

Assembly of Amyloid β -Protein Variants Containing Familial Alzheimer's Disease-Linked Amino Acid Substitutions

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Chapter Outline

Introduction: 'Minor' Changes have Major Effects	429	N-terminal and C-terminal Substitutions	436
Point Mutations Affecting the Aβ Sequence	430	The A2T Versus A2V A β (A673T, A673V APP) Mutations	436
Substitutions in Positions 21–23	430	The English H6R A β (H677R APP) and Tottori D7N A β (D678N APP) Mutations	436
The E22Q A β (E693Q APP) Dutch Mutation	430	The K16N A β (K687N APP) Mutation	437
The E22G A β (E693G APP) Arctic Mutation	431	The L34V A β (L705V APP) Piedmont Mutation	437
The E22K A β (E693K APP) Italian Mutation	434	Silent and Incomplete Penetrance Mutations	437
The E22 A β (E693 APP) Deletion – Osaka Mutation	434	Conclusions	438
The A21G A β (A692G APP) Flemish Mutation	434	Acknowledgments	439
The D23N A β (D694N APP) Iowa Mutation	435		

INTRODUCTION: 'MINOR' CHANGES HAVE MAJOR EFFECTS

In the amino acid sequence of amyloid β -protein (A β), not all amino acids are equal in their importance to assembly kinetics and biologic activity. The length of the sequence itself has a major impact on A β assembly and bioactivity. A β is a product of sequential cleavage of the amyloid β -protein precursor (APP), first outside the membrane by β -secretase to release the ectodomain of APP, called soluble APP β (sAPP β), and then within the membrane by γ -secretase to release A β and the APP intracellular cytoplasmic domain (AICD; Fig. 38.1). Another enzyme, α -secretase, cleaves APP within the A β region, after residue 16, leading to formation of a shorter peptide, p3, which is not associated with disease. β -Secretase consistently produces A β starting at D1 (APP672). In contrast,

γ -secretase is a promiscuous enzyme, which cleaves APP in several locations, leading to peptides ending at various C-terminal positions, most commonly from 38 to 43 (though never 41). Historically, the most studied forms of A β have been the most abundant ones—the 40- and 42-residue forms, but studies of the 38- and 43-residue forms are on the rise. Changes in the C-terminal length of A β have a major effect on the aggregation kinetics, toxicity, and role in Alzheimer's disease (AD), even though, regardless of the exact C-terminal position, A β isoforms form oligomers, which are believed to be the major neurotoxic form of A β [1], and then go on to make fibrils [2] that are found deposited in the brain.

In the AD brain, A β deposits are formed in both the brain parenchyma and vasculature. The amyloid plaques found in the parenchyma are one of the pathologic hallmarks of AD. The other hallmark, neurofibrillary tangles,

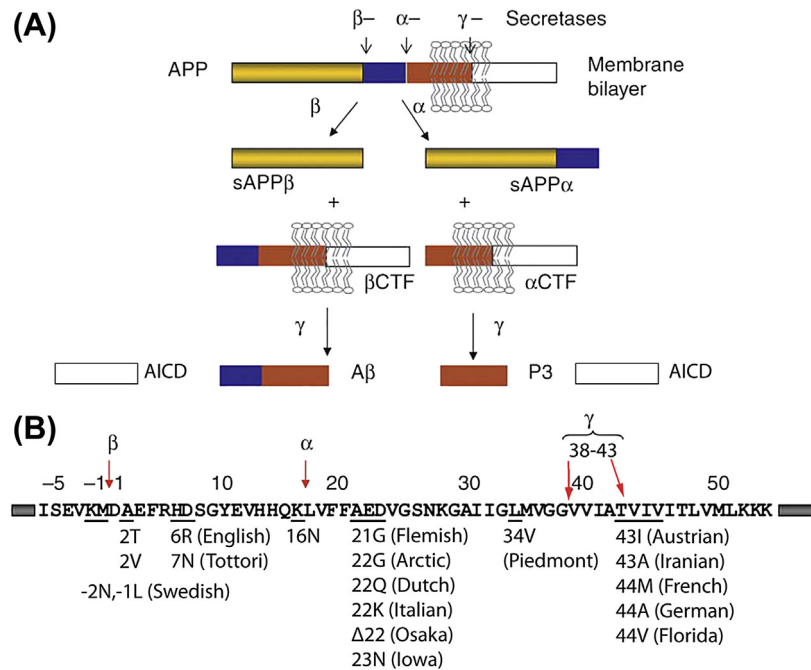


FIGURE 38.1 Processing of the amyloid precursor protein (APP) by α -, β - and γ -secretases and amino acid substitutions leading to familial AD. (A) Two pathways (β/γ and α/γ) of APP proteolysis. APP can be cleaved by either β - or α -secretase, which is then followed by γ -secretase cleavage. The designation of substrates and products are depicted. (B) Location and identity of amino acid substitutions caused by mutations in the *app* gene in or near the A β region. Residues are numbered according to A β sequence. D1 and A42 of A β correspond to APP672 and APP713, respectively. Adapted and reprinted with permission from Tian et al [80].

comprising hyperphosphorylated Tau, is discussed in Chapter 19: Polymorphism of Tau fibrils. Classic, dense-core amyloid plaques and diffuse plaques are composed primarily of A β 42 and A β 43 [3–5], whereas vascular amyloid consists mainly of A β 40 [6,7]. Levels of A β in the cerebrospinal fluid (CSF), which often are used as a biomarker for AD, differ between the A β isoforms. In non-demented persons, levels of CSF A β were found to be 40>38>42>39>37 [8]. In patients with AD, levels of A β 42 and A β 37 decreased, and levels of A β 38 and A β 40 increased [8] or remained unchanged [9,10]. A β 39 concentration levels were unchanged [8]. In plasma, levels of A β 38, A β 40, and A β 42 are indistinguishable between patients with AD and age-matched normal individuals [11]. Clinically, A β 42 has been most closely associated with AD because: (1) FAD-linked mutations in the *psen1* and *psen2* genes, which encode the catalytic unit of γ -secretase, presenilin-1 and presenilin-2, respectively, result in increased A β 42 levels [12,13]; and (2) A β 42 is more prone to aggregation [3,14,15] and is more neurotoxic [16–19] than A β 40. The two C-terminal amino acids of A β 42, I41 and A42, induce biophysical properties distinct from those of A β 40. Jarrett et al found that peptides of 39 or 40 residues remained kinetically soluble for days, whereas peptides of 42 or 43 residues aggregated within hours [15]. A β 42 also forms oligomers that are different from those formed by A β 40. For example, pentamer and hexamer ‘paranuclei’

are predominant A β 42 oligomers whereas dimer, trimer, and tetramer are more abundantly represented in the A β 40 oligomer population [20]. Multiple lines of evidence demonstrate that the difference in peptide length is one of the key components for controlling early oligomerization. In fact, paranuclei and several other types of oligomer form exclusively from A β 42 and not from A β 40 [21].

Many other regions of the A β sequence have been discretely studied for their unique characteristics, for example, the central hydrophobic cluster, A β 17–21 [22,23], and M35 [24–26]. Here we focus on mutations in *app* that confer amino acid modifications within A β and the differences in biophysical and clinical properties that result from these substitutions.

POINT MUTATIONS AFFECTING THE A β SEQUENCE

Substitutions in Positions 21–23

The E22Q A β (E693Q APP) Dutch Mutation

The Dutch mutation was the first one discovered to cause an intra-A β substitution [27]. The resulting disease is called hereditary cerebral hemorrhage with amyloidosis, Dutch type (HCHWA-D). Clinically, patients with HCHWA-D have A β deposition predominantly in cerebral vessel walls [27], severe cerebral amyloid angiopathy (CAA), and

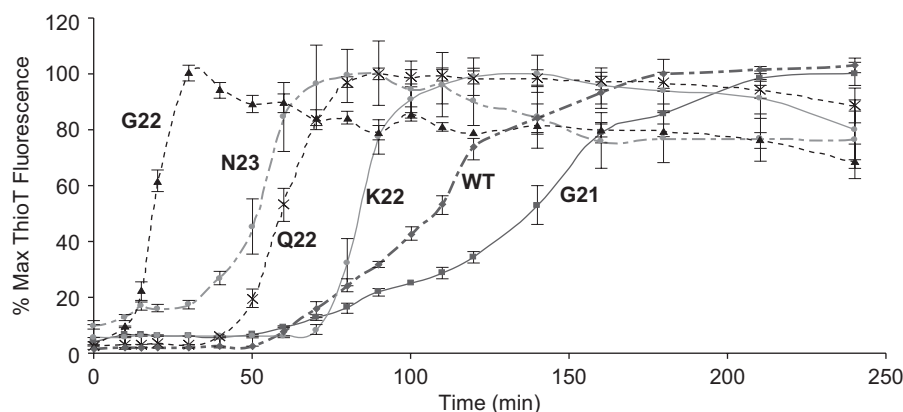


FIGURE 38.2 Most disease-associated intra-A β mutations increase A β aggregation kinetics. For continuous assessment of aggregation, A β variants were incubated with thioflavin T in the wells of a 96-well micro-titre plate, shaken at 650 rpm, and fluorescence measurements made at regular intervals. The Q22 data clearly show a lag phase between 0 and ~40 minutes, a growth phase, 40 to 80 minutes, and a plateau phase after 80 minutes. Adapted and reprinted with permission from Betts et al [33].

hemorrhages with early or diffuse plaques – but, unlike typical AD, minimal neurofibrillary tangles are observed. Likely, dementia develops as a result of the damage caused by multiple microinfarcts or hemorrhages because there is a strong correlation between hemorrhages and dementia. Because the location and degree of the infarcts are highly variable, there is a high degree of variability in the age of onset of dementia in this kindred [28,29].

Studies of the effects of wild-type (WT) A β 40 and [E22Q]A β 40 on cerebral microvascular endothelial cells suggest A β oligomer-mediated induction of apoptotic pathways through caspase signaling, with a correlation between the enhanced aggregation kinetics of the [E22Q]A β 40 variant and enhanced apoptosis levels [30]. The Dutch mutation not only enhances the aggregation kinetics relative to WT A β (Fig. 38.2) [31–33], it also increases cell toxicity [34,35]. [E22Q]A β 40 also polymerizes into protofibrils faster than WT A β 40 [36]. In a study that did not show increased oligomerization kinetics or β -sheet levels – but did show [E22Q]A β 40-induced apoptosis of primary human cerebral endothelial cells – the mechanism of apoptosis was attributed to the induction of the Bax mitochondrial pathway and was inhibited by the endogenous molecule tauroursodeoxycholic acid [37]. Similar to the human disease, transgenic mice expressing the Dutch mutant form of human APP develop few parenchymal plaques but exhibit prominent CAA [38] and early behavioral deficits [39].

The E22Q substitution replaces a negatively charged glutamate with a neutral glutamine, leading to a loss of negative charge. Sureshbabu et al [31] have suggested that, in the absence of other potentially confounding local environmental factors, mutations resulting in the loss of negative charge in A β facilitate aggregation [40] due to a decrease in the electrostatic repulsion among monomers [41]. This point is discussed in more detail in the *Conclusions* section, below.

The E22G A β (E693G APP) Arctic Mutation

Unlike some of the other cases of FAD caused by intra-A β substitutions leading to CAA, FAD caused by the Arctic mutation [42] is nearly indistinguishable pathologically from idiopathic AD. In Arctic FAD, A β is deposited mainly in the brain parenchyma and, to a lower extent, in the cerebral vasculature, resulting in few hemorrhages [43]. Interestingly, however, many of the plaques have a non-cored, ring-like character and are negative for Congo Red staining, unlike plaques from idiopathic AD brain (Fig. 38.3) [43], but are similar to FAD phenotypes linked to a deletion in exon 9 of *presenilin 1* [44], or to the Dutch mutation described above [45]. Similar to sporadic AD, reduced blood flow to the parietal lobe and general brain atrophy have been observed [43]. Disease onset is around the fifth or sixth decade of life, with cognitive dysfunction similar to other familial or sporadic AD phenotypes in multiple cognitive domains, including episodic memory, attention, cognitive speed, and visuospatial functions [43,46]. Carriers of the Arctic mutation, where the polar, negatively charged glutamic acid is replaced with a non-polar, neutral glycine, have lower plasma A β concentration levels than patients with sporadic AD, and decreased secretion of this variant, relative to WT A β , also has been observed in HEK 293 cells [46].

In vitro, [E22G]A β 40 has been shown to form fibrils at 3-times lower concentrations than WT A β 40 and at ≥ 2 -times faster rates (see e.g. Fig. 38.2) [33,47]. Similar results were observed with A β 42, where [E22G]A β 42 showed increased assembly into protofibrils and fibrils compared to WT A β 42. Correspondingly, the half-maximal concentration for neurotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay for the Arctic A β 42 isoform was ~15% that of WT A β 42 [34]. Though the Arctic mutation causes a decrease in the

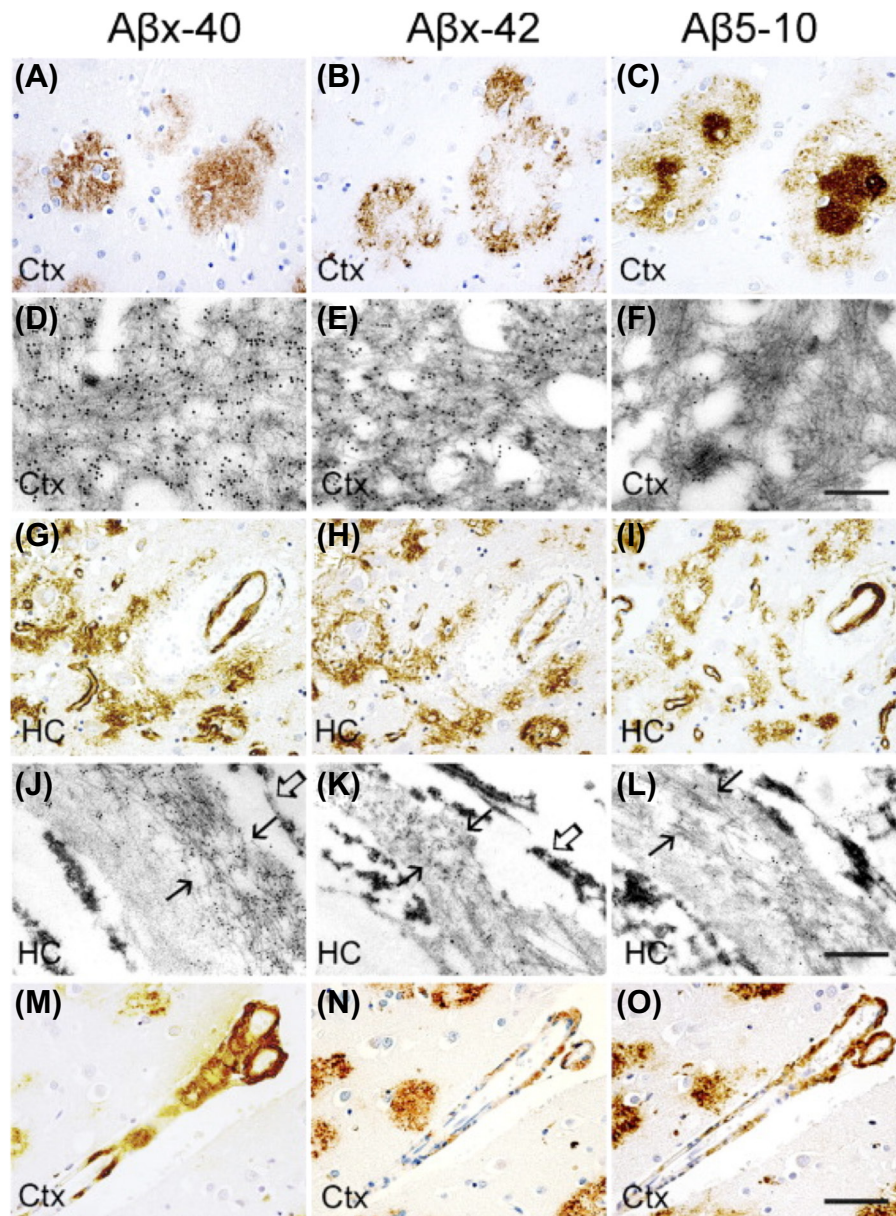


FIGURE 38.3 Ultrastructural analyses of immunostained parenchymal and cerebrovascular A β deposits in the brain of a patient with the Arctic mutation. A β immunoreactive parenchymal plaques in the temporal cortex (Ctx) examined with light microscopy (A–C) and immunoelectron microscopy (D–F) stained with A β 40-specific (A and D) A β 42-specific (B and E) and N-terminal A β antibodies (6E10; A β 5-10) (C and F). Adjacent sections with cerebrovascular amyloid angiopathy in the hippocampus (G–I) and temporal cortex (M–O) were stained with the three different antibodies and examined with light microscopy. Using electron microscopy of hippocampus (J–L), extensive positive staining (indicated by filled arrows) is found outside the basement lamina (open arrows) of a small vessel (likely a capillary) with the A β 40-specific antibody (J), whereas less labeling (filled arrow) is observed when using an A β 42-specific (K) or the 6E10 (A β 5-10; L) antibody. The scale bars represent 150 nm (D–F), 300 nm (J–L) and 150 μ m (M–O). Reprinted with permission from Philipson et al [107].

concentration levels of A β , both in patient plasma and in HEK 293 cell culture media, it enhances protofibril formation substantially (Fig. 38.4) [16,46,48,49], and this enhancement was proposed by Nilsberth et al [46] to be the cause for the FAD. Protofibrils are important intermediates in amyloid assembly [36], which cause selective neuronal death [50,51]. They have been shown to impair spatial

learning in transgenic mice [52] and thus have been suggested to be a crucial factor in disease etiology [53].

In studies examining the rates of nucleation and elongation using thioflavin T (ThT) fluorescence, among five A β 40 variants containing FAD-linked substitutions in residues 21–23 (E22G Dutch, E22G Arctic, E22K Italian, A21G Flemish, and D23N Iowa), the Arctic variant had the

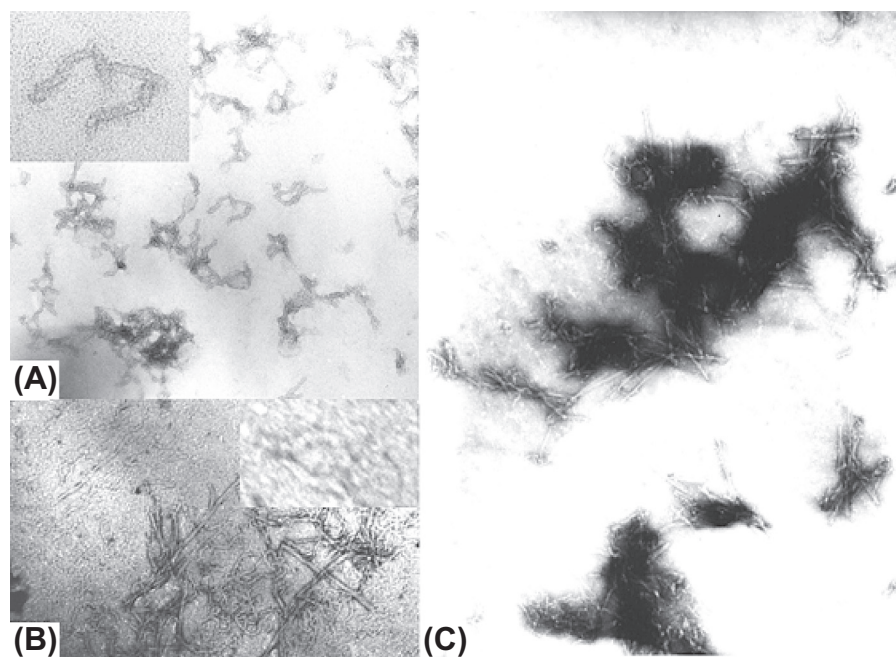
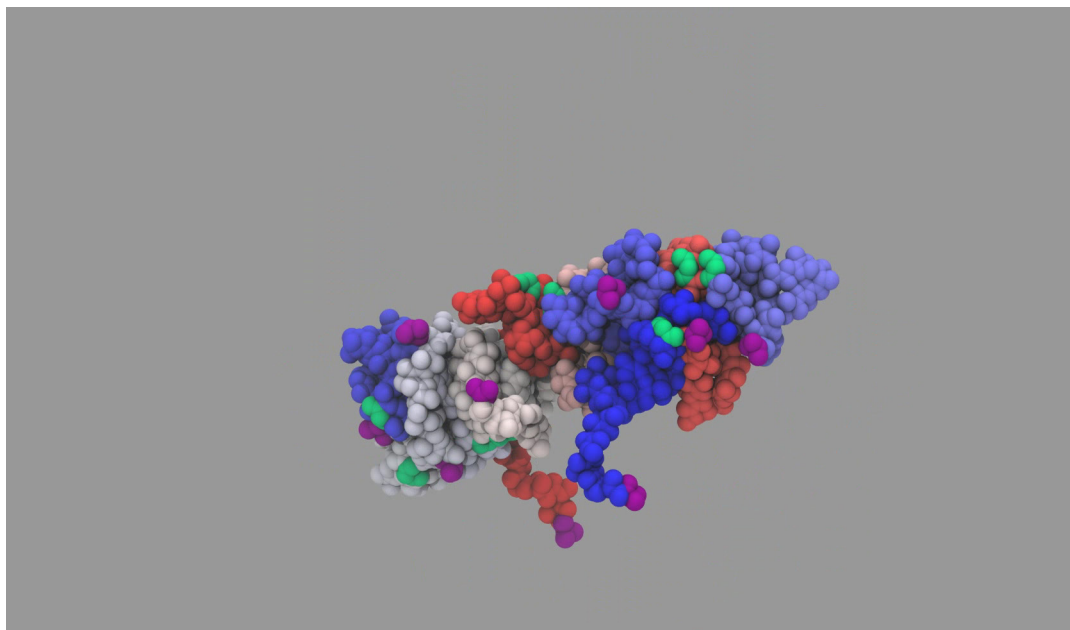


FIGURE 38.4 Electron micrographs of A β protofibrils. High-magnification transmission electron micrographs, 104 000 \times (insets, 630 000 \times), of sedimented negatively stained WT A β 40 (A) and A β 40Arc (B, C) samples. WT A β 40 protofibrils are seen with their typical curved appearance and with lengths of ~30–60 nm (A). Both curved (B, C) and straight A β 40Arc protofibrils can be discerned (C) in this preparation. The A β 40Arc protofibrils appear longer than the WT A β 40 protofibrils, and a larger percentage of straight, short protofibrils were noted. The diameters of the two protofibril types were similar (~4–6 nm; insets). A β 40Arc fibrils were also observed that exhibited large diameters (~10–18 nm) resulting from intertwining of two or more thinner fibrils (B). Scale bars, 100 nm, 20 nm (insets). Reprinted with permission from Nilsberth et al [46].

shortest lag phase—15 minutes. The lag phase is one of three phases of growth typically measured in ThT fluorescence experiments. It is followed by growth and plateau phases (Fig. 38.2). The lag phase often is seen in the early incubation times with A β 40 and is characterized by no ThT fluorescence increases. Presumably, nucleation events, similar to crystal nucleation, occur during this time and once they reach a critical concentration an exponential increase in fluorescence is observed during the growth phase. In comparison to the 15-minute lag phase of [E22G]A β 40, the lag time of WT A β 40 was 75 minutes. Similarly, the time to 50% of the maximum aggregation of Arctic A β 40, 18 minutes, was substantially lower than that of WT A β 40, 108 minutes, and was the shortest among the five variants studied [33]. Certain studies reported that oligomers, protofibrils, and fibrils of Arctic A β were indistinguishable from those of WT A β under free solution conditions (i.e. in the absence of a lipid surface) [54,55]. In contrast, an increase in fibril diameter of Arctic A β 42 was observed by Dahlgren et al relative to WT A β 42, with a corresponding decrease in the viability of neurons treated by the two A β 42 alloforms [16]. Additionally, fibrils of [E22G]A β 40 were found to be shorter and less rigid than those of WT A β 40 [55], and [E22G]A β 40 was found to give rise to higher-order oligomers than WT A β 40 or any of the other mutant forms studied, as observed by photo-induced cross-linking of unmodified proteins (PICUP) and sodium

dodecyl sulfate polyacrylamide gel electrophoresis [56]. In discrete molecular dynamics studies, the first seven amino acids in the N-terminus of [E22G]A β 40 within oligomers (Online Video 1) were found to be more flexible and solvent-exposed than in WT A β 40 oligomers [57]. Similarly, a flexible N-terminus has been observed in oligomers of WT A β 42, but not in those of WT A β 40. Thus, this is hypothesized to be important in mediating A β 42-induced toxicity [58–60] and it may play a role in the earlier onset of disease in carriers of the Arctic mutation.

When monomers of WT A β 40 or A β 40 with FAD-linked mutations at positions 21–23 were incubated for 12 hours on planar lipid bilayers formed on mica by fusion of total brain lipid extract vesicles, among all the mutants, the Arctic mutant disrupted the largest area of phospholipid bilayer [55]. Human SH-SY5Y neuroblastoma cells transfected with APP containing the Arctic mutation showed enhanced sensitivity to toxic stress, as compared to cells transfected with WT APP [61]. The Arctic mutation has been shown to increase A β production in transfected SH-SY5Y cells by favoring cleavage by β -secretase over α -secretase. This may occur due to a change in APP cellular localization from the plasma membrane – where it is accessible to α -secretase – to intracellular locations [62]. However, HEK 293 cells, transfected with APP containing the Arctic mutation, exhibited media protein concentrations with significantly lower A β 42 levels, yet similar

**ONLINE VIDEO 1** [E22G]A β 40 oligomer assembly simulation.

A discrete molecular dynamics trajectory showing the assembly process of [E22G]A β 40 from a monomer into an elongated undecamer. In this animation, only a part of the simulation box with 32 peptides is visible to allow for a close-up view of the assembly into a single elongated oligomer. The purple residue depicts the N-terminal amino acid D1, and the green residue represents glycine at position 22 of the Arctic mutant [E22G]A β 40. Distinct colors are used for individual peptide chains. Several events, in which a monomer or a smaller oligomer attaches and detaches from the oligomer under observation, can be observed during the simulation. The movie, which was created by using 400 equally-spaced time frames, spans 40 million simulation steps, equal to ~12 ms. The animation shows that the flexible N-termini play an active role in the oligomer assembly, possibly facilitating the assembly through interactions of A2 and F4 with the hydrophobic CHC and the C-terminal region.

A β 40 levels, compared to WT APP-transfected cells [46]. The cause of the discrepancy between increased A β production and decreased secreted A β 42 in these two cell culture systems requires further exploration. Mice expressing the [E693G]APP variant show increased and earlier intracellular A β immunoreactivity, and form parenchymal amyloid plaques more rapidly, than mice with comparable expression levels of WT APP [63,64]. These mice also show cognitive deficits in hippocampus-dependent learning [65].

The E22K A β (E693K APP) Italian Mutation

The Italian A β variant, in which a negatively charged glutamate is replaced by a positively charged lysine, leads to a disease similar to that caused by the Dutch variant. This variant causes severe CAA with a heterogeneous age of onset [28,66]. CAA caused by the Italian or Dutch A β variants is uniquely characterized by deposition of A β in the smooth muscle cells surrounding the cerebral vasculature as compared to the typical A β pathology in sporadic AD, which is found mostly in brain parenchyma [67]. Similar to both the Dutch and Arctic mutations, HEK 293 cells transfected with APP containing the Italian mutation show decreased secretion of [E22K]A β 42 levels, but similar levels of [E22K]A β 40 compared to cells transfected with WT APP.

[E22K]A β has a greater propensity to aggregate than WT A β [34], which, based on molecular dynamics simulations, has been suggested to be due to an increase in α -helix formation in the region A β 20–24 compared to WT A β 42, potentially leading to an increase in helix–helix interactions among monomers and thus an increase in the alignment of unstructured regions near the helices to facilitate oligomerization [68]. Lin et al suggested that the increase in α -helix formation might be attributable to a loss of α -helix destabilizing electrostatic repulsion between E22 and D23 and/or to the longer aliphatic side chain of lysine relative to glutamate, which might provide a favorable hydrophobic interaction with V18 [68]. The Italian mutation in A β 42 leads to increased toxicity in rat pheochromocytoma (PC-12) cells using the MTT assay [34] or primary cultured human cerebrovascular smooth muscle cells measured by the fluorescent Live/Dead cell assay [67] compared to WT A β 42. There is no consensus about the rate of aggregation of [E22K]A β compared to other variants containing substitutions at positions 21–23. The Italian A β variant has been reported to be among the fastest and among the slowest aggregators [31,32]. Betts et al [33] showed that the E22K substitution resulted in a decrease in the nucleation rate but an increase in the fibril-elongation rate of [E22K]A β 40 compared to WT A β 40 (Fig. 38.2), which may account for these conflicting results. [E22K]A β 40 showed larger oligomers [55,56] and shorter and less rigid fibrils relative to WT A β 40, yet these characteristics did not correlate with the degree of disruption of the phospholipid bilayers, which were similar for the Italian and WT A β forms [55].

The E22 A β (E693 APP) Deletion – Osaka Mutation

A FAD-linked A β variant lacking the glutamic acid residue at position 22 was identified in 2008 in a Japanese kindred [69]. Based on their study of this A β variant and the affected individuals, Tomiyama et al hypothesized that the E22 deletion (Δ E22) might represent the first recessive mutation linked to AD because only homozygote carriers showed AD-type dementia. Possibly, the mutation acts in a dose-dependent manner or has incomplete penetrance [69]. Δ E22 mutation carriers showed reduced levels of retaining the amyloid-specific Pittsburgh compound-B in positron emission tomography relative to patients with sporadic AD [69].

The deletion reduced total A β secretion levels significantly in HEK 293 cells transfected with a mutant APP construct, compared to WT A β , without affecting the A β 42/40 ratio. Synthetic [Δ E22]A β 42 assemblies inhibited long-term potentiation more potently than WT A β 42 [69], and induced synapse loss in mouse hippocampal slices [70], providing a clue about the mechanism by which this A β variant causes FAD even though A β levels are reduced.

In vitro, [Δ E22]A β 40 formed β -sheet structures 400-fold faster than WT A β 40 as measured by circular dichroism spectroscopy, and [Δ E22]A β 42 formed β -sheet conformation so fast that it was difficult to measure before the observation of large amounts of β -sheet [71]. The effect of the [Δ E22]A β 40 mutation on oligomer distribution, studied by PICUP, was reduced abundance of dimer, trimer, and tetramer relative to WT A β 40 (Fig. 38.5). The oligomer size frequency distribution of [Δ E22]A β 42 was distinct from that of WT A β 42 and was characterized by a relatively high abundance of dodecamers and octadecamers [71]. The Δ E22 variants of both A β 40 and A β 42 formed short protofibrils and fibrils immediately upon solvation from lyophilizates, whereas the WT peptides only showed globular and small, string-like structures [71]. The critical concentration for fibril formation of the deletion variants was approximately half that of their corresponding WT alloforms [71]. Inayatullah et al concluded that the primary biophysical effect of the Δ E22 mutation was to accelerate and stabilize conformational changes in monomeric A β [71], which has been suggested to be the rate-limiting step in fibril elongation [72,73]. Additionally, this variant was found to be more resistant to degradation by neprilysin and insulin-degrading enzyme, both of which are known A β -degrading enzymes [74], than WT A β 40 [69].

The A21G A β (A692G APP) Flemish Mutation

The Flemish mutation in APP was discovered in 1992 by Hendriks et al [75]. The mutation leads to the substitution of a nonpolar, neutral alanine residue in position 21 of A β by a nonpolar, neutral glycine. This substitution leads to a decrease in the hydrophobicity and loss of chirality of residue 21 with no net change in peptide charge [55]. Carriers of the Flemish mutation

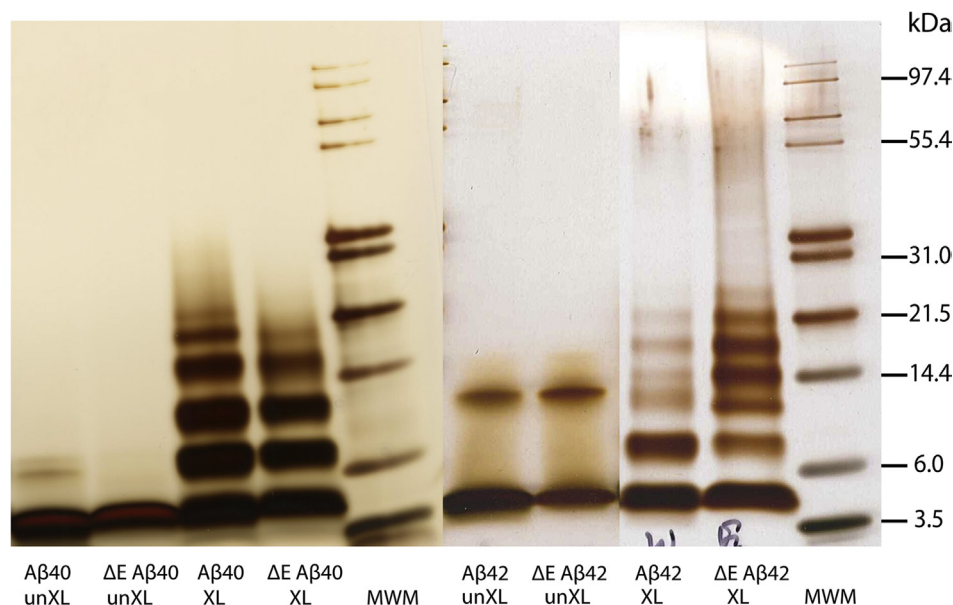


FIGURE 38.5 Oligomer distribution of A β and Δ E22 A β . SDS-PAGE and silver staining of PICUP-stabilized material was used to study the oligomer distribution of A β and the Δ E22 variant. Un-cross-linked = unXL, cross-linked = XL, molecular weight marker = MWM. Image courtesy of Drs. Mohamed Inayatullah and David Teplow.

develop FAD with presenile dementia characterized pathologically by unusually large plaque cores and cerebral hemorrhage due to CAA with an age of onset in the mid 40s [75–77]. Interestingly, unlike the other intra-A β mutation-caused substitutions, the A21G substitution was found to be associated with increased production of both A β 40 and A β 42 [46,78].

A 4-fold increased A β secretion was observed in HEK 293 cells transfected with APP harboring the Flemish mutation, compared to WT A β [79,80]. The A β (17–23) region has been found to inhibit γ -secretase cleavage by binding to an allosteric site within the γ -secretase enzyme complex. This inhibitory region is disrupted by the Flemish mutation, which reduces the inhibitory potency of the domain and leads to increased A β production [80]. Additionally, monomers of [A21G]A β 40 are degraded significantly more slowly by neprilysin, though not by insulin-degrading enzyme or plasmin, compared to WT A β 40 [33].

In cell culture experiments, the toxicity of [A21G]A β 40 was similar to that of WT A β 40 [34,81]. Unlike most other mutations leading to intra-A β substitutions, the Flemish A β 40 variant not only does not have a greater propensity to form fibrils but actually has slower fibrillogenesis kinetics than WT A β 40, including a longer lag phase and the longest time to half-maximal ThT fluorescence among five A β 40 variants containing substitutions in residues 21–23 (Fig. 38.2) [33,82]. Similarly, [A21G]A β 42 showed almost no increase in ThT fluorescence over 24 hours [34]. Experiments examining the oligomer size distribution of [A21G]A β 42 found a narrower distribution with increased percentage of paranuclei, which have been suggested to be important in the toxicity difference between A β 40 and A β 42 [20], compared to WT A β 42 [56],

potentially contributing to the etiology of Flemish FAD. A different study found that the extent of phospholipid bilayer disruption by the Flemish A β 40 variant was decreased compared to WT A β 40 *in vitro* [55]. Though this difference was attributed to the increased hydrophilic character of glycine relative to alanine [55], it is unlikely that one methyl group would be entirely responsible for such a difference. Rather, the increased flexibility of the glycine likely confers conformational changes that lead to the observed reduced interaction of [A21G]A β 40 with the membrane mimetic relative to WT A β 40.

Though the A21G substitution does not enhance the toxicity or aggregation kinetics of A β , it does increase the brain levels of A β by both increasing production [46,78] and decreasing clearance [33]. Thus, by comparing the Flemish mutation to other mutations we may gain insight into the role of A β concentration versus its aggregation kinetics in AD onset.

The D23N A β (D694N APP) Iowa Mutation

The Iowa mutation was described first in 2001 [83]. The substitution results in severe CAA with the addition of neurofibrillary tangles and an unusually larger proportion of A β 40 in amyloid plaques. This substitution replaces a negatively charged aspartate with a neutral asparagine, resulting in dementia around the sixth or seventh decade of life [83]. The Iowa A β variant leads to the formation of larger oligomers [55,56], has a greater propensity to aggregate, and is more potently toxic to PC-12 cells than WT A β [34]. [D23N]A β 40 fibrils grow with a much shorter lag period and shorter growth time to half-maximal β -sheet level, second only to the Arctic mutant on both traits (Fig. 38.2) [33].

Interestingly, the D23N substitution has a dramatic effect on the quaternary structure of A β within amyloid fibrils. Over the last decade, multiple solid-state NMR studies showed that within fibrils, A β was arranged in parallel, in-register β -sheets [84–86]. The Iowa variant is the first one that has been shown to form both parallel and antiparallel β -sheet fibrils [87]. This finding was interesting not only because it was novel but because it also raised many questions about how a one-amino-acid modification could result in a major change in the quaternary structure that had been consistently observed for other variants.

N-terminal and C-terminal Substitutions

The A2T Versus A2V A β (A673T, A673V APP) Mutations

In 2012, a mutation that replaces an alanine at position two with threonine, which previously had been partially described in a 1993 stroke patient [88], was identified in an Icelandic population to be protective not only against AD, but also against general cognitive decline in the elderly [89]. Jonsson et al discovered that the A2T substitution resulted in a reduction of APP cleavage by β -secretase by approximately half compared to WT APP, leading to ~40% less total A β concentration.

Interestingly, a mutation causing an A2V substitution had been identified in an Italian population, in which it causes recessive, very early onset FAD. The proband, in whom the disease was first diagnosed, was 36 years old at the onset of cognitive deficits [90]. This mutation increased A β production via a change in APP processing and led to enhanced formation of A β fibrils *in vitro*. Incubation of [A2V]A β 40 with WT A β 40 or [A2V]A β 42 with WT A β 42 resulted in instability of A β aggregates—more amorphous aggregates were observed by electron microscopy than in preparations of WT A β alone or [A2V]A β alone, and more low-molecular-weight peptide was observed by size-exclusion chromatography (SEC). In cell culture experiments, mutant:WT A β heteromers had diminished neurotoxicity, supporting the autosomal recessive pattern of inheritance [90].

The effect of substitutions at position 2 in A β , and of mutations N-terminal to the β -secretase cleavage site, i.e. outside the A β region (Fig. 38.1, Swedish double-mutation), which lead to increased A β levels, suggests that WT APP is a lower efficacy substrate for β -secretase and that at least the two residues N-terminal and the two residues C-terminal of the β -secretase cleavage site are critical for the enzymatic process [91].

The English H6R A β (H677R APP) and Tottori D7N A β (D678N APP) Mutations

Additional FAD-linked mutations in the N-terminal region of A β include the English mutation, which causes a H6R substitution [92], and the Tottori-Japanese mutation leading

to a D7N substitution [93]. Both the English and the Tottori mutations have been shown to enhance fibril formation through a reduction in lag time [94] and a facilitation of the elongation phase [95]. Ono et al [94] also noticed overall accelerated kinetics in transitions of secondary structures from statistical coil to α -helix to β -sheet. Both mutations lead to increased average oligomer size, and are substantially more efficient at seeding fibril formation than WT A β 40 or A β 42 oligomers. The English and Tottori variants also are significantly more toxic than the WT counterparts in PC-12 cell culture as measured by the LDH assay (Fig. 38.6) [94]. Interestingly, in different experiments where [H6R] A β 40 or [D7N]A β 40 were seeded with WT A β 42, in addition to eliminating the lag phase, substantial acceleration of the elongation phase also was observed [95]. When seeds made of the mutant forms or of WT A β 40 were compared for their nucleating ability side-by-side, ThT fluorescence increased more rapidly when mutant A β isoform seeds

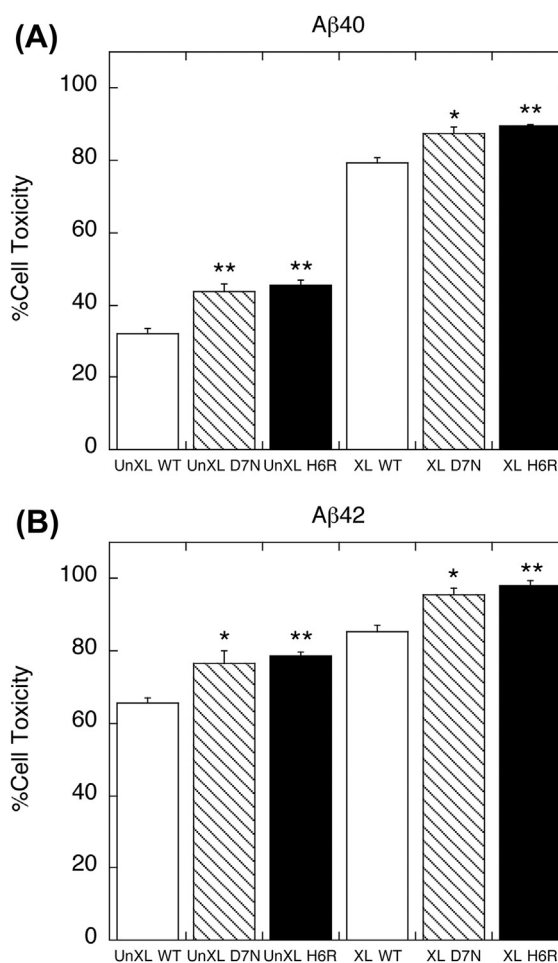


FIGURE 38.6 Toxicity of H6R and D7N measured by the lactate dehydrogenase assay. Differentiated PC-12 cells incubated for 48 hours with 25 μ M un-cross-linked (UnXL) or cross-linked (XL) A β 40 (A) and A β 42 (B) of WT, H6R, or D7N were subjected to the lactate dehydrogenase release assay of cell toxicity. Data represent three independent experiments (* p < 0.05; ** p < 0.01). Image courtesy of Drs. Kenjiro Ono and David Teplow.

were incubated with their respective monomers, suggesting that the mutant peptides have higher affinity for their homologous peptide monomers than for the WT monomers [95]. Surprisingly, the accelerated fibril formation was not accompanied by accelerated protofibril formation as measured by SEC [95]. The observation that seeding of fibrils is significantly more efficient with the English and Tottori A β variants suggests that the N-terminus is involved in the nucleation process [95]. This is consistent with *in vitro* biophysical experimental findings using scanning amino acid substitutions and PICUP, which showed that substitution of the first residue of A β from aspartic acid to tyrosine led to significant changes in the oligomer distribution pattern of both A β 40 and A β 42, possibly caused compaction of oligomer structures, and slowed assembly kinetics [96].

The K16N A β (K687N APP) Mutation

A mutation discovered in 2012 to result in dementia with an autosomal dominant inheritance pattern induces a lysine to asparagine substitution at position 16 [97], directly N-terminal to the cleavage site of α -secretase, which leads to the formation of the non-pathologic peptide p3 (A β 17–x; Fig. 38.1). In experiments using HEK 293 or SH-SY5Y cells, this mutation resulted in decreased sAPP α and sAPP β concentration levels and decreased turnover of full-length APP at the cell surface. These observations were hypothesized to be due to prolonged half-life of full-length APP caused by the reduction in α -secretase cleavage [97]. *In vitro* experiments demonstrated that [K16N]A β (11–28) was a poorer substrate for the ADAM10 α -secretase than WT A β (11–28). Additionally, cell culture experiments showed that α -CTF concentration levels were reduced significantly and that β -CTF increased significantly with a corresponding increase in levels of both A β 40 and A β 42 [97]. In SH-SY5Y cells and in rat primary hippocampal neurons, [K16N]A β 42 was found to be less toxic than WT A β 42 at equal concentrations. However, a mixture of the two adding up to the same concentration was equally or more toxic than WT A β 42 alone (Fig. 38.7). The mixture of [K16N]A β 40 and WT A β 40 showed higher toxicity than WT A β 40 alone. Interestingly, [K16N]A β 40 alone showed either slightly more or equal toxicity to WT A β 40 alone, depending on the cell type and oligomer size measured by SEC [97]. Examination of fibrillar morphology by electron microscopy suggested that neither [K16N]A β 40 nor [K16N]A β 42 formed rigid mature fibrils by 24 hours, and mixture of either of the mutant peptides with the corresponding WT A β inhibited the formation of mature fibrils and favored protofibrillar aggregates [97], providing one possible explanation for the high toxicity of these mixtures. Incubation of [K16N]A β 42 alone with neprilysin showed decreased degradation compared to WT A β 42 alone. Moreover, the mixture of [K16N]A β 42 and WT A β 42 also showed resistance to neprilysin

degradation, suggesting another way by which the mutation can be linked to FAD. A mechanism proposed for the increased stability of the mixed oligomers is the potential for K16 of one monomer to form a salt bridge with N16 of another monomer, which could locally stabilize the β -sheet structure [97].

The L34V A β (L705V APP) Piedmont Mutation

Similar to the disease phenotype of amino acid substitutions in the 21–23 region of A β , the Piedmont mutation that substitutes V for L at residue 34 is characterized primarily by CAA. Interestingly, carriers of this mutation showed an absence of parenchymal A β deposits and neurofibrillary tangles [98]. ThT studies comparing WT A β 40 with [L34V]A β 40 showed little increase in fluorescence for both A β types up to 2 days of incubation. On day three, [L34V]A β 40 fluorescence levels began increasing slowly but steadily and at a faster rate than WT A β 40 [99]. Electron microscopy studies of the morphology of the Piedmont A β variant showed protofibrils after 3 days of incubation, compared to only small globular structures seen with WT A β 40 [99]. In cell culture toxicity studies using immortalized human brain microvascular endothelial cells, [L34V]A β 40 showed higher toxicity levels than WT A β 40 and similar to those of [E22Q]A β 40. Toxicity was measured as the number of morphologically apoptotic cells at 72 hours of incubation. With cultures of vascular smooth muscle cells, 72 hours of incubation with [L34V]A β 40 resulted in even higher toxicity than [E22Q]A β 40 [99].

Silent and Incomplete Penetrance Mutations

In addition to the mutations mentioned above, which lead to amino acid substitutions inside the A β sequence, other mutations have been identified in the A β -coding region of *app* in AD-afflicted individuals that either do not cause an amino acid change, i.e. are silent, or do not have complete penetrance and thus are found also in non-AD individuals. Silent mutations have been found in AD-afflicted individuals at A β residues 34, 37, and 40 [42,100–102]. The mutation at residue 37 has been identified in several instances, including three cases with a family history of AD [42,102], one case of cerebral hemorrhage in a 41-year-old individual with no family history of AD [102], and two cases of healthy individuals, one 35-year-old with no family history of AD [102] and the other an 11-year-old child [101]. An A42V substitution has been observed in a person with schizophrenia with cognitive deficits [103] but also in a non-schizophrenia-afflicted person [100]. Finally, a double mutation that causes a substitution of A42T A β (A713T APP) and a silent base change at APP residue 715 also has been observed in an individual with early-onset AD, yet five relatives who carried this mutation did not present with AD [104]. It is difficult to hypothesize how silent mutations could cause disease, though the codon

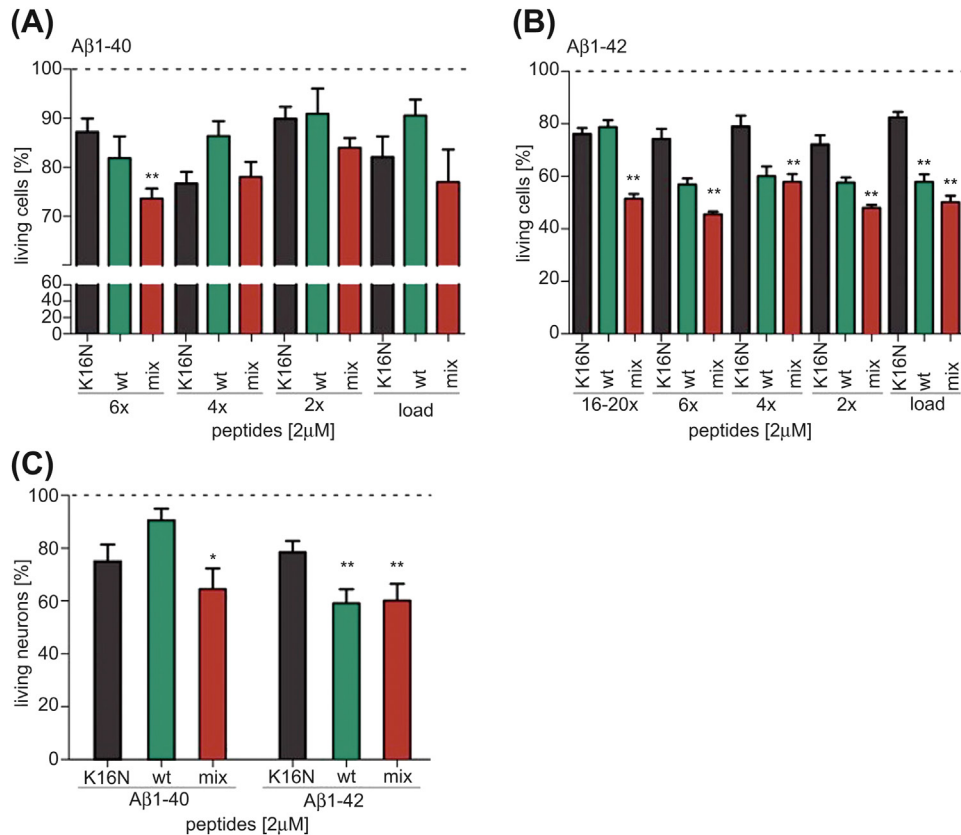


FIGURE 38.7 Oligomerization and toxicity of K16N substituted A β peptides. SH-SY5Y cells were incubated for 12 hours with either 2 μ M freshly dissolved peptides (load) of A β 40 (A) or A β 42 (B), or oligomers (2–20x) obtained by SEC. (C) Primary hippocampal neurons were incubated for 48 hours with 2 μ M freshly dissolved peptides. Toxicity was determined by percentage of living cells compared to untreated control cells ($n = 4-8$). The data are presented as mean \pm SEM. (* $p < 0.001$, ** $p < 0.0001$). Reprinted with permission from Kaden et al [97].

usage bias or organism preference for one of many codons that encode the same amino acid, may have relevant functionalities and thus consequences. It is possible that these mutations also are random and do not correlate with AD. If this is the case, study of silent mutations may help to define the limitations of tolerable change in the A β sequence [104]. If the substitution-causing mutations actually are relevant to AD pathology, but also can be found in non-afflicted individuals, further exploration will be required into incomplete penetrance and co-factors that lead to disease.

CONCLUSIONS

Studies of mutations affecting the A β amino acid sequence have been critical in illuminating the regions within A β where structural changes may lead to disease and the molecular interactions they are involved in.

As mentioned at the end of the section on the Dutch mutation (above), one factor that can facilitate A β self-assembly is the loss of electrostatic repulsion among monomers [41]. This theory is supported by the increase in aggregation properties of the Dutch [E22Q], Arctic [E22G], Tottori [D7N],

and Iowa [D23N] mutations, where a negatively charged amino acid is substituted by a neutral one, changing the net charge of the A β peptide from -3 to -2 . Similarly, the E22 deletion causes the loss of a negative charge. In the case of the Italian mutation, although local repulsion between the positively charged lysine residues in position 22 still exists, the overall charge of A β is reduced from -3 to -1 . The opposite situation happens with the K16N mutation, which leads to loss of local repulsion between the lysine residues in position 16, but an overall increase in net charge to -4 . This analysis suggests that local electrostatic repulsion, global changes in peptide net charge, and other factors affecting A β conformation and assembly, may be linked to FAD caused by the corresponding mutations.

Another factor may be the destabilization of 'native' metastable structures. In the A β monomer, a turn region has been identified within the decapeptide A β (21–30), which may be one of the earliest conformations formed [105]. This turn, which was hypothesized to nucleate A β folding and assembly, is stabilized by hydrophobic interactions between V24 and K28 and by long-range electrostatic interactions between K28 and either E22 or D23. The destabilization of

the turn by substitutions (or deletion) at positions 22 or 23, but not 21, and the positive correlation observed between such destabilization and higher oligomerization propensity of the Dutch, Arctic, Italian, and Iowa A β variants, have been implicated in the causation of the resulting FAD [106].

Many of the FAD-linked mutations that affect regions of APP outside of the A β sequence increase A β levels. In contrast, the Dutch, Arctic, Italian, Δ E22, and A2T modifications cause a decrease in secreted A β . Because both a decrease in A β levels, resulting from intra-A β substitutions, and an increase in A β levels, resulting from other FAD-linked mutations, cause disease, the concentration of A β may be only part of the problem. This conclusion is supported by the existence of non-demented individuals with extensive A β plaque pathology. The studies described here suggest that A β sequences that differ from one another by one amino acid can have substantially distinct aggregation kinetics and degrees of toxicity. Importantly, the differences in assembly kinetics and aggregate morphology likely correlate with differences in toxicity of the A β variants. Interestingly, the Flemish mutation increases A β levels, possibly due to loss of an inhibitory domain in A β (17–23) [80]. In view of the decrease in A β levels due to changes at residue 22, it is possible that A21G causes a loss of function of the inhibitory activity whereas E22G, E22K, E22Q, and Δ E22 cause gain of function of this inhibitory domain.

Mutations discovered in the *app*, *psen1*, and *psen2* genes leading to FAD suggested a causative role of A β in AD. The mutations affecting specifically the A β sequence provide particular insight into important regions, interactions, and structures involved in the way A β self-assembles and affects susceptible brain regions. These studies also highlight the paramount impact one amino acid change can have on multiple characteristics from protein function and folding to brain pathology and age of disease onset.

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