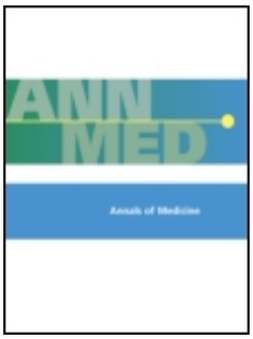

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REVIEW ARTICLE

Molecular genetics of Alzheimer's disease: An update

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Abstract
Alzheimer's disease (AD) is a complex disorder of the central nervous system (CNS). Molecular genetic research has provided a wealth of information regarding the genetic etiology of this devastating disease. Identification and functional characterization of autosomal dominant mutations in the amyloid precursor protein gene (*APP*) and the presenilin genes 1 and 2 (*PSEN1* and *PSEN2*) have contributed substantially to our understanding of the biological mechanisms leading towards CNS neurodegeneration in AD. Nonetheless, a large part of the genetic etiology remains unresolved, especially that of more common, sporadic forms of AD. While substantial efforts were invested in the identification of genetic risk factors underlying sporadic AD, using carefully designed genetic association studies in large patient-control groups, the only firmly established risk factor remains the ε4 allele of the apolipoprotein E gene (*APOE*). Nevertheless, one can expect that with the current availability of high-throughput genotyping platforms and dense maps of single-nucleotide polymorphisms (SNPs), large-scale genetic studies will eventually generate additional knowledge about the genetic risk profile for AD. This review provides an overview of the current understanding in the field of AD genetics, covering both the rare monogenic forms as well as recent developments in the search for novel AD susceptibility genes.

Key words: *Alzheimer's disease, causal genes, complex diseases, molecular genetics, neurodegeneration, susceptibility genes*

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder of the central nervous system (CNS) and is the most common dementia subtype in the elderly (50%–70% of demented patients). Clinically, AD is characterized by progressive deterioration of cognitive functions, ultimately leading to complete dependency and death. Most AD patients present with impairment of recent memory, but during disease progression other symptoms such as changes in personality and behavior become apparent. There is a substantial overlap in clinical symptoms between AD and other CNS degenerative brain diseases involving dementia (e.g. frontotemporal lobar degeneration (FTLD) and Creutzfeldt-Jakob disease). Therefore, a definite diagnosis of AD is obtained only by pathological examination of the autopsied brain. In addition to severe neuronal loss, AD brains show two distinct pathological lesions: extracellular plaques composed of aggregated amyloid β (Aβ) peptides, and intracellular neurofibrillary tangles consisting of filaments of hyperphosphorylated protein tau (1).

AD has a complex etiology involving the interplay of both genetic and environmental factors (2). Two major risk factors are increased age and a positive family history of dementia. An European population-based study calculated an AD prevalence of 5% in the age group 65 years and older, which increased to 22% among those aged 95 years and older (3). Although the majority of patients develop clinical symptoms at later age (>65 years; senile or late-onset AD), 1%–2% of patients have an earlier disease onset (presenile or early-onset AD). Independently of onset age, AD brain pathology is

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Key messages

- Monogenic forms have contributed significantly to the current understanding of the pathobiology of Alzheimer's disease (AD).
- The amyloid precursor protein is a key protein in developing novel therapies for AD.
- Novel genetic designs will provide clues for defining risk profiles for sporadic AD.

identical, though in young patients the disease progresses more rapidly, and brain pathology is more pronounced. Twin studies identified a substantial genetic component in AD, with an estimated heritability of up to 80% (4). Molecular genetic studies performed in the last 20 years have produced important new genetic data, though predominantly in studies of rare monogenic forms of early-onset AD. Highly penetrant mutations were identified in three genes, the amyloid precursor protein gene (*APP*) and the presenilin genes 1 and 2 (*PSEN1* and *PSEN2*) (2). Also, a fourth gene, the apolipoprotein E gene (*APOE*), a major risk gene was identified in late-onset AD families (5). Except for *APOE,* not much is known about other risk genes contributing to late-onset AD, though several such risk genes must exist with effect sizes equal to or even larger than the *APOE* ε4 allele (6,7).

Monogenic forms of AD

Amyloid precursor protein gene (APP)

APP, located on chromosome 21, was the first gene identified in autosomal dominant early-onset AD families (8). Instrumental here were the observation of AD brain pathology in Down's syndrome (DS) or trisomy 21 patients (9), the isolation and sequencing of the senile plaque Aβ peptide (10), and the mapping of its precursor gene *APP* (11) near the AD-linked chromosomal region on chromosome 21 (12,13). Mutation analysis of *APP* in AD was stimulated by the observation of linkage with *APP* (14) and the identification of a missense mutation in its Aβ sequence in hereditary cerebral hemorrhages with amyloidosis-Dutch type (HCHWA-D) (Dutch E693Q mutation, numbering according to the 770 amino acids isoform) (15). HCHWA-D is a rare autosomal dominant disorder characterized by recurrent cerebral hemorrhages due to extensive Aβ congophilic amyloid angiopathy (CAA) affecting small cerebral blood vessel walls (16). The first AD mutation in *APP* was identified near the C-terminal site of the Aβ sequence and was nicknamed London *APP* mutation (V717I) (8). A double-mutation was identified in AD near the N-terminus of the Aβ sequence, the Swedish mutation *APP* KM670/671NL (17). We identified a second mutation within the Aβ sequence, the Flemish *APP* mutation (A692G) located adjacent to the Dutch *APP* mutation, which associated AD and CAA (18).

Since then, 21 different missense mutations have been identified in *APP* in 68 families (AD Mutation Database; http://www.molgen.ua.ac.be/ADMutations/). Nonetheless *APP* mutations explain less than 1% of early-onset AD families (19).

Amyloid precursor protein. APP consists of 18 exons within a genomic region of 290 kb with part of exons 16 and 17 coding for the Aβ peptide (20) (Figure 1A). Alternative splicing produces three major isoforms (Figure 1B), of which APP695 is predominantly expressed in the brain, especially in neurons. APP is a single-pass type I transmembrane glycoprotein with a small cytosolic C-terminal domain and a large luminal N-terminus (11).

Contrary to what was initially assumed, the Aβ peptide is also formed under normal physiological conditions (21,22). APP is proteolyzed by α-, β-, and γ-secretases following one of two mutually exclusive ways: the constitutive or non-amyloidogenic pathway which precludes the formation of intact Aβ peptides and is the major APP processing pathway in most cell types (23); or alternatively the amyloidogenic pathway which is particularly enriched in neurons and gives rise to Aβ peptides (21,22,24–26) (Figure 1C). In the latter, APP is cleaved first by β-secretase at N-terminal position 1 of the Aβ sequence (β-site) (27). Of note is that β-secretase also cleaves APP at the adjacent β′-site, producing $A\beta_{11–40}$ and $A\beta_{11–42}$ (27). Next γ-secretase cuts the membrane-bound C-terminal fragment to produce Aβ peptides (28,29), a heterogeneous mixture of Aβ peptides with varying C-terminal lengths of 39–43 amino acids. Under physiological conditions two major Aβ species are present. The major form is $A\beta_{40}$, ending at position 40 (90%), whereas about 10% end at position 42, $A\beta_{42}$. It is $A\beta_{42}$ that is predominantly present in amyloid plaques in AD brains (30). Studies have shown that this more hydrophobic $A\beta_{42}$ has a higher aggregation propensity compared to $A\beta_{40}$.

In addition to the regular γ-sites, there are two other cleavage sites located downstream of residue 42, e.g. the γ-like cleavage site or ε-cleavage site

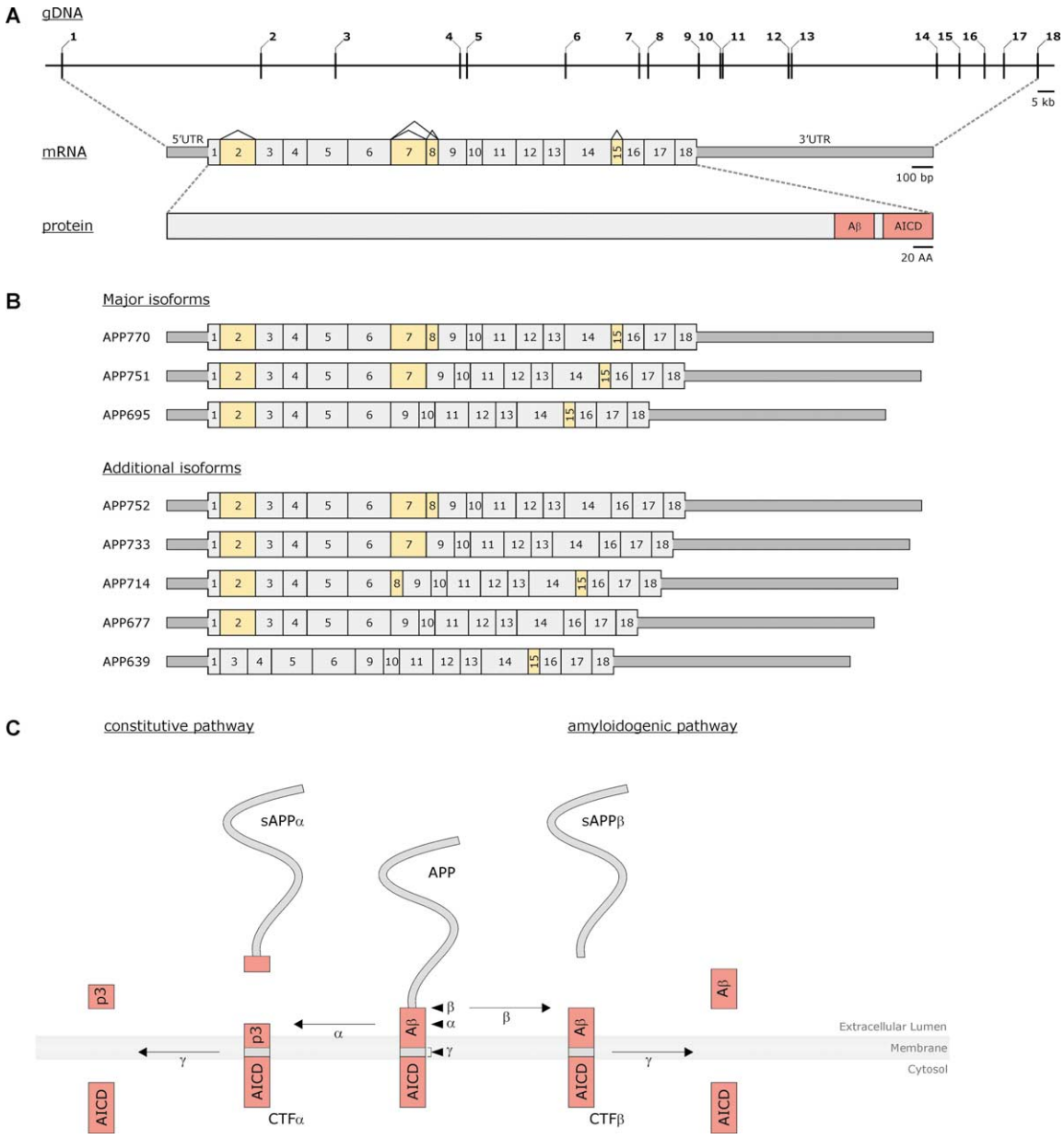

Figure 1. A: Schematic presentation of amyloid precursor protein (*APP*) at the genomic, transcript, and protein level. Numbers (genomic and transcript) indicate exons and yellow-colored exons (transcript) designate alternatively spliced exons. At the transcript level, untranslated regions (UTR) are represented as dark gray boxes; coding regions are shown in light gray. Pink boxes indicate the portion of the protein from which the Aβ peptide and the *APP* intracellular domain (AICD) are formed. B: Different *APP* isoforms, produced by means of alternative splicing, that have been isolated from human tissue. Each transcript is named according to the protein that can be translated from the transcript, i.e. APP770 encodes an isoform containing 770 amino acids. C: Schematic overview of the two major APP processing pathways, i.e. the constitutive and the amyloidogenic pathway. Arrowheads indicate the respective cleavage sites (α-, β-, and γ-site); arrows indicate the cleavage event by the respective proteases (α-, β-, and γ-secretase).

(31,32) and the ζ-cleavage site (33) (Figure 2). Proteolysis by γ-secretase at these sites generates longer Aβ peptides, e.g. $Aβ_{49}$ and $Aβ_{48}$ for ε-cleavage and $Aβ_{46}$ and $Aβ_{45}$ for ζ-cleavage, and studies showed that the γ-secretase cleavages of APP C-terminal fragments are likely sequential events. The first cut by γ-secretase occurs at the ε-site releasing the APP intracellular domain (AICD) and producing $Aβ_{49}$ and $Aβ_{48}$. These Aβ peptides stay in the enzyme's active site and are subsequently cleaved at the ζ-site ($Aβ_{46}$/$Aβ_{45}$) and the γ-site, ultimately releasing $Aβ_{40}$ and $Aβ_{42}$ (34–38) (Figure 2). In this manner APP is cleaved every three amino acids which fits well into an α-helical model of APP

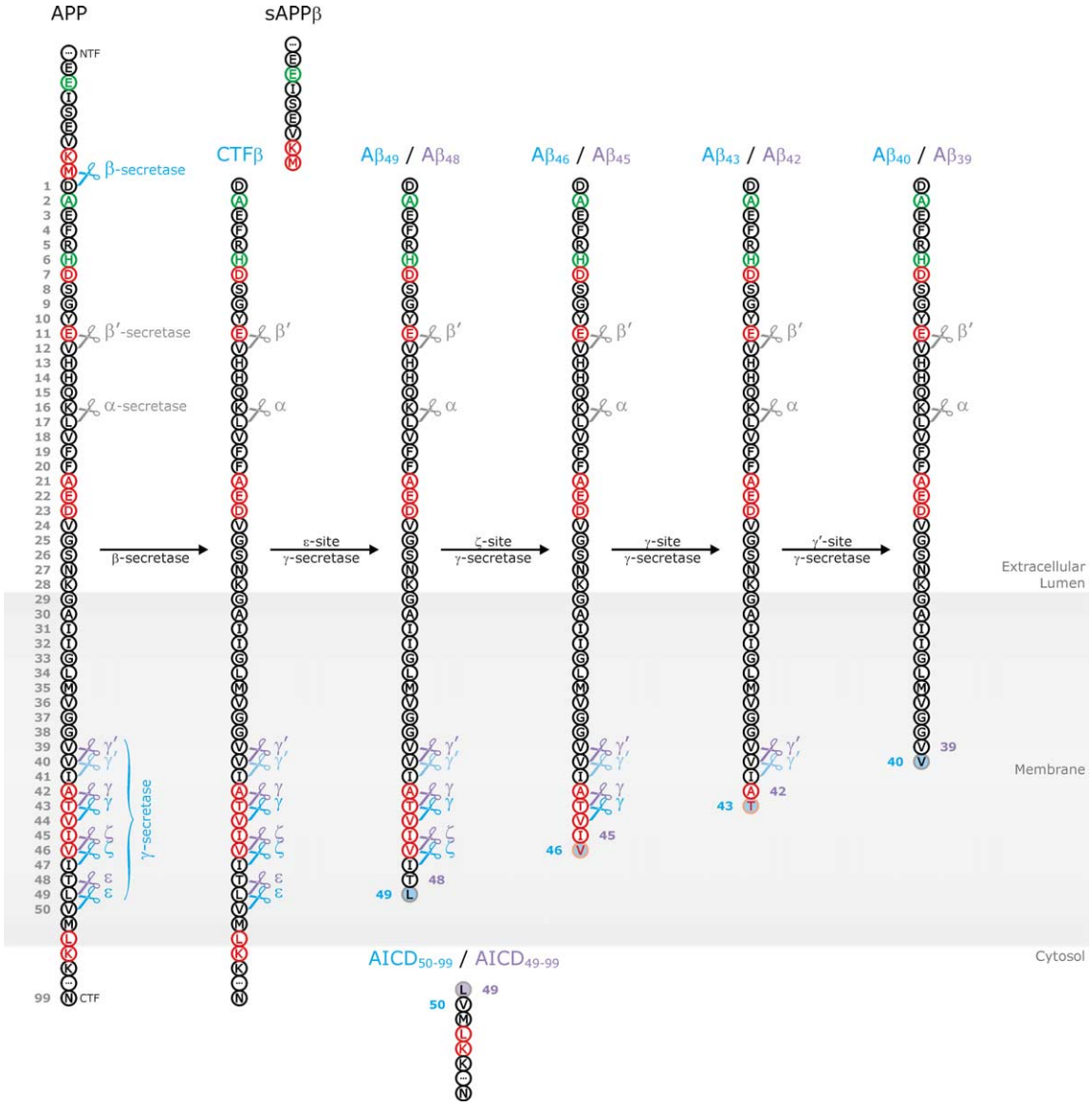

Figure 2. Schematical illustration of sequential APPcleavages producing amyloid Aβ peptide. In a first cleavage event, amyloid precursor protein (*APP*) is cut at the Aβ N-terminus (β-secretase action) releasing its large soluble ectodomain (sAPPβ) in the extracellular space. Subsequently, the membrane-bound C-terminal stub (CTFβ) is cleaved downstream of the C-terminal end of Aβ by the γ-secretase complex. The four possible cleavage sites (γ-, γ′-, ζ-, and ε-sites) are indicated by scissors. The first C-terminal cut occurs at the ε-site (ε-site γ-secretase activity) and releases the APP intracellular domain (AICD) into the cytosol. The majority (~90%) of CTFβ is cleaved between amino acids 49 and 50 (blue scissors) producing $A\beta_{49}$; however, an alternative cleavage occurs between amino acids 48 and 49 (purple scissors) leading to the formation of $A\beta_{48}$. Both membrane-bound peptides serve as substrates for subsequent cleavage events (ζ-site, γ- and γ′-site γ-secretase cleavage), cutting the peptides every three amino acids, ultimately producing $A\beta_{40}$ and $A\beta_{42}$ (or $A\beta_{39}$ in case $A\beta_{42}$ is not yet released into the extracellular lumen before the last cleavage event takes place). Amino acid positions (gray numbers) are numbered starting from the N-terminus of Aβ. Mutated amino acid positions are indicated in red, non-pathogenic variants are shown in green. Major cleavage events, giving rise to the production of $A\beta_{40}$, are indicated by blue scissors. Minor cleavage events, producing $A\beta_{42}$, are shown in purple.

processing, with $A\beta_{40}$ and $A\beta_{42}$ cleavages on opposite sites of the transmembrane domain (37).

APP missense mutations. All pathogenic APP missense mutations are located in or near the Aβ sequence and in the vicinity of protease cleavage sites, exerting their pathogenic effect by influencing APP proteolytic processing (for review see (39)) (Table I; Figure 2). The Swedish APP mutation KM670/671NL increased proteolysis at the β-site, thereby elevating total Aβ levels (40,41). Very recently we identified a novel mutation, E682K, in a familial early-onset probable AD patient (onset age 47 years), altering the highly conserved position 11

of the alternate β′-site (Van Broeckhoven et al. unpublished data). At this point, we have not yet completed the genetic studies, neither have we examined the effect of this mutation on Aβ processing. However, a study examining the selectivity of β-secretase cleavage of APP showed that blocking the β′-site by introducing the artificial double-mutation Y681K/E682K shifted β-secretase cleavage entirely to the β-site (42). Therefore, we can assume that also E682K affects β′-site processing of APP and potentially exerts its pathogenic effect by increasing $A\beta_{1-40}$ and $A\beta_{1-42}$ levels as in the Swedish APP mutation. On the other hand, we cannot exclude that this mutation renders Aβ a better substrate for β′-site cleavage, increasing the proportion of N-truncated peptides.

Mutations at the γ-site affect the positions where APP C-terminal fragments are cleaved, e.g. position 40 or 42 of the Aβ peptide, with different mutations exerting distinct effects on γ-secretase activity (43). Although results differ among studies and cell types used, the overall effect of these C-terminal mutations is an increase in the relative amount of fibrillogenic $A\beta_{42}$ (44,45). Therefore, the best predictor for pathogenicity of an *APP* mutation in an *in vitro* cell assay is the $A\beta_{42}/A\beta_{40}$ ratio. The two most C-terminal *APP* mutations, Australian L723P and Belgian K724N, also increased the $A\beta_{42}/A\beta_{40}$ ratio (39,46). Since ε- and ζ-cleavages generate intermediates for further γ-processing, it was predicted that alterations in APP processing at these sites would also result in a relative increase of $A\beta_{42}$ and the $A\beta_{42}/A\beta_{40}$ ratio. This was confirmed for several C-terminal *APP* mutations (T714I, V717F, and L723P) (35,37,47). For these mutations elevated levels of $AICD_{49-99}$ (numbering according to Aβ peptide) and its N-terminal counterpart $A\beta_{48}$ were formed, often combined with a concomitant decrease in the physiologically predominant species ($AICD_{50-99}$ and $A\beta_{49}$) (35,37,47). Interestingly, the latest model for APP γ-secretase processing proposed that $A\beta_{48}$ gives rise to the production of $A\beta_{42}$ while $A\beta_{49}$ is converted to $A\beta_{40}$, explaining how differential cleavage at the more C-terminally located cleavage sites would result in an increased $A\beta_{42}/A\beta_{40}$ ratio (36–38). Since these differences in ε-cleavages also generate different AICD fragments, it is plausible that mutations might affect AICD function. The normal function of AICD has not been unambiguously determined yet, but it is presumed to function in neurogenesis (48), transcriptional regulation of target genes (49), and signal transduction (49). Affecting either one or all of these processes could contribute to the mutation's pathogenicity.

Mutations at the α-secretase site interfere with processing of APP at its internal Aβ peptide cleavage site (α-site) (50). This was shown for the Flemish APP mutation (A692G) (18), which decreased α-cleavage (51,52) resulting in more substrate for the amyloidogenic processing into Aβ peptide. However, most α-site mutations do not result in increased Aβ secretion, in fact they are associated with reduced levels of Aβ peptides (52–54), suggesting a different mechanistic action. Since α-site mutations are

Table I. Overview of the effects of different types of mutations in Alzheimer's disease genes on the processing of amyloid precursor protein (*APP*) and the production of Aβ.

Gene	Type of mutations	Location of mutations	Effect of mutations
APP	missense	N-terminal of Aβ peptide (β-secretase site)	enhanced β-secretase cleavage → increased Aβ production
APP	missense	Aβ peptide encoding region	alteration of the Aβ sequence and its properties → increased aggregation propensity → increased protofibril and/or fibril formation reduced α-secretase cleavage → increase in β-secretase substrate
APP	missense	C-terminal of Aβ peptide (γ-secretase sites)	decreased cleavage at $A\beta_{40}$ and/or increased cleavage at $A\beta_{42}$ → relative increased production of $A\beta_{42}$ compared to $A\beta_{40}$
APP	gene/locus duplication	whole gene	increased levels of APP as substrate for Aβ production; relatively increased production of $A\beta_{42}$ compared to $A\beta_{40}$
APP	promoter mutations	5′ regulatory region	increased levels of APP as substrate for Aβ production
PSENs	missense mutations—insertions/deletions—genomic deletions	scattered over the protein	decreased γ-secretase activity alterations in the position of the cleavage site → relative increased production of $A\beta_{42}$ compared to $A\beta_{40}$

Abbreviations: APP, amyloid precurson protein; PSENs, presenilins.

located within the Aβ sequence they alter its amino acid composition and therefore its physicochemical properties. Several studies have shown an enhanced protofibril and/or fibril formation (E693Q, E693G, and D694N) (15,53,55–60) or increased fibril stability when formed (A692G) (61) for the mutant Aβ peptides. Also the affinity and toxicity towards cerebrovascular cells was altered for some mutant Aβ peptides (58,62–67), providing an explanation for the association of several α-site mutations with different clinical/pathological characteristics such as intracerebral hemorrhages due to extensive CAA (E693Q, E693K), intracerebral hemorrhages and dementia (A692G), or dementia only (E693G) (50).

APP regulatory and dosage mutations. More recently *APP* mutations were identified that affect *APP* copy number or transcriptional activity. An *APP* locus duplication was identified in five autosomal dominant early-onset AD families (68). All five duplications had different chromosomal break points and contained additional genes, implying that the *APP* locus contained a recombination hot spot. The *APP* duplication patients had a mixed phenotype of AD and/or intracerebral hemorrhages, caused by extensive CAA (68,69). In one Dutch early-onset family, we observed a similar phenotype of AD with CAA caused by a genomic duplication of only *APP* (70). Although there are only few studies, so far *APP* duplications account for about 8%–10% of autosomal dominant early-onset AD families and 3% of familial AD (68–71).

In the 5′ regulatory region of *APP* we identified mutations in probable early- or late-onset AD that significantly increased *APP* transcriptional activity *in vitro* with some mutations increasing expression by a factor of nearly two (72,73). Unfortunately, we had no autopsied brain available of AD patients carrying these promoter mutations and thus could not obtain a definite diagnosis of AD, nor could we examine *APP* expression *in vivo*. However, very recently one mutation carrier (*APP* -369C>G) died and neuropathological diagnosis confirmed AD pathology with a strong CAA component similar to AD duplication patients (Brouwers et al. unpublished data). Nonetheless, one genetic study was unable to replicate our findings (74), indicating that further studies are needed to define the pathogenic role of *APP* promoter mutations in risk for AD.

Higher levels of γ-secretase substrate (e.g. *APP* C-terminal fragments) have been directly correlated with an increased $A\beta_{42}/A\beta_{40}$ ratio (75). This suggests that elevated levels of *APP* in case of *APP* duplication or increased *APP* transcriptional activity (Table I), could ultimately result in an elevated $A\beta_{42}/A\beta_{40}$ ratio similar to that observed for *APP* γ-site mutations. Whether this is the case *in vivo* in patients carrying such a mutation awaits further investigation.

*Presenilin 1 and 2 genes (*PSEN1 *and* PSEN2*)*

Initial linkage studies showed that not all AD families could be explained by a genetic defect located on chromosome 21 (13), and mutation analyses showed that the majority of AD families did not segregate an *APP* mutation (19,76–80). Consequently, genetic heterogeneity of familial AD was further investigated, and significant linkage was obtained in multiple AD families supporting a major early-onset AD locus on chromosome 14 (14q24.3) (81–84). Eventually, positional cloning identified the chromosome 14 gene, and it was named presenilin 1 (*PSEN1*) (85), based on the observation of missense mutations in several linked pedigrees (86,87).

In the extended Volga-German AD family (88), a genome-wide study identified linkage to chromosome 1q31–42 (89). Following the identification of *PSEN1*, homology mapping identified a second *PSEN* gene, *PSEN2*, in the linked region (90,91), in which a missense mutation (N141I) segregated with AD in seven Volga-German kindreds (90). A second missense mutation (M239V) in *PSEN2* was identified in an Italian family (91). To date, 10 *PSEN2* mutations have been identified in 18 families, while 164 *PSEN1* mutations appear in 361 families (92) (AD Mutation Database). In a population-based epidemiological sample of early-onset AD, we estimated the overall mutation frequencies of *PSEN1* and *PSEN2* at, respectively, 6% and 1% (93).

Most *PSEN* mutations cause typical AD, clinically and pathologically indistinguishable from sporadic AD, except for the early onset age and a more rapid and pronounced disease progression. Nonetheless, *PSEN* mutations are occasionally associated with AD with onset age >65 years (93) (AD Mutation Database). In the Volga-German kindreds some *PSEN2* N141I carriers had late-onset AD (90,91), and two other *PSEN2* mutations (V148I and Q228L) were detected in patients with an onset >65 years (94,95). More recently, two *PSEN1* mutations (A79V and R269H), previously associated with early-onset AD, were also found in late-onset AD patients (96,97). We observed in Belgian AD patients, with onset ≤70 years, missense mutations in *PSEN1* (C263F), and *PSEN2* (R62C and R71W) in three AD patients with onset age >65 years (98). Together these observations

suggest that modifying factors influence onset age in *PSEN* mutation carriers.

Presenilins (PSENs). *PSEN1* covers a genomic region of ±84 kb and comprises 13 exons (99), whereas *PSEN2* is only ±25 kb in size and has 12 exons (100) (Figure 3A). Apart from differences in genomic size and number of exons, the *PSENs* have a similar gene structure. Same as *APP*, *PSENs* are expressed in a wide variety of tissues including brain, although expression of *PSEN2* is remarkably lower in brain (85,90,91). In brain, the *PSENs* are primarily expressed in neurons, with higher levels noted in the cerebellum and the hippocampus (101–103). Both genes produce a number of alternatively spliced transcripts.

The PSEN proteins share an overall amino acid sequence identity of 67% (90,91). Hydrophobicity plots suggested that PSENs represent integral membrane proteins containing at least seven transmembrane domains that are highly conserved between human PSENs and their orthologues (85,90,91), but the topology of PSENs remained a matter of debate (104–108). Recent evidence

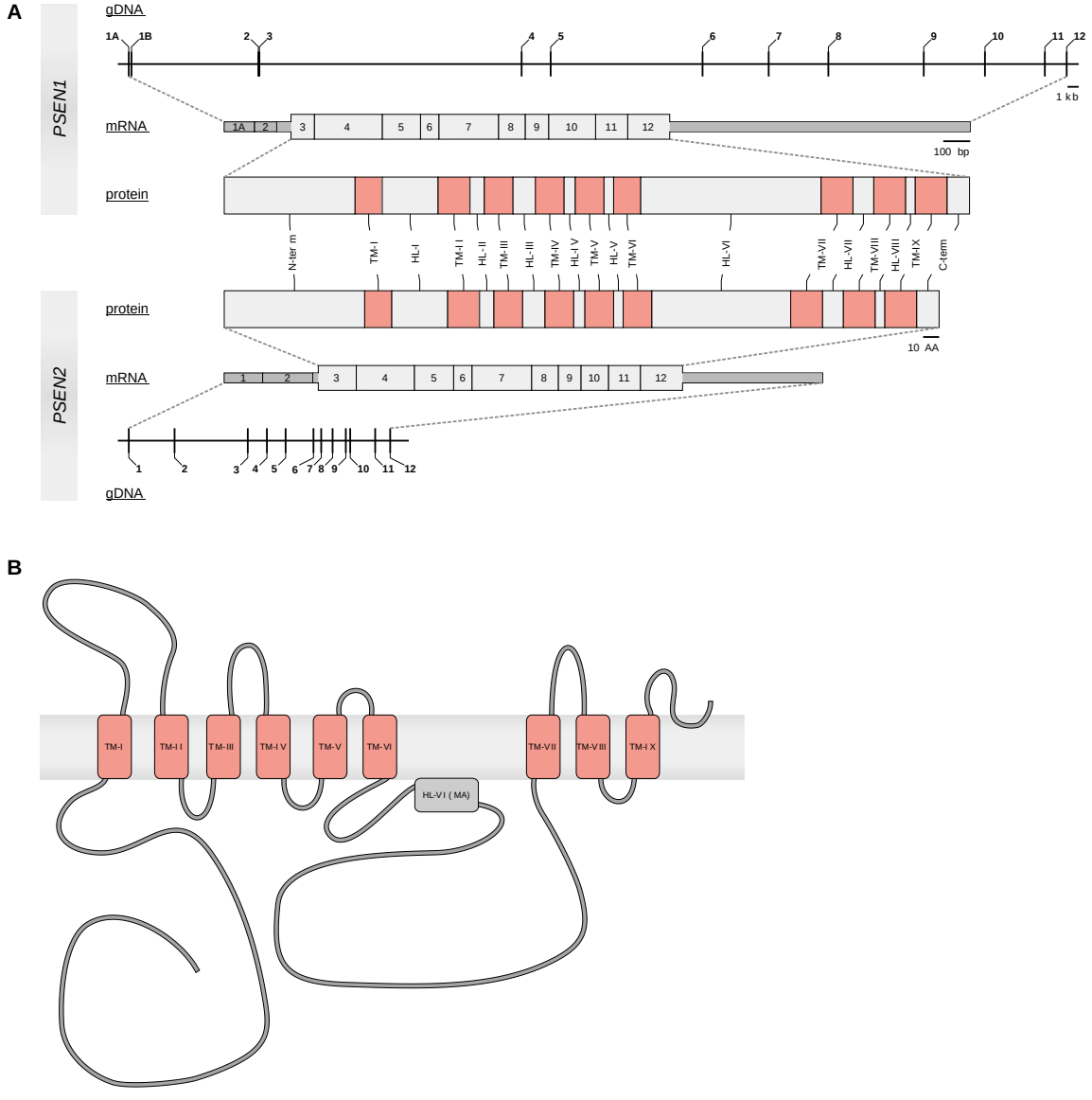

Figure 3. A: Schematic presentations of the presenilins (*PSEN1* and *PSEN2*) at genomic, transcript, and protein level. Numbers (genomic and transcript) indicate exons. At transcript level, untranslated regions are represented as dark gray boxes; coding regions are shown in light gray. Pink boxes indicate the transmembrane regions (TM) in both proteins, connected by hydrophilic loops (HL). B: Nine-transmembrane topology of PSEN1. Transmembrane regions (TM, pink boxes) are connected by hydrophilic loop structures. In hydrophilic loop VI a portion of the loop is associated with the membrane (MA, gray box).

indicated that PSENs most likely adopt a nine-transmembrane topology (109–111) (Figure 3B).

Under physiological conditions, the bulk of PSENs are endoproteolytically processed by cleavage within the large hydrophilic loop, yielding N- and C-terminal fragments that are present in a 1:1 stoichiometry, forming stable heterodimeric complexes (112–118). Endoproteolytic cleavage is a highly regulated process happening after intramolecular interactions have been established, but fragments derived from the different PSEN molecules do not associate with each other (119). Although endoproteolytic cleavage is not necessary to produce functionally active presenilin proteins (112), both fragments are essential to ensure presenilin activity (118,120,121). PSENs are predominantly located in the endoplasmic reticulum and the intermediate and early-Golgi compartment (101,122–124), implying that they are involved in protein processing.

PSENs are γ-secretases. The multiple transmembrane topology of PSENs indicated that they function as cell receptors, channel proteins, or in intracellular trafficking of proteins. Since PSENs show substantial homology with SPE-4, a *Caenorhabditis elegans* protein that is involved in storage and transport of proteins (85,90), it was presumed that the PSENs had a similar function, e.g. regulating intracellular transport of APP (85,90). Insights into the function of PSENs resulted from a genetic screen in *C. elegans* aiming at identifying proteins involved in Notch (LIN-12) signaling. Here a protein (SEL-12) with remarkable sequence similarity to PSENs was identified (125). Rescue by human PSENs of the *sel-12* phenotype in worms (125) and the Notch phenotype in *Psen1* null mice indicated a role for PSENs in Notch signaling (126,127). Furthermore, PSENs and APP interact with each other to form stable complexes (128–130). In neuronal cultures derived from *Psen1*$^{-/-}$ null mice, Aβ production was dramatically reduced and accompanied by an accumulation of APP C-terminal fragments (131). As α- and β-secretase cleavage of APP was not affected, these results implied that Psen1 is directly involved in γ-secretase cleavage of APP (131). Proteolytic cleavage of Notch, producing the Notch intracellular domain (NICD) that is involved in signal transduction, bore a striking resemblance to the γ-secretase processing of APP, and further studies indicated that PSEN1 is also required for the ligand-dependent cleavage of Notch (28,132–134). Taken together, these observations suggested that PSENs either function as necessary cofactors or regulators of γ-secretase activity or represent the actual protease activity. Supportive evidence of the latter was obtained using mutagenesis of either one of two highly conserved aspartic acid residues, and using transition state analog inhibitors directed to the active site of aspartyl proteases. Both experiments showed a potent inhibition of γ-secretase activity, similar to that observed in *Psen1*$^{-/-}$ cells (29,135,136). These data provided evidence that PSENs indeed offer the catalytic activity of γ-secretase, and function as diaspartyl proteases.

Even though it was generally accepted that PSENs are in fact γ-secretases, it was noted that γ-secretases exist as high-molecular-weight complexes in the cell suggesting that other proteins are implicated (137–139). Three PSEN interacting proteins, nicastrin (NCSTN) (140), presenilin enhancer 2 (PEN2) (141), and anterior pharynx-defective 1 (APH1) (141,142), were identified as members of the γ-secretase complex, and were shown to be essential components for its activity (143–145). APH1 and NCSTN are involved in stabilization of the PSEN holoprotein in the γ-secretase complex, whereas PEN2 is required for endoproteolytic cleavage of the holoprotein (146). Thus, the γ-secretase complex consists of four proteins, PSEN1 or 2, NCSTN, APH1 and PEN2, but PSENs are providing the catalytic active site. Also, γ-secretase was shown to process a series of different type I transmembrane proteins through a process nowadays known as 'regulated intramembrane proteolysis' (RIP) (for review see (147–149)).

Gain or loss of PSEN function. The *PSEN* mutation spectrum comprises primarily missense mutations that are scattered over the protein with some clustering around putative transmembrane domains, e.g. 63% and 67% in PSEN1 and PSEN2, respectively (92) (AD Mutation Database). Also most mutations occur at residues conserved among human PSENs (95% in PSEN1 and 100% in PSEN2) and often so among presenilin orthologues, supporting their functional relevance. Some but not all mutations interfere with the endoproteolytic processing (113,150–152), like mutations deleting exon 9 (Δ9) containing the sequence coding for the endoproteolytic cleavage site (112). Deletion of exon 9 inhibits endoproteolytic cleavage and maintains PSEN1 as a stable holoprotein though functionally active as γ-secretase. Therefore, the pathogenic nature of the Δ9 mutation is more likely the result of the introduction of a missense mutation at the junction of exons 8 and 10 (S290C) (153).

That *PSEN* mutations were involved in the pathogenic pathway leading to amyloid deposition was shown by Aβ peptide measurements in plasma

and in conditioned medium from fibroblasts of *PSEN1* and *PSEN2* mutation carriers. *PSEN* mutations resulted in relatively elevated levels of $A\beta_{42}$ compared to $A\beta_{40}$ (154,155). Higher levels of $A\beta_{42}$ were also observed in brains of *PSEN1* mutation carriers compared to sporadic AD patients (156–158). Further evidence was obtained in transgenic mouse models or transfected cells overexpressing mutant *PSENs*, where a relatively increased production of longer Aβ species was seen (159–163).

Since the vast majority of mutations identified in *PSENs* were missense mutations that lead to an increase in the $A\beta_{42}/A\beta_{40}$ ratio, it was hypothesized that *PSEN* mutations conferred their pathogenic effect by acquiring a toxic gain-of-function. However, it remained unclear how the many different missense mutations scattered over the entire protein were able to cause a similar gain-of-function. Detailed analyses of different mutant PSENs showed that some mutations increased the $A\beta_{42}/A\beta_{40}$ ratio by decreasing $A\beta_{40}$ rather than increasing $A\beta_{42}$ (164–168), implying a PSEN loss-of-function as disease causation mechanism. Also, APP processing at the ε-site was reduced for a substantial number of mutant PSENs (164–166,169–171), implying a general loss of γ-secretase activity and not only at the γ-site. Also comparable results were obtained for the release of the intracellular domain of other γ-secretase substrates, such as NICD (134,165,166,169,170, 172–174). In addition to less efficient processing at the ε-site, mutations were shown to alter the preferred cleavage site, since they often increased $AICD_{49–99}$ and/or decreased $AICD_{50–99}$ production (35,47), similarly to what was observed for *APP* γ-site mutations. Together with the current sequential APP processing model, this could explain the contradiction of a partial loss-of-function leading to an increased $A\beta_{42}/A\beta_{40}$ ratio, the apparent toxic gain-of-function. Mutant PSENs that less efficiently process their substrates also cut these substrates more often at the alternative minor ε-cleavage site. The less active mutant γ-secretase releases $A\beta_{42}$ before it is further processed to $A\beta_{39}$, resulting in increased amounts of $A\beta_{42}$ even though there is a general loss of function (37,175,176).

The apparent loss-of-function of PSEN mutations corroborates well the partial rescue of the *sel-12* phenotype in worms by mutant PSEN compared to the wild type (177). Although initial rescue experiments in *Psen1* null mice disproved this hypothesis (178,179), this was likely due to the rather mild effect on NICD production by the mutant PSEN (A246E) used (only 20% decreased) (165,178,179). Also, conditional *Psen1* and *Psen2* knockout mice displayed memory impairment, synaptic dysfunction, and age-dependent neurodegeneration in the absence of Aβ (180), suggesting a role for PSENs in neuronal survival. Thus loss of PSEN function might contribute to neurodegeneration.

Also, previously, several genetic studies reported association of AD with polymorphisms located in the 5′ regulatory region of *PSEN1* and *PSEN2* (181–184). The risk-conferring allele of the associated *PSEN1* promoter polymorphism (−22C) leads to a neuron-specific reduction of transcriptional activity of the *PSEN1* promoter, due to alterations in transcription factor binding sites (185). Moreover, two rare promoter mutations were identified in early-onset AD patients, of which one significantly reduced *PSEN1* transcription in neuronal cells (183). These data also suggested a pathogenic effect through a loss-of-function mechanism rather than a toxic gain-of-function.

Other monogenic loci/genes for AD

Novel locus on chromosome 7. The identification of mutations in *APP* and *PSENs* has substantially contributed to the genetic etiology of familial AD. Nonetheless, in a significant percentage of familial AD patients the genetic cause is still unknown (7). In these families, with early- or late-onset AD, the disease is often inherited in an autosomal dominant manner (186–189). In a Dutch family with mean onset age 66.8 years (range 47–77 years), we performed a genome-wide screen (186,189) and identified a novel locus on chromosome 7q36. Mutation analysis of all 29 known genes in the 5.44 Mb linked region did not unambiguously identify the underlying gene.

Association with non-AD genes. The mutation R406W in the microtubule associated protein tau gene (*MAPT*), causally related to FTLD (190), has been identified in several families diagnosed with clinical AD (190–196). A second mutation (ΔK281) was found in a patient with a clinical and pathological diagnosis of AD (197). Furthermore, the major tau isoform accumulating in neurofibrillary tangles in the patient's brain corresponded to the isoform predominantly produced as a result of the ΔK281 mutation (197). While most *MAPT* mutations are identified in FTLD patients, it is not surprising to find some in AD patients since both dementia subtypes share overlapping clinical symptoms and a differential diagnosis of AD or FTLD is often difficult to establish particularly in the later stages of the disease. Furthermore, it has been suggested that AD and FTLD represent

disease expressions in one common spectrum ranging from CAA (amyloid-positive, tau-negative) over AD (amyloid-positive, tau-positive) and tau-positive FTLD, to tau-negative, ubiquitin-positive FTLD (FTLD-U) (198).

Another example is the identification in two late-onset AD patients of a null mutation (IVS1 + 5G > C) in the progranulin gene (*PGRN*) underlying FTLD-U pathology (199–201). The intron 1 splice-site mutation IVS1 + 5G > C was originally identified in a large Belgian FTLD-U founder family, consisting of 10 branches with 39 patients. Strikingly, the disease in this large family is characterized by a wide range in onset age (from 45 to 78 years) and association of the *PGRN* mutation with different clinical phenotypes (FTLD, AD, and Parkinson disease (PD)) (201). A similar effect was observed for another *PGRN* founder mutation (R493X), with 30% of mutation carriers presenting with memory problems and a clinical diagnosis of AD in three patients (202). In both studies postmortem analysis revealed mixed pathologies of FTLD-U and AD (or PD) (201,202). As *PGRN* encodes a growth factor, it is conceivable that the protein could potentially function as a general neuronal survival factor. Null mutations in *PGRN* lower the threshold for neurodegeneration and formation of pathologic lesions of, for example, AD, occurring because of another disease mechanism or modifying factor (201). In this context, we identified several *PGRN* missense mutations in AD patients that likely disrupt PGRN protein by interfering with the characteristic granulin folds of the protein (203). Interestingly, similar missense mutations were observed in FTLD patients (204), and the corresponding mutant proteins were shown to be less efficiently secreted and more rapidly degraded (205). Taken together, these missense mutations might have a milder effect on the amount of functional protein produced, increasing an individual's risk of developing a neurodegenerative CNS disease at later age.

While these observations are exemplifying the clinical heterogeneity of mutations in dementia genes and their contribution to the complexity of the neurodegeneration process, *MAPT* or *PGRN* mutations remain infrequent causes of clinical diagnosed AD. Nevertheless, screening for mutations in these two genes might be warranted in clinically diagnosed AD patients in which mutations in known AD genes are absent.

Searching for AD susceptibility genes

The identification of genes in which mutations are responsible for monogenic early-onset forms of AD has contributed substantially to understanding the molecular mechanisms involved in AD pathogenesis. Nonetheless, the majority of AD patients develop the disease at older age and, although there is a clustering of patients into families, segregation of AD in these families does not follow a Mendelian inheritance pattern.

Apolipoprotein E gene (APOE)

A genome-wide linkage study in late-onset AD families identified a novel locus on chromosome 19 (19q13.1–19q13.3) (206). About the same time the apolipoprotein E (APOE) was shown to interact with the Aβ peptide in cerebrospinal fluid, and its gene (*APOE*) was located near the chromosome 19 linked region (207). Moreover, APOE had been associated with senile plaques and neurofibrillary tangles in AD brains (208), and *APOE* transcription was upregulated in brains of AD patients (209). Subsequently, it was shown that one of the three major APOE isoforms, APOE ε4, was overrepresented in familial late-onset AD patients compared to aged healthy control individuals (207). The *APOE* ε4 association was extensively confirmed in both familial as well as sporadic late-onset AD patients of different ethnic backgrounds (5,210,211), and later also in early-onset AD patients (212).

APOE ε4 primarily acts by lowering onset age in a dosage-dependent manner, increasing risk 3 times in heterozygotes and 15 times in homozygotes (211). The ε2 isoform, on the other hand, was shown to have a protective effect; however, this could not be consistently replicated (213). *APOE* genotype was also shown to modify onset age in carriers of causal AD mutations. This was extensively shown for *APP* mutations (73,214–217) but also for several *PSEN2* mutations (218). No effect could be observed for *PSEN1* mutations (219), with the exception of a large Colombian *PSEN1* pedigree (220). Apart from the three major protein isoforms, four promoter variants influencing *APOE* expression levels were shown to affect AD risk in several studies (221–226). Although an *APOE* ε4 independent effect seemed only present for one variant (− 491A/T), this finding is noteworthy given that *APOE* ε4 affects AD risk in a dose-dependent manner.

In vitro studies have indicated that the APOE ε4 isoform binds Aβ peptides with a higher avidity compared to APOE ε3 (227). Furthermore, there is a strong correlation between the presence of an *APOE* ε4 allele and a higher Aβ burden in the brains of AD patients (228,229), suggesting that APOE interacts with Aβ in enhancing its deposition in plaques. This is supported by the observation that homozygous *Apoe* knockout ($Apoe^{-/-}$) mice

develop fewer and more diffuse, non-fibrillar Aβ deposits (230–232). Some but not all studies assessing the effect of different APOE isoforms on Aβ fibrillization showed that the ε4 isoform leads to increased Aβ aggregation *in vitro* (227,233–236). Similarly, *in vivo* studies in *Apoe*$^{-/-}$ mice indicated that APOE ε4 increased Aβ fibrillization and plaques formation compared to APOE ε3 (237,238). Still, it is possible that APOE exerts its effects through different mechanisms, e.g. APOE is a major cholesterol transporter and high cholesterol levels have been associated with an increased Aβ load in animal models (239,240) and changes in APP processing (241–243). Thus APOE isoform-specific changes in cholesterol binding and transport in brain might also affect plaque formation in AD brains.

Other AD susceptibility loci/genes

Two approaches are being followed to unravel the genetic etiology of late-onset AD, i.e. hypothesis-driven candidate gene studies and hypothesis-free (in terms of biological function or position) genome-wide analyses. Genome-wide linkage and association studies in large samples consisting of late-onset AD families and sib pairs have identified several chromosomal loci harboring potential AD susceptibility genes (244). In candidate-gene-based studies researchers have focused on genes encoding functionally relevant proteins, e.g. proteins that belong to the γ-secretase complex (for example *PSEN1* (185,225, 245) or *NCSTN* (225,246)), regulate APP trafficking (*SORL1* (247,248)), or are involved in fibrillization or clearance of the Aβ peptide (*IDE* (225,249), *ACE* (225,250), *PLAU* (251), *MME* (252)). Also genes that are implicated in other neurodegenerative diseases (*PRNP* (253)) or genes located in linked regions have received attention (www.alzgene.org).

Genetic designs. Over the years genetic studies have experienced a marked evolution. Early studies investigated one or few single nucleotide polymorphisms (SNPs) at a time. Unless the functional polymorphism itself was tested, the success of these studies was limited due to a variety of factors, including absence of linkage disequilibrium (LD) between the marker tested and the underlying functional variant. Rapidly advancing technology allowed gene-wide studies utilizing the underlying LD pattern in haplotype-based approaches in order to capture a maximum of genetic information. However, positive results obtained in some of these studies proved difficult to replicate either because the original finding was a false positive, or it was attributable to heterogeneity at the genetic, allelic, mutational, or population level, or it was influenced by differences in study design or statistical power. Recently, geneticists went even further in their search for AD susceptibility genes, analyzing hundreds to thousands of SNPs spread throughout chromosomal regions showing linkage to AD (254,255), whole chromosomes (256,257), and even the complete genome (258–261).

It is not within the scope of this review to go into detail of the numerous studies on the even so numerous candidate AD susceptibility genes that were performed over the last 10–20 years. A regularly updated overview can be found on the Alzgene website (www.alzgene.org) (225). Here we will focus on recent developments in identifying novel AD risk genes.

Genome-wide linkage studies. Genome-wide linkage studies in late-onset AD families and sib pairs (244) (www.alzgene.org) generally used several hundreds of microsatellite markers spread throughout the genome. These studies identified a substantial number of chromosomal regions implicated in AD. Some of these loci, such as those on chromosomes 9, 10, 12, and 19, were repeatedly linked to AD (244), suggesting that these loci contained important AD susceptibility genes. Since multiple genes with small effect sizes were expected to contribute to late-onset AD, several groups performed genome-wide screens in isolated populations (188,261–267). Geographically and/or culturally isolated populations are assumed to be less genetically heterogeneous, with disease risk being determined by a smaller number of genes of which some are enriched in the population under study, thus facilitating their identification. These studies confirmed several of the loci identified in outbred populations, for example 1q21 and 1q25 (261), and 10q24 (261,267). Furthermore, they also extended the genetic spectrum with additional, new genetic loci implicated in AD, for example 8p12–q22 (262) and 3q22–q24 (261).

Locus- or chromosome-wide association studies. Genetic approaches based on microsatellite markers generally identify extended chromosomal regions harboring a large number of potentially causal genes. Although many candidate gene studies were performed, especially in the linked regions on chromosomes 9, 10, and 12, the proven causal genes have not yet been found (www.alzgene.org). In a renewed attempt researchers resorted to fine-mapping by genotyping large numbers of SNPs across these regions.

On chromosome 12, analysis of a large number of SNPs across the linkage region (12p11–13)

showed that the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) and some of its paralogues could be implicated in AD pathogenesis (254), but decisive arguments for a role of *GAPDH* as a risk factor for AD are still awaited. For the chromosome 10 AD locus, two independent studies were performed targeting either the locus (10q21.1–10q25.1; 47 cM) (255) or the whole chromosome 10 (256). Both studies also differed in study design in terms of ethnicity, stratification for *APOE* genotype, and SNP selection (LD-based versus a gene-centric approach in which SNPs were selected that have a higher chance of having a direct biological effect (e.g. non-synonymous and regulatory variants)). Not surprisingly different genes were identified, dynamin binding protein gene (*DNMBP*) (255) and a gene (*LOC439999*) encoding a protein similar to ribosomal protein S3a (*RPS3A*) (256). Similarly to chromosome 10, the locus on chromosome 9 was investigated using a chromosome-wide gene-centric approach (257), resulting in the identification of several significant SNPs, with the most significantly associated SNP located under the 9q22 linkage peak pointing to the death-associated protein kinase 1 gene (*DAPK1*).

Although these approaches in identifying risk genes underlying large linkage and association peaks provided some insights into novel pathways potentially involved in AD pathogenesis, replication of these findings in additional independent study populations proved to be hard (268–271). Nonetheless, the *DNMBP* and *GAPDH* findings could be confirmed in independent studies (272,273).

Genome-wide association studies. Locus- and chromosome-wide studies balance between a targeted candidate region approach and a hypothesis-free approach, as to possible biological pathways leading to AD. Candidate genes are usually selected to fit within existing hypotheses, the most prevailing being the amyloid cascade hypothesis. Several other pathways have been suggested, e.g. altered cholesterol metabolism, impaired axonal transport, increased oxidative stress, etc. The number of proteins fitting in existing hypotheses is enormous, making it practically impossible to analyze all the corresponding genes in detail one by one.

Since high-throughput genotyping platforms permit genotyping of large numbers of SNPs and since the completion of the human sequence allows studying common genetic variation across the genome, researchers started to apply genome-wide association studies. One of the promises is that this holistic approach will uncover candidate genes that otherwise would have escaped attention. The approach already proved fruitful in other complex genetic disorders, such as myocardial infarction (274), type 2 diabetes (275,276), and coronary heart disease (277). In AD, however, achievements are still limited. Currently two different approaches are used in AD research, on the one hand there is the gene-centric approach, utilizing genotype data of a large number of, potentially functional, SNPs located in and around genes (258,278), while the other consists of an LD-based approach, where SNPs are selected based upon SNP informativeness and the LD structure of the population under study (259,260,279). Although these studies already revealed several new genes and pathways potentially implicated in AD, they also generated some questions. It remains to be established whether these genes can be replicated using independent samples, as none of the studies performed to date seem to identify the same genes. Furthermore, genes identified in locus- or chromosome-wide studies are not detected in genome-wide studies on the same samples (254,256–258). In fact, the only gene that is clearly associated in different study populations and designs is still *APOE*.

Conclusions

After more than 20 years of extensive genetic research in the field of AD, we can conclude that AD is a complex and genetically heterogeneous disorder. Most of the insights we have gained so far came from genetic studies in large multigenerational families in which AD is inherited in an autosomal dominant manner. Linkage analyses in these families has led to the identification of three causal genes (*APP*, *PSEN1*, and *PSEN2*) and one susceptibility gene (*APOE*) that are consistently involved in AD genetic etiology. Yet, there are still additional AD genes to be identified. There is at least one autosomal dominant AD family, in which mutations in the known dementia-causing genes have been excluded, harboring a causal gene at chromosome 7q36 (186). For the more common, generally late-onset form of AD, a large number of chromosomal regions linked to or associated with the disease have been discovered, but their underlying genes have not yet been unequivocally identified. As has already been proven in the past with the identification of *APP* and the *PSENs* as causal genes, the discovery of novel genes could contribute significantly to our understanding of the disease process by revealing pathways that could provide access points for novel therapeutic strategies. Though much effort has been put into mapping novel AD genes, the search for genes contributing to

the risk profile of late-onset AD has been complicated by the many pitfalls of the designs and techniques used in the past, such as candidate-gene-based association studies. However, the field of complex genetics is rapidly evolving, and although strategies currently used to identify risk genes for AD (such as locus-, chromosome-, or genome-wide screens) yielded so far inconsistent data, they are promising since they have already been successfully applied in other complex diseases like myocardial infarction (274), type 2 diabetes (275,276), and coronary heart disease (277). In these studies much larger sample sizes are used compared to what is currently used in genome-wide screens of AD. This implies that in order to be successful, sample sizes used in genome-wide screens for identification of risk alleles with a small effect on the disease, as is expected for a heterogeneous, complex disorder such as late-onset AD, should be increased. Further, as genetic heterogeneity creates a problem in the reliable identification of novel risk factors, more effort should go into minimizing heterogeneity by using populations with fewer founders (isolated populations). Another way of reducing genetic heterogeneity can be established by utilizing endophenotypes in genome-wide studies. Here, phenotypes that are associated with the disease but are closer to the underlying biology and thus less prone to other modifying factors, for example environmental influences, are investigated. This strategy was already proven successful in a genome-wide association study on memory performance (280). Also, in the future, in addition to genotyping larger study populations and minimizing genetic heterogeneity, more effort could be put into combining data from different approaches, such as gene expression data, genome-wide association studies, and proteomics, in order to prioritize the number of potentially implicated genes and to discover novel pathways involved in AD pathogenesis.

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