MINI REVIEW

The multiple paradoxes of presenilins

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One of the main histological hallmarks observed in the cortex of Alzheimer’s disease-affected patients is the senile plaque, a proteinaceous deposit that derives mainly from the overproduction of a 39–43 amino-acid-containing peptide called β-amyloid (Aβ). The most striking evidence that Aβ overproduction is intimately linked to Alzheimer’s disease pathology comes from observations that all but one (Ancolio et al. 1999) of the pathological mutations located on the genes responsible for early onset aggressive forms of the disease, lead to an increase in Aβ production and, particularly, in the production of the more readily aggregable 42-amino-acid-long species (Van Broeckhoven 1995; Hutton and Hardy 1997). Whether Aβ is, per se, the etiological cause of the disease, or whether it behaves as an intermediate effector of a pathological cascade of events is still a matter of discussion. However, whatever the case, it remains clear that Aβ derives from its precursor, the β-precursor protein, by enzymatic digestion by so-called β- and γ-secretases, two proteolytic activities responsible for the generation of the N- and C-termini of Aβ, respectively (Selkoe 1991; Checler 1995). As the inhibition of these two activities is theoretically the easiest possibility for the generation of the N- and C-termini of Aβ, the double knock-out of both presenilins 1 and 2 fully abolished Aβ production and, particu-
larly in the production of the more readily aggregable 42-amino-acid-long species (Van Broeckhoven 1995; Hutton and Hardy 1997). Whether Aβ is, per se, the etiological cause of the disease, or whether it behaves as an intermediate effector of a pathological cascade of events is still a matter of discussion. However, whatever the case, it remains clear that Aβ derives from its precursor, the β-precursor protein, by enzymatic digestion by so-called β- and γ-secretases, two proteolytic activities responsible for the generation of the N- and C-termini of Aβ, respectively (Selkoe 1991; Checler 1995). As the inhibition of these two activities is theoretically the easiest possibility for slowing down or blockading Aβ-mediated Alzheimer neuropathology, several teams have targeted the presenilins. The most striking evidence that Aβ overproduction is intimately linked to Alzheimer’s disease pathology comes from observations that all but one (Ancolio et al. 1999) of the pathological mutations located on the genes responsible for early onset aggressive forms of the disease, lead to an increase in Aβ production and, particularly, in the production of the more readily aggregable 42-amino-acid-long species (Van Broeckhoven 1995; Hutton and Hardy 1997). Whether Aβ is, per se, the etiological cause of the disease, or whether it behaves as an intermediate effector of a pathological cascade of events is still a matter of discussion. However, whatever the case, it remains clear that Aβ derives from its precursor, the β-precursor protein, by enzymatic digestion by so-called β- and γ-secretases, two proteolytic activities responsible for the generation of the N- and C-termini of Aβ, respectively (Selkoe 1991; Checler 1995). As the inhibition of these two activities is theoretically the easiest possibility for slowing down or blockading Aβ-mediated Alzheimer neuropathology, several teams have targeted the β- and γ-secretases for identification and characterization. It is now generally believed that an unusual membrane-bound aspartyl protease (BACE; β-site APP cleaving enzyme, or memapsin2 or Asp2) is a β-secretase (Hussain et al. 1999; Sinha et al. 1999; Vassar et al. 1999; Yan et al. 1999; Lin et al. 2000). Concerning the elusive γ-secretase, several groups suggest that presenilins could act as the genuine γ-secretase, a hypothesis that is disputed by others. Here, we will present some objective experimental and theoretical arguments that lead us to argue against a direct role of presenilins as proteases.

Presenilins 1 and 2 are homologous proteins that most likely play a central role in Alzheimer’s pathology. First, when mutated, these proteins are associated with early onset forms of the disease and, indeed, appear responsible for most of the autosomal dominant familial cases of the disease (Van Broeckhoven 1995; Hutton and Hardy 1997). Similar to all but one of the mutations on βAPP, mutations on presenilins trigger the increased production of Aβ42, and specifically increase the Aβ42 : total-Aβ ratio (Checler 1999a). It is easy to envisage that mutations in βAPP, all located near the γ-secretase-targeted sequence, can introduce modifications of the recognition/catalytic parameters of γ-secretase for βAPP. Therefore, this may explain the phenotypical alteration of βAPP maturation at the γ-secretase site. At this stage, it is more difficult to conceive how mutations in presenilins, which appear widely distributed within the serpentine sequence of presenilins, i.e. in putative transmembrane domains as well as in the hydrophilic domains, could lead to the same selective increase in production of Aβ42. Before there was any definite explanation for these observations, this finding alone indicated that there was likely to be a control of γ-secretase-mediated βAPP maturation, mediated either directly or indirectly, by presenilins.

An important breakthrough in the elucidation of this βAPP/presenilins link came from the deletion of presenilin genes. Bart De Strooper and colleagues elegantly demonstrated that the knock-out of the presenilin 1 gene led to drastic reduction in the γ-secretase-mediated cleavage of βAPP (De Strooper et al. 1998). The residual secretion of Aβ40/42 in presenilin 1 knock-out cells appeared likely to be caused by the contribution of endogenous presenilin 2, as the double knock-out of both presenilins 1 and 2 fully abolished Aβ secretion (Herreman et al. 2000; Zhang et al. 2000b). These observations, however, did not elucidate the mechanism by which presenilins influence γ-secretase-mediated βAPP cleavage.

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Abbreviations used: Aβ, β-amyloid; FAD, familial Alzheimer’s disease; K_d, dissociation constant; K_i, inhibition constant; K_m, Michaelis constant; NICD, Notch intracellular domain; sAPPα, α-secretase-derived secreted APP fragment.

Interestingly, the knock-out of the presenilin 1 gene is lethal and leads to drastic abnormalities during embryogenesis, particularly in the caudal region as a result of skeletal defects (Shen et al. 1997; Davis et al. 1998; Donoviel et al. 1999). Strikingly, this phenotype appears reminiscent of that observed when the gene for Notch-1, a transmembrane protein involved in cell fate decision, is deleted. The similarity between the presenilin-1 and Notch-1 deletion phenotypes is consistent with earlier genetic evidence suggesting that presenilins correspond to the C. elegans homolog sel-12, which is involved in the signalling mediated by lin-12, the nematode counterpart of Notch (Levitan and Greenwald 1995; Baumeister et al. 1997). Notch signalling is dependent upon a proteolytic cleavage occurring inside the plasma membrane, close to the inner side of the membrane facing the cytoplasm (Schroeter et al. 1998). This cleavage liberates an intracellular fragment, the Notch intracellular domain (NICD), which translocates into the nucleus where it mediates Notch function. The knock-out of the presenilin gene also leads to the impairment of NICD production (De Strooper et al. 1999), thereby probably explaining the observed dramatic embryogenic alterations.

Taken together, these findings show that presenilins are associated with two cellular phenotypes, Aβ and NICD production, which involve key proteolytic events. This conclusion led several teams to suggest that both γ-secretase-mediated cleavage of βAPP and NICD production could be a result of the same activity, and the most ‘natural’ hypothesis was that this activity could correspond to the presenilins themselves. The most direct lines of evidence supporting this possibility, based on biochemical, pharmacological and mutational approaches, were the following: (1) presenilins co-purify with γ-secretase-like activity in a high-molecular-weight complex (Li et al. 2000b); (2) peptide-based transition state aldehyde inhibitors prevent Aβ and NICD production and interact with presenilins after cross-linking (Esler et al. 2000; Li et al. 2000a; Seiffer et al. 2000); (3) mutation of two critical aspartyl residues prevents presenilin endoproteolysis and abolishes both Aβ and NICD production (Ray et al. 1999; Wolfe et al. 1999; Berezovska et al. 2000; Capell et al. 2000; Kimberly et al. 2000). It has therefore been strongly suggested that presenilins could correspond to a novel type of autocatalytically activated aspartyl protease.

The mutation paradox

The hypothesis that presenilins are the genuine γ-secretases faces several theoretical and conceptual problems, and careful examination of some of the studies cited above leads to somewhat contradictory results. The very first report on presenilins as molecular entities bearing catalytic potential, came from a study by Wolfe and colleagues that indicated that the substitution of aspartyl residues 257 and 385 (by alanines) in presenilin 1 prevented γ-secretase-mediated cleavage of βAPP (Wolfe et al. 1999). Furthermore, it was reported that the endoproteolytic breakdown of presenilin 1 [a well-known cleavage that leads to N- and C-terminal presenilin 1 fragments, thought to associate and form the biologically active entity (for a review see (Checler 1999b)] was also abolished by mutation of the aspartyl residues (Wolfe et al. 1999). It was therefore suggested that presenilin 1 could be a novel transmembrane enzyme, the prototypic member of a novel class of aspartyl proteases, and that it could be activated by autocatalysis. The di-aspartyl residues involved in the catalytic site were proposed to be located in transmembrane domains 6 and 7, totally embedded in the membrane hydrophobic environment. We will not discuss here the fact that this model revolutionizes the concept of catalysis by implying the presence of water in a very unfriendly hydrophobic environment. Indeed, the localization of aspartyl 385 of presenilin 1 in the membrane has not been clearly demonstrated. However, it is important to emphasize here a theoretical concept about acidic proteases. In all acidic proteases [it should be noted here that a recent paper indicated that presenilin-mediated γ-secretase cleavage is sensitive to pepstatin (Xia et al. 2000), as are all ‘classical’ acidic proteases (Davies 1990)], the two aspartyl residues behave as an acid–base couple that require one protonated and one deprotonated aspartyl moiety. This means that mutation of one of the aspartyl residues should be sufficient to fully abolish catalysis. Nevertheless, it has been reported that mutation of the aspartate 257 residue of presenilin 1 abolishes NICD production without affecting Aβ recovery (Capell et al. 2000). This observation is hard to reconcile with the hypothesis of a di-aspartyl group directly involved in the catalytic events responsible for Aβ and NICD production.

The ability to discriminate between presenilin-mediated Aβ and NICD production was further demonstrated by Kulic and colleagues (Kulic et al. 2000) who introduced arbitrary mutations at position 286 of presenilin 1 (a well-described position responsible for familial forms of Alzheimer’s disease), and showed that Aβ42 recovery was greatly enhanced whereas NICD production was impaired (Kulic et al. 2000). The easiest way to reconcile these interesting data with other results is to envisage presenilins acting upstream of γ-secretase. Alternatively, to stick to the presenilin/protease hypothesis, it would be necessary to consider that presenilins would bear several catalytic sites selectively responsible for Aβ- or NICD formation. However, in this case, it would be difficult to understand the results from the mutagenesis studies that show abolition of the overall catalytic potential of presenilin 1/2 for both Notch and βAPP by mutating only the C-terminal aspartyl residue.

Another important result has arisen from a recent study showing that a glycine residue adjacent to aspartate 385
(glycine 384, using the presenilin 1 numbering) is conserved in bacterial acidic proteases and that its mutation affects Aβ and NICD recovery (Steiner et al. 2000). What does this finding teach us? This study demonstrates that mutations of residues other than the aspartyls can lead to the same phenotypic alterations. Here, the important point is that the nature itself of the mutated residue, i.e. a glycine, precludes the possibility of a direct contribution to catalysis, as glycine residues lack a side chain, an obligate structural element needed to contribute to the polarization of the scissile bond. It can be argued that this glycine residue contributes to the substrate binding/recognition site. It remains clear that mutating a residue that is not directly involved in catalysis can mimic the effect of the aspartyl residue mutations. As a corollary, the phenotypic alteration observed when mutating aspartyl residues is not sufficient per se to demonstrate a direct participation in catalysis.

What could we propose as a mechanism underlying the observed effect of the glycine mutation? It could be argued that this residue occurs in a functionally strategic structural zone of presenilins. In this context, it is noteworthy that mutation of this glycine is particularly efficient in decreasing Aβ and NICD production when a proline residue (an amino-acid that theoretically induces structural changes in the polypeptide backbone) or a lysine (that adds an additional ‘charge’) is introduced in the sequence (Steiner et al. 2000), i.e. when structural perturbations are likely to occur. In contrast, the substitution of the glycine residue by an aliphatic alanine drastically increased Aβ recovery (Steiner et al. 2000). This Gly → Ala transformation in fact corresponds to a natural familial Alzheimer’s disease (FAD)-linked mutation known to drastically exacerbate Aβ42 production.

The latter point leads us to a very general statement, that it is very striking that most of the FAD mutations on presenilins lead to increased production of Aβ, specifically Aβ42. As these mutations are widely distributed all along the presenilin sequence, i.e. in transmembrane domains as well as in hydrophilic structures, it is hard to envisage that all presenilin mutations similarly affect βAPP catalysis. Furthermore, a recent paper indicated that a mutation at cysteine 92 of presenilin 1, as expected, increased Aβ42 but impaired Notch/Lin12 signalling in C. elegans (Zhang et al. 2000a). A systematic assessment of the effects of a series of FAD-mutations on both Aβ and NICD productions should shed light on a further discrimination between these two paradigms.

The pharmacological paradox

Studies aimed at delineating the sensitivity of Aβ-generating activities into various classes of inhibitors would per se deserve a review. Among these studies, it is particularly interesting to note a report suggesting that the 40- and 42-residue species of Aβ could be generated by distinct γ-secretases (Citron et al. 1996) that are differentially sensitive to MDL28170, an aldehyde inhibitor formerly characterized as a potent calpain blocker (Mehdi 1991). This observation is puzzling. Theoretically, an enzyme can hydrolyse a given substrate at distinct sites with various affinities. This is reflected by the resulting ratio of the various catabolites. In the coupling of presenilins/APP, this possibility would explain the fact that a 9 : 1 (Aβ40/42) ratio is observed in many studies. However, an inhibitor acting on a single catalytic site should affect the absolute amounts of the products generated but not the ratio of their production, as was reported for MDL28170 (Citron et al. 1996). This theoretical consideration, although a very basic enzymologic statement, can be seen by itself as a definite argument against the presenilins/secretase hypothesis.

Recent studies on the design of peptide-based transition state analog inhibitors [referred to as MW167 (Wolfe et al. 1998) or commercially available under the nomenclature DFK167] have described these pharmacological agents as potent inhibitors of Aβ production (Wolfe et al. 1998; De Strooper et al. 1999) and Notch cleavage (De Strooper et al. 1999; Berezovska et al. 2000). If presenilins are autocatalytically activated secretases, these agents should be expected to prevent presenilin endoproteolysis. We recently established that DFK167 is unable to block endoproteolysis of endogenous or overexpressed presenilin (Petit et al. 2000), a paradigm that was not examined by others (Wolfe et al. 1998). These data agree well with another study showing that an engineered uncleavable presenilin, in which a pathological mutation had been introduced, still triggers increased production of Aβ42 (Steiner et al. 1999). Thus, despite previous reports (Wolfe et al. 1999), by pharmacological and mutagenesis approaches, it is clearly possible to distinguish between presenilin endoproteolysis and Aβ-generation.

Another problem for the presenilins/secretase hypothesis arises from our recent study establishing that novel non-peptidic inhibitors can fully block the production of secreted Aβ40 and Aβ42 from cells expressing wild-type βAPP, and increase the recovery of endogenous C83 and C99 C-terminal fragments of βAPP (derived from α- and β-secretase cleavage, respectively). In this study, we found that endoproteolysis of presenilin and Aβ generation are two cellular events that can be dissociated, as presenilin cleavage was not affected by our inhibitors. More strikingly, our inhibitors did not affect NICD production (Petit et al. 2000). It should be emphasized here that we were monitoring the direct production of NICD and not the final phenotype associated with NICD that could have been modulated downstream to the primary catalytic event, along the cascade of cellular intermediates. Here again, in classical enzymology, the enzyme/inhibitor complex is characterized by a unique inhibition constant (K_i) value, and this constant...
does not vary according to the type of substrate, even if a single enzyme (in our case γ-secretase/Notch cleaving activity) can display various affinities ($K_m$) for its various substrates. Therefore, mutational (aspartate 257 of presenilin 1) or pharmacological discrimination (our inhibitors) approaches converge to cast a shadow over the hypothesis of a presenilin enzymatic entity.

Independent studies have indicated that it is possible to cross-link radiolabeled inhibitors to presenilins and their fragments (Esler et al. 2000; Li et al. 2000a; Seiffer et al. 2000). It should be noted that because of methodological problems, probably related to the permeability of the inhibitors and the relatively low affinity for the ‘enzyme’, some of the cross-linking experiments were performed in vitro on cell extracts (Li et al. 2000a), and, in this case, whether the probes had reached their ‘real’ endogenous target remains questionable. Another study described cross-linking experiments in cell lysates and isolated microsomes as well as in situ, in living cells (Esler et al. 2000). However, it should be noted that the demonstration of the specificity of the covalent binding, i.e. the prevention of the labeling with unlabeled probe, was only partial in microsomes and was not documented in living cells (Esler et al. 2000). Finally, it is relatively difficult to envisage how an identical probe only differing by the location of the cross-linking group, could alternately label either the N- or the C-terminal fragment of presenilins (Li et al. 2000a). When considering the proposed model of a transmembrane ‘hydrophilic’ pore formed by the direct interaction between N- and C-terminal presenilin fragments, it is difficult to reconcile this proposition with the very small size of the inhibitor that would have to act as a ‘hook’ able to fish out both fragments. This last point of my argument can be still debated, as the putative structural model of presenilin organization in the membrane still awaits demonstration. One cannot definitely rule out the possibility that the N- and C-terminal fragments are so closely associated that cross-linking the two subunits would remain possible.

**The spatial and ‘cleaved site’ paradoxes**

Not so long ago, we would have taken as a very strong argument the ‘spatial paradox’ (a concept that is not ours!) concerning the cellular sites of Aβ and NICD production. It was previously thought that presenilins were proteolytically processed in the early secretory pathway, probably in the endoplasmic reticulum, and that no presenilin-like immunoreactivity was present in the plasma membrane (for a review see Checler 1999b), a cell compartment thought to be the site of NICD production. Now, several papers have provided evidence that presenilin immunoreactivity could be associated with the plasma membrane. Although this finding is still debated, the observation potentially solves a problem caused by the difference between the subcellular localization of Notch and presenilin. However, let’s consider that presenilins are indeed located at the endoplasmic reticulum/Golgi (where most of Aβ is generated) and also at the plasma membrane level (where NICD is produced). This means that an identical activity would occur in cell compartments displaying distinct physicochemical features. Significantly, this would imply that presenilins would be able to cleave distinct substrates efficiently in both very acidic or virtually neutral pH environments. This would be a totally novel feature for an aspartyl protease, because, as stated above, an absolute requirement for functional acidic proteases is the presence of one ionised and one protonated lateral chain of the two aspartyl residues (Pearl 1987). This explains why most acidic proteases display a maximal activity at a pH close to the pK value of the aspartyl carboxyl, i.e. around pH 4. In the environment of the plasma membrane, i.e. close to neutral pH, the two aspartyl residues would be in the –COO\(^-\) deprotonated forms. This chemical state would preclude any possible hydrogen bonding of the water molecule to the active aspartyl residues, an intermediate step necessary for further attack of the carbonyl carbon of the scissile bond of substrates.

More striking is the nature and intramembrane localization of peptide bonds targeted by γ-secretase and Notch-cleaving enzymes. There is no apparent amino acid sequence around the cleaved bonds that could be seen as a putative consensus recognized sequence. It is true that this is not an unusual feature for proteolytic activities, but very often, it is compensated for by another structural element. The apparent specificity could be driven by substrate recognition and accommodation within the catalytic site of the enzyme, which would be dependent upon the topological configuration of the enzyme near to its substrate. As an example, membrane-bound α-secretase clearly cleaves a number of substrates at very distinct peptide bonds, but always at the same distance from the membrane (Sisodia 1992), probably because there exists a molecular adaptation driven by the enzyme/substrate topology. It is not anecdotal to emphasize the fact that presenilins do not fall into any of these situations. Thus, not only are the sequences targeted totally unrelated, but the intramembrane site of cleavage of βAPP (middle of the membrane) is distinct from Notch cleavage (close to the inner leaflet of the plasma membrane).

**Is there an alternative explanation?**

The most likely alternative explanation is that presenilins behave as molecular chaperones of βAPP. γ-secretase or both. If presenilins bind to βAPP upstream of all secretase activities, this could explain why presenilin 1 was reported to also modulate the constitutive (Ancolio et al. 1997) and regulated (Murphy et al. 2000) the production of the α-secretase-derived secreted APP fragment (sAPPα). Many proteins have been shown unambiguously to interact directly...
with presenilins (for reviews see Checler 1999a,b). This leads to theoretical considerations. Chaperon proteins interact firmly with their binding partners because of their very high affinity (low nanomolar range). These affinities (or the dissociation constant, \(K_d\)) explain why it is possible to co-immunoprecipitate two interacting cellular partners. In contrast, the affinity of proteases for their substrates is usually more than one thousand-fold lower (the Michaelis constant, \(K_M\), is in the 1–10 \(\mu\)M range). This explains why, to our knowledge, there is no example of a protease co-immunoprecipitating with its physiological substrate, as has been demonstrated for presenilin and \(\beta\)APP. If the role of presenilins as a molecular transporter is privileged, it should be noted that Naruse et al. demonstrated nicely that deletion of presenilin 1 drastically impaired the neuronal intracellular transport of several proteins including \(\beta\)APP (Naruse et al. 1998). An important communication by Dr S. S. Sisodia (at the 30th annual meeting of American Neuroscience) documented the fact that single or double aspartate 257 or 385 mutations of presenilin 1 all drastically affect \(\beta\)APP trafficking, leading to considerable accumulation of intact \(\beta\)APP at the cell surface. This is obviously an important advance in the understanding of the alternative mechanistic explanations for the effect triggered by presenilin aspartyl mutations.

**Provocative digressions**

A few provocative issues could be raised at this stage. If presenilins are the genuine \(\gamma\)-secretases, are they cleaving all proteins with which they interact or are they selective for \(\beta\)APP? If increasing or abolishing A\(\beta\) production by mutating or deleting a given protein is a good clue to suggest that such a protein is \(\gamma\)-secretase, should we not also regard nicastrin as an additional putative candidate? Indeed, this novel protein interacts with presenilins, when mutated triggers increased production of A\(\beta\), and when inactivated by a small deletion (that could be seen as a molecular knock-out), A\(\beta\) production is impaired (Yu et al. 2000).

**Conclusion**

This debate would be solved if presenilin could be purified to homogeneity and could be shown to display in vitro catalytic activity towards \(\beta\)APP (or any other protein). Using current techniques, we are relatively pessimistic about this possibility, as transmembrane proteins are hard to solubilize, and their recovery in a detergent environment rarely allows the elimination of accompanying proteins. Particularly difficult in the case of presenilins is the fact that to be able to measure any catalytic function, the purification procedure presumably would have to preserve the heterodimeric association of the N- and C-terminal presenilin moieties thought to mediate the biological function of the protein.

Discussions about the nature of the \(\gamma\)-secretase are not only a fundamental or intellectual debate. The issue of whether or not presenilins are the genuine \(\gamma\)-secretase should drive future therapeutic strategies aimed at preventing \(\gamma\)-secretase-mediated A\(\beta\) production. If the same enzyme is responsible for the release of NICD, it would be difficult to envisage how Notch-mediated cellular functions would not also be affected, even if studies suggested that inhibiting A\(\beta\) production and Notch cleavage led to only partial impairment of Notch signalling (Berezovska et al. 2000). Differential inhibition of A\(\beta\) production and Notch function may perhaps be achieved in vitro but may be much more difficult to achieve in human studies.

However, if presenilins are not \(\gamma\)-secretase, the fact that novel non-peptide inhibitors drastically inhibit A\(\beta\) recovery without affecting NICD production leads me to be optimistic. This observation leaves intact a theoretical therapeutic intervention targeting the \(\gamma\)-secretase activity without ‘side-effects’ on cellular Notch-related functions.

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**References**


