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SPECIAL ISSUE

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Intracellular biology of Alzheimer's disease amyloid beta peptide

Abstract Strong evidence links excess production of a small peptide and the pathogenesis of Alzheimer's disease (AD). Originally this peptide, beta-amyloid 42 (Aβ42), was assumed to be released by a pathogenic event; it is now well established that Aβ42 is released from cells during normal cellular metabolism of the Alzheimer amyloid precursor protein. Recently, in a series of surprising reports it was discovered that Aβ42 is produced intracellularly, and what might have been regarded first as a strange abnormality of a few selected cell lines has now been recognized as an important cellular pathway for Aβ production. Moreover, the differences between secretory and intracellular Aβ production might hold the clues for brain specificity and cellular mechanisms of AD pathogenesis.

Key words Cell biology · Endoplasmic reticulum · Golgi trans Golgi network · Amyloid · Alzheimer's disease

Introduction

After the initial identification of the small 4 kDa peptide beta amyloid (Aβ), the major component of the amyloid plaque [1, 2] and the subsequent cloning of its parent molecule, the Alzheimer amyloid precursor protein (APP) [3], a lot of effort has been put into the identification of the basic cell biology and the molecular pathways of Aβ generation. Substantial evidence based on the results gained from this work has provided substantial evidence for a central role of Aβ in Alzheimer's disease (AD). Remarkably more than 60 familial AD mutations located in three different genes (APP, PS I, and PS II; reviewed in Sandbrink et al. [4]) apparently result in the overproduction of a specific Aβ, isoform, Aβ42, which was also shown to be the major Aβ isoform deposited in AD [5–8]. Still unresolved is whether Aβ42 might impair neuronal function in AD and how it might affect cellular function. Much attention has been focused on extracellular Aβ depositions, the amyloid plaque, which is typical but not limited to AD. The coincidence of Aβ plaque load and AD argues for an active role of the plaque in AD. However, the role of amyloid plaques on the etiology of AD is strongly debated. For example, it is mostly accepted that synthetic Aβ applied to a variety of cells is toxic or induces neurodegeneration and microglial activation in vivo [9], yet this is opposed by the fact that evidently healthy neurons are found in the proximity of the plaque. This example nicely illustrates that Aβ possesses properties of a toxin but the very route how Aβ accesses the neurons might decide whether it is a harmful or harmless substance. Aβ was originally described as an extracellular substance but recently has been recognized to be produced intracellularly [10–26] and on or close to the cell surface [27, 28].

Intracellular protein accumulation is not specific for Alzheimer's disease alone, but has been shown in other degenerative diseases such as the recently discovered toxic nuclear aggregates of Huntingtin in Huntington's disease [29, 30], and intracellular accumulations of α-synuclein in Parkinson's disease [31] were noticed. PrP accumulations in intracellular vesicles in Creutzfeld Jakob's disease and nuclear accumulations of Ataxin in Machado Joseph's disease (SCA-3, reviewed in [32]) have been known for a long time. Interestingly in IAPP-Diabetes II amyloidosis intracellular depositions precede the degeneration of pancreatic islet cells [33, 34]. Furthermore, Aβ is not the only protein that is deposited intracellular in AD. Hyperphosphorylated tau protein is accumulated in neurofibrillary tangles, a feature of AD that had been recognized early [35, 36]. *In vivo* intracellular Aβ is known to exist in vascular smooth muscle cells [37] and perivascular or neuropil glia [38] but evidence from neurons is still missing. This review deals with the recent knowledge that has been gained about the intracellular production and accumulation of Aβ and the possible consequences of intracellular Aβ on the etiology of AD.

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APP

APP is a ubiquitously expressed type one transmembrane protein. The Aβ region extends from the middle of the lipid bilayer into the luminal or ectodomain of APP. Two proteases are involved in the release of Aβ from APP. The cleavage of the Aβ domain within the transmembrane region of APP highlights the first problem of Aβ generation since cleavages inside a lipid bilayer is poorly understood and poses theoretical problems with regard to accessibility and protease activity. It was, therefore, originally proposed that Aβ is released only during a pathological process [39]; however, Haass et al. [40] showed that Aβ is secreted by normal cellular metabolism. Until now no cell type is known to express APP without accompanying Aβ production. Aβ was found initially only as a small peptide in conditioned media, biological fluids, and plaques. As initial attempts failed to show intracellular Aβ, Aβ was assumed to be produced either in early endosomes or at the cell surface [27, 28].

Secretory Aβ

The interpretation of the cellular pathways resulting in Aβ production is complicated due to the presence of three major proteolytic activities (APP secretases) involved in the degradation of APP. Most APP molecules are cleaved close to the cell surface by an activity named α-secretase [41, 42]. Cleavage by α-secretase occurs inside of the Aβ domain at amino acid 17 of Aβ (Fig. 1) and therefore does not result in Aβ production but in release of secretory APPa. It should be mentioned that neither α-secretase nor the other APP-secretases have yet been identified with certainty, and it appears that actually several different proteases are involved in the respective secretase cleavages [43, 44]. Aβ and Aβ-like peptides have been found to be produced with strong N- and C-terminal heterogeneity (Reviewed by Evin et al. [45]); for several cleavage sites it is actually difficult to associate them to a specific APP secretases proteolytic activity [46].

The N-terminus of Aβ is generated by β-secretase cleavage, releasing APPβ but not immediately Aβ. α- and β-secretases both leave the APP C-terminal fragment, a transmembrane stump, behind. A third protease, named γ-secretase, is required for production of Aβ (β) and p3(α) [27]. γ-secretase appears to be activated only after the previous cleavage by other secretases [47]. Therefore, only the successive action of β- and γ-secretase results in production of Aβ; any inhibition of one of these proteolytic activities results in the abolishment of Aβ production. Furthermore, increased activity of α-secretase reduces Aβ production as well. α-secretase was reported to be located mainly on the cell surface [41, 48] and in the late secretory pathway [10, 27, 49–52]. Although the relative contribution and exact localization of cellular α-secretase activity appears to differ between different cell types. In general, the localization of all APP secretase activities appears to be subject to variations between cell types, and a large difference seems to be between neurons and nonneuronal cells. As discussed below detailed re-examination of different cell lines mostly resulted in the discovery of the presence of APP-secretase activities in the same cellular compartments that were previously identified in other cell types. Reported differences seem to be more of quantitative rather than qualitative nature and cells appear to possess the same basic mechanisms for APP degradation but differ in the way that they are utilized.

Cell surface APP that escaped cleavage by α-secretase is internalized into endosomes and processed by β-secre-

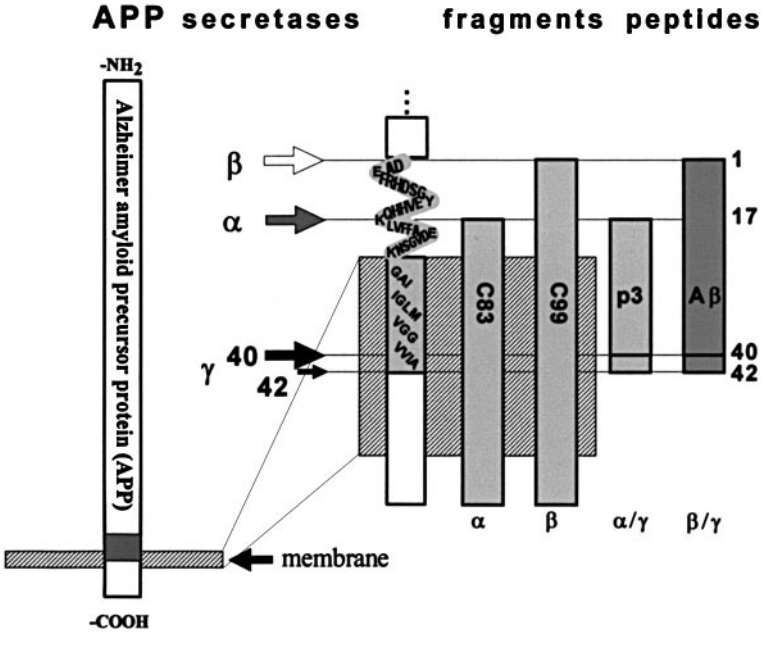

Fig. 1 APP structure and basic processing: APP is produced as type 1 transmembrane protein, cleavage at amino acids 1 and 17 results in the production of the respective secretory APP species APPα and APPβ (not shown) and of the C-terminal fragments C99 and C83 which are still membrane bound. The C99 and C83 fragments are further cleaved by γ-secretase at positions 40 and 42 of the Aβ region and results in the release of Aβ and p3. The relative abundance of Aβ42 cleavage depends on the cellular localization of γ-secretase activity and can be increased by the presence of familial AD mutations either in the APP or presenilin genes

tase, resulting in the C-terminal fragment APP-C99, or is transferred to lysosomes for final degradation [28, 53].

Following β- or α-secretase processing of APP, the remaining C99 or C83 APP fragments might become cleaved by γ-secretase either on the cell surface or possibly in endosomes [16, 27, 28, 48, 53]. The majority of the Aβ produced is Aβ40; only 5–10% of secretory Aβ is the disease linked isoform Aβ42.

In the special case of the "Swedish" mutant APP, the β-secretase cleavage site is mutated, resulting in very strong β-secretase activity [54]. Cleavage occurs during passage through the late secretory pathway and might involve an additional β-secretase enzyme [55].

Neurons, as opposed to peripheral cells, evidently possess higher levels of β-secretase activity while α-secretase activity is low [56, 57]. This results in a higher ratio of Aβ/p3 production for neurons.

Intracellular Aβ

Intracellular Aβ partially overlaps with secretory Aβ as some intracellular Aβ species might eventually become secreted. This is most likely for Aβ40 and TGN derived Aβ42 [16, 20, 24]. Intracellular Aβ production was first reported in cultures of NT2N cells by Wertkin et al. [58]. NT2 cells are human teratocarcinoma cells which irreversibly differentiate into neurons and nonneuronal cells when exposed to retinoic acid [59]. Differentiated neurons are called NT2N cells. During the differentiation, together with a change in the APP splice form from KPI containing APP to APP695, the amount of intracellular Aβ continuously increases, underlining the importance of the neuronal phenotype for intracellular Aβ production. Following this report, intracellular Aβ was found to be produced by a variety of other cell types including primary hippocampal neurons, human neuroblastoma cells Sy5y, mouse neuroblastoma cells Neuro 2a, and several peripheral cell lines including 293, COS7, and CHO cells [14, 16, 18, 20, 21, 48]. The estimates for the relative ratio between total secretory and intracellular Aβ vary between 1:3 (intracellular: extracellular Aβ) in neurons and 1:1000 in some peripheral cells (Hartmann, unpublished observation). These estimates are complicated by the facts that some intracellular Aβ is likely to become secreted by the different solubility of intracellular Aβ pools, and by the different biological half-life of intracellular and secretory Aβ. This is illustrated by the fact that NT2N cells after one week in cell culture contain approx. 26 fmol soluble Aβ/mg and secrete 53 fmol Aβ/ml/24h. After 6 weeks the cellular content increases to 44 fmol/mg and secretion to 266 fmol/ml/24h [13].

In spite of the fact that pulse chase experiments revealed that intracellular Aβ is produced before secretory Aβ [12–14], it might still be possible that Aβ is first produced at the plasma membrane and immediately internalized. For example, Sy5y cells are able to take up high amounts of synthetic Aβ. However, primary rat hippocampal neurons internalize relative small amounts of synthetic Aβ [60]. Since both cell types had been used for intracellular Aβ studies the true intracellular origin had to be confirmed. Co-incubation of APP overexpressing, metabolically labeled cells with nonlabeled, wild type cells did not show a relevant uptake of extracellular Aβ. The levels of internalized Aβ were in all cases reported below the levels of the intracellularly produced Aβ [11, 12, 16, 61]. Copurification of cell surface associated Aβ was ruled out by digestion of cell surface proteins with trypsin; no difference between trypsin digested cells and nondigested cells was found [11, 13, 48]. NH_4Cl alkalizing exposure to cells clearly demonstrated that intracellular Aβ generation is independent of endocytosis and that both pools are distinct and derived by separate mechanisms [14].

Since Aβ42 is the most important Aβ isoform for AD, it is compelling that intracellular Aβ has a high content of Aβ42; approx. 1/3 of the intracellular Aβ is Aβ42 [13, 14]. While Aβ42 has been studied in detail; it should be noted that due to lack of suitable antibodies the study of non Aβ40/42 Aβ peptides has thus far been mainly neglected. Familial AD mutations led to the discovery of the importance of Aβ42. If intracellular Aβ is involved in the generation of AD, one would expect to find increased intracellular Aβ42 production in the presence of AD mutations. APP containing the "London" mutation [62] increases Aβ42 levels without changing the overall Aβ production [63]; intracellularly the same is found [14]. APP containing the "Swedish" mutation [64] results in an overall increase of Aβ including both Aβ40 and Aβ42 [8, 54]. Intracellular Aβ produced from this protein shows a reduced 42/40 ratio but the net result was still an increased total amount of Aβ42. However, this effect differs strongly between the cell types tested [14, 19].

Compartments of intracellular Aβ generation

A lot of attention has been focused on the subcellular compartments involved in the production of intracellular Aβ. One major reason for this is that the compartment of Aβ42 production is identical to the preferred compartment for the presenilins. Presenilin 1 mutations induce the earliest onset of familial AD known [65], and since it was known that presenilins (PS 1 and PS 2) increase Aβ42 production [5, 54] a missing link was sought for Aβ42 and the presenilins. The presenilins are predominantly localized in the ER and to a lesser extend in the Golgi apparatus [66]. The presence of small amounts of presenilin in other organelles, however, is likely. Using a variety of control experiments including brefeldin A and temperature blocks, pulse chases, immunocytochemistry, and addition of an ER-retrieval signal to APP, it was surprisingly found that Aβ42 is produced in high amounts inside of the neuronal ER [16, 17]. The same processing pathway does exist in other cell types as well, though the overall rate of ER Aβ42 production is considerably smaller [18, 21, 26]. A prerequisite for Aβ production is the cleavage of APP by β-secretase; therefore, β-secretase activity should be

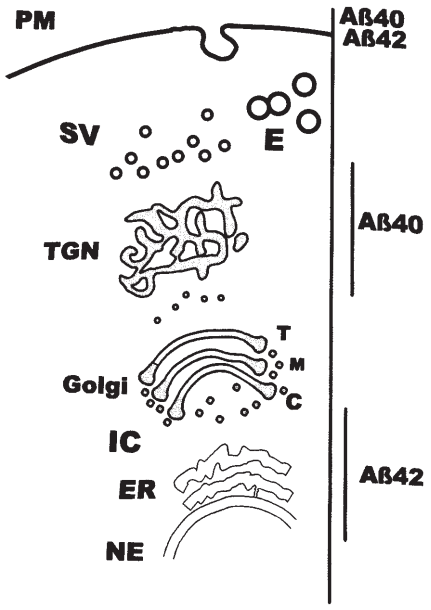

Fig. 2 Distribution of Aβ production. Intracellular Aβ42 is mainly produced in the ER/IC, Aβ40 in the TGN, and additional Aβ production was observed in the Golgi compartment. Results obtained with PS 1 wt transfected cells are not included in this figure since the relevance of PS overexpression on the localization of intracellular Aβ is not yet obvious, in general these cells showed a more relaxed distribution than non PS 1 transfected cells, however, not as strong as PS-1 mutated cells. Abbreviations: PM, plasma membrane; SV, secretory vesicles; E, endosomes; TGN, trans Golgi network; IC, ER Golgi intermediate compartment; ER, endoplasmic reticulum; NE, nuclear envelope

present in the ER to allow subsequent Aβ42 production. Data by Chyung et al. [15] prove that this is in fact the case. Interestingly this activity was not detected in non-neuronal cells possibly explaining the smaller intracellular Aβ42 production by these cells, emphasizing the preferential role of neurons for intracellular Aβ production.

The relevance of PS 1 to Aβ production goes far beyond this colocalization since knock out of PS 1 expression strongly reduces Aβ production with a simultaneous increase in APP C-terminal fragments [67] in the ER and Golgi [26]. Moreover, the introduction of two aspartate to alanine mutations in PS 1 transmembrane domains results in reduced levels of Aβ production. From these results it has been concluded that PS 1 is likely to be a gamma secretase itself [68]. Alternatively PS 1 might be an essential accessory factor for γ-secretase activity. It should be mentioned here that PS 1 knock out does not only decrease γ-secretase cleavage of APP but reduces Notch 1 cleavage as well. APP and Notch are both cleaved inside their transmembrane domain but differ as APP cleavage occurs in the middle of the transmembrane domain [69] and Notch cleavage occurs at the cytosolic side of the transmembrane domain [70]. Furthermore, the localization of γ-secretase activity at or close to the cell surface resulting in secretory Aβ does not appear to overlap with the main fraction of PS 1, but the absence of PS from the cell surface in non-PS transfected cells is controversial [71–73]. These observations are very recent and further work in this field will certainly result in very interesting insights into the mechanisms of APP processing. Taken together it can be concluded that intracellular Aβ42 is produced mainly in the ER and possibly in the ER Golgi intermediate compartment. Aβ42 is absent or – under the assumption that overexpression of wild type PS 1 does not change the localization of γ-secretase activity – can be present at least in CHO cells in Golgi and fades out in the later compartments of the secretory pathway. The exact localization of the major Aβ42 production is likely to differ slightly depending on the cell type analyzed.

The mechanism of how PS mutations increase the Aβ42 production is still unclear. An indication is given by the analysis of overexpression studies using PS 1 containing a familial AD mutation (M146L) [26]. In this case a strong increase of Golgi Aβ42 was detected. Therefore, the biological function of PS might be involved in the compartimentalization of Aβ 42 cleavage.

N-terminal truncations

Information about the N-terminus of intracellular Aβ peptides is limited; therefore, the name Aβ42 refers to all Aβ peptides ending with amino acid 42 irrespective of the N-terminus, while Aβ1-42 specifically refers to full length Aβ42. Aβx-42 refers to truncated Aβ peptides. Aβ n-42, N-terminal heterogeneity is typical for Aβ deposited in the AD plaque as well [74]. Several reports were able to show that intracellular Aβ42 is produced with N-terminal heterogeneity [14, 18, 20, 22, 24]. None of these peptides were sequenced, but since α-secretase activity does not result in intracellular Aβ formation [18, 61], it appears to be plausible that the N-terminal truncation extends up to a maximum length around amino acid 11 of Aβ [14]. From publications which allow one to draw conclusions about the N-terminus of Aβ42, the following picture emerges: In the ER, Aβ x-42 [18, 24] and Aβ1-42 [18, 26] were found, in Golgi Aβ1-42 was found using PS 1 transfected cells [26], and in TGN Aβ x-42 and Aβ 1-42 were identified [24]. Sudoh et al. [20] identified Aβ1-42 and Aβx-42 as well; however, conflicting with the previous results, all Aβ42 was found to be brefeldin A sensitive, thereby excluding the ER as compartment of origin. Aβ x-42 accumulated and Aβ1-42 decreased upon monensin treatment arguing for a separate localization of Aβx-42 and Aβ1-42 in Golgi and TGN. These results might be explained in part by the use of PS-I(M146L) overexpressing cells. With current knowledge the issue of the localization of truncated versus nontruncated Aβ42 is still unresolved and conflicting, which is further complicated by the fact that the exact N-terminus of these Aβ42 peptides is unknown.

Aβ40

One of the implications of Aβ42 localization to the TGN [24] would be that it might become secreted. In fact,

Aβ40 has been found to be present in the TGN in abundance [16, 24], to be packaged into coated vesicles [16], and found in post-TGN vesicles [24], strongly indicating that TGN derived Aβ can be transported and possibly becomes secreted. Production of Aβ in the TGN had been shown in a cell free assay; however, the C-terminus was not analyzed [75]. Since Greenfield et al. [24] found Aβ42 in TGN derived vesicles of PS 1 transfected N2a cells, this production and export pathway might exist for Aβ42 as well. In addition to the TGN, Aβ40 was also found to be present in smaller amounts in specific endosomes; however, it is not clear whether Aβ40 was generated in this compartment or transported towards this compartment from TGN [16]. Aβ, which is likely to contain Aβ40, was found in Golgi fractions of PS 1 transfected CHO cells [26]. Strikingly, no Aβ40 was found in the ER [16, 18, 24]. Furthermore, subcellular fractionations on density gradients show a continuous decrease of the Aβ42/total Aβ ratio from heavy [ER] fractions to lighter (Golgi and post-Golgi) fractions [26]. It was hypothesized that the increasing membrane thickness due to increasing cholesterol content along the secretory pathway [76] might be responsible for the inverse relationship between Aβ40 and Aβ42 [16]. In fact it was recently shown that the APP transmembrane domain is cleaved by γ-secretase in the middle of the lipid bilayer and mutations shifting the middle of the transmembrane domain result in cleavage at or close to the newly predicted middle of the lipid bilayer [69]. Cleavage in the thin ER membrane is therefore predicted to occur at amino acid 42 of Aβ. With increasing thickness of membranes along the secretory pathway, the Aβ40 production would therefore increase. Since membranes consist of lipid microdomains with differential thickness some variation is likely to take place.

Insoluble pools

Data from Skovronsky et al. [21] show that intracellular Aβ can be divided into two different pools by solubility in RIPA buffer versus formic acid. The insoluble Aβ increases during cultivation more than 10-fold and soluble Aβ constitutes then only 20% of total intracellular Aβ. Remarkably, most of this "old" Aβ is Aβ42 and the ratio of Aβ42:40 is changed to approx. 1:1. The increase in insoluble Aβ can most likely be explained by very slow degradation of insoluble Aβ; therefore, the degradation rate for insoluble Aβ has to be lower than the rate of addition of new insoluble Aβ from the soluble pool. The majority of the insoluble Aβ42 was produced in the ER/IC compartment. In other reports, Triton X-100 insolubility was used to assay for different pools of intracellular Aβ [22, 23]. Triton X-100 insolubility indicates the association of Aβ with low density cholesterol and ganglioside-rich lipid microdomains. Density gradient centrifugation could indeed show that insoluble Aβ is present in the low density fraction. Due to the different detergents used, this pool is not likely to be identical to the above mentioned insoluble pool. The correlation between the RIPA and Triton X-100 insoluble pools is unknown; since the later pool shows SDS stable Aβ dimers, this pool might be a precursor for the former insoluble pool.

Neurons are at high risk of suffering from detrimental Aβ production

AD is clearly a brain disease; however, peripheral cells express APP and also produce Aβ. Neurons produce the highest amount of APP, and β-secretase is more active in neurons than any other cell type. Neurons most intensively use the intracellular pathway for Aβ production. While mitotic cells are able to dilute intracellular Aβ accumulations, this is not possible for postmitotic neurons and accumulations should increase during aging. Brain specificity can, therefore, be considered to be caused by a coincidence of several neuronal features that make them prime candidates to suffer from Aβ production.

Aggregation of intracellular Aβ

Far less is known about intracellular Aβ than about extracellular Aβ. The picture about the role of intracellular Aβ in AD is still evolving and any assumption about its possible detrimental effects can only be a rough hypothesis. Intracellular Aβ possesses several features increasing its potential to harm cells. First, intracellular Aβ42 and Aβ40 are produced in different organelles [16, 24, 26]. Aβ42 aggregates faster if it is not mixed with Aβ40 [77, 78] and therefore the risk to acquire intracellular Aβ aggregates is increased. Aβ aggregates exhibit higher protease resistance and therefore cellular clearance of Aβ might be impaired. Data by Yang et al. [25] indicate that this is in fact the case. Aggregation of Aβ depends on the Aβ concentration [78], and Aβ production inside the small volume of the ER or any other organelle as compared to the large extracellular space of the brain will favor high Aβ concentrations. Aβ aggregation is also time dependent. Since intracellular Aβ42 has a long half-life and apparently does not become secreted [17], it will accumulate over time, increasing the risk of reaching critical threshold levels. The recently discovered pool of insoluble intracellular Aβ(21] appears to be derived from this process. Moreover, due to its exceedingly long half-life, this insoluble Aβ further increases the risk of intracellular Aβ.

A possible detrimental role for intracellular Aβ

Intracellular accumulation of Aβ, soluble or insoluble, might impair cellular functions. It appears that at least some cell types are unable to clear Aβ accumulates. Yet, the actual risk involved in intracellular Aβ accumulation, as well as Aβ accumulations in general, is not known. While the biological function of Aβ is not known, interestingly a deletion of the luminal domain of the Aβ region inside the APP molecule rendered these APP molecules

incapable of normal protein transport resulting in abolished axonal transport [61]. It is concluded from this finding that the biological function of the Aβ domain or parts of it is to interact with APP sorting receptors. The abundant presence of excess free Aβ in aging neurons might result in impaired APP transport, either due to interaction of Aβ with APP sorting receptors or, given the tendency of Aβ to form dimers and higher aggregates, due to interaction of Aβ with the Aβ domain in APP. In both scenarios APP would be incapable of binding its cognate sorting receptors. This might impair the biological function of APP and result in neurodegeneration. Moreover, accumulation of APP in the ER or other Aβ42 containing organelles would provide β- and γ-secretase with additional substrate resulting in further production of intracellular Aβ42, thus, eventually starting a vicious cycle. However, a γ-secretase independent pathway of APP degradation might exist as well [79], but the influence of intracellular Aβ accumulation specifically on this pathway is unknown. Intracellular accumulation of Aβ might impair the sorting of other proteins that use the same sorting receptors, and again this might lead to neuronal dysfunction. In the case of an ER overload, impaired ER protein transport is known to result in aberrant ER protein accumulation. Eventually cells respond to ER overload by downregulation of protein biosynthesis to restore proper ER function (for a review see Pahl [80]). In the case of the nonremovable Aβ as a cause for ER dysfunction, the ER overload response is unlikely to recover neuronal function.

As mentioned above a vicious cycle would be even more likely if Aβ accumulation could trigger amyloidogenic processing of APP. This seems in fact to be the case. Exposure of cells overexpressing APP to Aβ42 results in an intracellular accumulation of newly produced Aβ42. The majority of this new Aβ42 is already insoluble and exhibits a long half-life [25], showing the next step in the hypothetical vicious cycle.

Not mutually exclusive to the above mentioned hypothesis, intracellular Aβ could be present after neuronal death. Subsequent release of intracellular Aβ aggregates might provide Aβ seeding crystals for amyloid plaques.

Intracellular Aβ possesses a score of cell biological and biochemical features that make it a highly interesting risk factor for AD, but the final relevance for AD pathogenesis will most likely not be known before the toxic mechanism of Aβ42 in AD will be clarified.

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Note added in proof An APP β-secretase has been identified (Vassar R, et al. (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286: 735–741).

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