory activity, was also found in cell-free assays for γ-secretase activity.[280–282] Binding studies of NSAIDs to γ-secretase in enriched membrane fractions showed noncompetitive antagonism with transition-state inhibitors, thus suggesting binding to an allosteric site.[280] Binding studies with a fluorescently labeled γ-secretase complex suggested that presenilin is the target of NSAIDs.[283]

How their binding changes the specificity for the cleavage site is unknown, but models have been proposed based on a helical structure of the substrate in the γ-secretase complex.[263] This puts the Aβ40 cleavage site opposite to the Aβ42 and 38 sites, and a shift of the enzyme along the substrate axis would result in an increased cleavage of Aβ38 at the expense of Aβ42. However, an important element of uncertainty for such a model is the assumed helical conformation of the substrate, which may not reflect the structure of the substrate during the cleavage reaction. Interestingly, is was also shown that other structures such as, for example, selective COX2 inhibitors, can cause the opposite shift in cleavage specificity, that is, more Aβ42 and less Aβ38, which again shows the remarkable flexibility of the γ-secretase complex and options for its modulation.

It was noted before in epidemiological studies that prolonged usage of some NSAIDs reduced the risk of developing AD over the life-time, and is was speculated that this modulation of γ-secretase towards a decreased production of Aβ42 may underlie their protective effect. However, this is difficult to reconcile with the very low potency of NSAIDs as γ-secretase modulators (the EC_{50} is usually in the range of $>50\,\mu M$, far above their COX-inhibitory activity) and their poor permeation across the blood–brain barrier. Myriad Pharmaceuticals have entered a phase III study with R-flurbiprofen (flurizan). Since this compound is inactive towards COX targets and its conversion into the COX-active S form is very low in humans, it was assumed that even a very high dose of 800 mg twice daily would not result in adverse gastrointestinal toxicities, but result in therapeutically meaningful concentrations in the brain. However, the development of this drug was recently stopped because of a lack of efficacy. In the meantime γ-secretase modulators have been published which are structurally different from the NSAIDs but which are active in the sub-micromolar range.[284]

4.2.3. *Inhibition of β-Secretase*

The discovery of BACE1 inhibitors was kick-started with the purification of the protein, the cloning, and functional expression of its cDNA, followed shortly afterwards by the elucidation of the 3D structure of its catalytic domain—all benefits never enjoyed by the "γ-secretase community". Nevertheless, the discovery of potent and therapeutically useful inhibitors has been slower than initially anticipated, and at the time of writing this Review only one compound has entered the clinic (CTS-21166 of CoMentis). Potent peptidomimetic inhibitors based on scaffolds known from previous drug developments for aspartyl proteases (statines, norstatins, hydroxyethylenes, hydroxyethylamines) have been described in the first generation of BACE inhibitors (for a review see Ref. [205]). While these compounds showed nanomolar binding affinities in cell-free assays, they were often poorly active in cellular activity assays and unsuitable for in vivo experi-

ments. The difficulty in obtaining inhibitors which are active in cellular assays may explain in retrospect why earlier cell-based screens for Aβ-lowering compounds repeatedly yielded γ-secretase inhibitors, but failed to discover leads for BACE inhibitors.

A number of compounds derived from peptidomimetics with better pharmacological properties have since been described, and there are also some examples of nonpeptidic inhibitors of nanomolar potencies in cell-free and cellular assays (Figure 11; for a review see Ref. [285–287]). However, what is still lacking are published reports which demonstrate a robust activity in vivo after oral application in one of the models previously used for the development of γ-secretase inhibitors (such as the APP-transgenic mouse or the wild-type rat). Some of the compounds were active in vivo after intravenous application or when applied together with a Pgp inhibitor (P-glycoprotein; a common disadvantage of compounds with peptidic features is that they often are good Pgp substrates). This gives hope that true drug candidates are not too far away. These will be valuable tools for confirming the preclinical finding that the inhibition of BACE1 will indeed significantly reduce the Aβ level and amyloid deposition, and be free of major mechanism-based adverse effects.

4.3. Further Targets

In healthy and young people, Aβ peptides are rapidly cleared and not given the chance to accumulate to levels which cause the formation of stable and toxic aggregates. It is evident that a number of proteases can cleave Aβ peptides into smaller peptides that can no longer aggregate and are then subject to final degradation. However, it is less clear which of these proteases is the true physiological "executioner" of Aβ, or if several proteases are needed for this task, working in different compartments and attacking different Aβ species and aggregates. Monomeric Aβ is an easier target than large oligomers or even amyloid deposits. Substantial in vivo evidence, such as data from genetic ablation of the gene or from transgenic overexpression, is available for the insulin-degrading enzyme and for neprilysin to support their physiological role in Aβ clearance. While the first gene manipulation leads to an increase in the Aβ level in the CNS, the second can prevent or reduce the formation of amyloid even in mice which overexpress a human FAD-APP transgene.[240,241] Activation of these proteases could thus be a means for enhancing Aβ degradation. Allosteric activators could be found for IDE, whereas neprilysin activity seems to

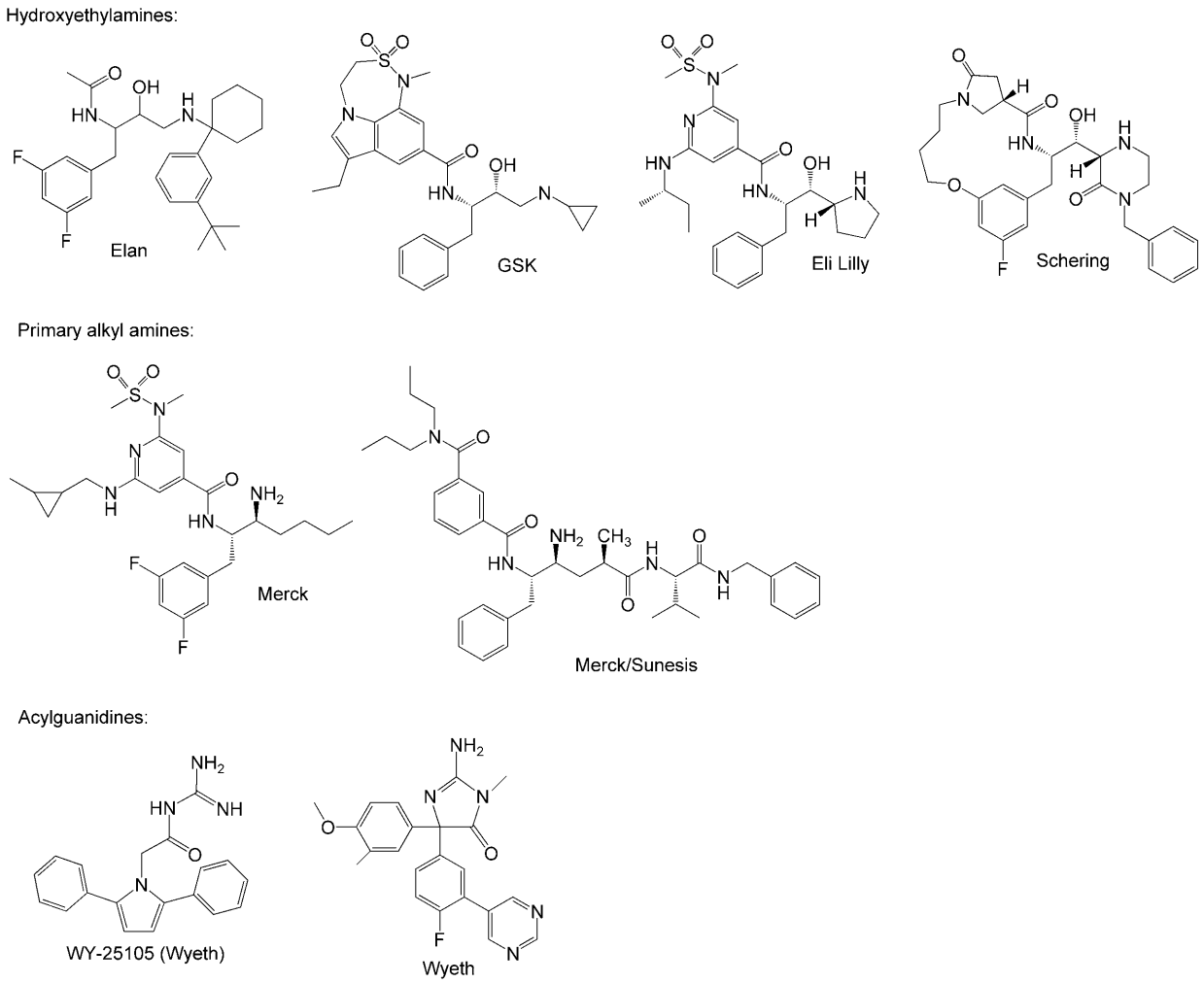

Figure 11. A selection of β-secretase inhibitors.

be under the control of the somatostatin pathway, which is accessible to pharmacological manipulation.[288–290] Such "degradation-enhancing" compounds are still in the exploratory stage, and have not entered the clinics.

An alternative pathway to prevent the stabilization of Aβ peptides and toxicity is through the use of aggregation inhibitors. A number of such inhibitors have been described over the years, some are plant-derived products such as PTI-777 (exebryl) of ProteoTech, while others are designed compounds such as alzhemed (tramiprosate; Figure 12) of

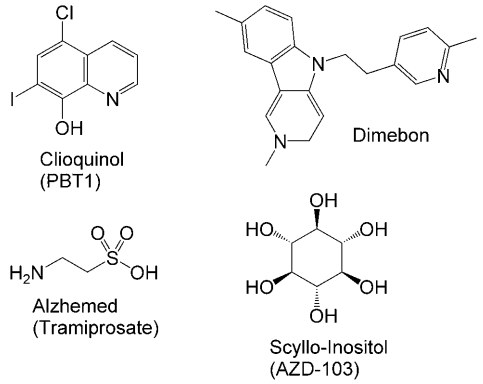

Figure 12. A selection of compounds which have been (clioquinol, alzhemed) or are currently in clinical trials (scyllo-inositol, dimebon).

Neurochem, which has in the meanwhile completed a phase III trial for AD—unfortunately without clear positive results. Interference with Aβ oligomerization and resulting toxicity was shown for scyllo-inositol (AZD-103 by Transition Therapeutics; Figure 12). This compound reportedly blocked the inhibitory effects of natural Aβ oligomers in the formation of LTP in slice cultures of mouse brain and blocked the learning impairments in rats which result when Aβ oligomers were injected in to their brain.[291] The effect appears to be specific, since the stereoisomer chiro-inositol offered no protection. ELAN recently announced the start of a phase II study with AZD-103 (ELND005) in patients with mild to moderate AD.

RAGE receptors in the endothelial cells lining blood vessels bind Aβ peptide and transport it across the blood–brain barrier into the CNS.[245] Transtech Pharma has developed small inhibitors of the RAGE/Aβ-peptide interaction (TTP488) and completed a phase IIa study with AD patients.

A reduction in the Aβ level in plasma as well as a normalization of plasma Zn^{2+} concentrations was reported in a double-blind, placebo-controlled, pilot phase II clinical trial with orally administered clioquinol (Figure 12) with 36 moderately severe AD patients for 36 weeks. A statistically significant slower rate of cognitive decline was also observed.[292] The compound had been shown in Tg2576 mice to increase Cu^{2+} and Zn^{2+} levels in the brain and to reduce cortical deposition of Aβ amyloid by 49%.[293] Clioquinol is presumed to form a complex with the Cu^{2+} and Zn^{2+} ions in the intestinal tract that can permeate the blood–brain barrier. This complex would mediate the transport of ions into cells, where they lead to a metal-dependent activation of matrix metalloproteases, which would degrade Aβ.[294]

4.4. Beyond Amyloid and Outlook

Even if the amyloid hypothesis posits that the accumulation of pathogenic Aβ peptides is the ultimate cause of the disease, it is evident that disease progression is driven by important co-pathologies, many of them appearing in the very early phases and even preceding large-scale amyloidosis. Tau pathologies play an important role, and their development offers additional targets for therapeutic intervention. In particular, the protein kinases, which cause the characteristic over-phosphorylation of tau protein as it occurs in the neurofibrillary tangles, are viewed as tractable drug targets. A major hurdle for this approach is the unsolved question as to which of the different phosphorylation sites in the tau protein are essential for the transformation of native tau to its pathologic form and which protein kinase accomplishes this phosphorylation step. The proline-directed protein kinases GSK3β and Cdk5 as well as the mitogen-activated protein kinases ERK, p38, and JNK have all been described as having important tau-phosphorylating activity. Some transgenic mouse models have become available which develop a tau pathology and which should become valuable tools for further evaluation of protein kinases as drug targets.

Type II diabetes is a frequent co-morbidity of AD, and epidemiological studies have suggested that diabetes is a risk factor for AD. The disease is commonly treated with thiazolidinedione (TZD) drugs, which activate the peroxisome-proliferator-activated receptor γ (PPARγ)—a nuclear receptor. Two of these drugs, rosiglitazone and pioglitazone, are on the market. A phase II study with rosiglitazone in patients with mild to moderate AD demonstrated significant improvement in cognition in a subgroup of those who did not have the ε4 allele of the ApoE gene. The mechanism of the drug is a matter of debate; the authors of the study suggested that rosiglitazone acts on mitochondria and increases their number and metabolic efficiency.[295,296] PPARγ agonists reportedly enhanced the clearance of Aβ peptide in cell-culture models.[297] Treatment of transgenic mice with pioglitazone reduced the expression of BACE1 in the brain.[298,299] A phase III study with rosiglitazone is in progress. However, it was also reported that TZDs have anti-inflammatory activities and suppress the production of inflammatory cytokines and reduce COX2 expression. Another compound hypothesized to stabilize mitochondrial function is dimebon. This compound improves cognition, memory, and behavior in patients with mild to moderate AD.[300]

Neuroinflammation, especially activated microglia, is a consistent feature of lesions associated with AD. Epidemiological studies have clearly shown that long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the risk of developing the disease, and accordingly a number of NSAIDs have been tried as therapeutics for the treatment of established disease. An early small study with indomethacin for the treatment of mild to moderate AD suggested a

protective effect on cognition. However, this finding was not confirmed in later larger studies with other NSAIDs or selective COX-2 inhibitors, and for the time being it has to be assumed that anti-inflammatory drugs can have preventive activity but lack therapeutic potential.[301]

A similar picture may hold true for statins. Epidemiological data show that lowering cholesterol by intake of statins reduces the risk of developing AD. Furthermore, studies in cell cultures and in transgenic mouse models have demonstrated that hypercholesteremia can increase the production of Aβ peptides and amyloidosis. However, clinical studies that show a clear therapeutic effect of statins in AD patients are still lacking.[302,303]

So what can we do while we wait for effective treatments to become available? The manipulation of two major risks, advancing age and genetic make-up, is still beyond our means. Population-based studies on aging, such as the Rotterdam study, suggest some life-style factors which may modify the risk for developing the disease, such as smoking (it increases the risk[304]) or moderate alcohol consumption (it decreases the risk).[305] The intake of antioxidants, such as vitamin C and E, either preventive or therapeutic, has received considerable attention because of the extensive oxidative pathology observed in the brain of AD patients, however, a proof of efficacy is missing.[306] Similarly, arguments have been proposed for polyphenolic compounds derived from plants, such as the green-tea constituents catechin and quercin, resveratrol in red wine, and curcumin in curry powder. A protective anti-amyloid effect was demonstrated for some of these compounds in the transgenic mouse model for AD.[307,308]

Physical exercise may be an important factor for attenuating the risk of Alzheimer's dementia, as was again demonstrated in the transgenic mouse model based on overexpression of the human APP. The typical memory and learning impairments observed in aged mice with extensive brain amyloidosis was significantly reduced when the animals were offered an enriched environment, that is, when their cage offered equipment for exercise, such as running wheels, and changing objects, such as colored pieces of plastic, used for play. Some of these studies even claimed that not only learning and memory was improved, but that the amyloid pathology was actually reduced, possibly through the induction of the Aβ-peptide-degrading enzyme neprilysin.[309,310]

Physical exercise was also demonstrated to increase BDNF (brain-derived neurotrophic factor) and its receptor TrkB (tyrosine kinase B receptor), CREB (pCREB, phosphorylated cAMP response-element-binding protein and CREB mRNA), IGF-1 (insulin-like growth factor-1), and synapsin-1, as well as also histone acetylation, in particular in the hippocampus.[311,312] In a mouse model of neurodegeneration (CK-p25 TG mouse), histone acetylation increased the sprouting of dendrites, generation of new synapses, and recovery of lost long-term memories.[313] Synapsin-1 connects synaptic vesicles to the actin cytoskeleton at the synapses. Circulating IGF-1 participates in the clearance of Aβ from the brain by modulating the function of the choroid plexus. Treatment of APP/PS2 mice with IGF-1 significantly ameliorated their AD-like symptoms.[314,315] The phosphorylation of the transcription factor CREB by MAP-K (mitogen-activated protein kinase) plays a key role in the formation of long-term memory where protein synthesis is involved.[316] The increase in BDNF is dependent on the NMDA receptor. The binding of CAMKII (calcium/calmodulin protein kinase II) to the NMDA receptor keeps CAMKII in an autonomously activated state when its initial activating Ca^{2+} stimulus has disappeared. The increase in these proteins through physical exercise contributes to the clearance of Aβ, promotes synaptic plasticity, and in this way improves cognitive processes.

While all these factors may be of little help for those where the disease has already struck, they may offer some incentives for those at risk—and this may well be everybody beyond a certain age—to minimize their personal risk. Nevertheless, it is evident that effective therapeutics are urgently needed, and it is hoped that anti-amyloid strategies will offer a significant step towards a causal therapy.

We thank our colleagues Manfred Brockhaus, Friedrich Metzger, and Hansruedi Loetscher at Hoffmann–La Roche for providing critical feedback on early drafts of the manuscript. We also thank Bernd Bohrmann for providing Figure 4.

Received: June 13, 2008
Published online: March 28, 2009

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