Rationalizing a pharmacological intervention on the amyloid precursor protein metabolism

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The treatment of Alzheimer’s disease remains a major challenge because of our incomplete understanding of the triggering events that lead to the selective neurodegeneration characteristic of Alzheimer’s brains. The rational design of a pharmacological intervention is therefore a great theoretical challenge. One approach involves the study of the pharmacological modulation of the amyloid precursor protein metabolism, in which the goal is to reduce the formation of β-amyloid in the hope of reducing the formation of a potentially neurotoxic peptide. Such an approach has led to the identification of a complex intracellular mechanism that can be regulated by neurotransmitters and other ligands.

Over the past 15 years, extensive research has provided a detailed knowledge of the biology and genetics of Alzheimer’s disease (AD)1 beginning with the demonstration that the major proteinaceous component of AD brain senile plaques is a peptide of 39–43 amino acids named β-amyloid or Aβ, which is derived from a larger precursor, the amyloid precursor protein (APP). The routes of cellular APP metabolism are complex and involve different pathways that lead to the proteolytic processing of the precursor, which is mediated by at least three unidentified proteolytic activities1 (see Fig. 1).

One of the leading, although controversial, hypotheses for the pathogenesis of AD is centered around the potential toxic role of excessive production of Aβ (Ref. 2). Several investigators favor the amyloid cascade as a working hypothesis, which considers Aβ formation as an early event in Alzheimer’s pathogenesis. Major support for the amyloid hypothesis has come from genetic studies that show that all gene mutations linked to familial forms of AD are connected with alterations in APP processing to form Aβ (Refs 1, 3). In addition, data obtained using peripheral tissues and body fluids from patients affected by the common sporadic form of the disease have contributed to the idea of altered APP metabolism, which includes a reduction in sAPPα release and increased levels of Aβ, which form Aβ, is a common feature of AD (Ref. 4). Altogether, these data support the hypothesis that an aberrant APP metabolism occurs during the development of the disease, which increases the production of a potentially neurotoxic peptide. At the same time, the release of sAPPα, a potentially neuroprotective molecule, is reduced and this could also contribute to the neurodegeneration1.

Defective signal-transduction network

A consistent feature of AD is the degeneration of cholinergic neurones, which leads to the impairment of acetylcholine transmission. There have also been reports of multiple neurochemical deficits in the cerebral cortex and hippocampus of the AD brain. The changes in neurotransmission in the AD brain appear to involve multiple events including impaired neurotransmitter-receptor-mediated signal transduction2.

G proteins are the first link between receptor activation and the modulation of intracellular effector systems. Receptor activation triggers the hydrolysis of phosphoinositides (PI) by phospholipase C (PLC) generating diacylglycerol and inositol (1,4,5) triphosphate [Ins(1,4,5)P3]. These molecules are second messengers for the mobilization of Ca2+ from internal stores and protein kinase C (PKC) activation. The AD brain shows alterations in all of these systems including changes in the activity7 and levels8 of PLC and impairment of the phosphoinositide signalling cascade following stimulation by an acetylcholine receptor agonist9, which could be related to the uncoupling of nicotinic M1 receptors from Gq proteins10. It has also been shown that the number of receptor sites for Ins(1,4,5)P3 are reduced in the AD brain11,12, and, more recently, these alterations have been correlated to the staging of neurofibrillary and amyloid pathology, which suggests a close relationship with the pathogenetic process13.

One of the most consistent findings from AD brain studies is the change in the levels, activity, and subcellular distribution of PKC (Ref. 14). As with the Ins(1,4,5)P3 receptor sites, the reduced PKC levels in AD brain have also been shown to be correlated with neuropathological staging15. Finally, widespread defective G-protein-stimulated adenylate cyclase activity was shown to be correlated with defective G-protein receptor coupling16. Altered signal-transduction mechanisms are also consistently found in peripheral tissues from AD patients, which suggests that these changes are not secondary to neuronal loss and could be directly involved in AD pathogenesis6.

These observations assume particular importance in the context of AD pathogenesis as the metabolic fate of APP is significantly regulated by events that involve the activation of second messengers (see Fig. 2). PKC was the first signal-transduction-related molecule to be implicated in the regulation of APP metabolism. Extensive studies on regulated APP processing established that the Aβ-forming amyloidogenic pathway and the s-secretase non-amyloidogenic pathway were mutually exclusive. This simple view of APP metabolism might not fully reflect the complexity of the system and the occurrence of such balanced metabolism in all cell types is being questioned17.
The target of PKC phosphorylation is not the APP molecule itself as originally suggested but possibly either the α-secretase or, more likely, a key cellular factor that targets APP to the α-secretase, perhaps by supporting the budding of APP-containing vesicles from the trans-Golgi network. Following this lead, it was also shown that PKA can induce the release of sAPPα from cells. Treatment with forskolin stimulated the release of sAPPβ from intact PC12 cells and the addition of purified PKA to cell-free Golgi-enriched membranes induced the budding of APP-containing secretory vesicles with a mechanism that was distinct and additive to that observed with PKC (Ref. 19). In a different cellular system, Marambaud et al.25 showed that forskolin and 8-bromo-cAMP induced the release of sAPPβ and suggested that the proteasome could be an intermediate target between the activation of the kinase and the activity of α-secretase. However, Efthimiopoulos et al.26 observed that following forskolin-mediated elevation of cAMP in C6 glioma cells, phorbol-ester-induced sAPPβ release was inhibited. Similar results were obtained using rat cortical astrocytes, thus the discrepancies on the effect of cAMP and PKA are still unresolved.

It has been suggested that the effect of PKC on APP processing is mediated, at least in part, by other kinase systems. The intervention of the mitogen-activated protein kinase (MAPK) cascade in the process has been suggested by the observation that the MAPK inhibitor PD98059 also blocked the phorbol-ester-mediated release of sAPPβ in different cellular systems. Interestingly, divergent results were obtained in the two studies regarding the effect of MAPK inhibition on Aβ production. In the study by Mills et al.,27 the MAPK inhibitor PD98059 also blocked the effect of phorbol esters on the release of Aβ, i.e. it blocked the reduced release of Aβ following direct PKC activation. By contrast, Desdouits-Magnen and co-workers failed to

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**Fig. 1.** The structure of the amyloid precursor protein (APP), the β-amyloid (Aβ) peptide and sites of APP processing. The sequence of the β-amyloid peptide is expanded to show the position of the known mutations of APP associated with the early onset, autosomal dominant forms of familial Alzheimer’s disease (FAD). All these mutations areanking or within the Aβ peptide sequence and modify the normal metabolism of APP towards an increase in the production of amyloidogenic fragments. The processing of APP into its most common derivatives is due to three proteolytic activities, α-secretase, β-secretase and γ-secretase, which generate the larger C-terminal products (CTFs) that can be subsequently cleaved by γ-secretase. This prevents the formation of amyloidogenic fragments and constitutes the non-amyloidogenic pathway. The β-amyloid peptide is formed and secreted as a physiological product of cell metabolism as a product of α- and γ-secretase, β-secretase activity forms the N-terminal side of Aβ, cleaving APP before Asp1 of the Aβ peptide sequence and leaving in the membrane a 10 kDa C-terminal fragment (10 kDa CTF). This prevents the formation of amyloidogenic fragments and constitutes the non-amyloidogenic pathway. The β-amyloid peptide is formed and secreted as a physiological product of cell metabolism as a product of α- and γ-secretase, β-secretase activity forms the N-terminal side of Aβ, cleaving APP before Asp1 of the Aβ peptide sequence and leaving in the membrane a 10 kDa C-terminal fragment (10 kDa CTF). Both the C-terminal fragments can be substrates of the γ-secretase activity. In particular, the 12 kDa CTF can lead to the formation of Aβ1–42 depending on which γ-secretase activity occurs. In some cases, the overall effect of a mutation is a net increase in the production of β-amyloid (e.g. the ‘Swedish’ mutation), in other cases such as the mutations at residues 17 and 717 (numbered according to the longest APP isoform of 770 amino acids), the effect is a shift in the normal processing of the precursor and leads to the formation of longer forms of the peptide (e.g. Aβ1–40). The presence of mutations in these proteins, whose function is yet to be determined, also contributes to altered APP metabolism and increases the levels of β-amyloid ending at amino acid 42.
observe an effect of PD98059 on the basal release of Aβ or on the Aβ-reducing effect of phorbol esters treatment. Although these differences might be caused in part by the different experimental schemes used, it could not determine whether or not the involvement of the MAPK cascade in the regulation of PKC-dependent APP metabolism is located downstream of the point of divergence between the amyloidogenic and non-amyloidogenic pathway.

Among the second messengers, the role played by Ca²⁺ in AD is extremely important. It is a cofactor for the activation of PKC and tyrosine kinases. Ca²⁺ ions flow through receptor-activated channels and are released by internal stores following the binding of Ins(1,4,5)P₃, to its receptor. The role of Ca²⁺ in APP metabolism has been studied using two strategies whereby intracellular Ca²⁺ levels were increased either by favouring the release from internal stores or by promoting the influx from the external milieu by the use of ionophores. The first indication of a precise role for Ca²⁺ in the regulation of APP metabolism came from experiments showing that thapsigargin, an irreversible inhibitor of Ca²⁺ reuptake from the ER, was able to stimulate sAPPα release as well as reduce the formation of Aβ (Ref. 25). The effect of thapsigargin was independent from PKC activation, which suggests for the first time the existence of alternative routes bypassing the kinase. A tyrosine phosphorylation mechanism has been demonstrated for the effect of the Ca²⁺ ionophore ionomycin, which increases the release of sAPPα in HEK293 cells. The effect of ionomycin was blocked only partially by specific inhibitors of PKC but completely abolished using tyrosine kinase inhibitors, thus allowing the hypothesis that protein tyrosine phosphorylation is either independent from PKC or a downstream target of PKC activation (Ref. 26).

In summary, a complex network of second messengers is involved in the regulation of APP metabolism (see Fig. 2), a network that is defective in AD brain but at the same time open to pharmacological modulation.
and holoprotein secrete sAPP from Down’s syndrome (DS) individuals. Fibroblasts from normal individuals. These cells, in vitro, provided a simpler system for studying APP metabolism compared to neurons. However, the regulation of APP metabolism was shown to be dependent on the maturation of the neurones in vitro. In rat fetal brain neurones, the release of sAPP increased over maturation of the cells in vitro reaching a plateau by seven days. By that time, the modulation of sAPP release by carbachol was no longer evident. These observations could, in part, explain the lack of responsiveness of rat cortical neurones to cholinergic treatment in vitro where neurones were kept in culture for 15 days. The findings introduce the concept of saturability of the secretory mechanism for sAPP release.

The cholinergic stimulation of brain slices elicited an increase in sAPP release with a pharmacological profile suggestive of a complex interaction between different muscarinic receptor subtypes. In fact, non-specific cholinergic agonists, such as carbamylcholine, and cholinesterase inhibitors, such as physostigmine, secrete sAPPs approximately twice as much as metabolotropic glutamate receptor agonists elicit an increase in sAPP release when stimulated with phorbol esters. The cholinergic stimulation of brain slices elicited an increase in sAPP release with a pharmacological profile suggestive of a complex interaction between different muscarinic receptor subtypes. In fact, non-specific cholinergic agonists, such as carbamylcholine, and cholinesterase inhibitors, such as physostigmine, secrete sAPPs approximately twice as much as metabolotropic glutamate receptor agonists elicit an increase in sAPP release when stimulated with phorbol esters.

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Receptor-mediated PKC-independent mechanisms

The activity of PKC is central to almost all the examples cited above. However, PKC-independent pathways can also participate in the stimulation of sAPPα release. The 5-HT<sub>1</sub><sub>G</sub> and 5-HT<sub>3</sub> receptors are further examples of receptors coupled to the positive regulation of sAPPα release from receptor-transfected cells. Nitsch and co-workers<sup>42</sup> have shown that downregulation of PKC by chronic treatment with phorbol esters, or inhibition of the kinase with chelengrine or staurosporine, did not interfere with the increase in sAPPα secretion following treatment with 5-HT of 3T3 cells transfected with these receptors. Bradykinin is an example of PKC-independent sAPPα secretory stimulus in human skin fibroblasts; this pathway, which possibly involves the release of Ca<sup>2+</sup> from internal stores mediated by Ins(1,4,5)P<sub>3</sub>, is not defective in fibroblasts from sporadic AD patients<sup>43</sup>, as opposed to a defective PKC-dependent APP metabolism<sup>44,45</sup>. The interpretation of these results is not always straightforward as they could be cell-type-specific events. Indeed, the bradykinin-mediated sAPPα release in NCF-differentiated PC12 cells is blocked by the kinase inhibitor staurosporine, whereas the sAPPα releasing effect of vasopressin in NRK-49F cells is not sensitive to chronic PKC downregulation<sup>46</sup>. In any case, it is clear that PKC-independent pathways could co-exist in parallel with PKC-dependent pathways in the same cell type.

Growth factors

Reports that tyrosine phosphorylation was also involved in the regulation of sAPPα release prompted the study of the response to a typical tyrosine-kinase-coupled receptor. The epidermal growth factor receptor (EGFR) has intrinsic tyrosine kinase activity and is also coupled to a variety of other effectors including phospholipase C<sub>y</sub>. Treatment of A431 cells with EGF induced a concentration- and time-dependent increase in sAPPα release that was paralleled by an increase in PI hydrolysis<sup>47</sup>. Studies with specific inhibitors of PKC and tyrosine kinase activities showed that the effect of EGF was partially dependent on PKC activation as previously suggested for M<sub>1</sub> and M<sub>2</sub> receptors, and completely blocked by tyrphostin AG1478, an inhibitor of EGFR receptor tyrosine kinase. NGF is yet another molecule that can greatly influence the metabolism of APP. Its use has also been suggested for the treatment of AD because of its effect on the cholinergic system. Its influence on APP metabolism is also connected with effects on the cholinergic system and these interactions have been extensively reviewed<sup>48</sup>.

Steroids

An important addition to the pharmacological regulation of APP metabolism comes from the fact that oestrogen treatment can increase the non-amyloidogenic processing of APP and reduce A<sub>b</sub> production, thus providing a potentially added value to the advocated use of oestrogen for the prevention of AD. It was first observed that long-term treatment of breast carcinoma cells with oestradiol increased the release of sAPPα without affecting cellular APP levels. More recently, the same effect has been observed by Xu and co-workers<sup>49</sup> following treatment of cells of neural origin including neuroblastomas and primary cultures of rat, mouse and human embryonic cerebrocortical neurones. These latter experiments demonstrated that oestrogen can also decrease the production and release of A<sub>j</sub>. As a mechanistic hypothesis, it is possible that oestrogens could affect the morphology and trafficking of post-trans-Golgi network secretory vesicles. Given that A<sub>j</sub> can be formed in the trans-Golgi network (Ref. 50) it could be that enhanced sAPPα release and consequent reduced A<sub>j</sub> formation are results of increased transport of APP from the trans-Golgi network to secretory vesicles.

Steroids, in general, have a significant effect on the activity of membrane-associated enzymes and processes. Given that APP is an integral membrane protein and that all of the enzymes regulating its metabolism are probably membrane-associated or integral membrane proteins, it is likely that fully functional cellular membranes are crucial to APP metabolism. Increasing the cholesterol content of cellular membranes of HEK293 cells<sup>50</sup> or COS cells<sup>51</sup> by either ethanol-dissolved cholesterol, methyl <i>β</i>-cyclodextrin-complexed cholesterol<sup>52,53</sup> or very low density lipoprotein-carried cholesterol<sup>54</sup> produced a dramatic decrease in sAPPα release. Consistent with the hypothesis that the levels of cholesterol in the membrane can influence the metabolic processing of APP, reducing the cellular cholesterol levels by 70% with statins or cyclodextrin has recently been shown to reduce (up to complete abolishment) the formation of A<sub>j</sub> in cultured hippocampal neurones<sup>55</sup>. These data strengthen the involvement of membrane cholesterol in the regulation of APP metabolism. In addition, the role of ApoE polymorphism can be, at least partially, reinterpreted in terms of differential ability to transport cholesterol in and out of the cellular membrane<sup>56</sup>.

Concluding remarks

All pharmacological means of stimulating sAPPα release have the ultimate goal of modulating APP metabolism towards an increased production of soluble <i>α</i>-secretase-derived APP and a consequent reduction in A<sub>j</sub> production, bypassing the occurrence of defective transduction systems. Knowledge of the modulability of APP metabolism has opened up new avenues for the treatment of AD and has provided investigational tools for the understanding of the biological role of APP and its metabolism. However, there are still important questions that need to be answered. In particular, as the review points out, almost all the experiments are focused on in vitro pharmacology, whereas in vivo experiments on normal or transgenic animals, overexpressing APP or its mutated forms, are still lacking. Moreover, with the exception of the experiments on oestrogens, the effects of the various drugs on APP metabolism have been studied acutely. In particular, it is not known whether the various pharmacological stimuli...
either acutely or chronically modify APP expression, an event that in turn could alter qualitatively and/or quantitatively the response to secretory stimuli. The resolution of these problems will finally allow the use of pharmacological interventions on the amyloid precursor protein metabolism for Alzheimer therapy.

Selected references