REVIEW

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CURRENT INSIGHTS INTO MOLECULAR MECHANISMS OF ALZHEIMER DISEASE AND THEIR IMPLICATIONS FOR THERAPEUTIC APPROACHES

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Short title: AD disease mechanisms and therapeutics

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ABSTRACT

During the last ten years, a lot of progress has been made in unravelling the pathogenic cascade leading to Alzheimer disease (AD). According to the most widely accepted hypothesis, production and aggregation of the amyloid β (Aβ) peptide plays a key role in AD and thus therapeutic interference with these processes is the subject of intense research. However, some important aspects of the disease mechanism are not yet fully understood. There is no consensus as yet on whether the disease acts through a loss- or a gain-of-function mechanism. While for many years, an increased production of Aβ42 was considered to be the prime culprit for the initiation of the disease process, and accordingly Aβ42 is elevated by AD-related *presenilin* (*PS*) mutations, recent data strongly suggest that *PS* mutations also lead to a loss-of-function of PS towards a plethora of its substrates including APP. How this PS loss-of-function, especially decreased Aβ40 secretion due to mutant *PS*, impacts on the disease pathogenesis is yet to be elucidated. Secondly, vascular abnormalities – frequently observed to co-occur with AD – might also play a critical role in initiation and aggravation of AD pathology given that the elimination of Aβ through a vascular route is an important brain Aβ clearance mechanism and its failure leads to formation of vascular amyloidosis and dense-core plaques. In this review, we will first focus on the important issue of a loss-of-function versus a gain-of-function mechanism for AD due to mutant *PS*, as well as on the possible role of vascular damage and reduced perfusion in AD. Special emphasis will be given to some of the AD mouse models that have helped to gain insights in the disease mechanism. Secondly, considering these mechanistic insights, we will discuss some therapeutic strategies which are currently in clinical or preclinical trials for AD.

KEY WORDS

Alzheimer disease; amyloid β protein; presenilin; transgenic mice; knock-out mice; loss-of-function; gain-of-function; vascular pathology; therapeutics; angiogenesis

Alzheimer disease (AD) is the most common form of dementia in the aged population and as life expectancy is continuously growing in Western countries, the major socio-economic impact of this illness will even increase in the future. AD is characterized by extracellular deposition of the amyloid β (Aβ) peptide and by intraneuronal hyperphosphorylated tau protein in neurofibrillary tangles, accompanied by a progressive loss of neurons in vulnerable brain regions that ultimately leads to dementia and death. Aβ is formed by cleavage of amyloid precursor protein (APP) by β-secretase (BACE, β-site APP cleaving enzyme) leading to the formation of a C-terminal fragment (CTF-β) that serves as a substrate for γ-secretase cleavage to yield full-length Aβ [1] (Fig. 1). During normal APP processing, however, the more common α-secretase cleavage occurs within the Aβ domain and the resulting CTF-α is further processed by γ-secretase to form a N-terminally truncated Aβ fragment, called p3 (Fig. 1). Aβ is normally secreted by healthy cells throughout life, but its normal physiological function remains largely unknown. There are two major Aβ species that are being formed: the 40 amino acids form (Aβ40) or the more aggregation-prone 42 amino acids long peptide (Aβ42). Under physiological conditions, Aβ40 constitutes about 90% of the total amount of Aβ. Mutations in *APP*, *presenilin 1* (*PS1*) and its homolog *presenilin 2* (*PS2*) have been identified that lead to early-onset, familial forms of AD (a database of these mutations can be found on http://www.molgen.ua.ac.be/ADMutations/). All these mutations influence APP metabolism to alter Aβ production, leading to a shift of the preferred cleavage site from position 40 to 42 [2;3] and Aβ42 is the first and predominant species accumulating in plaques in AD brain [4].

γ-secretase is a high molecular weight protein complex consisting of 4 components: Aph-1, Pen-2, Nicastrin and presenilins, the latter being the catalytic subunit [5]. Recently, another constituent of this complex has been identified, the TMP21 protein [6]. The γ-secretase complex is not only active in the intramembranous cleavage of APP, but also cleaves a number of other type I membrane proteins like Notch and N-cadherin. Upon ligand binding, Notch is cleaved at the S2 site and a membrane-bound fragment is generated which is subsequently cleaved at the S3 site by γ-secretase to generate Notch intracellular domain (NICD). An analogous PS-dependent cleavage also occurs in APP after leucine 49 of Aβ (ε-site), which is called S3-like γ-secretase cleavage-of-APP

leading to the production of APP intracellular domain (AICD) (Fig. 1). Moreover, PS1-dependent proteolysis occurs also at other Aβ positions, for instance Aβ37, Aβ38, Aβ39 and Aβ46 (ζ-site) [7].

In this review, we will first discuss the role of cellular and mouse AD models in understanding the "mechanism" of disease, focusing on the current debate of whether these models support or refute a gain-of-abnormal-function or a loss of physiological function(s) in the causation of AD. Secondly, we will discuss some examples of how some of these mouse models have been used as "disease models", where therapeutic strategies targeting Aβ have been successfully employed in at least preclinical trials. In addition to this we have also discussed a few compounds that might have a beneficial effect on vascular pathological abnormalities commonly observed in AD.

I. Progress in understanding the disease mechanism of AD: Clues from cellular and animal models

Because mutations in *APP* and *PS* cause familial forms of AD (FAD), the most logical way of understanding disease mechanism was to model these mutations in cells and mice. The study of transfected cells expressing *APP* or *PS* FAD mutations consistently showed that these mutations affect Aβ production, leading to increased levels of absolute or relative amounts of Aβ42 [2;3]. The first successful mouse models were APP transgenic models that progressively developed extracellular plaques, for instance, PDAPP and Tg2576 mice expressing the APP/Indiana (V717F) and APP/Swedish (APPK670M/N671L) mutations, respectively [8;9]. In contrast, transgenic mice overexpressing *PS1* mutations did not develop plaques while cerebral Aβ42 levels were elevated [10]. However, when PS1 transgenic mice were crossbred with APP transgenic mice, brain Aβ deposition was markedly accelerated [11;12]. While these transgenic overexpressing mouse models suggest a gain-of-function of increased Aβ42 (the role of Aβ40 will be discussed in the following paragraphs), a loss-of-function mechanism is more appropriately studied by gene knock-in and knock-out techniques which have some advantages when compared to overexpressing models. Firstly, heterozygous mice with one copy each of a wild-type and a mutant allele mimic the human situation better and

the knock-in alleles are expressed under the regulatory sequences of the gene itself. Secondly, in the overexpression models the observed effect might be due to overexpression itself rather than due to the mutation (e.g., by altering the physiological assembly of the γ-secretase complex or by leading to mistrafficking of the overexpressed proteins). Some of these knock-in and knock-out mouse models, together with *in vitro* studies, have provided exciting new insights into AD pathogenesis and will be discussed in the following sections.

A. Aβ-driven neurodegeneration caught amidst gain-of-function versus loss-of-function controversy for FAD mutations

Currently, it is still elusive whether FAD mutations act through a partial loss-of-function (LOF) or a toxic gain-of-function (GOF) mechanism. Many arguments were proposed to suggest that *PS* mutations cause a GOF. However, some of these arguments have been challenged by recent data suggesting that in addition to an Aβ42-related GOF, *PS* FAD mutations could also cause a decreased PS activity or a LOF of mutant PS1. Arguments in favor of a GOF or a LOF mechanism will be discussed in the following paragraphs and the salient features are summarized in Table 1.

(1) The dominant inheritance pattern of *APP* and *PS* mutations was initially argued to be suggestive of a GOF, however, haploinsufficiency or a dominant negative effect are also transmitted in a dominant way. In classical molecular pathology, presence of more than 150 mutations throughout the entire coding sequence of PS1 causing the same phenotype (AD) is more suggestive of LOF. Although most of these are missense mutations, some are also exonic deletions [13] and the latter definitely suggest LOF.

(2) A characteristic of a LOF point mutation is that it should cause the same pathological phenotype as a complete ablation of the gene. An increase in total Aβ originally thought to occur in transgenic mice and cells modeling missense *PS* mutations was against the phenotype of presenilin knock-out models where a 50% reduction of *PS* (in *ps*1 +/- mice) decreased Aβ40 and Aβ42 production [14;15]. However, new *in vitro* studies show that clinical *PS1* mutations decrease total Aβ, or more specifically Aβ40, thus suggesting a partial LOF [16;17] (see later).

(3) Initial data on PS1 A246E rescue experiments in *ps1* -/- mice showed that the mutated protein rescued the embryonic lethality as efficiently as the wild-type protein

[14;18]. However, a recent study showed that this particular mutation has a relatively mild effect on Notch processing [16] and this rescue might not have occurred had other mutations been studied that severely effect Notch processing (e.g., PS1 L166P or G384A [16]). This is also supported by a lower efficiency of mutant *PS* in rescuing the egg-laying deficit in *C. elegans* caused by loss of *PS* homolog *sel-12* [19;20].

(4) One of the most important reasons to believe that AD occurs through a GOF is the observation that all *PS* missense mutations linked with AD cause an increased Aβ42 over Aβ40 ratio (Aβ42/Aβ40), and Aβ42 has a much higher propensity to aggregate and be neurotoxic. Thus, an absolute or a relative increase in Aβ42 production represents a gain-of-toxic-function. In support of these data, all missense mutations in *APP* at both the β-secretase and γ-secretase sites increase total Aβ (as is the case with the only physiological β-secretase-site double-mutation, APP/Swedish) or specifically Aβ42 (as for all γ-secretase-site mutations). An increased amount of Aβ can also result from an APP gene dosage effect as observed in Down's syndrome patients or APP gene duplications causing AD [21;22]. Moreover, *APP* promoter polymorphisms linked to AD are also identified that lead to increased APP expression [23;24]. Although increased APP dosage is a solid evidence for a GOF, recent data suggest that an elevated Aβ production is not characteristic for all clinical *PS* or *APP* mutations [17;25;26]. It has been shown that some *APP* mutations, like the Austrian APPT714I (APP-Au) and the French APPV715M mutation [25;26], also cause a decrease in the amount of Aβ40 production. The *in vitro* decrease for the APP-Au mutation for instance, in both HEK cells and primary neurons was ≈ 80% for Aβ40 [26;27], with only a very modest Aβ42 increase, clearly suggesting a very severe LOF of PS in processing mutant APP. The *modest* Aβ42 increase is emphasized as reduced cleavage at the Aβ40 site might proportionally increase Aβ42.

Similar *in vitro* studies are more difficult to perform for *PS1* FAD mutations as it involves double transfection of both human *APP* and mutant *PS* leading to difficult normalization for two proteins. Thus, although earlier studies have shown that *PS1* FAD mutations increase Aβ42/Aβ40 ratio in transfected cells, measurement of this ratio did not allow dissecting whether this increase is caused by a reduction of Aβ40, a higher production of Aβ42, or a combination of both effects. To address to this issue, we

developed a novel, ELISA-based assay to study the absolute levels of the Aβ isoforms [17]. Using this tool we showed that not all clinical *PS* mutations cause an increase in Aβ42 production while importantly, they more consistently result in a decrease in absolute Aβ40 levels leading to an increased Aβ42/Aβ40 ratio [17]. Some of these data have also been confirmed in another recent study using a different assay and cell system, i.e., in *PS* double knock-out (DKO, *ps1* -/- *ps2*-/-) fibroblasts [16].

In support of these *in vitro* data, *in vivo* evidence for the role of reduced Aβ40 levels in AD pathogenesis has come from recent studies utilizing transgenic and knock-in mouse models. The first study models the APP-Au mutation (APPT714I) that has a drastic reduction of Aβ40. We showed that APP-Au mice deposit Aβ inside the neurons and this is consistent with other observations in mice and humans that Aβ not only accumulates in parenchyma but also in the intracellular compartment [28] (Figure 2). Despite having very low transgenic expression, APP-Au mice have reduced brain volumes on volumetric MRI, a sign of neurodegeneration [29]. The second study showed that loss of the wild-type presenilin 1 allele in mice expressing a knocked-in *PS* FAD mutation on Tg2576 APP background (APP/PS1M146/-) leads to a greatly accelerated plaque pathology [15]. This was due to reduced γ-secretase activity and concurrent loss of Aβ40 without an increase in Aβ42 levels. The third study used *PS1* knock-in mice with deletion of the hydrophilic loop domain of PS1 and showed a drastically reduced cleavage at the Aβ40 site, while Aβ42 production was not altered [30]. Interestingly, the reduction in Aβ40 production accelerated plaque pathology in APP FAD transgenic animals [30].

A possible protective role of Aβ40 is also derived from recent studies utilizing a fourth transgenic mouse model, the BRI-Aβ40 and BRI-Aβ42 transgenic mice that selectively produce Aβ40 or Aβ42 respectively, without utilizing human APP [31]. In these models, plaque formation was observed in transgenic mice expressing Aβ42 alone, while BRI-Aβ40 mice did not deposit plaques and did not form insoluble Aβ40. However, when BRI-Aβ42 mice were crossbred with BRI-Aβ40 mice, the bigenic offspring accumulated massive amounts of detergent-insoluble Aβ40 in brain [32]. On the other hand, when BRI-Aβ40 mice were crossed with Tg2576 mice, the resulting bigenic BRI-Aβ40/Tg2576 mice had significantly less amyloid deposition in both

parenchyma and cerebrovasculature than Tg2576 littermates [32]. These data all suggest that although Aβ42 has a major role in initiating plaque deposition, Aβ40 also has an important function in amyloidosis and depending upon the critical level of Aβ42, might even be anti-amyloidotic.

(5) LOF of mutant PS is also observed in the reduced cleavage of a plethora of other γ-secretase substrates including Notch and N-cadherin and will be more appropriately discussed in the next section (see *Aβ-independent neurodegeneration*).

(6) Finally, mutations in *APP* at the α-secretase site are also known that alter the primary Aβ sequence and therefore alter the fibrillogenic properties of Aβ [33]. This is definitely supporting a GOF of the mutant Aβ, however, most of these mutations also have additional phenotypes like vascular amyloidosis which also plays a role in neurodegeneration and this is different from classical AD pathology where only wild-type Aβ deposits in predominantly Aβ plaques [34;35].

To conclude the controversy of LOF vs. GOF mechanism in AD pathogenesis, it is very likely that the actual disease mechanism is situated in between the extremes of a pure loss- or gain of function mechanism. For instance, several studies describe a reduced function of *PS* mutations towards the cleavage of APP, Notch and other γ-secretase substrates, while at the same time these mutations also cause an increase in Aβ42/Aβ40 ratio. Thus FAD mutations might cause AD by the combined effect of a partial LOF in Notch signaling, AICD production and signaling or some other LOF, as well as a toxic gain-of-misfunction (increased Aβ42/Aβ40 ratio). It was also suggested that γ-site and ε-site and S3-cleavage function can be differentially affected. This was shown in a study where two investigated *PS1* mutants caused increased Aβ42 production while in contrast, they inhibited both ε-cleavage of APP and S3-cleavage of Notch [36]. This differential effect on γ-site, ε-site and Notch signaling was also observed in other studies utilizing a different set of *PS* mutations [16;37]. This dual effect could be explained by the fact that although they are both mediated by presenilins, cleavage at γ-site and ε-site of Aβ are independent catalytic events. The independent regulation of these cleavages has indeed been suggested recently [6].

B. Aβ-independent neurodegeneration

ALTERED CELLULAR SIGNALING IN DEMENTIA

A number of studies suggest that neurodegeneration caused by *PS* FAD mutations can occur via an Aβ-independent mechanism with the involvement of a loss of PS function. The proof of principle that (partial) loss of presenilin function can induce neurodegenerative processes, came from a study using forebrain-specific conditional *ps* double knock-out mice (PS cDKO mice) in which it was shown that loss of PS function leads to progressive synaptic dysfunction, memory impairment and neurodegeneration [38]. A complete loss of presenilin function would most likely result in embryonic lethality [39], but a partial loss is probably able to cause neurodegeneration over time, independently of changes in amyloid production but by reducing NMDA receptor-mediated responses and decreasing CREB/CBP dependent expression of target genes [38]. Moreover, it was shown that *PS1* itself is a CREB/CBP target gene [40] and we previously showed that a neuron-specific decrease in *PS1* expression increases the risk for AD [41]. It is important to remark that while considerable neurodegeneration could not be observed in all APP transgenic mice, this effect was seen in PS cDKO mice, supporting a mechanism of neurodegeneration independent of Aβ but caused by loss of PS activity. Also, it has been shown that *PS* FAD mutations cause a LOF in the PI3K/Akt cell survival pathway independent of γ-secretase [42]. This LOF activates GSK-3 and thus leads to tau overphosphorylation.

PS1 is involved in the processing of multiple proteins besides APP, and in mammalian cells *PS1* FAD mutations were shown to inhibit γ-secretase-mediated cleavage of several substrates. For instance, *PS* FAD mutations cause a loss of γ-secretase-mediated Notch cleavage related nuclear signaling [43], N-cadherin cleavage [44] and ephrinB2 cleavage [45]. For at least 1 mutation, PS1 L166P, it was shown that both formation of NICD and of AICD was impaired [46]. LOF towards ε-cleavage resulting in a lowered production of AICD was shown for a number of *APP* as well as *PS1* mutations [47] (see previous section). Similar effects towards presenilin function were also observed for *PS2* FAD mutations, which were causing a decreased formation of Aβ40, AICD and NICD [48]. These multiple effects ask for careful examination to determine which of these factors are relevant to AD pathogenesis.

The hypothesis that *PS1* mutations can lead to neurodegeneration independent of Aβ, is also supported by the finding that *PS1* mutations can cause frontotemporal lobar degeneration (FTLD) with tauopathy but in the absence of Aβ deposits in patient brain. We previously identified a *PS1* splice-site-G183V mutation in a family with ″Pick's disease tauopathy″ [49]. Brain PS1 mRNA analysis showed that ≈ 20% of the brain transcripts were alternatively spliced and PS1 protein was also reduced compared to AD and aged control individuals (Tolia A., Wils H., Theuns J., Dermaut B., De Strooper B., Van Broeckhoven C., Kumar-Singh S.; unpublished data). Because of the loss of PS1 protein, splice-site-G183V mutation is more complicated than a simple missense mutation. For instance, this partial loss of PS1 protein might lead to reduced PS1 function which could directly cause increased tau phosphorylation involving the PI3K/Akt pathway and leading to neurodegeneration [38]. The *PS1* splice-site-G183V mutation is different from *PS1* insR352 mutation identified in a nontauopathy FTLD family, which is caused by mutations in the progranulin (PGRN) gene [50;51]. *PGRN* is a recently identified gene for nontauopathy FTLD where frameshift or other null mutations cause loss of progranulin protein or haploinsufficiency [52;53]. As expected, a *PGRN* mutation is absent in splice-site-G183V mutation carriers [53]. The only other FTD family associated with a *PS1* mutation has again a splice-site mutation (L113P) [54] where both pathological and genetic analysis is eagerly awaited. These results all suggest that partial loss of many functional proteins can eventually lead to neurodegeneration.

To conclude, it is becoming obvious that Aβ-independent pathway(s) are also an important mechanism of neurodegeneration. This is especially important for *PS1* FAD mutations where perhaps a combination of Aβ-dependent and Aβ-independent neurodegeneration causes an earlier age-at-onset compared to *APP*-related FAD. Future research will try to estimate the contribution of each of these pathways which will especially be important for the more common, sporadic form of AD.

VASCULAR DEFICITS IN AD

AD is often accompanied by cerebrovascular pathology like congophilic amyloid angiopathy (CAA) and disrupted microvascular integrity and hypoperfusion [55],

suggesting that vascular deficits might play a causative role in the pathogenic mechanism of AD. A wide variety of structural microvascular abnormalities have been observed, like loss of endothelium, basement membrane thickening, astrogliosis and pericyte degeneration.

The majority of secreted Aβ that is not degraded or deposited in diffuse plaques is cleared from the brain through two major vessel-related pathways: direct transport across the blood brain barrier (BBB) via LDL receptor-related protein-1 (LRP-1) or alternatively, along the periarterial interstitial fluid drainage pathways to the CSF and eventually the systemic circulation. We recently showed in 2 mouse models (Tg2576 [9] and PSAPP [12]) that ≈ 90% of the dense-core but not diffuse plaques are centered on vessel walls or in the immediate perivascular regions [56]. These models have been widely used in AD research and show progressive development of plaques similar to that observed in AD, making them a good model to study plaque formation and its effects in brain. We showed in these models considerable ultrastructural microvascular abnormalities that occur in vessels in the direct vicinity of dense plaques or in vessels that deposited Aβ in their walls. For instance, we observed loss or thinning of endothelium, basement membrane thickening or splitting to accommodate Aβ, loss of smooth muscle cells, pericyte degeneration and sometimes even a complete degeneration of microvessels [56]. These effects show a close resemblance to microvascular deficits observed in AD. Also, micro hemorrhages were detected using Prussian blue staining for iron and leakage of the BBB was shown by infiltration of serum proteins, which are normally restricted by the BBB, into the parenchyma in association with dense plaques (Fig. 3). The pathology described for Tg2576 and PSAPP mice shows a high similarity to human Flemish AD pathology, caused by the Flemish APPA692G mutation, which is characterized by vascular hemorrhage and dementia. These patients have the largest dense-core plaques in AD, and the majority of the plaques also enclose vessels or are associated with vessel walls [26]. The Tg2576 and PSAPP mouse models as well as the Flemish AD brain show the formation of large dense cores associated with vessels, while diffuse plaques are less abundant, as well as a preponderance of Aβ40. The crucial role of Aβ40 in vascular amyloidosis was also demonstrated by a study of mice overexpressing the APPE693Q mutation (APP/Dutch) that showed extensive CAA, smooth muscle cell degeneration,

hemorrhages and neuroinflammation [57]. When these mice were crossed with transgenic PS1 mice, to increase the amount of Aβ42 in the brain, the amyloid pathology redistributed from the blood vessels to the parenchyma [57].

It is very likely that the nidus for seeding dense plaques at the vessel wall is either provided directly by vascular components or by association with specific chaperones that sequester Aβ, especially Aβ42, at vascular sites or by interaction with Aβ-assembly-promoting molecules. The mechanism of clearance of Aβ by transport along or across the vessels/BBB can be disturbed by down-regulation of LRP expression or Aβ-mediated proteasome degradation of LRP in vascular endothelium. The less efficient clearance could lead to deposition of Aβ at vascular sites, explaining the association of dense plaques and vessels. In addition, BBB dysfunction can further contribute to the less efficient clearance as well as to the growth of the vessel-associated plaques. Because soluble Aβ is known to exert a toxic effect on endothelial cells, the high local concentration of Aβ at the vessel wall might also lead to degeneration of endothelium, which in turn might cause even increased deposition by perturbing vascular transport [56].

The second process by which vessels are implicated in neurodegeneration is vascular insufficiency [55]. In ageing brain, hypoperfusion is a common problem and as yet it is difficult to know if it is the cause or the effect of neurodegeneration. Decreased global cerebral blood flow (CBF) compared to age-matched, non-demented controls is a characteristic of AD, which is consistently seen in the parietal and temporal cortices [55]. Methods like PET, SPECT and gas inhalation contrasted CT are used to detect regional CBF in outlined brain regions and show this reduced brain perfusion. The regional distribution and the degree of CBF decrease are dependent on the severity and particular symptoms of dementia, age of the patient and onset and duration of dementia. Reduced CBF consequently lowers glucose supply, the brain's fundamental energy source, and oxygen supply to neurons, possibly leading to neurodegeneration. A model to investigate the effects of hypoperfusion on neurons was made by the permanent ligation of major arteries supplying the brain in rats, leading to a drastic drop in CBF. Histological examination of the brain after a prolonged period of hypoperfusion revealed a significant loss of hippocampal CA1 neurons and an increased gliosis [58]. These results suggest a

335 link between reduced CBF and neuronal pathology. Also, transgenic mice expressing reduced levels of VEGF, a growth factor that is known for its angiogenic properties and produced by vascular mural cells, show degeneration of motor neurons [59].

It has been observed that only dense-core plaques but not diffuse plaques are surrounded by neuritic pathology. This is against the notion that not the fibrillar Aβ, 340 found in dense plaques, but non-fibrillar Aβ species, as found in diffuse plaques, are neurotoxic. A possible explanation for this controversy might be found in the fact that neurites surrounding dense plaques degenerate as a consequence of the leakage or the hypoperfusion of the vessels associated with the plaques.

345

II. THERAPEUTIC TARGETS FOR AD

Insights into the pathogenesis of AD are essential to identify therapeutic targets. While knock-in and knock-out models are indispensable for investigating AD disease mechanism, other models mimicking AD neuropathological changes are valuable tools for testing therapeutic strategies. Currently, symptomatic treatments, e.g. acetylcholine esterase inhibitors, are available but these can only temporarily ameliorate some symptoms and the treatment does not act on the underlying pathogenic process. In the following sections we will discuss some recently tested or potential therapeutic strategies for retarding or treating AD.

A. Therapy directed against Aβ

According to the amyloid cascade hypothesis, formation, aggregation and deposition of Aβ is responsible for initiating the pathogenic cascade of AD [1] and hence most currently conducted therapeutic trials are designed to interfere with these processes (Fig. 4).

REDUCED Aβ PRODUCTION VIA SECRETASE INHIBITION

The use of inhibitors of the Aβ-forming enzymes β- and γ-secretase is one possibility to reduce the amount of Aβ. An important remark here is that γ-secretase is a multifunctional molecule involved in the processing of multiple substrates and completely abolishing its function has detrimental consequences not only on embryonic development, but also in adult life [38;39]. When using γ-secretase inhibitors, it is of crucial importance to investigate the effect on other APP processing products (besides Aβ) because, for example, *PS* FAD mutations were shown to consistently reduce the amount of AICD produced (ε-CTF) [16]. This means that approaches that cause a similar accumulation of CTF are likely to promote AD, whatever the effect on Aβ formation is. A study in the Tg2576 mouse model revealed that acute treatment with a γ-secretase inhibitor leading to modest Aβ reduction (15-30%) was sufficient to reverse Aβ-induced cognitive deficits [60]. Unfortunately, γ-secretase inhibitors were also shown to cause abnormalities in the gastrointestinal tract and in lymphocyte development in rodents

when administered for a period of 15 days [61]. These changes likely resulted from the inhibition of Notch cleavage. In order to solve this issue, it will be very important to understand the mechanism of independent regulation of γ- and ε-cleavage, which will be helpful in the design of γ-site specific inhibitors that do not alter ε-site cleavage. Using such compounds, there would be no interference with essential physiological signal transduction mechanisms such as Notch signaling.

In contrast to γ-secretase inhibitors, BACE inhibitors might have a higher therapeutic potential because BACE knock-out mice do not show an abnormal phenotype compared to wild-type littermates [62]. For example, a study on Tg2576 mice revealed that administration of a BACE inhibitor resulted in a significant decrease of the Aβ level in plasma and in brain [63].

An important requirement for both β- and γ-secretase inhibitors, as for any drug that has to act in brain, is that they should be small enough to cross the BBB and moreover, they should be administered in early phases of the disease to have the desired effect. The latter also emphasizes the need for sensitive techniques for an early diagnosis of AD.

Some specific caveats should also be considered. For instance, if loss of Aβ40 is indeed an important factor in AD pathogenesis as suggested by recent studies [15-17;30], caution is needed because the reduction of the amount of Aβ40 might mimic the effect of presenilin mutations and in this way worsen instead of ameliorate the phenotype. In this eventuality, strategies aimed at altering APP processing to cause a selective increase of Aβ40 could be useful.

Some nonsteroidal anti-inflammatory drugs (NSAIDs) like ibuprofen were shown to modulate γ-secretase leading to a reduction of Aβ42 [64], making these drugs particularly interesting to specifically target the pathogenic Aβ42 species.

REDUCED Aβ OLIGOMERIZATION AND AGGREGATION

Because Aβ oligomers and/or fibrils are believed to be neurotoxic, interference with the formation of these structures might lead to a useful therapy. The major advantage of this approach is that the therapeutic target is a pathological process instead of normal enzymatic activities. However, it is extremely important to first decipher the exact

contribution of the different aggregation states of Aβ to AD pathology, because for example blocking of the fibril formation might lead to the accumulation of its intermediates, the potentially neurotoxic oligomers. As an example, it was shown recently that administration of cyclohexanehexol inhibitors that prevent formation of high-molecular weight oligomers leads to improved cognition, synaptic physiology and Aβ pathology in an AD transgenic mouse model [65].

INCREASED Aβ CLEARANCE

Besides acting on the APP anabolism, therapies can also be designed towards increasing the breakdown of Aβ. An increased activity of Aβ-degrading enzymes like neprilysin (NEP) and insulin degrading enzyme (IDE) could be a possibility to fasten Aβ clearance. It was shown that mice overexpressing NEP and IDE had reduced levels of Aβ in brain and showed retardation or even complete prevention of plaque formation [66].

Secondly, several successful anti-amyloid immunotherapies have already been developed that significantly reduce plaque burden in the brains of transgenic mice. Active immunization of APP transgenic mice with human Aβ peptide reduced plaque burden and associated pathologies in brain [67]. Also cognitive deficits were improved [68;69]. A passive approach, with peripheral administration of anti-Aβ antibodies, showed that these antibodies were able to cross the BBB and to induce clearance of plaques [70]. In this study it was shown that the antibodies bind Aβ and the resulting complex is cleared by microglia by Fc-receptor-mediated phagocytosis. Also, anti-amyloid antibodies circulating in the body might bind Aβ and in this way sequester it in the periphery, leading to less accumulation in brain. However, caution is needed when using immunotherapeutic approaches for treatment of AD as it was shown in a human clinical trial using an Aβ1-42 vaccine that a subset of the immunized patients developed meningoencephalitis and as a consequence this study was discontinued. A possible explanation for these adverse effects might lay in the activation of cytotoxic T cells and passive immunization trials would probably lead to fewer side effects.

B. Therapy directed against aberrant cell signaling

As described, aberrant cell signaling caused by *PS* mutations leads to tau phosphorylation and neurodegeneration, suggesting that intervention with tauopathy might have a beneficial effect in the treatment of AD. Modulating tau phosphorylation and aggregation can be achieved by inhibition of tau kinases like GSK3 and Cdk5, activation of phosphatases, blocking of tau assembly (e.g. with Congo red derivatives) or stabilization of microtubule integrity (e.g. with taxol derivatives). As discussion of these therapeutic strategies is beyond the scope of this review, readers are referred to appropriate references [71;72].

C. Therapy directed against vascular damage

Because damage to cerebral blood vessels is a prominent feature in AD [55], it can be anticipated that administration of angiogenic factors might be beneficial against the occurrence of vascular deficits. Such factors have already been studied in relation to the treatment of other disorders like for example amyotrophic lateral sclerosis (ALS) [73]. The combined use of these angiogenic agents with anti-Aβ immunotherapy might even enhance the therapeutic effect. Some of these modalities are discussed here.

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

A well-known pro-angiogenic factor is vascular endothelial growth factor (VEGF) which is a key regulator of vascular and lymphatic endothelial cell (EC) sprouting and also has a neuroprotective effect [74]. Intracerebroventricular injection of VEGF in two rat models for ALS delays disease onset, improves motor performance and prolongs survival [75]. It was observed in this study that VEGF acts most efficiently in close proximity of its injection site, suggesting that in order to have a beneficial effect on the cerebral vascular system, this compound should preferably be injected directly into the brain.

The level of VEGF is upregulated in AD patients, specifically in reactive astrocytes of the neocortex, cerebral vessel walls, as well as in cerebrospinal fluid [76;77]. It is not yet known if this upregulation is due to a compensatory neuroprotective effect and/or in response to hypoperfusion. However, it was also shown that VEGF binds Aβ with high affinity and colocalizes with plaques in the brains of AD patients [78]. It

was suggested that this binding might lead to a local deficiency of available VEGF around plaques, preventing its vasoprotective function and thus aggravating instead of counteracting AD progression. Administration of the VEGF molecule to AD patients might thus have an advantageous effect on vascular abnormalities via stimulation of EC growth and on neuronal cell survival through its neuroprotective effect.

PLACENTAL GROWTH FACTOR (PlGF)

Another vasoactive compound is placental growth factor (PlGF), a homolog of VEGF. It is able to revascularize ischemic tissues as efficient as VEGF [79], while it did not exert an effect on healthy tissues. Adenoviral PlGF gene transfer into skin of ears caused the formation of mature, non-leaky vessels that persisted for more than 1 year and interestingly, no complications as edema, fibrin deposition and growth of unstable vascular tangles were observed, as was the case for VEGF. Also, PlGF stimulates both EC and smooth muscle cells (SMC), while VEGF preferentially stimulates ECs only. This means that PlGF will not only promote the formation but also the stabilization of nascent vessels and since both ECs and SMCs are affected in AD brain, this should have a beneficial combined effect on vascular pathology. It was also shown that intravenous injection with VEGF/PlGF heterodimers or a combination of PlGF and VEGF increased ischemic myocardial angiogenesis in a mouse model where VEGF or PlGF administration alone was not effective [80]. It was suggested that PlGF therapy would amplify the angiogenic activity of VEGF, which is already locally upregulated in AD, and thus use VEGF as a downstream effector in addition to its own angiogenic signaling [80].

PLATELET DERIVED GROWTH FACTOR (PDGF) FAMILY

PDGF-BB is a molecule that binds to receptors on mural cells and stimulates the growth and migration of mural cells around endothelial channels. It is secreted by ECs for the maturation of blood vessels and the stimulation of mural cells to produce VEGF. A member of the same family is PDGF-CC. This factor was shown to mobilize endothelial progenitor cells in ischemic conditions while it did not affect blood perfusion in quiescent vessels. It also induced VEGF release and in this way it would exert an indirect effect on

ECs [81]. It was shown that both PDGF-CC and VEGF stimulate EC progenitor differentiation, while PDGF-CC but not VEGF also induces SMC differentiation and myofibroblast outgrowth. In a comparative study of 3 PDGF family members, it was shown that PDGF-CC is superior to PDGF-AA and PDGF-BB in stimulating EC migration, EC chemotaxis, microvascular sprouting and myofibroblast outgrowth [81]. Dose and duration of administration of this growth factor have to be carefully optimized since too high doses might lead to organ fibrosis by uncontrolled stimulation of fibroblasts. Moreover, when PDGF-C is overexpressed in mouse heart, this also leads to vascular defects like vascular leakage and loss of microvessels [82].

BASIC FIBROBLAST GROWTH FACTOR (FGF-2)

Basic fibroblast growth factor (bFGF or FGF-2) not only stimulates angiogenesis [83] but additionally, also was shown to have a protective effect against degeneration of neurons and to upregulate neurogenesis after traumatic brain injury (TBI) in the adult hippocampus of mice [84]. In this study, it was shown that dividing, neuron marker-labeled cells were more abundant in the brain of FGF-2 +/+ mice than in FGF-2 -/- mice after TBI, suggesting that FGF-2 enhances neurogenesis. Moreover, FGF-2 -/- mice had a greater decrease in the number of granule cell layer neurons of the dentate gyrus after TBI, suggesting that FGF-2 attenuates neurodegeneration. The dual role of FGF-2 was also confirmed through gene transfer experiments that showed that FGF-2 supplementation after the onset of TBI was able to upregulate neurogenesis and to reduce granule cell degeneration [84]. However, a number of other studies do not support the neuroprotective and neurogenesis-inducing role of FGF-2. In a first study, FGF-2 overexpressing mice were crossed with inbred APP transgenic mice, leading to an enhancement of the lethal effects of APP overexpression [85]. As a possible mechanism, it was suggested that the hypertrophy of vascular SMC observed in FGF-2 transgenic mice [86] would lead to a decrease in blood flow due to increased vasoconstriction. In combination with the effect of Aβ on the vasculature, this would lead to increased hypoxia, possibly explaining the enhanced mortality in the double transgenic animals [85]. Another possible explanation comes from a recent study, where it was shown that elevated FGF-2 concentrations enhance the division of immature cultured adult rat

hippocampal progenitors, but have a negative effect on neuronal lineage determination and neuronal maturation [87]. This is especially important as FGF-2 immunoreactivity within neurons, astrocytes and the vasculature is elevated in AD brain [88], suggesting that impaired neurogenesis caused by elevated FGF-2 levels might play a role in AD pathogenesis. Moreover, in proliferating adult rat hippocampal progenitor cells, FGF-2 was also shown to upregulate expression of tau and the activity of GSK-3, leading to increased phosphorylation of tau [89].

OTHER ANGIOGENIC AGENTS

As shown in Table 2, a number of other factors are also known to have a stimulating effect on angiogenesis. Among these compounds, there are quite a number of growth factors and transcription regulators that induce expression of angiogenesis-related genes like for example VEGF.

Most factors have been tested in models of wound healing, ischemia or cancer, but relatively little information is available on the effects in brain. An additional complicating factor for this kind of studies is that the compound should be able to cross the BBB if it is to be administered peripherally. A solution to overcome this problem is direct intracerebral injection of the compounds, but this kind of invasive technique is not without danger. This is especially important if this procedure is to be considered for the treatment of patients. An alternative is to engineer the compounds to increase brain uptake, for example by binding them to a compound that is actively transported through the BBB.

Recently, it was shown that the growth factor progranulin (PGRN) is implicated in neuronal survival [52;53]. Moreover, PGRN stimulates the production of other growth factors including VEGF [90], making it a potent inducer of angiogenesis. PGRN is a secreted growth factor that is expressed in many tissues and is involved in the regulation of multiple processes including development, wound repair and inflammation. Moreover, increased expression of PGRN is linked to tumorigenesis so when used for treatment purposes, caution is needed to find an optimal dose that does not cause detrimental side effects, a remark which is applicable to all growth factors which would be used in such studies.

CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, cellular and animal models have provided a number of challenging new insights into the pathogenesis of AD that have impacted on the current therapeutic strategies for AD. Although gain-of-toxic-function by Aβ still remains a favorite hypothesis and concurrently, most of the therapies being tested are directed against Aβ, AD occurring by an Aβ-independent mechanism or the so-called loss-of-function mechanisms are also getting more attention. Although loss-of-function of PS due to mutations in *PS* has been clearly shown, PS loss-of-function could be a more general mechanism in AD also occurring in patients carrying *APP* mutations and in the far more common sporadic patients of AD.

One of the consequences of the loss-of-function of (mutant) PS also impacts amyloidosis by which total Aβ, or the most abundantly secreted Aβ40 isoform, is drastically reduced. This was not only shown in cellular models [17], but also in mouse models where increasing the level of Aβ40 in brain while keeping the Aβ42 level constant decreased plaque burden. For instance, crossbreeding of BRI-Aβ40 mice with Tg2576 mice resulted in significantly reduced amyloid deposition compared to Tg2576 littermates [32]. These data suggest that Aβ40 might be a protective molecule, although its mechanism of action is currently unknown. Either Aβ40 interferes with the Aβ42 seeding or it sequesters Aβ42 and facilitates its clearance from brain. In addition to this Aβ-dependent loss-of-function mechanism, loss-of-function also impacts through Aβ-independent ways by affecting other PS-dependent substrates and other signaling pathways such as CREB/CBP and PI3K/Akt, which might also impact on AD pathogenesis. In turn, these mechanisms have therapeutic implications. For instance, the possible protective role of Aβ40 is extremely crucial to study as the current anti-amyloid therapies (like γ-secretase and BACE1-inhibitors) also reduce Aβ40 production, in addition to the already known caveats of γ-secretase inhibitors of interfering with essential physiological processes, causing additional pathologies. To correct PS loss-of-function caused by mutant *PS* or reduced expression, instead of interfering at a number of steps, gene therapy to supplement *PS* should be considered.

And finally, because vascular pathology is an important feature in AD pathogenesis leading to vascular leakage (extravasation of serum proteins in parenchyma)

and hypoperfusion, treatment directed towards the cerebral vascular system might have beneficial effects on AD. PlGF and PDGF-CC seem to appear as very interesting molecules to test in AD mouse models because they stimulate both EC and SMC growth, leading to the development of new, stable vessels, avoiding some complications associated with VEGF that causes the formation of unstable vessels. Because all angiogenic factors have specific effects on certain vascular cell types, it seems logical that the best therapeutic effect will probably be achieved by combining several substances that complement each other. Indeed, in a number of studies it was shown that treatment with a combination of substances has a greater effect than treatment with individual compounds, for example for the combination of VEGF and PlGF [80]. However, extensive *in vivo* studies in AD mouse models will be required to investigate the specific effects of angiogenic compounds on AD pathogenesis and to find optimal treatment procedures to minimize side effects.

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TABLE LEGENDS

Table 1: Summary of arguments favoring a gain-of-function or a loss-of-function mechanism for FAD mutations.

See section I A for more details.

Table 2: List of angiogenic factors as potential therapeutic targets against vascular deficits in AD.

FIGURE LEGENDS

Figure 1: Proteolytic processing of APP.

Schematic representation of the longest APP isoform (APP770) with indication of the position of the Aβ sequence and the single transmembrane domain of APP (Tm). NH2 and COOH indicate N-terminus and C-terminus of the protein, respectively. An enlarged view of the Aβ sequence is shown below with indication of β-, α- and γ-secretase cleavage sites (amino acid numbering for Aβ is shown above the sequence and for APP770 below the sequence). Aβ40 and Aβ42 are formed by subsequent cleavage of β- and γ-secretase, while p3 arises from α- followed by γ-secretase cleavage. While Aβ is generated by γ-secretase cleavage in the middle of the transmembrane domain (γ-site), APP intracellular domain (AICD) is formed by cleavage close to the cytoplasmic border of the transmembrane part (ε-site).

Figure 2: Intraneuronal Aβ accumulation in APP-Au transgenic mouse model.

A transgenic mouse model expressing the APPT714I mutation at levels lower than endogenous murine APP showed progressive accumulation of 4G8-immunoreactive intraneuronal deposits in CA1 and subiculum (arrows). The picture shown is of a 12 month-old APP-Au +/- mouse. 4G8 antibody is directed against amino acids 17-24 of Aβ. Scale bars, 20 μm.

Figure 3: Vascular abnormalities in Tg2576 transgenic mouse model.

Vascular deficits in Tg2576 mice lead to extravasation of serum proteins, which are normally restricted by the BBB, into the parenchyma. Serum proteins (Alb, albumin) co-localized with Aβ plaques in a hemorrhagic area (asterisk) of a 17-month-old Tg2576 mouse as studied by fluorescent microscopy. Arrowhead points to a plaque distant from hemorrhage not infiltrated by serum proteins. Scale bars, 40 μm.

Figure 4: Therapeutic strategies directed against Aβ.

Reduction of the amount of Aβ accumulating in brain can be achieved by 3 major routes as shown in the balloons: (1) reduction of the formation of Aβ by inhibition of the secretase enzymes; (2) inhibition of Aβ aggregation; (3) increase of the Aβ clearance from brain by (a) stimulating the activity of Aβ-degrading enzymes like insulin degrading enzyme (IDE), neprilysin (NEP) and endothelin-converting enzyme (ECE-1-); (b) treatment with antibodies against Aβ; (c) preserving cerebral vessel integrity because vascular pathology leads to less efficient clearance of Aβ.

Table 1

	In favor of gain-of-function (GOF)	In favor of loss-of-function (LOF)
Nature of *PS1* mutations	Missense mutations are usually GOF	Missense mutations can also cause LOF; Exonic deletions are LOF
Distribution of *PS1* mutations		Mutations distributed over the entire protein
***PS1* rescue experiments**	PS1A246E is as efficient as wild-type in rescuing lethality of *ps1* -/- mice. Other mutations not studied.	Many *PS* mutations are less efficient than wild-type *PS* in rescuing egg-laying deficit in *C. elegans*
Aβ production	Increased Aβ42 *in vitro* and *in vivo* by FAD *APP* and *PS* mutations	Decreased Aβ40 *in vitro* by *PS* and *APP* γ-secretase site mutations
		Decreased Aβ40 alone causes accelerated plaque pathology
Aβ aggregation	Increased aggregation by *APP* α-secretase site mutations	
APP dosage	*APP* gene duplications or *APP* promoter polymorphisms leading to increased Aβ production and AD	
AICD production		Reduced by *PS* mutations
Notch, N-cadherin and ephrinB2 processing		Reduced by *PS* mutations

Table 2

Compound	Animal model tested	Methods	Results	Reference
Vascular endothelial growth factor (VEGF)	Ischemia model	Intraperitoneal VEGF injection	Protection against permanent paralysis	[91]
	SOD1 G93A rat model of ALS	Intracerebroventricular VEGF injection using osmotic pumps	Delayed onset of paralysis, improved motor performance and prolonged survival	[75]
Placental growth factor (PlGF)	Nude mice	Adenoviral PlGF gene transfer into skin of ears	Formation of mature, non-leaky vessels that persisted for more than 1 year	[79]
Platelet-derived growth factor (PDGF-CC)	Ischemia models	Subcutaneous PDGF-CC injection using osmotic minipump	Enhanced postischemic revascularization of heart and limb	[81]
Basic fibroblast growth factor (FGF-2 or bFGF)	FGF-2 -/- and FGF-2 +/+ mice	Experimental TBI	Decreased neurogenesis and increased neuronal loss in FGF-2-/- mice	[84]
	C57BL/6 mice	Overexpression of FGF-2 by intracerebral injection of herpes simplex virus-1 amplicon vectors after TBI	Increased neurogenesis and reduced neurodegeneration	[84]
	FGF-2 overexpressing mice	Crossed with APP overexpressing mice	Enhanced lethal effect of APP overexpression; brain Aβ levels unchanged	[85]
Hypoxia-inducible factor-1alpha (HIF-1alpha)	FVB mice	Adenoviral HIF-1alpha gene transfer to skeletal muscle	Increased capillary sprouting and proliferation; transactivation of the VEGF promoter	[92]
Cardiac ankyrin repeat protein (CARP)	Wound healing animal models	Adenoviral CARP gene transfer	Induction of neovascularization (sponge implantation model of experimental granulation tissue) and increased blood perfusion (rabbit excisional wound model and rat ischemic wound model)	[93]
Del-1	Ischemia models (mouse and rabbit ischemic hind-limb muscle)	Intramuscular injection with Del-1 encoding expression plasmids	Induction of angiogenesis and improved muscle function	[94]
Sonic hedgehog (Shh)	Diabetic mouse model	Topical gene therapy with the use of naked DNA encoding for Shh	Acceleration of wound recovery by increased wound vascularity (partially by enhanced recruitment of bone marrow-derived endothelial progenitor cells)	[95]
Rosiglitazone	C57/BL6 mice	Treatment with rosiglitazone and femoral angioplasty	Promotion of differentiation and maturation of APCs to ECs and inhibition of the differentiation to SMCs	[96]
Endothelial PAS domain protein 1 (EPAS1)	Wound healing mouse models	Adenoviral EPAS1 gene transfer	Induction of expression of VEGF and endothelial-specific receptors Flt-1, Flk-1, and Tie2 mRNA at the wound site resulting in promotion of mature angiogenesis	[97]
Oxygen-regulated protein 150 (ORP150)	Diabetic mouse model	Adenoviral ORP150 gene transfer to wounds of diabetic mice	Accelerated wound repair and increased VEGF antigen in wounds	[98]

APCs, angiogenic progenitor cells; EC, endothelial cells; SMC, smooth muscle cells; TBI, traumatic brain injury