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Review

Evidence For and Against a Pathogenic Role of Reduced γ-Secretase Activity in Familial Alzheimer's Disease

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Abstract. The majority of mutations causing familial Alzheimer's disease (fAD) have been found in the gene *PRESENILIN1* (*PSEN1*) with additional mutations in the related gene *PRESENILIN2* (*PSEN2*). The best characterized function of PRESENILIN (PSEN) proteins is in γ-secretase enzyme activity. One substrate of γ-secretase is encoded by the gene *AMYLOID BETA A4 PRECURSOR PROTEIN* (*AβPP/APP*) that is a fAD mutation locus. AβPP is the source of the amyloid-β (Aβ) peptide enriched in the brains of people with fAD or the more common, late onset, sporadic form of AD, sAD. These observations have resulted in a focus on γ-secretase activity and Aβ as we attempt to understand the molecular basis of AD pathology. In this paper we briefly review some of the history of research on γ-secretase in AD. We then discuss the main ideas regarding the role of γ-secretase and the *PSEN* genes in this disease. We examine the significance of the "fAD mutation reading frame preservation rule" that applies to *PSEN1* and *PSEN2* (and *AβPP*) and look at alternative roles for AβPP and Aβ in fAD. We present a case for an alternative interpretation of published data on the role of γ-secretase activity and fAD-associated mutations in AD pathology. Evidence supports a "PSEN holoprotein multimer hypothesis" where *PSEN*

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fAD mutations generate mutant PSEN holoproteins that multimerize with wild type holoprotein and dominantly interfere with an AD-critical function(s) such as autophagy or secretion of Aβ. Holoprotein multimerization may be required for the endoproteolysis that activates PSENs' γ-secretase activity.

Keywords: Amyloid precursor protein secretases, familial Alzheimer's disease, gamma-secretase, human APP protein, human PSEN1 protein, human PSEN2 protein

DISCOVERY OF THE AUTOSOMAL DOMINANT FAMILIAL ALZHEIMER'S DISEASE LOCI AβPP, PSEN1 AND PSEN2

In 1906, Alois Alzheimer first identified foci (plaques) in the cerebral cortex staining in a similar manner to starch (hence "amyloid") in the brain of the late Auguste Deter, a patient who had suffered from presenile dementia [1]. Nearly 80 years later (in 1985), the discovery that Alzheimer's disease (AD) brains and Down syndrome brains share vascular deposits of amyloid-β (Aβ) led to the suggestion that AD may be caused by a genetic defect on Chromosome 21 [2, 3]. The subsequent discovery that Aβ is also found in neuritic plaques in AD brains [4, 5] founded the amyloid hypothesis that Aβ is central to the pathological process causing the disease. Great weight was lent to the amyloid hypothesis by the subsequent identification of a mutation causing familial AD (fAD) in the *AβPP* gene [6] and, after the *PSEN1* and *PSEN2* fAD loci were identified in 1995 [7, 8], by the realization that mutations in the *PSEN* genes could alter Aβ synthesis [9–12]. (Other, rarer fAD loci have since been identified, e.g., *SORL1* [13, 14]). Subsequent molecular analyses including inhibitor studies have shown that the PSEN proteins form the catalytic core of the γ-secretase enzyme complexes that cleave AβPP to form Aβ (e.g., [15–17]). While the effects of fAD mutations on Aβ production have been seen to be variable (for example, see summaries in [18, 19]), a consistent characteristic is a shift in the ratio of longer versus shorter forms of Aβ and this is thought to favor the formation of purportedly toxic aggregates [19, 20]. Nevertheless, during the past decade, the amyloid hypothesis has come under increasing criticism for a number of reasons including the lack of overt neuronal loss following deposition of human Aβ in mouse brains [21] (although synaptic toxicity is apparent [22]) and the failure of therapeutic approaches based on reduction of Aβ production or aggregation [23] (although this may be due to the advanced disease stages at which treatment has been attempted [24]). However, it is not the purpose of this paper to review the vast literature that now addresses the role of Aβ in AD. (Interested readers can refer to a number of excellent reviews on that topic such as [25–27].) Instead we will focus on the effects of mutations in the *PSEN* genes that represent the overwhelming majority of known fAD mutations.

Before γ-secretase can cleave a type I transmembrane domain protein (having a luminal/extracellular N-terminus and a cytosolic C-terminus), the luminal/extracellular domain must be almost entirely excised [28]. The luminal/extracellular domain of AβPP can be cleaved by a number of enzyme activities (Fig. 1A, 1B) but only cleavage at AβPP's "β-site" by a "β-secretase" allows production of the Aβ peptide. Subsequent cleavage by γ-secretase of the remaining transmembrane fragment of AβPP, C99, releases Aβ and the AβPP intracellular domain, AICD (see later and Fig. 1A).

ARE PRESENILINS THE ONLY γ-SECRETASES?

For a decade following the identification of the *PSEN* loci, there was uncertainty regarding whether PRESENILIN proteins were γ-secretases or the only source of γ-secretase activity. For example, in 2001, Armogida et al. [29] showed that mouse embryonic fibroblasts that apparently lacked *Psen1* and *Psen2* gene activity, indeed lost the ability to cleave within the transmembrane domain of an introduced Notch1 protein γ-secretase substrate. However, they could still produce 40 and 42 amino acid residue forms of Aβ ($Aβ_{40}$ and $Aβ_{42}$) from endogenous mouse AβPP. In 2002, Wilson et al. [30] showed that neurons harvested from mouse embryos lacking *Psen1* and *Psen2* activity could still produce, specifically, $Aβ_{42}$ in the endoplasmic reticulum (ER)/intermediate compartment indicating the presence of an additional γ-secretase activity in the early secretory pathway. In 2003, Taniguchi et al. fractionated membranes from cells lacking *PSEN1* and *PSEN2* activity and identified a fraction with apparent γ-secretase activity but lacking PSEN protein [31]. Lai et al. [32] in 2006 analyzed γ-secretase-like activity in the membranes of blastocyst-derived cells and saw continued

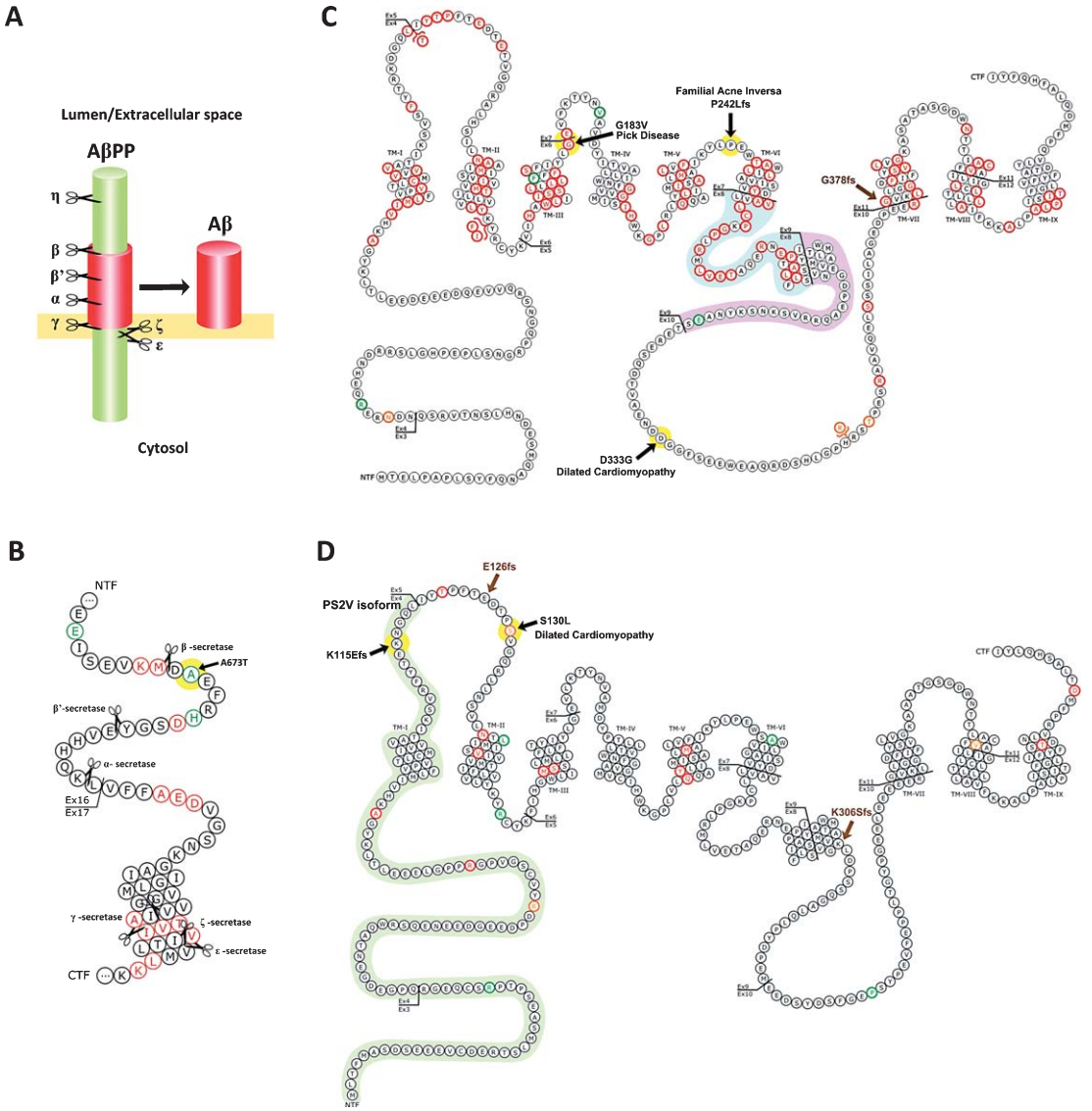

Fig. 1. Disease-causing mutations in PSEN1, PSEN2 and AβPP. A) Cleavages of AβPP. β-Secretase cleavage at the β-site, not the β'-site, followed by γ-secretase cleavage is required to produce Aβ peptides. Panels B, C, and D are modifications of diagrams taken from the Alzheimer Disease & Frontotemporal Dementia Mutation Database [47]. Red amino acid residues are sites of pathogenic mutation causing dementia. Green residues are non-pathogenic variants. The pathogenic nature of orange residues is unclear. Transmembrane domains (TM) and residues encoded by particular exons (Ex) are indicated and numbered. B) Mutations and cleavage sites in AβPP in the region of Aβ. Mutations are thought to affect the level of production of Aβ by changing cleave site preference or affect the structure of Aβ, or both. The production of AICD may also be affected. The position of the "protective" A673T mutation [159] is highlighted with a yellow background. C) Mutations in PSEN1. Residues affected by mutations causing Pick Disease, Familial Acne Inversa and Dilated Cardiomyopathy are highlighted with a yellow background. Residues deleted by fAD mutations causing loss of exon 9 are highlighted with a pink background. The L271V mutation causes increased formation of a naturally-occurring PSEN1 isoform lacking residues encoded by exon 8 (highlighted by a blue background) but it is the simultaneous expression of full-length PSEN1 containing the valine residue at position 271 that apparently causes fAD [51, 91]. D) Mutations in PSEN2. Residues included in the hypoxia-induced isoform PS2V are highlighted with a green background. The position of the unique frame-shift fAD mutation K115Efs and of the S130L mutation causing Dilated Cardiomyopathy are highlighted with yellow background. The "mutations" putatively identified by Kadmiri et al. [121, 122] are upstream of the region shown in B or are indicated with brown arrows and text in C and D.

production of Aβ that could not be inhibited with the γ-secretase inhibitor L-685,458. However, the activity could be inhibited with the general aspartyl protease inhibitor pepstatin A. The production of Aβ was also observed in membranes from blastocyst-derived cells lacking PSEN proteins. Interestingly,

the optimal pH for production of $A\beta_{40}$ and $A\beta_{42}$ by this activity was pH 6.0 compared to the measured optimum pH for Aβ production when the L-685,458 was not present of closer to pH 7.0. This suggests that the two activities may be distributed in different subcellular compartments. The PSEN-independent γ-secretase-like activity was also responsible for a greater proportion of total $A\beta_{42}$ production than of total $A\beta_{40}$. Other evidence that PRESENILINs might not be essential for production of Aβ included that expression of human AβPP in a yeast species apparently lacking PRESENILIN orthologs, *Pichia pastoris*, nevertheless permitted formation of Aβ peptides [33]. However, the PRESENILINs were, ultimately, conclusively shown to function as γ-secretases by the demonstration that transition state analogue inhibitors of γ-secretase bind to the PSENs [34], by reconstruction of the enzyme complex (consisting of the four proteins NCSTN, PSENEN, APH1 and PSEN) in the yeast *Saccharomyces cerevisiae* [17] and by the demonstration that recombinant PSEN alone, when expressed in and purified from bacteria and then reconstituted into liposomes, retained some ability to cleave AβPP [35].

The studies above suggest that a non-PSEN-dependent form of γ-secretase-like activity can be present in animal cells and can produce Aβ. One protein that may provide this activity is the acidic aspartyl protease Cathepsin D (CTSD). CTSD is capable of producing Aβ in assays where AβPP fragments equivalent to those produced after cleavage by β-secretase are provided [36, 37]. In human brain, BACE1 protein appears to provide most of the β-secretase activity generating Aβ [38] (although *in vitro* studies have indicated that CTSD may also cleave AβPP at its β-site raising the possibility that this one protease may be sufficient to produce Aβ from AβPP [39]). CTSD shows maximum activity at pH 3.8 [40] similar to BACE1's optimum of pH 4.5 [41]. Curiously, BACE1 is nearly inactive at pH 6.0 which is the optimum for the γ-secretase-like activity described by Lai et al. above [32] who also showed that γ-secretase has a pH optimum closer to 7! This is despite the fact that a protein complex has been detected containing both PSEN1 and BACE1 (together with OPIOID RECEPTOR, DELTA-1; OPRD1) [42] indicating that the γ-secretase and β-secretase enzyme activities may (at least in some situations) be spatially co-located. However, to confirm that CTSD provides the γ-secretase-like activity in cells lacking PRESENILINs it will be necessary to analyze Aβ production in triple-mutant cells lacking all of *PSEN1*, *PSEN2*, and *CTSD*.

Finally, it is worth mentioning the fascinating case of another γ-secretase substrate, the NERVE GROWTH FACTOR RECEPTOR, NGFR (p75(NTR)) and its close homolog NRH1. NRH1 is found in non-mammalian vertebrates while a truncated form, NRH2 (also known as NRADD) exists in mammals [43]. The sequence similarity between NGFR and NRH1 points to a relatively recent evolutionary origin by a gene duplication event in vertebrates. However, unlike NGFR, the apparent intramembraneous cleavage of NRH1 (and NRH2) cannot be inhibited by the γ-secretase inhibitors DAPT and WPE [43]. Unpublished preliminary investigation of the zebrafish orthologue of NRH1 in Lardelli's laboratory supports these observations. However, another report on NRH2/NRADD did see inhibition of cleavage of NRH2 with γ-secretase inhibitors L685,458; DAPT and Compound E [44] and resolution of these conflicting observations is required. Was the common ancestor of the NGFR and NRH1 proteins cleaved by γ-secretase or the "other" unidentified γ-secretase-like enzyme? What determines the susceptibility of a transmembrane protein to γ-secretase cleavage when no clear cleavage site recognition sequence or motif has been identified? [45].

CURRENT THINKING ON THE ROLE OF PRESENILINS IN AD

Less than 1% of AD cases are thought to be due to autosomal dominant mutations [46]. Nevertheless, research focused on how these mutations cause AD at the cellular and molecular level has had enormous influence on how we view the etiology of both early onset fAD and the far more common late onset sporadic AD (sAD). Over 205 different fAD mutations have been identified in the *PSEN1* gene [47]. In a large French study of 130 families showing autosomal dominant early-onset AD [14], 82 families (63%) possessed missense mutations in *PSEN1*. A further 7 families (5%) showed missense mutations in *PSEN2*. Therefore, mutations in the *PSEN* genes accounted for over two-thirds of autosomal dominant fAD in the study. 14 missense mutations in *AβPP* and 11 duplications of *AβPP* (i.e., 19% of families) were also found. The predominant focus on Aβ as the cause of AD pathology has meant that most research on the role of *PSEN1* and *PSEN2* mutations in fAD has

examined their effects on cleavage of AβPP and Aβ production.

It is a very striking characteristic of the fAD mutations in *PSEN1*, *PSEN2*, and *AβPP* that none of the hundreds of these fAD mutations cause loss of the gene (e.g., by deletion) or truncation of the coding sequences (e.g., by nonsense or frameshift mutations) [47]. Even mutations causing exon loss from transcripts due to deletion of exon DNA [48] or through effects on splicing [49–51] result in transcripts with open reading frames coding for normal C-terminal sequences. (There are some rare exceptions to this "fAD mutation reading frame preservation rule" that are discussed below.) The concentration of the AβPP fAD mutations near the α-, β-, and γ-secretase cleavage sites in the protein supports that the mutations alter these cleavage events (reviewed in [52]). However, the fAD mutations in the PSEN proteins are scattered throughout their peptide chains with a somewhat higher frequency in their numerous transmembrane domains (see Fig. 1C, D). Three basic ideas have been suggested to explain this lack of any focus to the position of the fAD mutations in PSEN1 or PSEN2:

1. Mutations in PSEN Proteins Shift the Profile of Aβ Production toward Longer Peptides

γ-Secretase cleavage of AβPP occurs after the luminal/extracellular domain of the protein is excised by α– or β-secretase activity. If AβPP is cleaved by β-secretase at its β-site (rather than at its β'-site [41, 53] or at the α-site, see Fig. 1), then Aβ peptides of around 40 amino acid residues length can subsequently be formed. Following α– or β-secretase cleavage of AβPP, the membrane-bound fragment is further cleaved by the γ-secretase enzyme complex at what is termed the ε-site near the cytosolic extent of AβPP's transmembrane domain [54–56] (Fig. 1A, 1B). This "endopeptidase" cleavage by PRESENILIN releases AβPP's cytosolic "intracellular domain" (AICD) that interacts with a number of intracellular signaling pathways and acts as a transcription factor (reviewed in [57, 58]). Subsequent "carboxypeptidase" cleavages by PRESENILIN are thought to shorten the remaining, membrane-embedded portion of AβPP until its hydrophobicity is sufficiently reduced that it can escape from the lipid bilayer as soluble Aβ [59, 60]. The position of the initial ε-site cleavage is variable [56] and subsequent cleavages by PRESENILIN may occur progressively from the carboxy-terminal (the "progressive proteolysis hypothesis" [61, 62]) along either of two "product lines" governed by the initial cleavage [61, 63]. An attractive aspect of this idea is that the many and widely distributed fAD mutations in the PSEN proteins can be thought of as generally reducing the efficiency of γ-secretase cleavage of AβPP resulting in the commonly cited phenomenon of increased Aβ peptide length in fAD (e.g., [9, 10]) and sAD [64] brains. This idea also aligns the apparent effects of fAD mutations in both of the PSENs with mutations in AβPP that affect its cleavage by γ-secretase, i.e., either type of mutation leads to formation of longer Aβ forms ([65] see below).

In support of this apparently unifying molecular mechanism underlying fAD are the results of cell-free γ-secretase assays published in 2012 by Chávez-Gutiérrez et al. [63]. These suggest that fAD mutations in the PSENs need not alter the initial endopeptidase cleavage of AβPP but consistently decrease the rate of the final carboxypeptidase cleavage. These researchers also saw that fAD mutations in AβPP apparently shifted the γ-secretase cleavage of AβPP into the product line favoring formation of $A\beta_{42}$. However, despite the apparent consistency of these results there are also uncertainties. First, within cells, γ-secretase is concentrated in cholesterol- and sphingolipid-rich lipid rafts [66, 67] but it is doubtful that cell-free γ-secretase assays involving solubilisation of cell membranes preserve this environment. This may be significant, since the thickness of lipid bilayers has been shown to affect the site of γ-secretase cleavage of AβPP with thinner membranes promoting formation of $A\beta_{42}$ over $A\beta_{40}$ [68]. Reassuringly, recent research published by Chávez-Gutiérrez and co-workers assaying AβPP cleavage in detergent-resistant membranes (lipid-raft rich) extracted from the prefrontal cortices of fAD brains, showed a consistent decrease in carboxypeptidase cleavage but not endopeptidase cleavage [69]. However, no such change was observed when these assays were performed on detergent-resistant membranes from sAD brains. The researchers explained this by citing that Aβ clearance rather than production is thought to be defective in late onset sAD [70] but, nevertheless, it implies the existence of different mechanisms underlying early onset fAD and late onset sAD. Also perplexing is the idea that the "endopeptidase" and "carboxypeptidase" activities of the γ-secretase complex are differentially affected by mutations in the PSENs when, essentially, these are most likely an identical enzyme activity imposed on different lengths of the same substrate protein. Why does this differential effect occur?

Another troubling aspect of the idea that promotion of $A\beta_{42}$ over $A\beta_{40}$ production is critical in AD is the fact that some fAD mutations in PSEN1 dramatically decrease the ability of γ-secretase to produce Aβ while the shift in the $A\beta_{42}/A\beta_{40}$ ratio need not be dramatic (e.g. [71]). It is difficult to understand why a slight isoform ratio shift should be important in promoting pathology via Aβ aggregation when, presumably, aggregation would be inhibited by a decrease in the total Aβ concentration (including, as observed in [71], either no significant change or a decrease in the concentration of $A\beta_{42}$).

2. Changes in the Level of γ-Secretase Activity are Critical

The γ-secretase enzyme cleaves over 90 substrate proteins including those involved in critical forms of cell signaling such as Notch receptors and NGFR (reviewed by [72]). In 2007, Shen and Kelleher proposed the Presenilin hypothesis which states that changes in the activity of the PRESENILINs underlie fAD and, possibly, sAD [18]. Aβ is incorporated into the model by postulating that it may inhibit normal γ-secretase activity (something that has actually been observed under non-physiological conditions in cell culture [73]) and that it interferes with synaptic function and signal transduction. While acknowledging the diverse functions of the PRESENILINs including in regulation of Wnt signaling (e.g., by regulating phosphorylation of β-catenin [74]) and in Ca^{2+} ion homeostasis, the hypothesis is primarily γ-secretase focused.

In 2013, Shen, Kelleher, and co-workers published an analysis stated to support a dominant negative interaction between fAD mutant forms of PSEN1 and the wild type (non-mutant) form as would be found in heterozygous individuals possessing dominant fAD mutations [75]. In the study, they co-tranfected constructs expressing mutant and wild type PSEN1 in a 1:1 ratio at (purportedly) sub-saturating levels into mouse embryonic fibroblasts lacking mouse *Psen* genes. They saw reductions in γ-secretase activity as evidenced by reduced production of AICD from co-transfection with the C99 fragment of AβPP or of the Notch intracellular domain, NICD, from a truncated form of the Notch1 receptor. (N-terminally truncated forms of transmembrane protein such as C99 are commonly used in γ-secretase assays since these do not require prior membrane-proximal, extracellular/luminal cleavage in order to become γ-secretase substrates.) However, the reductions in AICD and NICD were not >50% as one might expect for a dominant negative effect. While this may be explained by the claim that the levels of PSEN1 were not saturating, one needs to remember that, in cell transfection experiments, only a subset of the cells present are competent to absorb the expression vector DNAs [76] and the expression levels of the vectors in these individual cells can be quite high, while the expression measured over the total cell population will be lower. Also, as Thinakaran et al. showed in 1997 [77], cells appear to possess a mechanism that limits total PSEN protein to a particular level.

In 2015 the debate around the importance of changes in γ-secretase activity gained additional intensity [78] with the publication of another paper by Shen, Kelleher, and co-workers with direct relevance to the question of whether fAD mutations in the PSENs have dominant negative effects on γ-secretase activity. Xia et al. [71] had introduced fAD mutations of human *PSEN1* (L435F or C410Y) into the endogenous *Psen1* gene of mice and found that levels of *de novo* Aβ production (reflecting γ-secretase activity) were reduced by approximately half in *in vitro* γ-secretase assays of brain tissue from embryonic and 3 month old heterozygous L435F mice. This does not support a dominant negative action of L435F mutant Psen1 on the γ-secretase activity of wild type Psen1. The L435F or C410Y mutant alleles showed almost complete loss of AβPP cleavage when homozygous. Interestingly, both mutations cause great increases in Psen1 holoprotein levels in heterozygous mouse brains (presumably due to decreased auto-endoproteolysis of Psen1 that activates the γ-secretase activity of the protein [79]). Endogenous levels of both $A\beta_{40}$ and $A\beta_{42}$ were decreased in the detergent-resistant ("insoluble") fraction from 3-month-old heterozygous L435F mouse brains but little change was seen for the soluble fraction. This suggests either that clearance of Aβ was reduced (as might be expected from, e.g., inhibited lysosome function) or even that a homeostatic mechanism exists to maintain a particular level of Aβ expression in brain. However, the $A\beta_{42}/A\beta_{40}$ ratio was significantly increased in both fractions. We note also that in the recent study by Chávez-Gutiérrez and co-workers examining detergent resistant membranes from AD brains, they state, "Undoubtedly, our data do not support a mutant-mediated "dominant-negative effect" on the healthy allele".

The 2015 Xia et al. paper is important, since it provides evidence of a loss of γ-secretase activity in *PSEN1* fAD mutant brains. However, it does not resolve whether this or changes in the $A\beta_{42}/A\beta_{40}$

ratio are critical to the disease. Indeed, another Psen1 mutation knock-in model from the laboratory of Shen and Kelleher — the G183V mutation [80] that apparently causes Pick disease (a frontotemporal dementia) without Aβ deposition [81, 82] — also shows decreased γ-secretase activity and Aβ production. Why should the same phenomena (decreased γ-secretase activity and Aβ production) produce two different diseases? This does not support that decreased γ-secretase specifically causes fAD. However, both Pick disease and AD are tauopathies where neurons show inclusions containing MICROTUBULE-ASSOCIATED PROTEIN TAU (MAPT) [83] and aberrant splicing of *PSEN1* transcripts has been observed in sporadic frontotemporal dementia [84]. Takashima et al. [85] showed that PSEN1 protein binds MAPT together with a kinase that phosphorylates the latter, GLYCOGEN SYNTHASE KINASE 3-BETA (GSK3B). They also showed that transfection of fAD mutant forms of PSEN1 into COS-7 cells could increase phosphorylation of MAPT. Saura et al. [86] showed that loss of both PSEN1 and PSEN2 from the postnatal forebrain of mice causes hyperphosphorylation of MAPT. These observations suggest that decreased γ-secretase activity may be involved with the formation of MAPT inclusions although Watanabe et al. [80] did not observe these (or changes in the phosphorylation of MAPT) in the brains of their homozygous G183V mouse model.

We should note that the G183V mutation of human *PSEN1* is unusual since it affects the spice donor site of exon 6 and produces both normally spliced transcripts and transcripts with truncated open reading frames [81, 83]. The Watanabe et al. paper shows that these aberrant transcripts are not completely degraded in brain tissue although nonsense-medicated mRNA decay appears to remove them completely in other tissues. We have analyzed the putative protein products of these aberrant transcripts using assays in zebrafish embryos and have seen dominant negative effects specifically on Notch signaling but not on γ-secretase cleavage of an AβPP orthologue [87]. Zebrafish Psen1 peptides equivalent to those putatively translated from the aberrant transcripts also incorporate specifically and avidly into detergent-resistant higher molecular weight complexes and bind to both Psen1 and Psen2 proteins [87]. Therefore, it is possible that the action of truncated forms of PSEN1 protein may underlie the frontotemporal dementia pathology of the G183V mutation of human *PSEN1*.

A very significant advance in our understanding of γ-secretase came in 2015 with publication of the first detailed atomic structure of the enzyme complex by Bai et al. [88]. Notably, these researchers introduced ten different fAD mutations into PSEN1 and saw no consistent change in γ-secretase activity. Some mutations abolished cleavage of C99, some had little influence, while other actually increased γ-secretase activity! As Bai et al. stated, "These observations strongly suggest a disconnection between the total protease activity of γ-secretase and the development of fAD". They also observed, "All eight mutations for which the $A\beta_{42}/A\beta_{40}$ ratio can be calculated led to increased $A\beta_{42}/A\beta_{40}$ ratios compared with wild-type γ-secretase . . . The generally increased ratios of $A\beta_{42}$ over $A\beta_{40}$ may suggest a causal relationship for development of fAD, but could also be explained by other possibilities." We describe two such possibilities later in this paper.

3. Changes in the Non-γ-Secretase Activity(ies) of PSEN Underlie AD Pathology

The γ-secretase enzyme is a complex of four proteins and, if reduced γ-secretase activity was the cause of fAD, then we might expect fAD mutations to occur in the other γ-secretase complex components. Remarkably, these have never been found but mutations in *PSEN1*, *NCSTN*, and *PSENEN* have been found in another disease, inherited acne inversa [89] (also known as hidradenitis suppurativa). It is also very significant that the single known mutation in *PSEN1* causing acne inversa, P242LfsX11 (Fig. 1C), does not follow the "fAD mutation reading frame preservation rule" since it causes a frameshift in exon 7 and truncation of the open reading frame. This mutation does not appear to cause fAD [89].

The existence of 205+ fAD mutations in *PSEN1* following the "fAD mutation reading frame preservation rule" and a single *PSEN1* mutation that does not preserve the open reading frame and that causes a completely different disease, strongly supports that mutations in *PSEN1* can only cause fAD when they preserve the "full-length" of the protein. A parsimonious explanation for this phenomenon may have been revealed by the Heilig et al. paper [75], that showed strong association between mutant and wild type forms of the PSEN1 holoprotein (a phenomenon also noted by others [90, 91]), and by work from Nixon and co-workers demonstrating an effect of fAD *PSEN1* mutations on autophagy [92]. In 2010, Lee et al. showed that heterozygous fAD mutations in *PSEN1* inhibit macroautophagy by reducing

N-glycosylation of the V0a1 subunit of vacuolar [H^+] ATPase leading to reduced acidification of lysosomes. Subsequently, two other laboratories disputed these findings [93, 94] leading to a very "vigorous" debate at the AlzForum online site [95]. However, increased lysosomal pH due to mutations in *PSEN1* has since been supported by additional work [96].

The Lee et al. paper notes three remarkable phenomena: a) It is the PSEN1 holoprotein, not its endoproteolysed form, that complexes with the proteins required for N-glycosylation of the V0a1 subunit of the vacuolar [H^+] ATPase; b) loss of the γ-secretase complex component NCSTN does not affect macroautophagy; and, c) chemical inhibition of γ-secretase activity does not affect macroautophagy. These three phenomena exactly align with what we know of mutations in PSEN1 causing fAD, i.e., a) only mutations that preserve the full length of the PSEN1 holoprotein cause fAD; b) only mutations in PSEN1 and not other γ-secretase complex components cause fAD and; c) decreased γ-secretase activity does not cause fAD (since it is observed in mouse models of human *PSEN1* mutations causing either fAD or FTD and so does not differentiate between them and since reduced γ-secretase activity causes acne inversa without fAD). This idea is also consistent with a single, fundamental mechanism for both fAD and sAD since disturbances of lysosomal function have been observed in human fAD brains [97] and fibroblasts [92] and disturbances of autophagy have been observed in sporadic late onset AD brains [98]. Therefore, lysosomal dysfunction provides a unifying pathological mechanism underlying AD. Such a unifying mechanism is important because, at the genetic level, there is little overlap between mutations causing fAD and the genetic risk loci for sAD identified in genome-wide association studies (GWAS). To date, only the gene *SORL1* has been identified as a locus in both fAD and sAD, although other connections are apparent. For example, a search using human *PSEN1* in the gene co-expression database COXPRESdb [99] shows that the most tightly co-regulated gene is the sAD risk locus *PICALM* (Ebrahimie, Lardelli et al., unpublished results), a locus repeatedly identified in GWAS [100–103] and that influences autophagy [104]. Also, a truncated isoform of PSEN2, "PS2V", is upregulated in sAD brains [105]. PS2V is induced by hypoxia/oxidative stress, is associated with increased production of Aβ [106, 107], can suppress the unfolded protein response (UPR) and can boost γ-secretase activity [107, 108] through an unknown mechanism that may involve interaction with full-length PSEN protein [87].

Other activities of the PSEN proteins have also been seen to be dependent on the holoprotein rather than the endoproteolytically cleaved, γ-secretase-active form. PSEN holoproteins have been proposed to act as Ca^{2+} "leak channels" in the ER with fAD mutants unable to perform this function [65, 109]. In 2009, Schon and co-workers showed that the PSEN proteins are concentrated in a particular, specialized functional domain of the ER where it is closely juxtaposed to mitochondria, the "mitochondria associated membranes" (MAM, see [110]). Formation of protein disulphide bonds occurs in the MAM involving relatively large amounts of H_2O_2 formation (oxidative protein folding, reviewed by [111]). The MAM is comprised of detergent resistant membrane with the characteristics of a lipid raft [110, 112]. It is responsible for cholesteryl ester and phospholipid synthesis [112], controls mitochondrial activity and apoptosis via Ca^{2+} signaling (reviewed in [113]), is a site of autophagosome initiation [114] and is required for innate immune inflammatory responses [115]. Therefore, the MAM is a nexus for cellular activities and phenomena commonly associated with AD pathology. Indeed, Schon's laboratory has shown that fibroblasts from both fAD and sAD individuals exhibit increased MAM formation leading to the hypothesis that it is dysfunction in communication between the ER and mitochondria that causes AD (the "MAM hypothesis", [116]). While changes in PSEN expression and function can alter the degree of MAM formation in cells, MAM formation does not appear to be dependent on γ-secretase activity, implying that it is changes in PSEN holoprotein function that are critical.

In summary, we suggest that a parsimonious explanation for the PSEN "fAD mutation reading frame preservation rule" and the dominant nature of these mutations is a "PSEN holoprotein multimer hypothesis" whereby fAD mutations in *PSEN1* or *PSEN2* cause disruption of a holoprotein function (or functions) that are critical for maintenance of cellular homeostasis in an aging brain. The mutations are dominant due to inhibitory multimerization of mutant holoproteins with wild type holoprotein. The hypothesis is summarized in Fig. 2 (showing the possibility that the fAD-critical function disrupted by mutation of the holoprotein is N-glycosylation of the V0a1 subunit of vacuolar [H^+] ATPase as suggested by Lee et al., [92]).

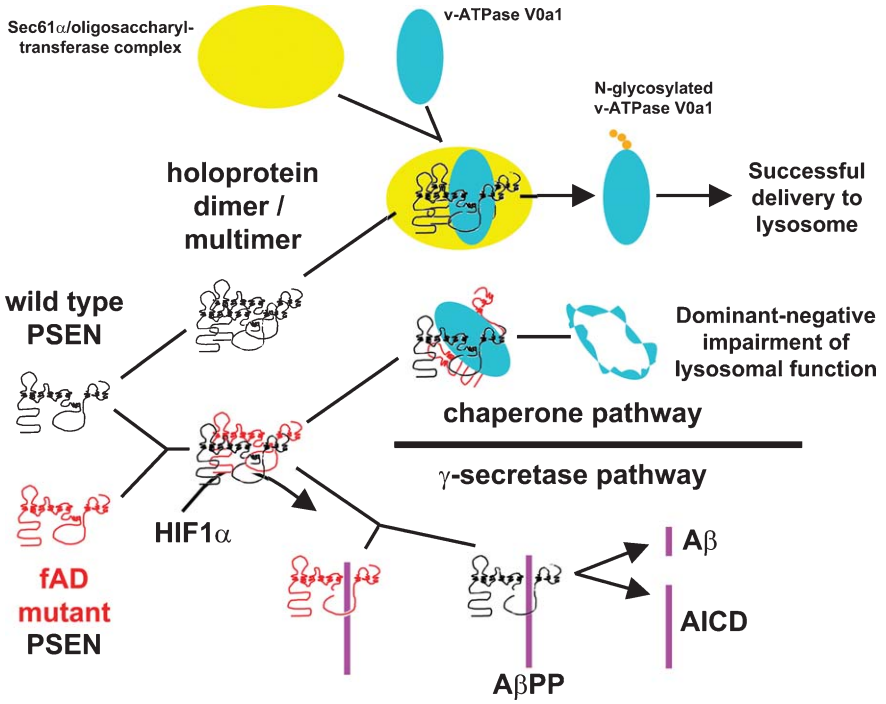

Fig. 2. Hypothetical model of action of PSEN1 holoprotein including multimerization and a possible interaction with HIF1α. PSEN holoproteins can form multimers and these multimers may be necessary for the endoproteolysis that activates γ-secretase activity. Mutations that preserve the open reading frames of PSENs may allow mutant holoproteins to bind to wild type holoproteins and inhibit holoprotein-dependent activities that are critical to cellular homeostasis in ageing brains. Under hypoxic conditions HIF1α may normally interact with multimeric holoprotein complexes to stimulate formation of active γ-secretase. Normal lysosome acidification may be required for correct metabolism and/or secretion of AβPP, AICD and Aβ.

REAL AND ARTEFACTUAL EXCEPTIONS TO THE "FAD MUTATION READING FRAME PRESERVATION RULE"

Until recently no possible exceptions were known to the rule that fAD mutations preserve the open reading frames of *PSEN1*, *PSEN2*, and *AβPP*. However, in 2010, Jayadev et al. published a report on fAD mutations in *PSEN2* that included discovery of a two nucleotide deletion causing a frameshift near the downstream end of exon 4 followed shortly thereafter by a premature termination of the open reading frame. If this mutant allele, K115Efs, produces a protein product then it would resemble the PS2V isoform mentioned previously that can both increase γ-secretase activity (and hence Aβ production) and inhibit the UPR by an unknown mechanism [107, 108] (see Fig. 1D). (The two phenomena may be mechanistically related [117].) Since PS2V is observed to be upregulated in sAD brains, the K115Efs mutation may provide another link between the pathologies of fAD and sAD [108]. However, K115Efs may be mechanistically unique among the PSEN fAD mutations since it may enhance Aβ production (or AβPP signaling) via increasing γ-secretase activity or it may promote Aβ oligomerization via suppression of the UPR (or both) rather than inhibiting Aβ breakdown and/or secretion via autophagy [118–120].

In 2014, Kadmiri et al. published two papers reporting the discovery of frameshift mutations in Moroccan fAD families [121, 122]. In both papers it is claimed that 17 sporadic cases of AD and eight families with AD were examined, so presumably the same patient cohort forms the basis for both studies. In one paper they claim to have discovered seven novel frameshift mutations in *AβPP*, three of which occur in families [121]. Remarkably, one particular mutant allele was discovered in three apparently separate families and, in one family, the proband possessed two separate frameshift alleles while an unaffected relative had a mutation affecting an *AβPP* transcript splice site! No mis-sense mutations in *AβPP* were reported. If we consider that the Alzheimer Disease & Frontotemporal Dementia Mutation Database lists 26 different AβPP mutations discovered for

AD (10 complete gene duplications and 16 missense mutations) from tens of separate publications, the likelihood that so many novel, unprecedented fAD mutations in AβPP would be discovered in one study is infinitesimally small. In their second paper [122] Kadmiri et al. claim to have found frameshift mutations in exon 10 of *PSEN1* (Fig. 1C) and in exons 5 and 9 of *PSEN2* (Fig. 1D). The mutations were apparently found in three different families with no mutations found in sAD cases. However, no analysis of DNA from two or more affected individuals within a family is reported. Once again, with over 220 different mutations found collectively in *PSEN1* and *PSEN2* in dozens of separate studies, none of which destroy the open reading frame of the genes, the probability is extremely small that three different such mutations could be found within only eight AD families. For these reasons we consider it highly unlikely that the data in these two papers is reliable and verification of the data is essential before they can be regarded as part of the fAD mutation knowledge base. Unfortunately, the "mutations" the papers report have already been cited uncritically elsewhere in the scientific literature [123].

WHAT THEN OF AβPP, Aβ, AND THE $A\beta_{42}/A\beta_{40}$ RATIO?

The fact that mutations in the three major fAD genes all affect production of the Aβ peptide has been the most convincing evidence for the centrality of Aβ in the etiology of fAD. Therefore, if we are to discount changes in γ-secretase activity as being causative of fAD, another mechanism must be found by which mutations affecting the PSENs and AβPP can have inter related effects. In this light it may well be significant that AβPP is a substrate of chaperone-mediated autophagy [124] and that autophagy is involved in both the secretion and breakdown of Aβ [119]. AβPP has also been shown to have a very wide range of cellular functions. AβPP influences Fe ion homeostasis via its interaction with Ferroportin (SLC40A1, [125]) and AβPP affects embryo development, including vascular development, possibly via secretion of its extracellular/luminal domain [126, 127] and Aβ [128]. AβPP also influences the rate of protein synthesis [129], neurite outgrowth [130], and axonal pruning [131] as well as LPS-mediated innate immune responses [132]. Signaling via AICD regulates genes including *MEMBRANE METALLOENDOPEPTIDASE* (*MME*, also known as neprilysin) [133] and *TRANSTHYRETIN*, *TTR* [134] that encode proteins involved in degradation and clearance of Aβ from the CNS, respectively. One or more of these functions may interact with PSEN holoprotein activities. For example, alterations in *PSEN* gene expression affect metal homeostasis [135] and *PSEN2* is also known to influence LPS-mediated innate immune responses while *PSEN1* may not [136].

There are many different lengths of peptide cleaved from the membrane-proximal region of AβPP. These can also carry various forms of chemical modification that may influence their role in AD pathogenesis (reviewed by [137]). However, the most intensively studied peptides derived from AβPP are $A\beta_{40}$ and $A\beta_{42}$. For many years these forms of Aβ were regarded as unfortunately toxic but otherwise functionless by-products of AβPP cleavage [138]. However, numerous lines of evidence now argue against this. The sequence of Aβ is highly conserved in species as distantly related as primates (humans) and lobe-finned fishes (Coelacanth) [128] implying that mutations in Aβ are selectively disadvantageous. Aβ has neuroprotective properties [139] and may act variously as an antioxidant [140–145], in vascular development [128, 146] and function [147], as a regulator of long term potentiation [148] and even as an antimicrobial peptide of the innate immune system [149]. Aβ has also been implicated as a transcription factor. In 2011, Lahiri and colleagues claimed the existence of a DNA sequence motif binding Aβ in the promoters of the *AβPP*, *BACE1*, and *APOE* genes [150, 151]. In 2012, Piccini et al. [152] showed upregulation of *BACE1* expression specifically by $A\beta_{42}$ (but not $A\beta_{40}$) applied to neuroblastoma cells apparently via the JNK signaling pathway and in 2014 Baracker et al. [153] showed that cells can absorb Aβ and transport it into nuclei where, specifically, $A\beta_{42}$ (but not other isoforms) can form transcriptional complexes to repress expression of the genes *LRP1* and *KAI1*. Aβ is upregulated by cellular stress, in particular hypoxia [154–156]. This is a selectively advantageous protective response since the molecular mechanism for upregulating Aβ under hypoxia/oxidative stress appears conserved in both zebrafish and humans, species that shared a common ancestor almost half a billion years ago [157]. The production of Aβ is very responsive to the level of expression of BACE1 [158] which itself is sensitive to hypoxia [154–156]. The "protective mutation" in AβPP (A673T) identified in studies of the Icelandic population and that reduces BACE1 cleavage

of AβPP by around 40% [159] can be seen in this light. The mutation may well be reducing the cellular dysfunction that can accompany an over-vigorous protective response, much as reducing inflammatory responses can reduce cancer risk [160] or improve wound healing [161]. This is supported by the work of Weihong Song and colleagues [162] who showed that, in fact, the most common site of cleavage of AβPP by BACE1 is the β' site that prevents Aβ formation by forming a shorter peptide (see Fig. 1). They also showed that the fAD-causing "Swedish" double mutation of AβPP inhibits this cleavage so that cleavage at the alternative β-site producing Aβ becomes more frequent. In this case the Swedish mutation may be seen as causing an upregulated stress response.

Recently, Villa et al. [163] showed that the master regulator of cellular responses to hypoxia, HYPOXIA-INDUCIBLE FACTOR 1, ALPHA SUBUNIT (HIF1α), binds directly to γ-secretase complexes to upregulate γ-secretase activity under low oxygen. This upregulation did not require increased transcription of γ-secretase complex components. Rather it appeared to be due to activation of existing "inactive γ-secretase". It is possible that, under hypoxic stress, HIF1α promotes the endoproteolysis of a pre-existing pool of PSEN holoprotein to form active γ-secretase. Hypoxia is a form of cellular stress increasingly identified in AD [164, 165] and one implication of Villa and colleagues' analysis is that fAD mutations might affect the effectiveness of this cellular response via alteration of an interaction (direct or indirect) between PSEN holoprotein and HIF1α. Hypoxia also upregulates autophagy [166] consistent with the role of PSEN1 holoprotein in lysosomal acidification and the concentration of PSEN proteins in the MAM. It is particularly intriguing that, in transgenic mice, expression of human fAD mutant forms of *PSEN1* exclusively in neurons can cause a microvascular phenotype of abnormally looped vessels as well as "string vessels" [167]. Aβ peptides are known to be vasoactive [168] and their secretion requires autophagy [118] making them a candidate for a mechanism of communication between neurons and brain microvasculature. Since Aβ levels are so responsive to hypoxia (e.g., [169, 170]), we can speculate that the increase in brain Aβ deposition seen decades before onset of sAD [171] may be driven by the inability of aging brain vasculature to deliver sufficient oxygen. *PSEN* fAD mutations that promote abnormal microvascular phenotypes may thereby predispose to, and accelerate the onset of, AD. (See also a recent comprehensive review of the role of vasculature in AD by Di Marco et al., [172]).

Lastly, we should examine another of the strongest pieces of evidence used to support the pathological role of $A\beta_{42}$ (and hence the pathological role of apparent changes in the endopeptidase activity of γ-secretase): The close and highly statistically significant inverse-correlation of variation in $A\beta_{42}/A\beta_{40}$ ratio with the mean age of onset of various fAD mutations in *PSEN* [173, 174]. As ever, we must remember that correlation is not causation and that change in the $A\beta_{42}/A\beta_{40}$ ratio may be acting as a very tight marker of the severity of another change in PSEN function that is actually causing the disease. In 1997, Cook et al. [175] used three independent methods to show that preventing the movement of AβPP from the ER/intermediate compartment to the Golgi in neurons greatly reduced $A\beta_{40}$ while leaving $A\beta_{42}$ production unaffected. In the same year, Hartmann et al. [176] used labeling of subcellular structures with monoclonal antibodies specifically binding either $A\beta_{42}$ or $A\beta_{40}$ to show the specific production of $A\beta_{42}$ in the ER of neurons. As Area-Gomez et al. [110] showed, most of the γ-secretase activity in the ER is located in the MAM so, if change in PSEN function leads to increased association between the ER and mitochondria (increased MAM), we could expect the ratio of $A\beta_{42}$ to $A\beta_{40}$ to increase even if overall γ-secretase activity is decreased. Alternatively, Lai et al. [32] showed that the PSEN-independent, γ-secretase-like activity they detected specifically favors $A\beta_{42}$ production so reduction in PSEN-dependent γ-secretase activity would again be expected to increase the ratio of $A\beta_{42}$ to $A\beta_{40}$. As quoted previously, Bai et al. saw increases in the $A\beta_{42}/A\beta_{40}$ ratio for all of the various fAD mutations that they introduced into human PSEN1 and that showed detectable Aβ production. However, their data support that the increase in the ratio is an intrinsic property of the mutant PSEN molecule itself since they apparently analyzed the activity of highly purified γ-secretase in an *in vitro* assay system [88].

CONCLUSION

If we accept that there is strong evidence implicating a primary role for PSEN holoproteins in fAD then particular questions arise. Is lysosomal acidification the relevant function of PSEN holoproteins affected in fAD, or is it changes in Ca^{2+} homeostasis or some other, yet unknown function? Where

within cells do pools of PSEN holoprotein exist and what affects their equilibrium with γ-secretase complexes? What controls holoprotein stability? Do PSEN holoproteins function as dimers/multimers? How do fAD mutations affect holoprotein multimerization and function? With what molecules do holoproteins specifically interact and what different forms of holoprotein complex exist? Does the presence of mutant PSEN1 holoprotein increase the association of the ER with mitochondria and, if so, how does this occur? In what cell types are the function of PSEN holoproteins critical for fAD? Is the fAD-critical function of PSEN holoproteins mainly in neurons or are there critical functions in astrocytes, microglia and other neural cell types? How do changes in the rate of α-, β-, and γ-secretase cleavage of AβPP influence the fAD-critical PSEN holoprotein functions? It is clear that there is still a long way to travel to understand how mutations in the *PSEN* and *AβPP* genes cause AD but testing of this "PSEN holoprotein multimer hypothesis" may make the path a little clearer. A 2015 paper from Nixon and coworkers has shown that raised lysosomal pH due to loss of PSEN1 contributes to failure of cytosolic Ca^{2+} ion homeostasis. Lee et al., 2015, *Cell Reports* **12**, 1430–1444.

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