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# The pharmacology of amyloid precursor protein processing

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**Abstract**

The possibility to understand the causes and treat the symptoms of Alzheimer's disease patients is still a great challenge. The triggering events leading to the selective neurodegeneration observed in Alzheimer's brains are not completely understood. This lack of understanding of the pathophysiological processes posses an important theoretical challenge for the rational design of pharmacological intervention. The scientific community is divided over the pathogenesis of the disease which is historically divided between 'baptists' and 'tauists'. Baptists suggest that β-amyloid, the peptide deposited in neuritic plaques, is the cause of all damages while tauists suggest that hyperphosphorylated tau, the cytoskeletal protein that forms neurofibrillary tangles, is the culprit for the disease. This review will be focused on the pharmacological modulation of the amyloid precursor protein metabolism, with the goal of reducing the formation of β-amyloid. Over the years such an approach has led to the identification of a complex intracellular mechanism, which may be regulated by neurotransmitters and other ligands. More recently, these efforts have contributed to the characterization of the enzymes which regulate the formation of β-amyloid.


*Keywords:* Amyloid precursor protein; Secretases; Signal transduction; Alzheimer's disease

## 1. Alzheimer's disease and the amyloid cascade hypothesis

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that affects a large proportion of the elderly population. Clinically, AD is characterized by a gradual onset of memory loss followed by progressive cognitive and physical deterioration. Post-mortem evaluation of brain tissue from AD patients is characterized by neurofibrillary tangles and neuritic or senile plaques. The former is mainly composed by a cytoskeletal protein named tau, which becomes hyperphosphorylated and self-aggregates into neurofibrillary tangles. The latter are characterized by the accumulation of proteins in the form of β pleated sheet fibrils.

The major component of senile plaques is the β-amyloid peptide [Aβ]. Currently, one of the leading, although controversial, hypothesis for the pathogenesis of AD is focused on the potential toxic role of an excessive production of Aβ. As a working hypothesis (Fig. 1), a number of investigators favour the 'Amyloid Cascade' which considers Aβ formation as an early event of Alzheimer's pathogenesis. In the mid 80s, identification of the β-amyloid peptide as the major proteinaceous component of AD brain senile plaques was reported. Aβ is a peptide of 39–43 amino acids, derived from a larger precursor, the amyloid precursor protein or APP. APP is a multiple isoform integral membrane protein with a large extracellular domain, a membrane anchoring domain and a short intracellular C-terminal tail (Fig. 2). The immature form of APP undergoes several post-translational modifications including N-glycosylation, O-glycosylation, and Tys sulfation to give the mature form of APP (reviewed in Nunan and Small (2000) and Selkoe (2001)). Following these steps, the routes of APP metabolism become more complex and result in different pathways leading to proteolytic processing of the precursor by at least three proteolytic enzymes (Nunan and Small, 2000). Near the cell surface or in a secretory vesicle a protease, i.e. α-secretase, cleaves APP in the extracellular domain and releases the ectodomain (sAPPα or soluble APPα) into the extracellular space. This proteolytic cleavage occurs within the Aβ sequence, therefore preventing the formation of amyloidogenic fragments and constitutes the non-amyloidogenic pathway. The β-amyloid peptide, is formed following cleavage by 'β-' and 'α-secretases' that cleave at the N and C terminus of Aβ, respectively (Fig. 2).

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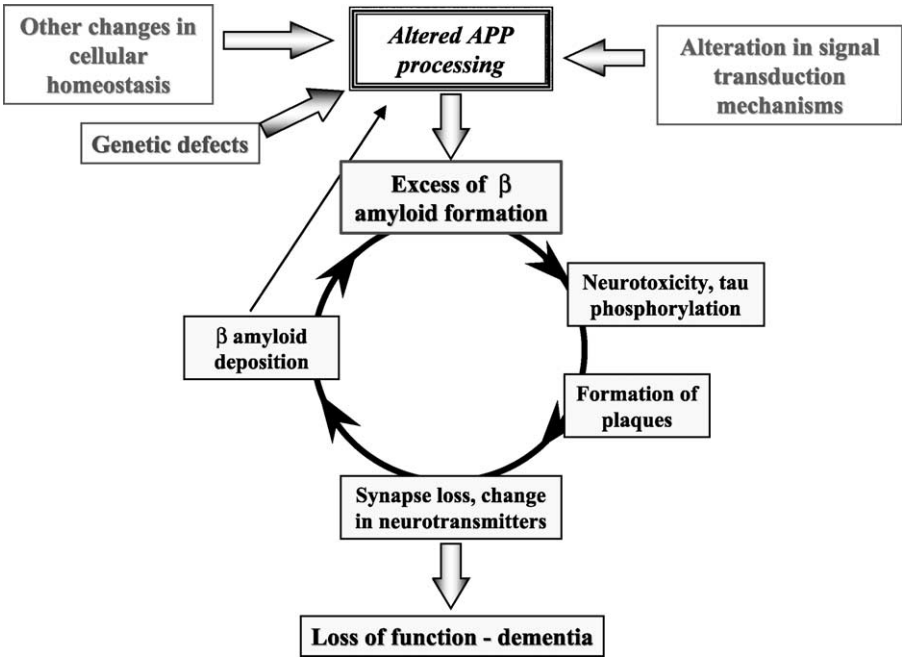


Fig. 1. Working hypothesis based on the amyloid cascade. As detailed in the text, the central event for the amyloid cascade hypothesis is the excessive formation of Aβ. The source of excessive Aβ formation is the amyloid precursor APP and multiple factors may contribute to its aberrant processing. Here, we have indicated genetic alterations since it has been largely demonstrated that mutations either on the APP molecule itself or on other proteins, namely PS, thought to be involved in APP processing, results in aberrant metabolism and excess production of amyloidogenic products. Defective signal transduction mechanisms, neurotransmitter changes and other perturbations of normal cellular homeostasis may also contribute to aberrant APP processing. Most of these alterations have been detected and characterized in the brain and/or peripheral tissues of AD patients and constitute a significant example of the pharmacological modulability of the regulated processing of APP.

Major support to the 'amyloid' hypothesis comes from genetic studies. The APP gene is found on chromosome 21 and Down's syndrome patients [trisomy of chromosome 21] invariably develop prematurely the characteristic lesions (neuritic plaques) of AD. The first familial cases described in AD were linked to mutations in the APP gene. All of these mutations either flank or are found within the Aβ peptide sequence and modify the normal metabolism of APP, which increases the production of amyloidogenic fragments. Other genes, when mutated, are associated with an autosomal transmission of early onset forms of AD. These genes give rise to the presenilins (PS) (Levy-Lahad et al., 1995; Sherrington et al., 1995) and include two related genes PS1 and PS2 that account for almost all of the autosomal dominant forms of familial AD (FAD). Some authors have suggested that these proteins (in particular PS1) may have a direct role in APP processing (see later). In all cases, the result of these genetic alterations is a dysregulation of APP processing. In some cases, the effect seems to be a net increase in the production of β-amyloid, i.e. the 'Swedish' mutation (Citron et al., 1994). Other mutations at amino acid 717 (numbered according to the longest APP isoform of 770 amino acids), or the mutations in PS, alter normal processing of the precursor and results in the formation of longer forms of the peptide (Kosaka et al., 1997; Scheuner et al., 1996; Suzuki et al., 1994) which are more susceptible to aggregation and deposition.

Finally, data using peripheral tissues and body fluids from patients affected by the common sporadic form of the disease have elucidated that altered APP metabolism is a common feature, since 'α-secretase' derived sAPPα release

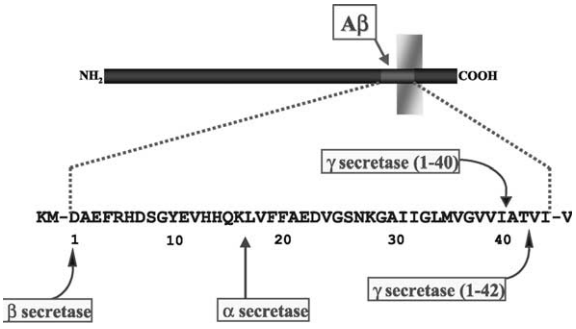


Fig. 2. Structure of the amyloid precursor protein (APP), the β-amyloid peptide and the sites of APP processing. The sequence of the β-amyloid peptide is expanded to evidence the sites of cleavage due to a, b and g secretases. Alpha-secretase cleaves within the Aβ sequence between Lys (K)16 and Leu (L)17 producing the large secreted sAPPα fragment and leaving in the membrane a 10 kDa C-terminal fragment (10 kDa αCTF). Beta-secretase is the activity that forms the N-terminal side of Aβ cleaving APP before Asp (D)1 of the Aβ sequence. The activity results in the liberation of sAPPβ and leaves in the membrane a 12 kDa C-terminal fragment (12 kDa βCTF). Both the C-terminal fragments can be substrates of the γ-secretase activity. In particular, the 12 kDa βCTF can lead to the formation of Aβ 1–40 or Aβ 1–42 depending of which γ cleavage occurs (see text for more details).

from fibroblasts is defective (Bergamaschi et al., 1995). Taken together, these data support the hypothesis that aberrant APP metabolism occurs during the development AD, increasing the production of Aβ.

## 2. The proteases involved in APP processing

### 2.1. Alpha-secretase

Cleavage of APP by α-secretase occurs between residues Lys16 and Leu17 of the Aβ sequence (Sisodia, 1992), and results in the release of the soluble ectodomain of APP (sAPPα). Following cleavage and release of sAPPα, a second enzymatic product, the C-terminal fragment (αCTF), which can be a substrate for α-secretase yields a secreted non-amyloidogenic 3 kDa fragment known as p3 (Haass et al., 1993). Although the proteolytic event occurs most likely in the trans-Golgi network or other downstream compartments of the protein secretory pathway (De Strooper et al., 1993; Kuentzel et al., 1993; Refolo et al., 1995; Sambamurti et al., 1992; Sisodia, 1992), including compartments at the plasma membrane called caveolae (Ikezu et al., 1998), the exact subcellular location of α-secretase remains unclear. This is possibly due to the presence of distinct enzymes with similar functions.

Numerous molecules have been implicated as α-secretase, in particular a general family of proteases that release soluble ectodomains of integral membrane proteins (Hooper et al., 1997). One member of this family, TNFα converting enzyme (TACE; ADAM-17), cleaves membrane-bound tumour necrosis factor alpha (TNFα) to release a soluble form of TNFα (Black et al., 1997; Moss et al., 1997). TACE exhibits α-secretase activity since in vitro studies have shown that TACE is able to cleave APP within the Aβ sequence (Buxbaum et al., 1998), and the experiments conducted on embryonic fibroblasts derived from mice knockout for the TACE enzyme show that phorbol esters-mediated release of sAPPα is completely blocked by the knockout of the protease (Buxbaum et al., 1998). Importantly, however, these cells do secrete basal levels of sAPP demonstrating that constitutive sAPPα release is not affected by the TACE knockout (Buxbaum et al., 1998). Further support of the possibility that two different enzymes play roles in constitutive and regulated secretion of sAPPα comes from the experiments of Racchi et al. (1999) showing that the hydroxamic acid-based compound KD-IX-73-4 inhibits the phorbol ester-mediated sAPP release as well as receptor-mediated sAPP release induced by carbachol or by bradykinin, the latter being a Protein kinase C-(PKC)independent mechanism. Consistent with the data presented by Buxbaum et al. (1998), the inhibitor KD-IX-73-4 was effective in blocking stimulated but not constitutive sAPP release (Racchi et al., 1999).

Candidates for α-secretase activity include other members of the 'disintegrin and metalloprotease' [ADAM] family (Lammich et al., 1999). The ADAMs family consists of membrane-anchored zinc metalloproteinases composed of several distinct protein domains (Lammich et al., 1999). The major candidate is ADAM 10 which has been implicated in both the constitutive and PKC-regulated α-secretase pathways (Lammich et al., 1999; Skovronsky et al., 2000). Overexpression of ADAM-10 increases both basal and phorbol ester stimulated α-secretase cleavage several fold (Lammich et al., 1999), while inhibiting ADAM 10 with a point mutation in the zinc-binding site, inhibits endogenous α-secretase activity (Lammich et al., 1999). This suggested that unlike TACE, ADAM 10 is involved in both the regulated and constitutive secretion of APP. Other members of the ADAM family have been implicated in APP processing including ADAM 9 (Koike et al., 1999) and it is possible that several ADAMs may have partially overlapping α-secretase activities.

### 2.2. Beta-secretase

The cleavage of APP at residue 1 of the Aβ sequence liberates a truncated form of sAPP [sAPPβ] and produces a C-terminal fragment of 12 kDa (βCTF). This cleavage is the first step in the production of Aβ and the beginning of the amyloidogenic pathway. The beta cleavage occurs in the endosomal/lysosomal pathway (Koo and Squazzo, 1994), however, the involvement of other intracellular compartments (ER and Golgi) has been described (Chyung et al., 1997; Haass et al., 1995). Initially, the enzyme was known as 'β-secretase' but it is now referred to as BACE following the identification, cloning and characterization of the enzyme (Hussain et al., 1999; Lin et al., 2000; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). BACE is a type 1 transmembrane protein, containing two active site motifs with a conserved sequence, similar to aspartic proteases. The enzyme is expressed in the brain by a rather uniform pattern across all subregions and across all neurons, with glial cells expressing little or no BACE (Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). BACE is also expressed at low levels in most peripheral tissues with the exception of the pancreas (Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). The subcellular localization of BACE is consistent with the known sites of APPβ cleavage such as endosomes and the Golgi apparatus. Since its initial discovery many studies have been directed towards the characterization of BACE. An extensive review of the specific literature is beyond the scope of this paper. It is interesting to point out that experiments of overexpression of BACE in cellular models have demonstrated an increase in the levels of sAPPβ and βCTFs with reduction of site cleaved fragments (Vassar et al., 1999). This increase in sAPPβ and βCTFs was accompanied by an increase in both Aβ40 and Aβ42 levels, and consequently inhibition of BACE activity, using antisense oligonucleotides, leads to increase of site cleaved products and reduction of total Aβ (Vassar et al., 1999). BACE knockout mice exhibit no other

phenotype, with the exception of reduced levels of Aβ (Cai et al., 2001; Luo et al., 2001). These findings may indicate that a therapeutic strategy directed at lowering BACE activity can be achieved with relative safety. A second enzyme, BACE2, apparently is not involved in Aβ processing, however, its function has not been characterized. Unlike BACE, BACE2 is highly expressed in heart, kidney and placenta (Farzan et al., 2000; Yan et al., 1999). Hence pharmacological agents that selectively block BACE but not BACE2, may provide valuable insights towards addressing these respective roles.

### 2.3. Gamma-secretase

The final step in the amyloidogenic pathway is the cleavage of the product of BACE, βCTF, to liberate Aβ. The protease, γ-secretase, has the unusual property of cleaving APP in a region that is localized within the membrane bilayer, which is not common among cellular proteases. A further complication is the fact that γ cleavage can produce two different C-termini, one ending at amino acid 40 (numbering according to the sequence of Aβ, see Fig. 2) and one ending at amino acid 42. An additional product, C-terminal fragment of APP termed γCTF has been recently described (Nunan and Small, 2000; Pinnix et al., 2001; Selkoe, 2001; Wolfe and Haass, 2001). Other proteins follow a complex mechanism of intramembrane proteolysis including the sterol regulatory element-binding protein (SREBP) and Notch (reviewed in Brown et al. (2000)). In all cases, the intramembrane proteolysis can occur only following primary processing of the lumenal or extracellular portion of the protein. Gamma-secretase activity has been proposed to rely on the presence of presenilin1 (PS1) (reviewed in Wolfe and Haass (2001)) either as an accessory element or as the secretase itself. Cleavage of membrane spanning segments poses a conformational problem. Because transmembrane segments are generally thought to form α-helices, it is known that these structures are resistant to attack by proteases (Paetzel et al., 1998). For the γ-secretase, it would need to be able to unfold the α-helix, however, no evidence yet has been provided to show that this occurs during cleavage of APP by γ-secretase (see review by Wolfe and Haass (2001)).

## 3. The network of intracellular signalling pathways affecting APP processing

The effect of gene mutations on APP metabolism is a powerful tool by which to define the pathogenesis of the familial form of AD. However, for the more common sporadic forms of the disease, a key event leading to altered APP metabolism may reside in more complex neurochemical and signal transduction alterations. These biochemical and neurochemical abnormalities are present both at the brain level and in peripheral tissues and constitute a major model for the study of the physiological and pharmacological modulation of APP processing.

The initial finding on the pharmacology of APP processing was the demonstration that the non-amyloidogenic pathway can be regulated through phosphorylation processes. PKC was the first signal transduction system described (Gillespie et al., 1992; Buxbaum et al., 1993; Caporaso et al., 1994). Alteration of PKC levels and activity is one of the most consistent finding in AD brain tissue. In addition, altered signal transduction mechanisms, particularly PKC, are found consistently in peripheral tissues from AD patients suggesting that these changes are not secondary to neuronal loss and may be directly involved in AD pathogenesis (reviewed by Gasparini et al. (1998)). As a result of deficiencies in PKC activity (Govoni et al., 1993), we have demonstrated that the non-amyloidogenic processing of APP is altered in AD fibroblasts (Bergamaschi et al., 1995). These findings have been replicated (see Gasparini et al. (1998)) and Vestling et al. (1999) demonstrated that alterations are not present in cells derived from patients with FAD mutations. Overall these findings provide further support that altered APP metabolism is a key event in the amyloid cascade hypothesis.

The studies on the role of PKC in the regulated APP processing have established that the Aβ forming amyloidogenic pathway and the α-secretase non-amyloidogenic pathway appear to be balanced, although there were exceptions (LeBlanc et al., 1998) which may be related to the particular cellular and experimental model. It is now know that the target of PKC phosphorylation is not the APP molecule itself, yet the possibility that PKC targets directly the α-secretase or other key cellular factors possibly related to the vesicular trafficking (Xu et al., 1995) of APP and/or the α-secretase, has not been resolved.

Particular importance has been given to which specific PKC isoform is involved in APP processing. PKC is a multigene family of enzymes with at least 12 different isoforms. The groups of isoforms are divided into calcium-dependent (also known as conventional PKCs: α, βI, βII and γ) and calcium-independent, distinguished within the group in novel PKCs: δ, ε, η, θ, μ; and atypical PKCs ζ, τ and λ isoforms (Dekker and Parker, 1994; Nishizuka, 1992). In AD fibroblasts, one of the calcium-dependent PKC isoforms (PKCα) has been found to be defective (Bergamaschi et al., 1995). The role of PKCα and other isoforms in the regulated secretion of APP has been suggested. Initially, it was demonstrated that overexpression of PKCα in Swiss 3T3 fibroblasts increased the sensitivity of APP processing to phorbol esters (Slack et al., 1993). Subsequently, other investigators using either down regulation or inhibition of PKCα (Benussi et al., 1998; Jolly-Tornetta and Wolf, 2000) or overexpression of PKCα (Kinouchi et al., 1995) have indicated that PKCα plays a significant role in APP metabolism.

Others have suggested a specific role for another isoform such as PKCε. Overexpression of PKCε induces an increase

of sAPPα (Kinouchi et al., 1995), while other strategies pursuing the inhibition of PKCε provided evidence of a role of such isoform in APP processing (Yeon et al., 2001). More recently, Zhu et al. (2001) reported that expression of a peptide inhibitor of PKCε reduced phorbol ester-mediated sAPP release. This result ruled out the involvement of PKCα because of the ineffectiveness of Gö6976, which is a specific inhibitor of PKCα, β and γ isoforms. Using an antisense strategy, we have recently reported that PKCα is specifically involved in phorbol ester-induced APP metabolism and that its contribution is specific for direct PKC-mediated pathways (Racchi et al., 2002). Whereas PKCα down regulation can significantly impair phorbol ester-induced sAPPα release from SH-SY5Y cells, the down regulation of the kinase isoform does not affect receptor-mediated sAPPα release. Further support to PKC isoform specific roles in APP processing come from studies using benzolactam, a novel PKC activator with selectivity for the calcium-dependent isoforms α, β, and γ. Ibarreta et al. (1999) demonstrated an enhanced secretion of sAPPα in fibroblasts of AD patients and PC12 cells.

Indeed multiple intracellular second messengers contribute to the regulation of APP metabolism and they have extremely complex interactions (reviewed by Racchi and Govoni (1999)). At least five major kinase systems have been described that modulate the processing of APP. These data are derived from experiments either pursuing the direct stimulation of the respective signal transduction pathway of a pharmacological dissection of the pathways downstream of ligand–receptor interaction (see later).

Cyclic AMP-dependent protein kinase (PKA) was implicated since experiments with forskolin demonstrated that treatment of PC12 cells resulted in increased sAPPα release. The mechanism suggested was the modulation of vesicular budding from Golgi membranes of secretory vesicles containing APP (Xu et al., 1996). In a different cellular system, Marambaud et al. (1996) demonstrated that forskolin and 8-Bromo-cAMP could induce the release of sAPPα and suggested the involvement of the proteasome as an intermediate target between the activation of the kinase and the activity of α-secretase. However, others (Efthimiopoulos et al., 1996; Lee and Wurtman, 1997) observed an opposite effect of forskolin-mediated elevation of cAMP showing that such treatment induced inhibition of phorbol ester-induced sAPPα release. These discrepancies are still not resolved.

The role of calcium in second messengers activity is important, in particular as a cofactor for the activation of PKC and Tyr kinase (Tyr-K). The role of calcium in APP metabolism has been studied using two strategies. Intracellular calcium levels were increased by either favouring the release from internal stores or promoting the influx from the external mileu by the use of ionophores. Thapsigargin, an irreversible inhibitor of calcium reuptake from the ER, was able to stimulate sAPPα release as well as reduce the formation of Aβ (Buxbaum et al., 1994), with an effect independent from PKC activation. This suggested for the first time of the existence of alternative routes bypassing the kinase. The calcium ionophore ionomycin was able to increase the release of sAPPα in HEK293 cells (Petryniak et al., 1996). These authors suggested the involvement of a Tyr-K dependent mechanism, because the effect of ionomycin was blocked only partially by specific inhibitors of PKC while completely abolished using Tyr-K inhibitors (Petryniak et al., 1996). The stimulation of a wide range of receptors includes an intrinsic or associated Tyr-K activity, and many of them have been shown to regulate APP processing. Some examples and the implication of the complex interaction of downstream signals will be considered later.

The intervention of the mitogen-activated protein kinase (MAPK) cascade has been initially suggested by the use of selective inhibitors such as PD98059. Two different research groups have shown that inhibition of MAPK could block the effect of PKC activators on the release of sAPPA. It is interesting to notice that divergent results were obtained in the two studies concerning the effect of MAPK inhibition on Aβ production. In the study by Mills et al. (1997), the MAPK inhibitor PD98059 blocked also the effect of phorbol esters on the release of Aβ, while Desdouits-Magnen et al. (1998) did not observe an effect of PD98059 on the basal release of Aβ as well as on the Aβ reducing effect of phorbol esters treatment.

The last signal transduction system involved in the regulation of APP processing is dependent on phosphatidyl inositol 3 kinase (PI3-K) which was described for the first time as the kinase effector downstream of the insulin receptor, responsible for the induction of sAPPα release (Solano et al., 2000) (see later) from SH-SY5Y neuroblastoma cells.

Since a complex network of second messengers is involved in the regulation of APP metabolism, the neurochemical alterations described in AD brain are likely to involve multiple levels including impaired neurotransmitter receptor-mediated signal transduction (Cowburn et al., 1996). A significant number of alterations of these systems have been described in AD brains and peripheral tissues (Cowburn et al., 1996; Gasparini et al., 1998; Racchi and Govoni, 1999). Starting with neurotransmitters, deficits have been reported involving the noradrenergic, serotonergic, GABAergic and glutamatergic systems in the cerebral cortex and the hippocampus of AD brain tissue, however, the most consistent feature of AD is the degeneration of cholinergic neurons leading to the impairment of cholinergic transmission.

## 4. Cholinergic regulation of APP processing

A clear connection between the cholinergic system and APP/Aβ metabolism has been indicated over the years by several authors beginning with a pivotal paper (Nitsch et al.,

1992) which showed that in human embryonic kidney (HEK) cell lines stably transfected with individual muscarinic receptor subtype, that activation by carbachol of m1 and m3, but not m2 and m4 receptor subtypes increased sAPPα release. Stimulation of sAPP secretion from m1 receptor-expressing HEK cells as well as in human NT2nN cells was accompanied by a reduction of Aβ release (Wolf et al., 1995), suggesting that cholinergic agents may activate the non-amyloidogenic pathway with the potential to prevent amyloid formation. The effect of carbachol was directly dependent on ligand–receptor interaction and on protein kinase activation. Stimulation of sAPPα release by cholinergic receptors is blocked by staurosporine (Nitsch et al., 1992) or GF109203X (Racchi et al., 2001; Slack et al., 1995). In addition to PKC, other pathways activated downstream of the cholinergic receptor, are linked to the activation of APP processing. In fact, the inhibition of sAPPα secretion by GF109203X is not complete (Slack et al., 1995) and thus, some authors suggested the involvement of a Tyr-K (Slack et al., 1995) because Tyr-K inhibitors such as tyrphostin A25 and genistein were effective blockers of stimulated sAPPα release. Other authors have also demonstrated the involvement of a MAP-K pathway (Haring et al., 1998). The signalling pathways downstream of the m3 muscarinic receptor clearly involve both PKC-dependent and -independent mechanisms coupled to the activation of the MAP-K pathway (Slack, 2000). However, for the latter mechanism as it relates to APP metabolism, there are contrasting reports. Desdouits--Magnen et al. (1998) was not able to demonstrate a block of carbachol-mediated sAPPα release in PC12-M1 cells treated with the MEK inhibitor PD98059. Using the same cellular model, however, Haring et al. (1998) showed a significant inhibition of carbachol-mediated sAPPα release by PD98059. We have observed (unpublished data) using SH-SY5Y which endogenously expresses both APP and m1/m3 muscarinic receptors, a reduction of carbachol-mediated sAPPα release by PD98059 suggesting at least a partial involvement of MAP-kinase pathways.

A further step was the demonstration that cholinergic modulation of APP metabolism could occur in rat foetal brain neurons (Salvietti et al., 1996) in culture and in superfused brain slices (Nitsch et al., 1993). Electrical depolarization of rat brain slices provided strong that neuronal activity could determine a modulation of sAPPα release. This study also demonstrated that cholinergic stimulation could elicit an increase in sAPPα release with a pharmacological profile suggestive of a complex interaction between different muscarinic receptor subtypes (Farber et al., 1995).

Particularly relevant to these studies is the fact that therapy currently available for AD patients is limited to drugs which improve central cholinergic neurotransmission (Frisoni, 2001; Gauthier, 2002; Giacobini, 2001) such as acetylcholinesterase inhibitors (AChEI). These drugs control the key symptoms of AD, namely memory and cognitive impairment. Since it has been demonstrated that AchEIs may have an effect on APP processing, the potential to modulate the biochemical pathways involved in the pathogenesis of AD may be realized.

The initial finding using superfused brain slices, demonstrated an increase of sAPPα release in response to stimulation by physostigmine, eptastigmine and dichlorvos (Mori et al., 1995; reviewed by Giacobini (2001)). We have investigated the effect of treatment with metrifonate (MTF) and dichlorvos (DDVP), respectively, the prodrug and active cholinesterase inhibitor drug, on the processing of APP in SH-SY5Y neuroblastoma cells, which demonstrated that the acute treatment with both compounds resulted in an increased secretion of sAPPα. Finally, we demonstrated that cholinesterase inhibitors such as metrifonate were able to induce sAPPα release by an indirect cholinergic mechanism (Racchi et al., 2001) as shown by inhibition with atropine (Fig. 3). On the other hand, others have observed that the secretion of sAPPα in a number of cell lines was inhibited using the AchEI drug tacrine (Lahiri et al., 1994, 1998), or phenserine (Shaw et al., 2001). In a recent paper, Shaw et al. (2001) showed that phenserine treatment of SH-SY5Y cells rapidly reduced levels of endogenous APP. This action was not connected to the AchEI activity of phenserine, since

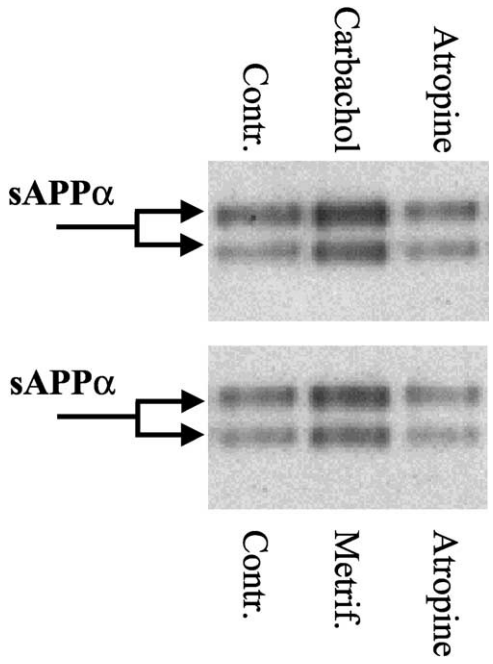


Fig. 3. Indirect cholinergic effect of AchE inhibitors on the processing of APP. Cells (neuroblastoma SH-SY5Y) were treated either with carbachol or with the cholinesterase inhibitor metrifonate, in the presence or absence of atropine (Racchi et al., 2001). The data with the antagonist atropine demonstrate that cholinesterase inhibition results in increased sAPPα release secondary to cholinergic stimulation, thus suggesting a direct relationship between the activity of cholinesterase inhibitors and APP processing. Although not all AchE inhibitors have demonstrated the same quantitative effect, we have always observed stimulation of APP non-amyloidogenic processing via indirect cholinergic stimulation (Racchi et al., unpublished data).

the effect was also observed following treatment with the stereoisomeric (+)-phenserine, which lacks ChEI activity. Consistently, with our previous experience (Racchi et al., 2001), other AchEI drugs (physostigmine and metrifonate) are unable to alter APP cellular levels in various neuronal cell lines (Lahiri and Farlow, 1996; Lahiri et al., 1997). Thus, phenserine, independent from its activity as AchEI, may influence the pathogenetic pathway in AD by modifying APP expression and thus the Aβ generating pathway. However, the concentrations of phenserine required to obtain modulation of APP expression are an order of magnitude higher than those that exert AchEI activity. Furthermore, it should be emphasized APP and its α-secretase-derived proteolytic product sAPPα may have significant neuroprotective and neurotrophic functions (Boyt et al., 2000a) thus reducing their expression and levels may not be completely favourable.

## 5. Other neurotransmitters and G protein coupled receptors ligands

A number of other neurotransmitters and various receptor ligands have been involved in the regulation of non-amyloidogenic processing of APP. Glutamate, an important neurotransmitter released during neural activity, can promote the release of sAPPα via metabotropic receptors and by activation of a signalling cascade involving PI hydrolysis and PKC activation as well as phospholipase A2 (PLA2). This has been demonstrated in cells transfected with individual metabotropic glutamate receptors (Lee et al., 1995; Nitsch et al., 1997). The effect of glutamate can be reproduced in cells with endogenous expression of mGluR1α receptors. In NT2N cells, agonists of glutamate metabotropic receptors, induce activation of PI hydrolysis and sAPPα release (Jolly-Tornetta et al., 1998). Primary hippocampal neurons secrete sAPPα in response to mGluR agonists (Lee et al., 1995), independent of activation by PLA2. The major signalling system coupled to sAPPα release in these cells is therefore PKC was demonstrated by experiments conducted on hippocampal and cortical brain slices. Metabotropic glutamate receptor agonists but not ionotropic receptor glutamate agonists stimulate sAPPα release via a PKC-dependent mechanism (Ulus and Wurtman, 1997).

Astrocytes in addition to neuronal cells have been used as cellular models, since they express a number of receptors coupled to ion channels or second messengers. Lee and Wurtman (1997) demonstrated that metabotropic glutamate receptor agonists increase the secretion of sAPPα following the activation of the PI hydrolysis/PKC signalling cascade. These authors demonstrated that cAMP is negatively coupled with sAPPα release since treatment with forskolin or dibutyryl cAMP inhibited the increase in sAPPα secretion caused by metabotropic glutamate receptor agonists.

In virtually all of the examples cited earlier, activation of a PKC-dependent pathway was involved. Serotonin receptors are an example of positive regulation of sAPPα release which is apparently independent of PKC activation. Nitsch and co-workers demonstrated that in cells transfected with either $5HT2_A$ or $5HT2_C$ receptors, the treatment with serotonin can induce a release of sAPPα which is not sensitive to PKC downregulation or selective inhibition (Nitsch et al., 1996).

More recently, another serotonergic receptor has been implicated in APP processing. The use of cells transfected with 5HT4 receptor (CHO cells) or endogenously expressing the 5HT4 receptor (IMR32 neuroblastoma cells), has provided evidence that serotonin and 5HT4 receptor agonists can promote the non-amyloidogenic secretory processing of APP. The intracellular signalling pathway activated is the formation of cAMP and PKA activation (Robert et al., 2001). In this study, the direct stimulation of adenyl cyclase resulted in the promotion of sAPPα release, which has also been repeated by others (Lezoualc'h et al., 2000; Marambaud et al., 1996). However, a surprising finding was the observation of a sAPPα secretion promoting effect induced by PKA inhibitors. These data suggested that PKA-dependent and -independent regulatory effects are present, downstream of 5HT4 receptors and compete with each other, the latter having the greatest effect.

Bradykinin is another stimulant of PKC-independent sAPPα secretion although these observations may relate to cell type specific events. The bradykinin-mediated sAPPα release in nerve growth factor (NGF) differentiated PC12 cells is blocked by the relatively non-specific kinase inhibitor staurosporine (Nitsch et al., 1998). Human skin fibroblast endogenously expresses the B2 type of bradykinin receptors. The pathway downstream involves a G protein coupled activation of PLC and initiation of the inositol cascade with the release of calcium from internal stores mediated by IP3. These pathway are known to result in the activation of PKC, however, whereas the PKC-dependent APP metabolism is clearly defective in AD fibroblasts (Bergamaschi et al., 1995) we demonstrated that bradykinin stimulated sAPPα release is not defective in fibroblasts from sporadic AD patients (Racchi et al., 1998). In all cases presented it is clear that PKC-independent pathways may co-exist in parallel with PKC-dependent pathways in virtually all cell types.

## 6. Regulation of APP processing by growth factors and Tyr-K coupled receptors

A pathway involving Tyr-K has been indicated in various systems important in the regulation of APP processing. This led to the study of the response to typical Tyr-K coupled receptors. The initial studies in this field demonstrated that treatment of A431 cells with EGF induced a concentration- and time-dependent increase in sAPPα release (Slack et al.,

1997). The epidermal growth factor receptor (EGFr) has intrinsic Tyr-K activity and is also coupled to a variety of other effectors including phospholipase Cγ; indeed the experimental treatments also produced a concentration dependent increase in PI hydrolysis. The activation of the secretory processing of APP by EGF is, at least partially, mediated by PKC, because the treatment of the EGF stimulated cell with the specific PKC inhibitor GF-109203X deceased the response by approximately 35% (Slack et al., 1997). On the other hand, the effect of EGF was completely blocked by Tyrphostin AG 1478, and inhibitor of EGF receptor Tyr-K.

NGF can also influence the metabolism of APP, and implicated in the modulation of the cholinergic system. These interactions and the role of NGF and other growth factors in APP processing and AD have been reviewed (Rossner et al., 1998; Isacson et al., 2002).

The roles of Tyr-K receptors and insulin in APP processing were investigated since several lines of evidence suggested a link between glucose utilization and defects in metabolism (Gasparini et al., 1997, 1998, 1999; Curti et al., 1997) associated with brain aging and the pathogenesis of AD. We tested the ability of insulin to regulate APP processing in cells that express endogenously insulin receptors and demonstrated that insulin modulates APP secretion by a Tyr-K dependent mechanism. Deprivation of glucose from the culture medium produced a reduction in the basal release of sAPPα, however, insulin was still able to induce a relative increase of sAPPα secretion, thus indicating that glucose is a necessary step of APP processing. Independent of glucose, insulin appeared to be acting as growth factor through a mechanism similar to EGF and other Tyr-K receptor ligands. The detailed dissection of the signal transduction network demonstrated that PKC and MAP-K were not involved in the effect of insulin on APP processing. Rather we demonstrated a novel intracellular pathway that increases the rate of secretion of sAPPα through the activity of phosphatidyl-inositol 3 kinase (PI3-K). Because of the physiological role of PI3-K in the translocation of glucose transporter-containing vesicles, we speculated that PI3-K involvement in APP metabolism might act at the level of vesicular trafficking. These speculations were later confirmed (Gasparini et al., 2001) who showed that insulin reduced intracellular accumulation of Aβ by accelerating APP intracellular trafficking. At variance with our observations of the action of insulin on APP metabolism was the report of mediation of insulin via a receptor Tyr-K/MAP-K pathway. The results of Solano et al. (2000) were also confirmed by Petanceska and Gandy (1999) who demonstrated that wortmannin, a PI3-K inhibitor could decrease the release of both Aβ and sAPPα from N2a neuroblastoma cells expressing either wild-type APP or the Swedish FAD-associated mutant variant of APP. These results suggest the possible involvement of a recently described trans-Golgi network (TGN)-associated PI3-kinase (Jones and Howell, 1997).

Following ingestion of glucose (Craft et al., 1999; Boyt et al., 2000b) or intravenous insulin administration (Craft et al., 2000) showed a reduction of plasma soluble APP concentration. Interestingly, the decrease in plasma APP concentration was affected by the ApoE genotype of the subject. Whereas insulin reduced APP levels for AD patients without an ApoE ε4 allele, it produced a raise of APP concentrations for AD patients with an ApoE ε4 allele. These results document ApoE-related differences in insulin metabolism in AD. Additional studies are required to determine the clinical significance and the implications for AD pathogenesis of these changes in plasma APP.

## 7. Steroids regulate APP metabolism

### *7.1. Steroid hormones*

Steroid hormones, e.g. estrogens, have been suggested in the treatment and/or prevention of AD. While mostly epidemiological, there has been a significant amount of data indicating a specific role of estrogens in the modulation of APP processing resulting in an increase in the non-amyloidogenic processing of APP and reduction of Aβ. The first finding was obtained in breast carcinoma cells (MCF7) following long-term treatment of with estradiol. The experiments demonstrated an increased release of sAPPα without effects on cellular APP levels, suggesting a mechanism regulating the actual processing and release of APP rather than its synthesis (Jaffe et al., 1994). These results did not provide an explanation on the mechanism of action of estrogen and did not clearly define whether or not the effect was dependent on the interaction with estrogen receptors. Subsequently, Xu et al. (1998) demonstrated a significant Aβ lowering effect of estradiol following long-term treatment of neuronal cells of various origin. The hypothesis upon the mechanism of action of estradiol was focused on the possible modification of intracellular trafficking of APP and in particular of APP-containing secretory vesicles. More recently (Manthey et al., 2001) it was shown that estradiol causes a very rapid increase in sAPPα release in neuroblastoma cell lines with a mechanism mediated by the MAPK pathway. In addition, the data show that the activation of MAPK-signalling pathway and the enhancement of the sAPPα release is independent of the presence of estrogen receptors since it could be induced to a similar extent in neuronal cells either lacking or over-expressing a functional estrogen receptor. The in vivo confirmation of the potential effect of estrogens on Aβ metabolism came from experiments using guinea pigs (Petanceska et al., 2000). Ovariectomized animals showed a 1.5-fold average increase in total brain Aβ, while animals treated with estradiol demonstrated a reversal of the ovariectomy-induced increase in brain Aβ levels. More recently, the same data were obtained in AD transgenic mice (Zheng et al., 2002). Brain Aβ levels were found to be

higher in estrogen-deprived mice than intact mice, and this effect could be reversed through the administration of estradiol. Overall these results provide evidence that post-menopausal estrogen depletion may be linked to an increased risk of AD through Aβ modulation, and estrogen replacement models do have significant effects, at least in experimental models, on the processing of APP and the production of Aβ.

Other examples of steroid modulation of APP processing come from the experiments of Gouras et al. (2000) showing that treatment of neuroblastoma cells and primary neurons with testosterone increases the secretion of the non-amyloidogenic sAPPα and decreases the secretion of Aβ peptides. Others (Goodenough et al., 2000) have suggested that the effect of testosterone follows a rapid time course, regulated by the aromatase-mediated conversion of testosterone to estrogen and is dependent on the MAPK signalling pathway.

### 7.2. Cholesterol

Cholesterol can significantly affect the activity of membrane associated enzymes and processes. APP is an integral membrane protein and the enzymes regulating its metabolism are membrane associated or integral membrane proteins. Cholesterol metabolism and AD are genetically linked because of the implication of the Apolipoprotein E genotype as a risk factor in the development of the disease. Epidemiologically, there are recent indications that there is a decreased prevalence of AD associated with the use of cholesterol-lowering drugs such as statins (reviewed in Simons et al. (2001) and Wolozin (2001)). The first experimental evidence that cholesterol may affect APP processing came from experiments in cell culture where membrane cholesterol enrichment was obtained by non-esterified cholesterol carried by rabbit VLDL (Racchi et al., 1997). The increase in cellular cholesterol caused a dose-dependent inhibition of sAPPα release, reproduced also by treatment with human LDL or by delivery of cholesterol dissolved in ethanol or cyclodextrin (Bodovitz and Klein, 1996; Racchi et al., 1997). More recently, others (Kojro et al., 2001) have suggested that the α-secretase enzyme ADAM 10 is a major target of the cholesterol effects on APP metabolism. Following treatment with lovastatin or with methyl-beta-cyclodextrin, a drastic increase of secreted sAPPα occurred, accompanied by reduced secretion of Aβ. Increased membrane fluidity and impaired internalization of APP, or higher expression of ADAM 10, may have been responsible for these findings.

Consistently, these data revealed that reducing the cellular cholesterol levels of hippocampal neurons by 70% with lovastatin or methyl-beta-cyclodextrin, inhibited almost completely the formation of Aβ, without affecting sAPPα release (Simons et al.,1998).

Several studies have investigated the fine mechanism of interaction between cholesterol and the machinery of APP processing. Initial suggestions came from the microdomain membrane localization described for APP. Ikezu et al. (1998) suggested that caveolae, which are plasma membrane invaginations where key signalling elements are concentrated, are the resident membrane microdomain where α-secretase processing takes place. Although other authors have suggested that APP (Hayashi et al., 2000) and also BACE (Riddell et al., 2001) are localized in cholesterol-enriched membrane microdomains different from caveolae, it is nevertheless clear that cholesterol plays a crucial role in maintaining the functional state of these microenvironments. Indeed it has been shown that alterations in cholesterol transport from late endocytic organelles to the endoplasmic reticulum have important consequences for both APP processing and the localization of α-secretase-associated PS, suggesting that the subcellular distribution of cholesterol may be an important factor in how cholesterol alters Aβ production and the risk of AD (Runz et al., 2002).

The effects of cholesterol on APP processing has been suggested also by studies in vivo. Transgenic mice expressing the Swedish FAD APP mutation, fed a high cholesterol diet caused a significant reduction of sAPPα as well as Aβ species in the brain (Howland et al., 1998).

Another model demonstrated that a diet-induced hypercholesterolemia resulted in significantly increased levels of Aβ peptides in the CNS. The hypercholesterolemic mice had significantly decreased levels of sAPPα, suggestive of a defective APP processing. The levels of total Aβ were strongly correlated with the levels of both plasma and CNS total cholesterol. Furthermore, the neuropathological analysis indicated that the hypercholesterolemic diet significantly increased amyloid deposit both in number and size (Refolo et al., 2000). More recently, the same transgenic mice were treated with the cholesterol-lowering drug BM15.766 and tested for modulation of β-amyloid levels. Such treatment significantly reduced plasma cholesterol, and in particular both brain amyloid load and levels of Aβ (Refolo et al., 2001).

Such data strengthen the involvement of membrane cholesterol in the regulation of APP processing. These intriguing relationships raise the possibility to pursue a cholesterol-lowering strategy to influence the progression of AD.

## 8. Concluding remarks

The critical point of the amyloid cascade hypothesis is altered APP processing which is a crucial event in AD pathogenesis. We have described multiple signal transduction mechanisms, neurotransmitter receptors and other receptor ligands involved in regulated APP processing. All pharmacological means of stimulation of sAPPα release have the ultimate goal of modulating APP metabolism with the resultant increase in production of soluble α-secretase

derived APP and a consequent reduction of Aβ production. Many of the transduction signals involved in APP processing have been described as defective in the AD brain and peripheral tissues, however, the redundancy of these systems in the regulated APP metabolism is such that alternative strategies are possible, which may bypass the defective pathways. All of these systems are potential targets for pharmacological modulation, beginning with AchE inhibitors and their effect on the APP processing pathway to the use of cholesterol-lowering drugs. Finally, the identification of secretases with distinctive enzymatic characteristics has boosted the research on novel specific protease inhibitors.

The task in future years will be to identify specific and unique mechanisms involved in APP processing and Aβ formation. In neurodegenerative diseases such as AD, the progression of degeneration can be slowed, however, never reversed. Thus, the goal is to find strategies and applications which if used early in the course of the disease may prevent the occurrence of further neurodegeneration.

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