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Amyloid Precursor Protein, Presenilins, and α-Synuclein: Molecular Pathogenesis and Pharmacological Applications in Alzheimer's Disease

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***Abstract*——Alzheimer's disease (AD) is the most common cause of dementia that arises on a neuropathological background of amyloid plaques containing β-amyloid (Aβ) derived from amyloid precursor protein (APP) and τ-rich neurofibrillary tangles. To date, the cause and progression of both familial and sporadic AD have not been fully elucidated. The autosomal-dominant inherited forms of early-onset Alzheimer's disease are caused by mutations in the genes encoding APP, presenilin-1 (chromosome 14), and presenilin-2 (chromosome 1). APP is processed by several different proteases such as secretases and/or caspases to yield Aβ and carboxyl-terminal fragments, which have been implicated in the pathogenesis of Alzheimer's disease. Alzheimer's disease and Parkinson's disease are associated with the cerebral accumulation of Aβ and α-synuclein, respectively. Some patients have clinical and pathological features of both diseases, raising the possibility of overlapping pathogenic pathways. Recent studies have strongly suggested the possible pathogenic interactions between Aβ, presenilins, and/or α-synuclein. Therefore, treatments that block the accumulation of Aβ and α-synuclein might benefit a broad spectrum of neurodegenerative disorders. This review covers the trafficking and processing of APP, amyloid cascade hypothesis in AD pathogenesis, physiological and pathological roles of presenilins, molecular characteristics of α-synuclein, their interactions, and therapeutic strategies for AD.**

I. Introduction

Aging is a major risk factor for neurodegenerative disorders, such as Alzheimer's disease (AD[1]) and Par-

[1] Abbreviations: AD, Alzheimer's disease; β-APP, β-amyloid precursor protein; Aβ, β-amyloid; ACh, acetylcholine; AChE, acetylcholinesterase; ACID, amyloid intracellular domain; ADAM, a disintegrin and metalloprotease; APLP, amyloid precursor-like protein; ApoE, apolipoprotein E; APP, amyloid precursor protein; BACE, β-secretase; BBB, blood-brain barrier; Ccas-PS2, PS2 fragments derived from alternative transcription or caspase-3 cleavage; cdk, cyclin-dependent kinase; CHO, Chinese hamster ovary; CNS, central

kinson's disease (PD), and the number of people with these conditions is increasing rapidly. Alzheimer's disease is the most common cause of dementia. United Nation population projections estimate that the number of people older than 80 years will approach 370 million by the year 2050. Currently, it is estimated that 50% of people older than age 85 years are afflicted with AD. Therefore, if these statistics hold true, in 50 years, more than 100 million people worldwide will suffer from dementia. The vast number of people requiring constant care and other services will severely strain medical, monetary, and human resources.

First described by Alois Alzheimer in 1906, the disease that bears his name largely remained an enigma until the twilight of the 20th century. Along with descriptions of progressive loss of memory and general cognitive decline, Alzheimer noted the presence of intraneuronal tangles and extracellular "amyloid" plaques in the disease-damaged brain, but he could not decipher whether the tangles or plaques were causative or merely markers of the disease. In 1991, the search for genetic linkages yielded a major clue: missense mutations in APP caused autosomal-dominant, early-onset (familial) AD, and these mutations occurred in and around the β-amyloid (Aβ) region of the precursor protein (Chartier-Harlin et al., 1991; Goate et al., 1991; Murrell et al., 1991; Hardy and Higgins, 1992)

The pathological hallmark of AD includes widespread neuronal degeneration, neuritic plaques containing β-amyloid (Aβ), and τ-rich neurofibrillary tangles (NFT) (Glenner and Wong, 1984). By the fourth decade of life, individuals with Down's syndrome display many of the same neuropathological features as do individuals with AD, and many of these individuals develop dementia early in life (Casanova et al., 1985; Wisniewski et al., 1985; Mann and Esiri, 1989; Sendera et al., 2000; Head et al., 2001). AD is multifactorial, with both genetic and environmental factors implicated in its pathogenesis. To date, mutations in three genes—the presenilin gene (PS1) on chromosome 14, the presenilin 2 gene (PS2) on chromosome 1, and the amyloid precursor protein gene (APP) on chromosome 21—all serve to transmit AD via autosomal-dominant inheritance. This form of AD is referred to as familial Alzheimer's disease (FAD) and is characterized by earlier onset of symptoms. There are other genes that are considered susceptibility or risk factors for AD. These include apolipoprotein E (ApoE ϵ4 variant) (Poirier et al., 1996), α2-macroglobulin (Blacker et al., 1998), a gene for a component of α-ketoglutarate dehydrogenase (Ali et al., 1994), the K-variant of butyryl-cholinesterase (Lehmann et al., 1997a), and several mitochondrial genes (Law et al., 2001). Epidemiological studies have demonstrated risk factors for AD that include age, gender (females are at greater risk), previous head injury, and cardiovascular disease (Law et al., 2001). Much work remains to be done to fully elucidate environmental factors that can influence both the onset and the progression of AD.

To date, the cause and progression of both familial and sporadic (late-onset) AD have not been fully elucidated. Proteolytic processing of APP by β-secretase, γ-secretase, and caspases generates Aβ peptide and carboxyl-terminal fragments (CTF) of APP, which have been implicated in the pathogenesis of Alzheimer's disease (Checler, 1995; Suh, 1997; Selkoe, 1999). The missense mutations in the gene encoding APP, as well as those in the genes encoding PS1 and PS2, share the common feature that they alter the γ-secretase cleavage of APP to increase the production of the amyloidogenic $A\beta_{42}$, a primary component of amyloid plaques in both familial and sporadic AD. All but one mutation triggers this phenotype. Ancolio et al. (1999) reported that V715M-APP significantly reduced total Aβ and $A\beta_{40}$ production without affecting $A\beta_{42}$ production, but it increased $A\beta_{X\text{-}42}$.

For the last decade, two major hypotheses on the cause of AD have been proposed: the "amyloid cascade hypothesis", which states that the neurodegenerative process is a series of events triggered by the abnormal processing of the amyloid precursor protein (Hardy and Higgins, 1992), and the "neuronal cytoskeletal degeneration hypothesis" (Braak and Braak, 1991), which proposes that cytoskeletal changes are the triggering events.

The most frequent sporadic forms of AD and PD are associated with an abnormal accumulation of Aβ and α-synuclein, respectively (Spillantini et al., 1997; Takeda et al., 1998, Selkoe, 2001). Human cases with clinical and neuropathological features of both AD and PD raise the possibility that these diseases involve overlapping pathways. Approximately 25% of patients with

nervous system; AChEI, AChE inhibitor; COX, cyclooxygenase; CSF, cerebrospinal fluid; CT, carboxyl terminal; CTF, carboxyl-terminal fragments; DFO, desferrioxamine; DHED, dehydroevodiamine hydrochloride; DLB, dementia with Lewy bodies; ERT, estrogen-replacement therapy; FAD, familial Alzheimer's disease; FDA, Food and Drug Administration; GSK-3β, glycogen-synthase kinase-3β; HEK, human embryonic kidney; IDE, insulin-degrading enzyme; IL, interleukin; iNOS, inducible nitric-oxide synthase; IP3, inositol phosphate-3; JNK, c-Jun NH_2-terminal kinase; kb, kilobase; KPI, Kunitz protease inhibitor; L-685,458, {1*S*-benzyl-4*R*[1-(1*S*-carbamoyl-2-phenylethylcarbamoyl)-1*S*-3-methylbutylcarbamoyl]-2*R*-hydroxy-5-phenylpentyl}carbamic acid *tert*-butyl ester; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MMP-9, matrix metalloproteinase-9; MW167, difluoroketone peptidomimetic 1; NAC, nonamyloid component; nAChR, nicotinic acetylcholine receptor; NF-κB, nuclear factor κB; NFT, neurofibrillary tangle; NICD, Notch intracellular domain; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NSAID, nonsteroidal anti-inflammatory drug; PD, Parkinson's disease; PDAPP, amyloid precursor protein V717F transgenic mice; PKC, protein kinase C; PS, presenilin; RIP, regulated intramembrane proteolysis; sβ-APP, soluble β-amyloid precursor protein; sα-APP, soluble α-amyloid precursor protein; sAPP, soluble amyloid precursor protein; SREBP, sterol regulatory element binding protein; TACE, tumor necrosis factor-α converting enzyme; TGF, transforming growth factor; TNF, tumor necrosis factor; uPA, urokinase-type plasminogen activator; UPR, unfolded-protein response.

AD develop frank PD (Galasko et al., 1994), and α-synuclein-immunoreactive Lewy-body-like inclusions develop in most cases of sporadic AD and FAD, as well as in Down syndrome (Lippa et al., 1999; Hamilton, 2000). Moreover, Lewy bodies contain APP (Arai et al., 1991; Van Gool et al., 1995; Halliday et al., 1997). The possible pathogenic interactions between Aβ and α-synuclein suggest that drugs aimed at blocking the accumulation of Aβ or α-synuclein might benefit a broader spectrum of neurodegenerative disorders than previously anticipated.

II. Amyloid Precursor Protein

A. Structure of Amyloid Precursor Protein

A partial amino acid sequence of Aβ was used to clone a cDNA encoding a protein now referred to as the APP, which has features of an integral type I transmembrane glycoprotein (Kang et al., 1987). The APP gene contains 18 exons spanning more than 170 kb (Yoshikai et al., 1990). The region encoding the Aβ sequence comprises part of exons 16 and 17 and contains between 40- and 43-amino acid residues that extend from the ectodomain into the transmembrane domain of the protein (Fig. 1).

Several APP mRNAs arise from alternative splicing and encode forms that differ mainly by the absence (APP-695) or presence (APP-751 and APP-770) of a Kunitz protease inhibitor (KPI) domain located toward the NH_2 terminus of the protein (Kitaguchi et al., 1988; Tanzi et al., 1988; De Sauvage and Octave, 1989; Oltersdorf et al., 1990; Sinha et al., 1990; Konig et al., 1992). There also exists a set of proteins called APLPs with structure similar to APPs (including forms containing or lacking a KPI domain), except that APLPs lack the Aβ sequence (Wasco et al., 1993; Slunt et al., 1994; Webster et al., 1995) (Fig. 1).

B. Trafficking and Proteolytic Processing of Amyloid Precursor Protein

The APP is an integral membrane protein processed by the three proteases α-, β-, and γ-secretase, which have been implicated in the cause of AD (Fig. 2). β-Secretase generates the NH_2 teminus of Aβ, cleaving APP to produce a soluble version of APP (β-APPs) and a 99-residue COOH-terminal fragment (CT_{99}) that remains membrane-bound. In contrast, α-secretase cuts within the Aβ region to produce APPαs, an 83-residue COOH-terminal fragment (CT_{83}).

Both CT_{99} and CT_{83} are substrates for γ-secretase, which performs an unusual proteolysis in the middle of the transmembrane domain to produce the 4-kDa Aβ and $CT_{57–59}$ [amyloid intracellular domain (AICD)] from CT_{99}, and a 3-kDa peptide called p3 and $CT_{57–59}$ from CT_{83}. Proteolysis by γ-secretase is heterogeneous: Most of the full-length Aβ species produced is a 40-residue peptide ($Aβ_{40}$), whereas a small proportion is a 42-residue COOH-terminal variant ($Aβ_{42}$). The longer and

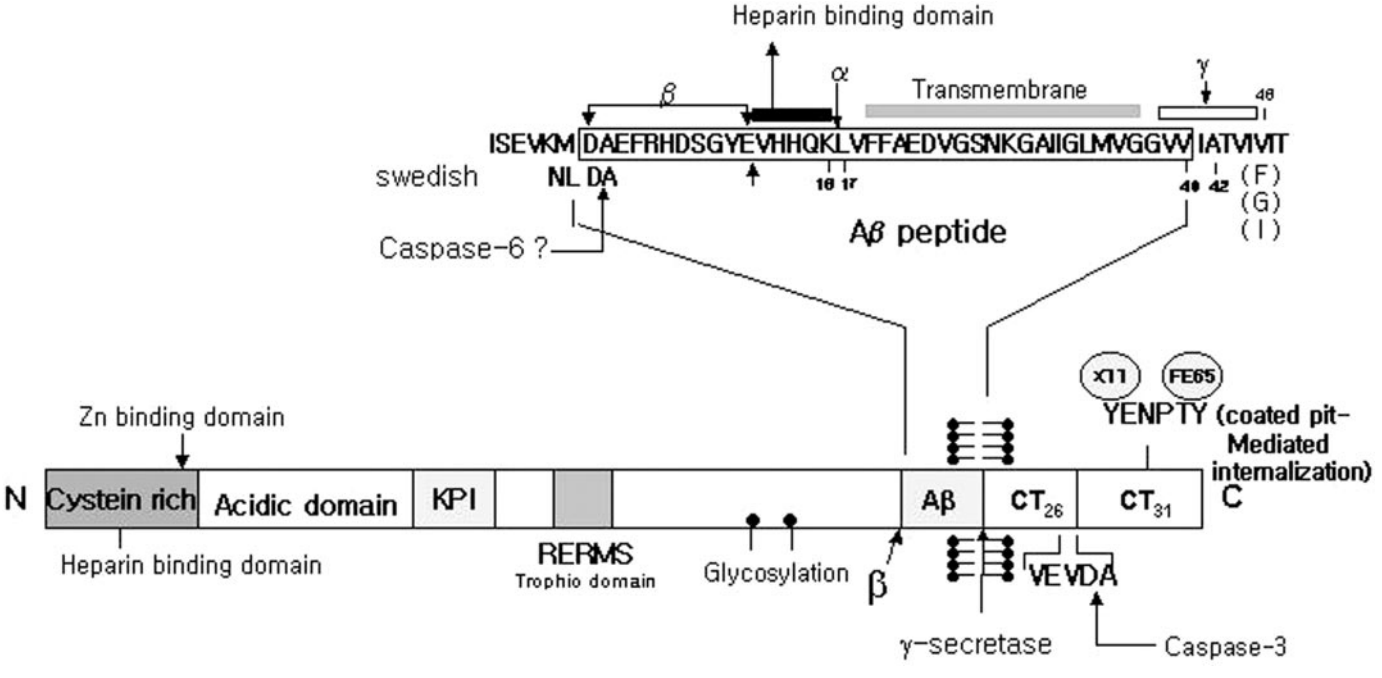

FIG 1. Structure of human β-APP. β-APP consists of a large extracellular domain, a hydrophobic transmembrane domain, and a short cytoplasmic carboxyl terminus. Some isoforms of β-APP (APP_{751} and APP_{770}) contain a KPI domain near the N terminus (Ponte et al., 1988). The Aβ sequence (amino acids 597–639 of APP_{695}) lies partially outside the cell membrane (amino acids 1–17 of Aβ; amino acids 597–613 of APP_{695}). A heparin-binding domain lies within the NH_2 terminal portion of Aβ (VHHQK) (Multhaup et al., 1994; Small et al., 1994). A heavily glycosylated region of APP lies NH_2-terminal to the Aβ sequence. This sequence is highly conserved and is nearly identical in species from *Drosophila* to human. Amino acid sequences of human and rodent Aβ differ by three amino acids located in the NH_2-terminal portion of peptide: amino acids 5 (human R, rodent G), 10 (human Y, rodent F), and 13 (human H and rodent R) of Aβ.

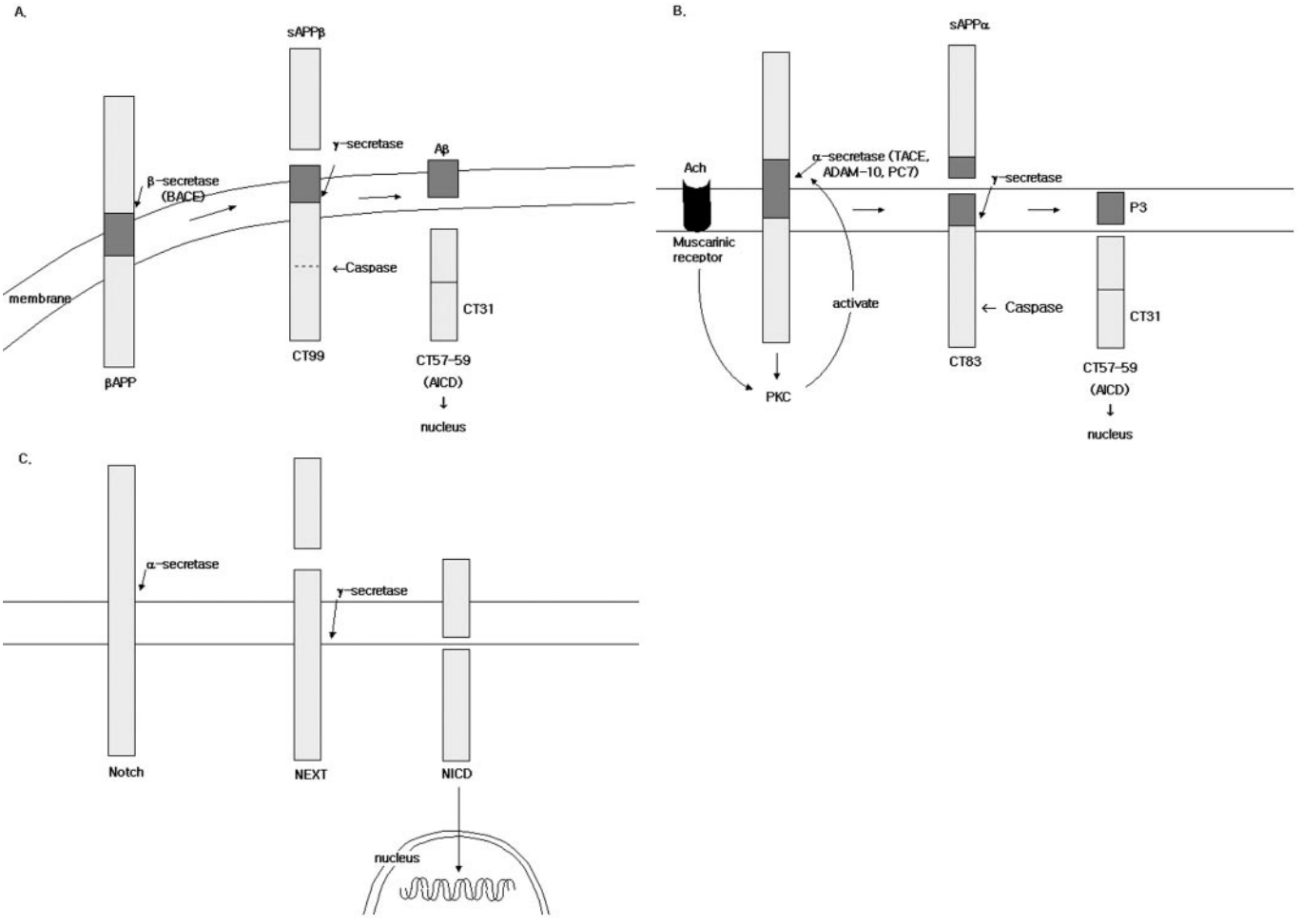

FIG 2. Processing of β-APP and Notch. A, β-APP is cleaved sequentially by β-secretase (BACE) and γ-secretase and/or caspases to generate Aβ, CT_{99}, $CT_{57–59}$ (AICD), and/or CT_{31}. CTF might translocate to the nucleus and affect the transcription of target genes. B, alternatively, β-APP can be cleaved by α-secretase and γ-secretase to produce P3 and $CT_{57–59}$ and/or CT_{31}. C, upon ligand binding, Notch is also cleaved sequentially by α-secretase and γ-secretase to produce NICD, which translocates to the nucleus and affects the transcription of target genes.

more hydrophobic $Aβ_{42}$ is much more prone to fibril formation than is $Aβ_{40}$ (Jarrett et al., 1993), and even though $Aβ_{42}$ is a minor form of Aβ, it is the major Aβ species found in cerebral plaques (Iwatsubo et al., 1994), Moreover, AD-causing mutations in APP near the β- and γ-secretase cleavage sites all increase $Aβ_{42}$, those near β-secretase cleavage site augment β-site proteolysis, leading to the elevation of both $Aβ_{40}$ and $Aβ_{42}$ (Citron et al., 1992; Cai et al., 1993), whereas those near the γ-site specifically increase production of $Aβ_{42}$ (Suzuki et al., 1994). Taken together, these findings implicated Aβ in the pathogenesis of AD and spurred AD researchers to identify the Aβ-releasing proteases.

α-Secretase displays characteristics of certain membrane-tethered metalloproteases, and β-secretase is a membrane-anchored protein with clear homology to soluble aspartyl proteases. The identification of the α-, β-, and γ-secretases provides potential targets for designing new drugs to treat AD.

1. α-Secretase. A major route of APP processing is via the α-secretase pathway, which cleaves on the C-terminal side of residue 16 of the Aβ sequence, generating an 83-residue C-terminal fragment (CT_{83}) (Figs. 1 and 2) (Esch et al., 1990). α-Secretase activity has both constitutive and inducible components. The constitutive activity has not yet been identified, but inducible α-secretase activity seems to be under the control of protein kinase C (PKC).

Two members of a disintegrin and metalloprotease (ADAM) family, tumor necrosis factor-α (TNF-α)-converting enzyme (TACE or ADAM-17) and ADAM-10, are candidate α-secretases (Fig. 2). TACE cleaves pro-TNF-α, releasing the extracellular domain (TNF-α) in a manner similar to that of APP. TACE apparently processes a spectrum of type 1 membrane glycoproteins, including TNF-α, the p75 TNF receptor, L-selectin adhesion molecule, and TGF-α.

The inhibition or knockout of TACE decreases the release of the α-cleaved product sα-APP (Buxbaum et al., 1998b). Mice lacking TACE die in utero, emphasizing the importance of ectodomain shedding during embryonic development (Peschon et al., 1998). However, cells deficient in TACE still have a residual α-secretase activity that cannot be increased by phorbol esters (Bux-

baum et al., 1998b). Thus, TACE may play a role in regulated PKC-dependent α-secretion.

TACE also seems to process Notch receptor. Upon ligand activation, Notch is processed by TACE (Brou et al., 2000; Mumm et al., 2000) (Fig. 2C) The membrane-associated carboxyl terminus is then cut by a PS-dependent r-secretase to produce the Notch intracellular domain (NICD), which translocates to the nucleus in which it interacts with and activates the CSL family of transcription factors (Schroeter et al., 1998). Such signaling is essential for cell fate determinations and tissue patterning during embryonic development.

Another metalloprotease, ADAM-10, also seems to process APP in an α-secretase-like manner (Lammich et al., 1999; Lopez-Perez et al., 2001). Overexpression of ADAM-10 increased both basal and phorbal ester-inducible α-secretase activity (Lammich et al., 1999). A dominant-negative form of ADAM-10 with a point mutation in the zinc-binding site was found to inhibit basal and inducible α-secretase activity, but it did not totally abolish sα-APP production (Lammich et al., 1999). ADAM-10 exists in a proenzyme form in the Golgi, but it becomes activated at the plasma membrane (Lammich et al., 1999)

ADAM-10 is also implicated in the Notch signaling pathway (Wen et al., 1997). Thus, TACE (ADAM-17) and ADAM-10 may both be α-secretases, which have very similar roles with respect to APP and Notch processing. Lopez-Perez et al. (1999, 2001) showed evidence for a role of the prohormone convertase PC7 in the constitutive α-secretase pathway. Definitive proof that they are α-secretases and whether other proteases also contribute to α-secretases activity remain to be determined. Because it is likely that several proteases contribute to α-secretase activity, it may be difficult to regulate APP processing pharmacologically through this pathway.

2. β-Secretase. In 1999, β-secretase was identified as a protein with homology to the pepsin family of aspartyl proteases (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000). β-Secretase contains a single transmembrane domain near the COOH terminus, a signal sequence and propeptide region at the NH_2 terminus, and two aspartates in its ectodmain, Asp_{93} and Asp_{289}, that are required for activity. Mutation of either aspartate does not affect removal of the propeptide region, indicating that β-secretase does not proteolytically cleave itself. Instead, the responsible protease seems to be a furin-like protease (Bennett et al., 2000). β-Secretase RNA is highly expressed in the brain and is also found in a variety of human tissues (Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000), consistent with the finding that Aβ is normally produced by many cell types and in accordance with that expected for β-secretase (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992; Busciglio et al., 1993). The intracellular localization of β-secretase protein is expressed primarily in the Golgi and in endosomes, whereas only a small amount of it can be detected in endoplasmic reticulum, lysosomes, and the plasma membrane (Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000). BACE is phosphorylated within its cytoplasmic domain at serine residue 498 by casein kinase 1, and the phosphorylation exclusively occurs after full maturation of BACE by propeptide cleavage and *N*-glycosylation and drives the localization of BACE to Golgi compartments and endosome (Walter et al., 2001). The gene for β-secretase is located on chromosome 11, but no AD-causing mutation in this gene has been identified so far (Saunders et al., 1999). A β-secretase homolog, BACE-2, maps to chromosome 21, raising the possibility that BACE-2 contributes to Down syndrome. Down syndrome patients secrete more Aβ from birth and invariably develop AD by age 50 years (Saunders et al., 1999). A1though BACE-2 cleaves APP and short-model peptides in a β-secretase-like manner (Farzan et al., 2000), there is very little of this protease in the brain, suggesting that it may play little if any role in the formation of cerebral plaques seen in AD. Instead, the AD associated with Down syndrome is probably caused by the presence of an extra copy of the APP gene, which is also located on chromosome 21.

BACE-2 is strongly expressed in heart, kidney, and placenta, suggesting that it may be important in highly vascularized systemic tissues (Farzan et al., 2000). It will be critical to develop drugs that selectively block BACE but not BACE-2. BACE knockout mice seemed to abolish Aβ production totally and to develop normally, healthy, and fertile (Luo et al., 2001; Roberds et al., 2001), showing that the therapeutic of BACE for treatment of AD may be free of mechanism-based toxicity.

3. γ-Secretase. After either α- or β-secretase releases the bulk of APP, the remaining carboxyl-terminal fragments, CT_{83} and CT_{99}, undergo proteolysis within their plasma membrane domain—regulated intramembrane proteolysis (RIP)—and the intracellular portion moves to the nucleus where it may affect the transcription of target genes (Fig. 2).

Proteins that undergo RIP include APP; Notch, a receptor involved in fate decisions during embryonic development; sterol regulatory element binding proteins (SREBPs), transmembrane proteins of the endoplasmic reticulum that regulate lipid metabolism (Brown et al., 2000); and ErbB-4, a member of the epidermal growth factor tyrosine kinase receptor family (Ni et al., 2001). How hydrolysis takes place in what is otherwise a water-excluded environment is unclear.

There is firm evidence that the intracellular portions of Notch and SREBP modulate gene transcription (Brown et al., 2000). Notch interacts with the coactivator p300 (Oswald et al., 2001), and SREBPs contain well-characterized DNA binding and transactivating domains that interact with a number of other transcription factors and coactivators (Edwards and Ericsson, 1999). Furthermore, the intracellular portion of APP forms a

complex with the nuclear adaptor protein Fe65 and with Tip60, which has histone acetyltransferase activity like p300, and stimulates transcription when fused to the DNA binding domains of the heterologous transcription factors Gal4 or LexA (Cao and Sudhof, 2001; Kimberly et al., 2001). However, the term RIP may be misleading, because proteolytic cleavage within a membrane has never been demonstrated directly. For example, it is possible that α- or β-secretase cleavage of APP results in movement of the C-terminal protein and exposure of the γ-secretase sites to the aqueous environment (Nunan and Small, 2000).

The enzyme that catalyzes the secondary cleavage of APP, Notch, ErbB-4, and SREBPs is γ-secretase. This γ-secretase has pharmacological characteristics of an aspartyl protease and remarkably loose sequence specificity for its substrate because many mutations in APP near the γ-secretase site still allow Aβ production in transfected cells (Maruyama et al., 1996; Tischer and Cordell, 1996; Lichtenthaler et al., 1997, 1999; Wolfe et al., 1999a–c) and seem to be a multiprotein complex, making its identification through expression cloning unlikely to succeed.

A crucial question is how protein cleavage itself is regulated. It seems that the triggering event is the removal of most of the extracytoplasmic part of the protein. This seems to be a prerequisite for the intramembrane cleavage by γ-secretase (Heldin and Ericsson, 2001).

4. Caspases. Not everyone agrees that nerve cells die by apoptosis in AD, but if the findings are confirmed, they could provide new targets for drugs aimed at slowing the progression of the disease. Reports that apoptosis might be involved in AD began emerging in the early to mid-1990s. Ivins et al. (1998) and Forloni et al. (1996) showed that Aβ causes neurons in culture to die by apoptosis.

Researchers found many more terminal deoxynucleotidyl transferase dUTP nick-end labeling-stained nerve cells in Alzheimer's patients' brains than in those from people who had died of other causes. The problem, however, was that the number of terminal deoxynucleotidyl transferase dUTP nick-end labeling-stained neurons was so large that it was not consistent with the time course of the disease.

Several investigators have looked at some of the 14 caspases so far identified. Stadelmann et al. (1999) showed that brain tissue from Alzheimer's patients had more nerve cells with activated caspase-3 than did samples from people who died of other causes, and the number of apoptotic neurons with the active enzyme was small—only approximately 1 in 1100 to 5000 neurons was affected—to be consistent with the slow course of AD.

Su et al. (2001) reported a similar percentage of neurons with active caspase-3 in brain with Alzheimer's disease and that the enzyme tends to be located in and around the amyloid plaques and neurofibrillary tangles. Neurons with caspase-3 were found in brains of mouse models of Alzheimer's or nerve cells in culture.

Yuan and Yankner (1999) showed that cortical neurons taken from the brains of mice in which the caspase-12 gene had been knocked out resist Aβ apoptosis-inducing effects. Caspase-12 is located in the membrane of endoplasmic reticulum, which regulates cellular responses to stresses such as protein misfolding and aggregation, free radicals, and the high concentrations of calcium ions and chemical toxins. Yuan and Yankner (1999) and Troy et al. (2001) found that hippocampal neurons from mice with an inactivated caspase-2 gene were completely resistant to apoptosis when exposed to Aβ.

Raina et al. (2001) showed that upstream caspases such as caspase-8 and -9 are activated in the brains of patients with AD, but they did not find activation of downstream caspases such as caspase-3. Therefore, they proposed that although apoptosis may be initiated in the neurons, it is aborted before it can kill them. Gervais et al. (1999) have evidence, from both cultured cells and examination of Alzheimer's brains, that caspases cut APP releasing Aβ (Fig. 3).

In addition, Lu et al. (2000) found that caspase cleavage of APP releases a second apoptosis-promoting peptide called CT_{31}, because it contains 31 amino acids from APP's carboxyl end. If brain caspases attack APP to release the toxic products Aβ and CT_{31}, then there may be a vicious cycle in which Aβ, by triggering caspase activation, fosters its own production, and thus further caspase activation and cell death. However, Soriano et al. (2001) found that caspase cleavage of APP actually decreases Aβ secretion by cells because it removes a signal sequence that would direct the peptide into the cell's secretory pathway.

The researchers found that the antibody which recognizes a caspase-3-cleaved fragment of fodrin, a major component of the fibers that form the cell skeleton of neurons, stained many more neurons in brains from Alzheimer's patients than in control brains. Dumanchin-Njock et al. (2001) showed that overexpression of CT_{31} triggers selective increase of $A\beta_{42}$ but not $A\beta_{40}$ production and elicits a concomitant cell toxicity that is caspase-independent. Rohn et al. (2001) reported that as the disease progressed, the brains received more of the fodrin staining and the tangles. de Boer et al. (2000) show that actin, another prominent protein of the cytoskeleton, undergoes caspase cleavage in brain with Alzheimer's disease. The researchers localized the caspase-cleaved actin to the degenerating nerve terminals. Mattson et al. (1998) showed that staining for caspases activated by Aβ can be found in the dendrites.

Several of the researchers suggested that Alzheimer's begins with such nerve terminal degeneration, consistent with findings that patients' degree of dementia is more highly correlated with the loss of nerve terminals

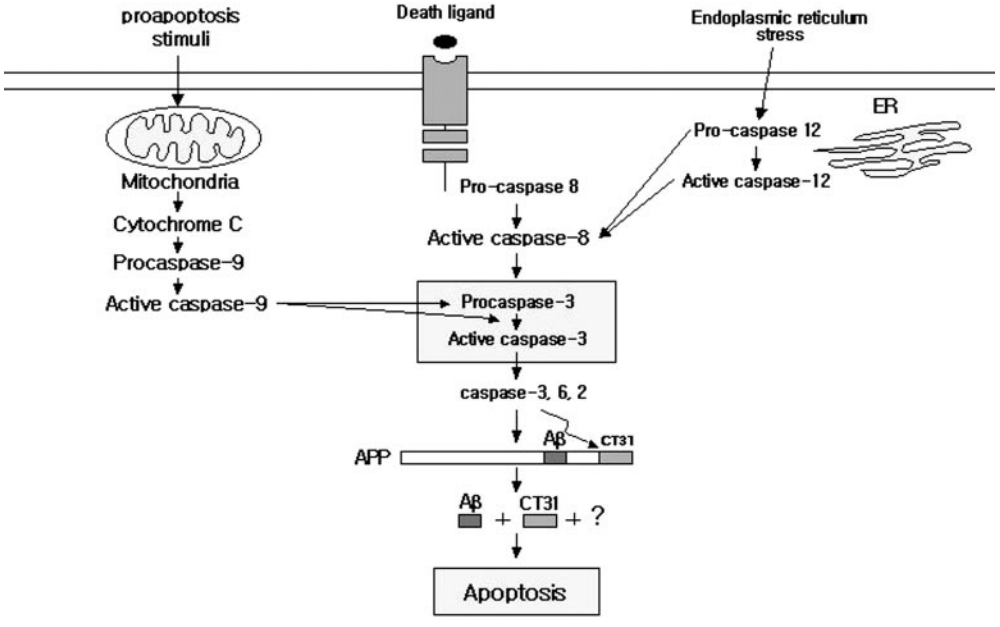

FIG 3. Probable processing of APP by caspases.

in their brains than with other pathological features, such as plaque formation. Ultimately, as a nerve cell loses more and more of its terminals, it will die.

A big question concerns how Aβ might trigger caspase activation. Troy et al. (2001) report that in cultured neurons, Aβ seems to act through c-Jun NH_2-terminal kinase (JNK). JNK, as well as the caspases, would be a potential target for drugs aimed at stopping or at least slowing Alzheimer's development. However, developing such drugs will not be easy, given the fact that both the caspases and the kinases play key roles in cell regulation throughout the body. For example, by helping eliminate cells with damaged DNA, the caspases protect against cancer. Caspase or kinase inhibitors aimed at stopping apoptosis in Alzheimer's could have unacceptable side effects. Antiapoptosis drugs by themselves may not be sufficient.

5. Amyloid-Degrading Enzymes. The most important question yet to be answered is why Aβ is deposited in sporadic AD, which accounts for more than 90% of all AD cases (Saido, 2000). Aβ is a physiological peptide, the steady-state levels of which are determined by the balance between the anabolic and catabolic activities (Selkoe, 1993; Saido, 1998). Because the rate of Aβ deposition is primarily a function of the steady-state level of Aβ in brain, the focus should be placed on how Aβ is metabolized in vivo.

The large percentage of familial or sporadic AD are not currently explained by abnormalities of Aβ production, so efforts to identify a candidate for Aβ-degrading proteases are important. Because an increase of only 50% in the production of a particular form of Aβ, caused by the majority of familial AD cases, leads to aggressive presenile Aβ pathology (Hardy, 1997; Selkoe, 1998), subtle alterations in this metabolic balance over a long period of time are likely to influence not only the pathological progression but also the incidence of the disease.

Reduced catabolism may account for the unresolved mechanism of late-onset AD development (Saido, 2000). Studies in mice show that newly generated Aβ is rapidly turned over in the brain (Savage et al., 1998), suggesting that Aβ-degrading proteases regulate its levels. However, the mechanism of Aβ catabolism has been less well understood than that of anabolism.

There are numerous proteases in the brain that could potentially participate in Aβ turnover. From the tube and tissue culture paradigms, Aβ degrader candidates include cathepsin D and E, gelatinase A and B, trypsin- or chymotrypsin-like endopeptidase, aminopeptidase, neprilysin (enkephalinase), serine protease complexed with α2-macroglobulin, and insulin-degrading enzyme (Saido, 2000). However, a single, dominant protease that is responsible for Aβ degradation has not yet been found.

It is necessary to distinguish between proteases that can degrade Aβ only in its monomer state and those that can degrade oligomeric and/or highly aggregated fibrillar forms of Aβ. Among the former class, neprilysin and insulin-degrading enzyme (IDE) have been focused on to date. Very few proteases belonging to the latter class have been documented because Aβ becomes resistant to proteases as a result of structural changes associated with its polymerization into fibrils. Biochemical experiments in which purified proteases are tested on synthetic Aβ peptides are of limited value. The ability of a particular protease to degrade naturally produced Aβ species at physiological concentrations of enzyme and substrate is important. Each candidate protease will need to be tested in transgenic and knockout mice to determine its effects on normal Aβ clearing and deposits. Human brain tissue should also be studied, taking

into account in which subcellular locus and under which conditions a protease is expected to cleave Aβ. Pharmacologically up-regulating certain Aβ-degrading proteases or interfering with the production or processing of their natural inhibitors could have great therapeutic potential.

a. Insulin-Degrading Enzyme. The enzyme occurs principally in a soluble form in the cytoplasm and is also present on intracellular membranes (Vekrellis et al., 2000). It occurs abundantly in a soluble, extracellular form in the nervous system as documented in human CSF and neuronal and microglial culture media (Qiu et al.,1998; Vekrellis et al., 2000). The existence of a membrane-anchored form of the protease suggests that it may regulate insulin-signaling at the plasma membrane and can participate in the degradation of both soluble and membrane-associated forms of Aβ. IDE degrades insulin, glucagon, atrial naturetic peptide, TGF-α, amylin and Aβ (Bennett et al., 2000). IDE has been shown to degrade rat and human amylin peptides similarly, despite the fact that only human amylin can form amyloid fibrils, suggesting that the motif recognized by IDE is not the β-pleated sheet region per se, but it is a conformation of the monomer in a preamyloid state (Bennett et al., 2000).

The cleavage products of Aβ by IDE are not neurotoxic and are not prone to depositing amyloid plaques, and recombinant IDE reduces Aβ toxicity in cortical neuronal cultures (Mukherjee et al., 2000). Endogenous IDE has been shown to degrade synthetic Aβ monomers in homogenates and membrane fractions of human brain (Perez et al., 2000; K. Vekrellis and D. J. Selkoe, unpublished data).

Naturally occurring oligomers of secreted Aβ in culture medium are resistant to IDE, whereas Aβ monomers are avidly degraded by the enzyme (Qiu et al., 1998; Vekrellis et al., 2000). These findings suggest that IDEs mediate much of the degradation of soluble monomeric Aβ but have less ability to degrade Aβ once it becomes insoluble and/or oligomeric (Selkoe, 2001b).

IDE gene is located on chromosome 10q, and different sets of late-onset AD pedigrees have shown linkage to DNA markers in the vicinity of this gene (Tanzi and Bertram, 2001). In the National Institute of Mental Health registry of 435 families with late-onset AD, genetic linkage to markers near l0q23-q25 and an allelic association of one of these markers with AD have been documented (Tanzi and Bertram, 2001). However, any association of the AD phenotype with polymorphisms in the IDE gene itself remains to be examined. Missense mutations in IDE that decrease its ability to degrade insulin in muscle have been discovered in the inbred Goto-Kakizaki diabetic rat, a compelling model of type 2 diabetes mellitus (Fakhrai-Rad et al., 2000).

b. Neprilysin. Neprilysin, a type 2 membrane protein on the cell surface and a neutral endopeptidase sensitive to both phosphoramidon and thiorphan, plays a major rate-limiting role in $A\beta_{1-42}$ catabolism (Iwata et al., 2001c). Neprilysin occurs almost exclusively in a membrane-anchored form and hydrolyzes several circulating peptides, such as enkephalin, atrial natriuretic peptide, endothelin, and substance P, and has wide tissue distribution and substrate specificity (Turner and Tanzawa, 1997). The intracerebral injection of synthetic Aβ peptides provided evidence that neprilysin is a major $A\beta_{42}$-degrading protease in rat brain, although the enzyme did not mediate $A\beta_{40}$ degradation in this paradigms (Iwata et al., 2001c).

Degradation of Aβ in the soluble fraction of brain seems not to be decreased by inhibition or deletion of neprilysin (Iwata et al., 2001c), whereas degradation of Aβ in the membrane fraction of brain is decreased ~25% to 35% by neprilysin inhibitors and ~70% by IDE inhibitors (K. Vekrellis and D. J. Selkoe, unpublished data), suggesting that neprilysin has little role in degrading soluble Aβ but can degrade buffer-insoluble, SDS-extractable Aβ associated with membranes (Selkoe, 2001b).

Steady-state levels of endogenous Aβ are elevated in the brains of young neprilysin-deficient mice (Iwata et al., 2001c), but the increase was not large and plaque formation was not observed. Given the rapid turnover of Aβ in brain (Savage et al., 1998), if neprilysin were the major degrader of Aβ, its deletion should produce an even greater accumulation. Therefore, other proteases may compensate in part for the loss of neprilysin. Long-term thiorphan infusion, which should inhibit several proteases, led to actual plaque formation in rats (Iwata et al., 2001c), a more robust effect than that of deleting neprilysin (Selkoe, 2001b).

The regional levels of Aβ in the neprilysin-deficient mouse brain were in the distinct order of hippocampus, cortex, thalamus/striatum, and cerebellum, with hippocampus having the highest level and cerebellum the lowest, correlating with the vulnerability to Aβ deposition in brains of humans with AD (Iwata et al., 2001c). Neprilysin mRNA levels were lowest in the hippocampus and temporal gyrus of brains with AD, which are vulnerable to senile plaque development, but levels were highest in the caudate and peripheral organs, which are resistant to senile plaque development (Yasojima et al., 2001). Work is underway to find evidence of linkage between AD and markers on chromosome 3, where the neprilysin gene is found.

c. Plasmin. The plasmin proteolytic cascade, known to be crucial for fibrinolysis and cell migration, has recently been implicated in Aβ clearance. In this cascade, tissue-type plasminogen activator or urokinase-type plasminogen activator (uPA) can be activated by binding to fibrin aggregates and then cleave plasminogen to yield the active serine protease, plasmin, which proteolyses fibrin and other substrates. Tissue-type plasminogen activator and uPA can be activated by Aβ aggregates to generate plasmin (Tucker et al., 2000).

Plasmin can significantly decrease the amount of neuronal injury induced by aggregated Aβ (10–30 μM) (Tucker et al., 2000). In vitro biochemical assays indicate that plasmin can proteolyze fibrillar Aβ, although at an efficacy that is approximately 100-fold less than that for freshly dissolved (largely monomeric) Aβ. The reaction was approximately 20-fold less efficient than that involving aggregated fibrin (Tucker et al., 2000). Moreover, in vivo evidence for a role of plasmin in regulating Aβ monomer or polymer levels has not yet appeared. uPA gene is mapped to a position near the center of the linkage region near 10q23-q25 (Tanzi and Bertram, 2001).

d. Endothelin-Converting Enzyme. This integral membrane zinc metalloprotease, with its active site located in the lumen and extracellularly, can cleave the endothelin precursors, bradykinin, substance P, and the oxidized insulin B chain (Eckman et al., 2001). Cellular overexpression of ECE-1 leads to a marked reduction in the levels of naturally secreted $A\beta_{40}$ and $A\beta_{42}$ in CHO cells. The enzyme directly proteolyzes both synthetic peptides in vitro (Eckman et al., 2001). Whether this protease can alter Aβ levels in vivo remains to be determined.

e. Other Candidate Proteases. Other proteases that have been reported to digest synthetic Aβ under in vitro conditions include matrix metalloproteinase-9 (Backstrom et al., 1996) and cathepsin D (McDermott and Gibson, 1996). The metalloendopeptidase 24.15 has been reported to indirectly regulate Aβ degradation. Whereas this enzyme does not proteolyze synthetic Aβ, decreasing its activity via antisense treatment leads to increased Aβ levels in cell culture (Yamin et al., 1999), suggesting that MP 24.15 processes a zymogen of an Aβ protease or degrades its endogenous inhibitor (Selkoe, 2001). Chevallier et al. (1997) showed that Aβ production or degradation is not affected by very selective inhibitors of endopeptidase 24.15, indicating that this enzyme is not involved in the genesis and degradation of Aβ. α1-Antichymotrypsin, the serine protease inhibitor, can increase Aβ deposition in APP transgenic mice (Mucke et al., 2000; Nilsson et al., 2001)

C. Amyloid Cascade Hypothesis: Two Major Amyloid Precursor Protein Metabolites Involved in Alzheimer's Disease Pathogenesis

The amyloid cascade hypothesis was first formulated more than a decade ago and centers around the Aβ peptide that is the main component of plaques (Glenner and Wong, 1984). There is a wealth of evidence to support this hypothesis (Selkoe, 1994; Checler, 1995; Mudher and Lovestone, 2002). The amyloid proteins involved in the pathogenesis of AD are Aβ- and CT- (carboxyl terminal peptides of APP) peptides (Fig. 4).

Classic Aβ is the marker for AD, and it has been linked to the accompanying neurodegeneration (Sisodia et al., 1990). Several lines of evidence suggest that the overexpression of β-APP and the subsequent production of Aβ could be linked to the genesis of AD (Checler, 1995). Furthermore, studies of plasma and fibroblasts from subjects with mutations in the genes encoding β-APP have established that they all alter β-APP pro-

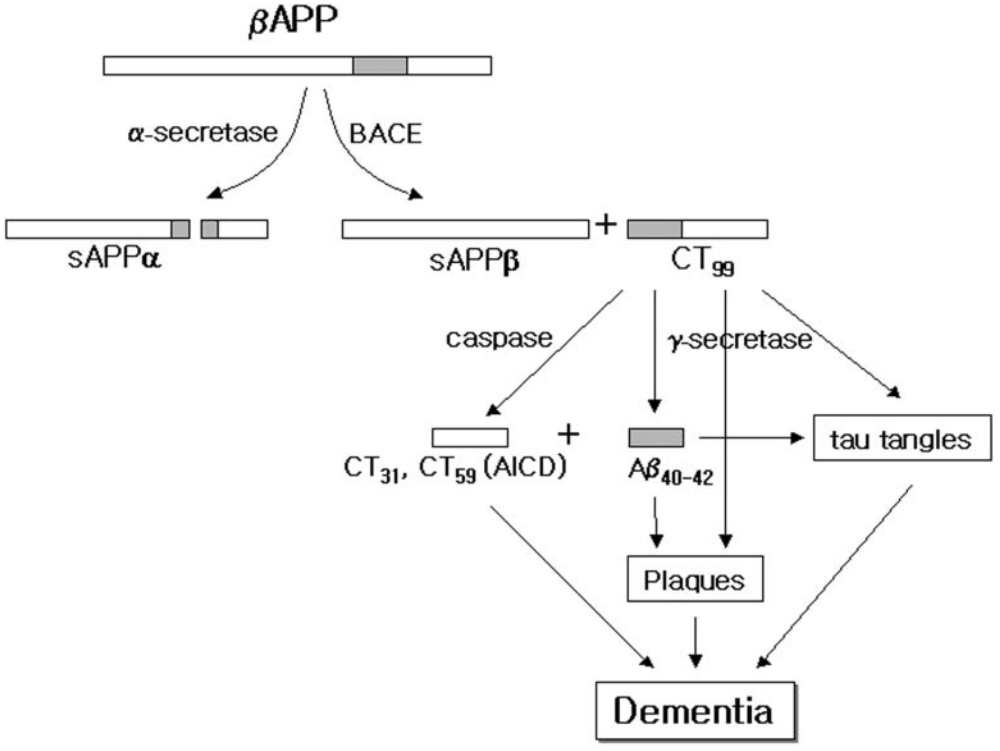

FIG 4. The amyloid cascade hypothesis.

cessing, which normally leads to the secretion of Aβ [relative molecular mass, 4,000; M_r, 4 K; ~90% $A\beta_{1-40}$, 10% $A\beta_{1-42(43)}$], so that the extracellular concentration of $A\beta_{1-42(43)}$ is increased (Suzuki et al., 1994). These results indicate that the β-APP mutations probably cause AD through an increase of $A\beta_{1-42(43)}$ in the brain.

Most obviously, mutations in APP are a rare cause of early-onset familial AD with all of the neuropathological and clinical features of AD. All but one of these mutations result in increased $A\beta_{1-42}$ generation in cell and animal models and in fibroblasts from affected families (Ancolio et al., 1999).

Trisomy 21 (Down syndrome), which leads to an overproduction of APP and Aβ, invariably leads to the early emergence of AD neuropathology (Selkoe, 1994). In addition, the E4 allele of ApoE promotes the precipitation of Aβ into insoluble plaques (Yankner, 1996). Even more persuasively, a locus on chromosome 10 associated with late-onset AD (Myers et al., 2000) is associated with increased Aβ generation (Ertekin-Taner et al., 2000).

In line with this are those studies showing that Aβ is neurotoxic to cultured cells and, at least in some conditions, induces tau phosphorylation (Takashima et al., 1993, 1998a; Alvarez et al., 1999). Amyloid vaccine (both passive and active immunization against Aβ) arrests and even reverses both plaque pathology and behavioral phenotypes in the transgenic animals (Schenk et al., 1999; Bard et al., 2000; Morgan et al., 2000).

Increasingly, attention is turning away from the deposits of extracellular insoluble aggregated amyloid in plaques and toward soluble, oligomeric and even intracellular $A\beta_{1-42}$ (Wilson et al., 1999; Klein et al., 2001). There are also some puzzling observations which hint that this hypothesis is not complete. For example, whereas transgenic mouse models bearing the FAD mutations do not show evidence of significant neuronal loss (Hsiao et al., 1995; Irizarry et al., 1997; Holcomb et al., 1998), little tau phosphorylation, and no tangle formation (Games et al., 1995). A relatively high concentration (two or three orders of magnitude) of Aβ was needed to exert toxicity, and some studies still failed to demonstrate Aβ toxicity in vivo (Clemens and Stephenson, 1992; Games et al., 1992; Stein-Behrens et al., 1992; Podlisny et al., 1993). Furthermore, it was reported that under certain culture conditions, Aβ promoted neurite outgrowth (Yankner et al., 1990; Koo et al., 1993) instead of exerting toxic action. Most important is that Aβ deposition has been observed in various brain areas without accompanying neurodegeneration (Joachim et al., 1989; Gearing et al., 1993; Einstein et al., 1994), whereas neurodegeneration can occur in areas with no Aβ deposition (Cochran et al., 1991).

It is also possible that the critical factor is not Aβ itself but that Aβ is a marker for proteolytic cleavage of APP, and it is the transcriptionally active carboxyl terminal of APP itself that is involved in the pathogenesis (Cao and Sudhof, 2001). Thus, Aβ may not be the sole active fragment in AD, and some other factor could be involved in inducing neuronal loss. The recent concentration on other potentially amyloidogenic products of β-APP has produced interesting candidates, the most promising of which are the amyloidogenic CT fragments of β-APP. The transgenic mice expressing β-APP and presenilins were not, however, examined for the presence of CT_{100}.

First, CT peptides have been found not only in various cultured cells (Maruyama et al., 1990; Wolf et al., 1990; Dyrks et al., 1992; Estus et al., 1992; Gandy et al., 1992a,b; Golde et al., 1992; Haass et al., 1992) but also in paired helical filaments (Caputo et al., 1992), in senile plaques (Selkoe et al., 1988), in microvessels (Tamaoka et al.,

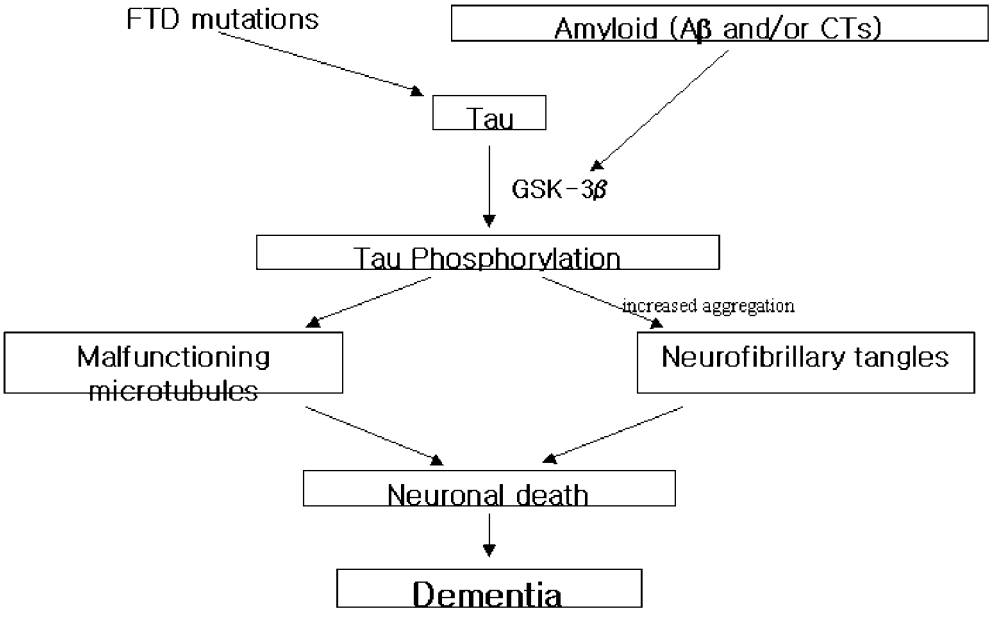

FIG 5. The tau and tangle hypothesis.

1992), in choroid plexus from human brain (Tokuda et al., 1995), and in human platelets (Gardella et al., 1992) (Table 2). CT fragments with molecular masses of 12 to 16 kDa have also been found in media and cytosol of lymphoblastoid cells obtained from patients with early- or late-onset FAD (Matsumoto, 1994) and Down syndrome (Kametani et al., 1994). Finally, several transfection studies have correlated production of the Aβ-bearing CT fragment with neurotoxicity (Yankner et al., 1989; Fukuchi et al., 1992a,b, 1993; Hayashi et al., 1992; Neve et al., 1992; Yoshikawa et al., 1992), whereas recent transgenic animal experiments using CT_{100} peptide have linked CT fragment production with neurodegeneration (Kammesheidt et al., 1992; Howland et al., 1995; Oster-Granite et al., 1996; Nalbantoglu et al., 1997).

In addition, this amyloidogenic CT peptide is not only expressed in the extracellular fluid of some FAD and Down syndrome cells, it is also secreted in the media of PC12 cells transfected with CT_{104} and human mixed brain-cell cultures (Yankner et al., 1989; Seubert et al., 1993; Matsumoto, 1994; Matsumoto and Matsumoto, 1994; Kim and Suh, 1996) (Table 2).

It has been recently demonstrated that either extracellular or intracellular application of CT_{105} elicited strong nonselective inward currents and toxic effects in *Xenopus* oocytes (Fraser et al., 1996), in rat Purkinje neurons (Hartell and Suh, 2000), and in PC12 and cultured rat cortical cells (Kim and Suh, 1996). The channel-inducing and toxic activity of CT_{105} was much more potent than that of any Aβ fragments (Table 3).

The synthetic peptide $APP_{713–730}$ of APP_{770} is highly fibrillogenic and interacts with tau in vitro and causes apoptotic neuronal death, suggesting that APP sequences other than Aβ may play a role in nerve-cell degeneration in AD (Marcon et al., 1999). Taken together, these lines of evidence postulate that the CT fragment is an alternative toxic element important in the generation of the symptoms common to AD. If clearing amyloid neither reverses dementia nor affects tangles, then the hypothesis needs considerable revision. Overall, however, the Aβ amyloid cascade hypothesis has fared remarkably well and has had few serious challenges.

1. Amyloid β-Peptide Hypothesis.

a. Neurotoxic Mechanisms of Amyloid β-Peptide: Free Radical Accumulation, Altered Calcium Homeostasis, and Inflammatory Response. AD researchers have mainly focused on determining the mechanisms underlying the toxicity associated with Aβ proteins. Aβ is a normal physiological product of APP processing (Estus et al., 1992; Golde et al., 1992) and a soluble component of the plasma and the cerebrospinal fluid (Seubert et al., 1992). The aggregation of soluble Aβ peptide into fibrillar cross-β pleated-sheet conformation is generally considered to be a critical event in the pathology of AD (Dumery et al., 2001). Aβ peptides may begin their toxic actions even before fibril formation. Increasing evidence suggests that soluble Aβ levels, and not Aβ plaques, are the best Aβ correlates of cognitive dysfunction in AD (McLean et al., 1999).

Several reports indicate that synaptic, physiological, and behavioral abnormalities precede Aβ plaque deposition in AD transgenic mice, supporting the possibility that Aβ plaques may not be the critical pathogenic en-

TABLE 1
Neurotoxic mechanisms of Aβ

Toxic Mechanisms	Effects	Reference
Alteration of ion homeostasis		
Potentiation of calcium channels or formation of calcium permeable channels	$[Ca^{2+}]\uparrow$	Mattson et al., 1992; Weiss et al., 1994; Vitek et al., 1994; Lin et al., 1999; Zhu et al., 2000; Green and Peers, 2001; Lin et al., 2001
Enhancement of glutamate-mediated excitotoxicity	$[Ca^{2+}]\uparrow$	Harkany et al., 2000
Inhibition of Na^+/Ca^{2+} exchanger	$[Ca^{2+}]\uparrow$	Wu et al., 1997
Selective inhibition of potassium channel	$[Ca^{2+}]\uparrow$	Good et al., 1996
Enhancement of K^+ current density	$[K^+]\uparrow$ $[Ca^{2+}]\downarrow$	Colom et al., 1998
Impairment of Na^+/K^+ ATPase	$[Ca^{2+}]\uparrow$	Mark et al., 1995
Generation of reactive oxygen species		
Production of free radical	Lipid peroxidation, DNA oxidation, and protein oxidation	Behl et al., 1994; Butterfield et al., 1994; Mattson et al., 1995; Varadarajan et al., 2000; Koppaka and Axelsen, 2000; Monji et al., 2001
	Release of lipid	Michikawa et al., 2001
Activation of inflammatory response		
Activation of microglia/astrocytes	Release of cytokines and chemokines	Hu et al., 1998; Tan et al., 1999; Lue et al., 2001
	NO generation	Akama et al., 1998; Tran et al., 2001
Activation of signaling pathway		
Activation of MAPK	Tau phosphorylation	Rapoport and Ferreira, 2000
Activation of JNK	Induction of Fas ligand	Morishima et al., 2001
Activation of calpain 1 and Cdk5	Tau phosphorylation	Patrick et al., 1999; Lee et al., 2000b
Activation of Src family tyrosine kinase	Tyrosine phosphorylation of cytoskeletal proteins (tau, microtubule-associated protein 2c)	Williamson et al., 2002

TABLE 2
In vivo evidence of amyloidogenic CT fragment generation

Tissue	Source	Reference
Human lymphoblastoid cells	Normal and FAD patients	Matsumoto and Fujiwara, 1991, 1993
Human platelets and megakaryocytes	Down syndrome	Matsumoto and Matsumoto, 1994
Human brains	Normal and FAD patients	Gardella et al., 1992; Ghiso et al., 1992; Kametani et al., 1994
Microvessels and leptomeningeal vessels		
Senile plaques	Normal and AD patients	Nordstedt et al., 1991; Estus et al., 1992; Ghiso et al., 1992; Tamaoka et al., 1992
Paired helical flaments		
Cerebral gray matter		
White matter	AD patients	Selkoe et al., 1988
Optic nerve	AD patients	Caputo et al., 1992
Human brain	Aged individuals	Nordstedt et al., 1991
	Down syndrome patients	Tokuda et al., 1995
	Healthy individuals	Amaratunga and Fine, 1995
	Healthy individuals	Golde et al., 1992
Neurites of amygdala and hippocampus	Down syndrome patients	Estus et al., 1992; Kametani et al., 1994
Transgenic mice	AD patients	Daly et al., 1998
CA 2/3 region of hippocampus of CT transgenic mice	Kammesheidt et al., 1992; Oster-Granite et al., 1996	
Brain of transgenic mice with β-APP-containing native and genetic mutations	Transgenic mice carrying CT_{100}	Howland et al., 1995; Araki et al., 1995; Nalbantoglu et al., 1997; Sato et al., 1997

tity. Potential roles for preamyloid protrofibrils and intraneuronally accumulated Aβ may prove to be important for the pathogenic process (Wilson et al., 1999; Selkoe, 2001a).

There are two major carboxyl terminal variants of Aβ. $Aβ_{1–40}$ is the major species secreted from cultured cells and found in cerebrospinal fluid, whereas $Aβ_{1–42}$ is the major component of amyloid deposits in brain with AD (Younkin, 1995). Increases in $Aβ_{1–42,}$ which is more prone to aggregation and formation of fibrils, have also been detected in transgenic mice and cells expressing FAD mutations of both APP and presenilins (Neve et al., 2000). These results suggest a link of this variant of Aβ to AD pathogenesis in that polymerization of Aβ into protease-resistant fibrils is a significant step in the pathogenesis of the disease (Tjernberg et al., 1999, Neve et al., 2000). The neurotoxicity exerted by aggregated Aβ can be mediated by several mechanisms, such as the generation of reactive oxygen species, dysregulation of calcium homeostasis, inflammatory response, and activation of some signaling pathways (Table 1 and Fig. 6).

One major area of researches has been calcium-mediated neurotoxicity. Aβ peptides can 1) increase calcium influx through voltage-gated calcium channels (N- and L-type channels); 2) form a cation-selective ion channel after Aβ peptide incorporation into the cellular membrane; 3) reduce magnesium blockade of NMDA receptors to allow increased Ca^{+2} influx; and 4) inhibit K^+ channel and Na^+/Ca^{2+} exchanger. Other major areas of research into Aβ peptide toxicity include free radical

TABLE 3
Summary of various effects of Aβ and CT

	Aβ	CTF	Reference
Neurotoxicity			
Cultured cells	+	+10	Kim and Suh, 1996; Marcon et al., 1999; Lee et al., 2000a
in vivo i.c.v.	+	++++	Song et al., 1998; Choi et al., 2001
Transgenic mice			Kammesheidt et al., 1992; Nalbantoglu et al., 1997; Sato et al., 1997
Neuritic plaques	+++	+++	
Channel effect			
Xenopus oocytes	−	+++	Fraser et al., 1996; 1997; Kim et al., 2002
Purkinje cells	−	+++	Hartell and Suh, 2000
Lipid bilayer	+	++	Arispe et al., 1993; Kim et al., 1999
Intracellular Ca^{2+}	+	+++	Kim et al., 1999, 2002; McKeon-O'Malley et al., 1999
LTP hippocampus	+	+++	Cullen et al., 1997; Nalbautoglu et al., 1997; Kim et al., 2001
Learning and memory impairment	+	+++	Kammesheidt et al., 1992; Nalbantoglu et al., 1997; Sato et al., 1997; Song et al., 1998; Choi et al., 2001
Cholinergic deficit	+	+++	Choi et al., 2001
Free radical generation	+++	−	
NO generation	+	+++	Rah et al., 2001; Bach et al., 2001
MAPK signaling	+	+++	Rah et al., 2001; Chong et al., 2001; Bach et al., 2001
NF-κB	+	+++	Rah et al., 2001; Chong et al., 2001; Bach et al., 2001
Nuclear translocation	−	+++	DeGiorgio et al., 2000; Cao and Sudhof, 2001; Kimberly et al., 2001; Gao and Pimplikar, 2001; Kim et al., 2002
Inflammatory cytokines and chemokines	+	+++	Rah et al., 2001; Chong et al., 2001
Gliosis and astrocytosis	+	+++	Song et al., 1998; Rah et al., 2001; Bach et al., 2001

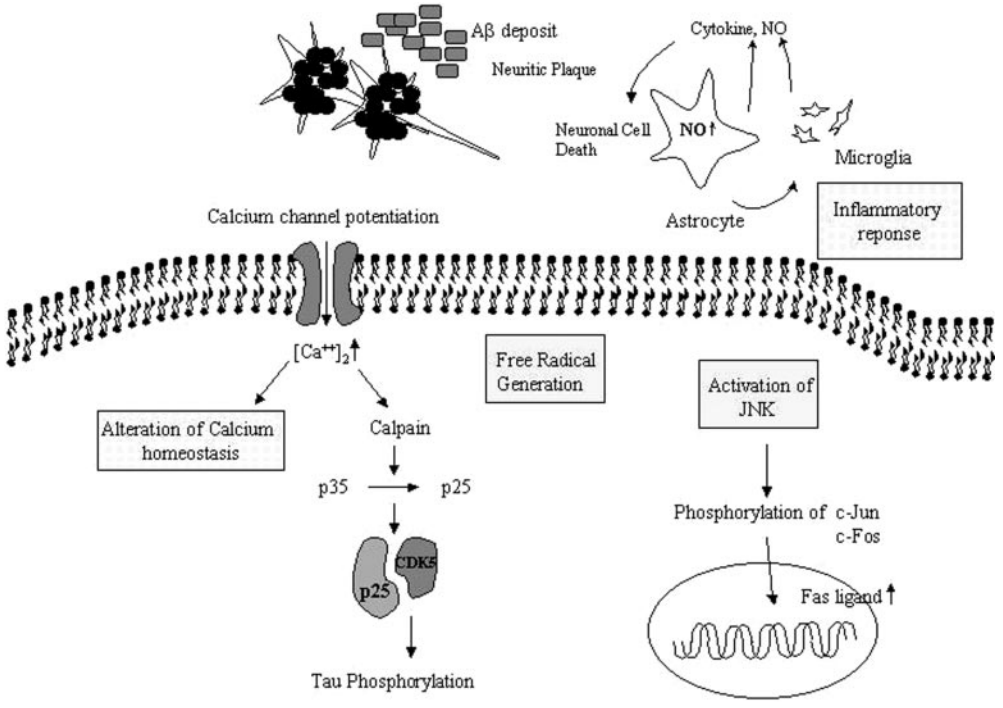

FIG 6. Summarized neurotoxic mechanisms of Aβ. Aβ is the main component of neuritic plaques found in brains with AD. Aβ exert neurotoxicity via several mechanisms: the alteration of calcium ion homeostasis, free-radical accumulation, inflammatory response, and activation of signaling pathways. Free radicals, generated by the interactions between the cytosolic membrane and Aβ, may lead to cellular dysfunctions via the inhibition of various enzyme activities, disruption of signaling pathways, and an activation of nuclear transcription factors. Aβ can disrupt cellular ion homeostasis by the potentiation of calcium channels and formation of ion pore. Inflammatory responses may be triggered by glial cells activated by Aβ, releasing a variety of proinflammatory cytokines, chemokines, and NO, exerting toxic effects on neighboring neurons. Aβ has been shown to activate some signaling pathways such as MAPK pathway in in vitro experiments. Recent report show that in cortical neurons exposed to Aβ, activated JNK phosphorylated and activated the c-Jun transcription factor, which in turn stimulates the transcription of several key target genes, including the death inducer Fas ligand. Application $A\beta_{1-42}$ induces the conversion of p35 to p25 in primary cortical neurons, leading to the activation of cdk-5 and tau phosphorylation.

formation, induction of inflammatory responses, and enhancement of excitotoxicity (Table 1).

i. Free-Radical Accumulation. The AD brain is subjected to increased oxidative stress resulting from free-radical damage (Markesbery, 1997). The sites in the AD brain in which neurodegeneration occurs and in which oxidative stress exists are reported to be associated with increased Aβ deposits (Hensley et al., 1995). Although the mechanism of Aβ-associated free-radical formation is not fully understood, Aβ is believed to contact or insert into the neuronal and glial membrane bilayer and generate oxygen-dependent free radicals that then cause lipid peroxidation and protein oxidation (Varadarajan et al., 2000). It has been shown that Aβ causes H_2O_2 accumulation in cultured hippocampal neurons (Mattson et al., 1995) and in neuroblastoma cultures (Behl et al., 1994). Electron paramagnetic resonance analysis of gerbil synaptosomes, using a 12-nitroxyl stearate spin probe, demonstrate that Aβ induced lipid peroxidation (Butterfield et al., 1994).

Increased oxidative modifications of proteins, such as advanced glycation endproducts have been found to increase DNA oxidation, and increased peroxidation of membrane lipids has been found in the brains of patients with AD upon autopsy (Halverson et al., 1990). Furthermore, the results by Koppaka and Axelsen (2000) indicate that oxidatively damaged phospholipid membranes promoted β-sheet formation by Aβ, and they suggest a possible role for lipid peroxidation in the pathogenesis of AD.

In addition, it has been shown recently that oligomeric Aβ, but not monomeric or fibrillar Aβ, promoted the release of lipid, cholesterol, phospholipids, and monosialoganglioside from cultured neurons and astrocytes in a dose- and time-dependent manner. These findings indicate that oligomeric Aβ promotes lipid release from neuronal membrane, which may lead to the disruption of neuronal lipid homeostasis and the loss of neuronal function (Michikawa et al., 2001).

Loss of membrane integrity resulting from Aβ-induced free-radical damage may lead to cellular dysfunction, such as inhibition of ion-motive ATPase, loss of calcium homeostasis, inhibition of glial cell Na^+-dependent glutamate uptake system consequences on neuronal excitatory NMDA receptors, loss of protein transporter function, disruption of signaling pathways, and activation of nuclear transcription factors and apoptotic pathways. Aβ-associated free radical generation might be strongly influenced by the aggregational state of the peptides (Monji et al., 2001). Neuronal death may be the ultimate consequence of these cellular dysfunctions (Varadarajan et al., 2000).

ii. Altered Calcium Homeostasis. Several lines of evidence have shown that Aβ can disrupt cellular ion homeostasis. Alterations in calcium influx is involved in Aβ toxicity via ion pore formation and also potentiation of calcium channels (Mattson et al., 1992; Vitek et al., 1994; Weiss et al., 1994) (Fig. 6). Neurons exposed to Aβ exhibit calcium responses sensitive to excitatory amino acids and membrane depolarization (Mattson et al., 1992; Hartmann et al., 1993; Mattson, 1994).

The elevation of $[Ca^{2+}]$ and increased sensitivity to excitatory stimuli induced by Aβ also seems to be mediated by free radicals, as described above. Aβ associates with the plasma membrane and induces inactivation of membrane-associated enzymes (Dumery et al., 2001). Several hypotheses on the mechanisms of Aβ-mediated ion homeostasis disturbances have been proposed, including the potentiation of calcium channels, ion pore formation, impairment of Na^+/K^+ ATPase (Mark et al., 1995) and Na^+/Ca^{2+} exchanger (Wu et al., 1997), and blockade of the K^+ channel (Good et al., 1996). In contrast, Colom et al. (1998) reported that a K^+ channel with delayed rectifier characteristics might play an important role in Aβ-mediated toxicity for septal cholinergic cells.

Cation channels are induced by both fresh and globular Aβ peptides (Bhatia et al., 2000; Kawahara and Kuroda, 2000; Zhu et al., 2000). $Aβ_{1–40}$ forms Ca^{2+}-permeable, Zn^{2+}-sensitive channel in reconstituted lipid vesicles (Lin et al., 1999, 2001). In addition, Green and Peers (2001) reported that hypoxia induced enhancement of Ca^{2+} channel, which is mediated by increased Aβ formation (Table 1).

Calcium is one of the most important intracellular messengers in the brain, being essential for neuronal development, synaptic transmission, plasticity, and the regulation of various metabolic pathways. Associations between the pathological hallmarks of AD (neurofibrillary tangles and amyloid plaques) and perturbed cellular calcium homeostasis have been established in studies of patients, and in animal and cell culture models of AD (Mattson and Chan, 2001). Central to the neurodegenerative process is the inability of neurons to properly regulate intracellular calcium levels (Mattson and Chan, 2001).

iii. Inflammatory Response. Early association of activated microglial cells and reactive astrocytes in neuritic plaques and the appearance of inflammatory markers indicate a state of chronic inflammation in AD (Chong et al., 2001). Immune activation and/or inflammatory activity have been shown to be significantly elevated in the brains of AD patients compared with age-matched control patients (Dumery et al., 2001). Continuous neuroinflammatory processes including glial activation may play a role in the pathogenesis of AD (Calingasan et al., 2002). Microglia and astrocytes would be activated, perceiving Aβ oligomers and fibrils as a foreign material, because these kinds of Aβ assemblies are apparently never observed during brain development and in the immature nervous system (Selkoe, 2001a).

A recent report showed that CD40 expression was increased on cultured microglia treated with freshly solublized Aβ and on microglia from a transgenic murine model of AD (TgAPPsw) and that CD40-CD40L interaction is necessary for Aβ-induced microglial activation (Tan et al., 1999). Activated microglia participate in the triggering of the classic complement pathway and in a variety of proinflammatory cytokine productions. Indeed, β-pleated fibrillar Aβ has been shown to directly activate the classic complement pathway fully in vitro (Chen et al., 1996). Complement proteins are integral components of amyloid plaques and cerebral vascular amyloid in AD brains. They can be found at the earliest stages of amyloid deposition, and their activation coincides with the clinical expression of Alzheimer's dementia.

Recent studies demonstrated the activation of mitogen-activated protein kinase (MAPK) pathways in response to Aβ fibrils after a subsequent downstream tyrosine kinase-dependent inflammatory signaling event in microglia (McDonald et al., 1997, 1998). Recently, it was reported that microglia from human AD brain exposed to Aβ produced and secreted a wide range of inflammatory mediators, including cytokines, chemokines, growth factors, complements, and a reactive intermediate (Lue et al., 2001). Significant dose-dependent increases in the production of prointerleukin-1β, interleukin-6, tumor necrosis factor-β, monocyte chemoattractant protein-1, macrophage inflammatory peptide-1β, IL-8, and macrophage colony-stimulating factor were observed after exposure to preaggregated $Aβ_{1–42}$ (Lue et al., 2001). Mounting evidence for inflammatory changes observed in the brains of APP transgenic mice and AD brain (Table 4) and results with in vitro experiments treated with Aβ suggest that the inflammatory response may be an important mediator of subsequent neuronal injury in AD.

iv. Activation of Signaling Pathways. Aβ has been shown to activate some signaling pathways, including the MAPK pathway and others in in vitro experiments. MAP kinase pathway participates in a number of reactions of the cell when responding to various external stimuli. It has been reported that fibrillar Aβ induced tau phosphorylation by the progressive and sustained activation of the MAPK in mature hippocampal neurons (Rapoport and Ferreira, 2000) (Table 1).

Cyclin-dependent kinase-5 and its neuron-specific activator p35 are required for neurite outgrowth and cortical lamination. Proteolytic cleavage of p35 produces p25, which accumulates in the brain of patients with AD. Application of $Aβ_{1–42}$ induces the conversion of p35 to p25 in primary cortical neurons, leading to the activation of cdk-5 and tau phosphorylation (Patrick et al., 1999; Lee et al., 2000b) (Fig. 6).

TABLE 4
Neuropathology in APP Tg mice

Tg Line	Major Neuropathology	Behavioral Deficits	Neuronal Loss	Electrophysiological Alterations
Tg2576	Extracellular plaque deposits with Congo Red, astrocytosis, microgliosis, Aβ elevation	Spatial, cued	N.D.	N.D.
PDAPP	Extracellular diffused and compacted plaques, astrocytosis, microgliosis	N.D.	N.D.	N.D.
TgAPP23	Congophilic and diffused plaques, astrocytosis, microgliosis	N.D.	Up to 25%	N.D.
C_{100}	Intracellular Aβ immunoreactivity, lysosomal abnormalities, microglia proliferation, normal NMDA and AMPA receptors	Spatial	+	N.D.
C_{104}	Extracellular Aβ immunoreactivity, gliosis, microglial activation	Spatial	+	+

N.D., not determined; +, detected; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid.

Recently, it was reported that in cortical neurons exposed to Aβ, activated JNK is required for the phosphorylation and activation of the c-Jun transcription factor, which in turn stimulates the transcription of several key target genes, including the death-inducer Fas ligand. The binding of Fas ligand to its receptor Fas then induces a cascade of events that lead to caspase activation and ultimately to cell death (Morishima et al., 2001) (Table 1 and Fig. 6).

In addition, $A\beta_{25-35}$ and $A\beta_{1-42}$ have been shown to induce the tyrosine phosphorylation of numerous neuronal proteins, including tau and microtubule-associated protein 2c, activating the Src family tyrosine kinase in primary human and rat brain cortical cultures (Williamson et al., 2002).

2. C-Terminal Fragment Hypothesis.

a. Neurotoxic Mechanisms of C-Terminal Fragment of β-Amyloid Precursor Protein.

i. In Vivo Generation of Amyloidogenic Carboxyl-Terminal Fragments of β-Amyloid Precursor Protein. It is possible that substantial amounts of β-APP expressed in cells are metabolized into nonamyloidogenic fragments as long as lysosomal proteases function normally but that potentially amyloidogenic fragments start to accumulate when lysosomal protease activities are lowered (Hayashi et al., 1992). A 16- to 22-kDa amyloidogenic CT fragment of β-APP was immunochemically detected in membrane-bound fractions of healthy unstimulated platelets as well as in similar fractions obtained from a Dami megakaryocytic cell line (Ghiso et al., 1992; Gardella et al., 1993; Li et al., 1995). It has been reported that Aβ-bearing CT fragments are found in media and cytosol of lymphoblastoid cells obtained from healthy donors and patients with early- or late-onset FAD (Matsumoto and Fujiwara, 1991, 1993; Matsumoto and Matsumoto, 1994) and Down syndrome (Kametani et al., 1994) (Table 2).

Several antibodies to the CT of β-APP label tangles (Yamaguchi et al., 1990) and dystrophic neurites of senile plaques (Ishii et al., 1989; Arai et al., 1990; Shoji et al., 1990; Yamaguchi et al., 1990). Aβ-bearing CT fragments have been detected in leptomeningeal vessels and microvessels and choroid plexus purified from the brain of aged healthy individuals and patients with AD (Nordstedt et al., 1991; Estus et al., 1992; Ghiso et al., 1992; Tamaoka et al., 1992; Kalaria et al., 1996) (Table 2). The 19-kDa amyloidogenic Aβ-bearing CT fragment was also found in gray matter, and the levels of the mature β-APP isoform (133 kDa), and the 19-kDa amyloidogenic CT fragment increased 2- to 3-fold with age (Nordstedt et al., 1991). Nordstedt et al. (1991) suggest that the age-dependent increase in content of either a mature β-APP isoform and/or a putative amyloidogenic CT fragment could explain why AD is associated with advanced age.

Tokuda et al. (1995) found secretory forms of β-APP (truncated β-APPs) that lack the entire Aβ sequence and CT fragments that contain the full-length Aβ in cerebral white matter. They suggested that amyloidogenic CT fragments can be produced by secretory cleavage of β-APP, which is antegradely transported through axons in human brain. Recent work has shown that there is rapid processing of β-APP in the optic nerve to generate a 14-kDa terminal membrane-associated fragment that contains the Aβ sequence (Amaratunga and Fine, 1995; Nalbantoglu et al., 1997).

CT_{100} amyloidogenic fragments were produced by transgenic mice and rats with full-length human β-APP containing native and genetic mutations linked to FAD and hereditary cerebral hemorrhage with amyloidosis of the Dutch type (Howland et al., 1995). It has been clearly shown that transfection of human neuroblastoma with a β-APP cDNA bearing the KM-NL double mutation leads to overproduction of Aβ and Aβ-bearing CT products (Citron et al., 1992; Cai et al., 1993; Golde et al., 1993; Felsenstein et al., 1994). However, Iizuka et al. (1996) reported that the KM-NL double mutation promoted the production of intracellular Aβ and its release with no increase in the level of CT fragments. Their results suggested the presence of a distinct pathway in which Aβ is directly cleaved at both N and C termini from the β-APP fragment intracellularly to release Aβ.

Potentially, amyloidogenic CT fragments in cultured human embryonic kidney 293 cells transfected with $\beta\text{-APP}_{751}$ or $\beta\text{-APP}_{695}$ (Knops et al., 1992) or β-APP

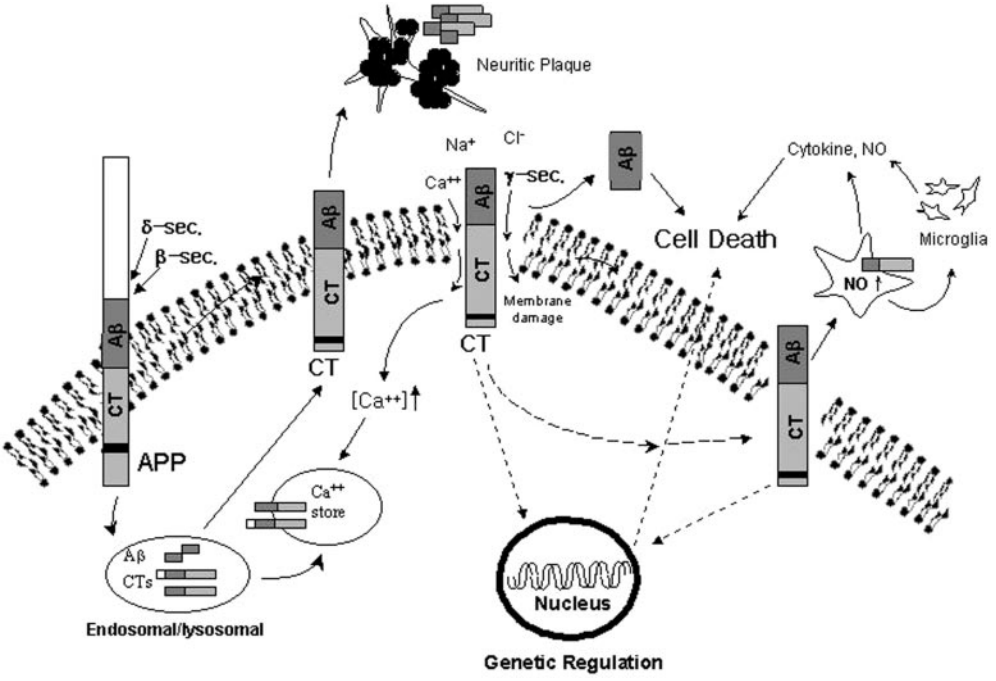

FIG 7. Hypothesis of an etiological role of amyloidogenic CTF of β-APP in AD. β-APP is internalized and processed in the endosomes/lysosomes where CTF and Aβ are produced. In AD and related disorders such as Down syndrome, excessive production of β-APP and/or reduction of some endosomal/lysosomal activities may induce accumulation of amyloidogenic CTF of β-APP in the neuron and/or near the membrane. Intracellular CTF and Aβ may form ion channels or pores in the cell membrane or puncture holes in Ca^{2+} stores. Both actions could result in a large increase in intracellular Ca^{2+} concentration and cell damage, leading to cell death. CTF may attack mitochondria, which leads to the increased release of cytochrome *c* and the activation of caspase-3 and may enter the nucleus to affect genetic regulation of some genes and finally to death. In addition, CTF may increase the production of NO in astrocytes and microglia, which may induce cell death. CTF may be released from the cell and/or more easily released from the damaged neurons into the extracellular space. Extracellular CTF form de novo ion channels, and this may induce neuronal death from outside the cells.

mutant (Golde et al., 1992; Johnstone et al., 1994) were detected. Baskin et al. (1991) reported an increased release of an amyloidogenic CT from stressed PC-12 cells.

Zhao et al. (1995) demonstrated that neuronal and endothelial cells expressing mutant β-APPV717F generated higher levels of large, potentially amyloidogenic CT fragments, which were enhanced upon treatment of the cells with leupeptin. These results suggest that mutations in the APP gene shift the protein processing toward the amyloidogenic pathway, possibly involving the endosomal-lysosomal system.

A CT product and aggregated species have been detected in HeLa cells (Dyrks et al., 1991), in PC12 cells (Yankner et al., 1989; Maestre et al., 1992, 1993), in CV-1 cells, in SK-N-MC cells (Wolf et al., 1990), in COS-1 cells (Maruyama et al., 1990), and in P19-derived neurons (Fukuchi et al., 1992b; Yoshikawa et al., 1992) when CT_{100} is expressed with and without a signal peptide. Expression by the baculovirus system of full-length β-APP transcripts in insect cells leads to processed intermediates, including a 17-kDa fragment (Gandy et al., 1992a,b).

Exogenous $A\beta_{1-42}$ is internalized selectively by hippocampal field CA1 and causes neurons to accumulate amyloidogenic carboxyl-terminal fragments of the amyloid precursor protein accompanied by slow deturioration of central synapses (Bahr et al., 1998). Kammesheidt et al. (1992) found abnormal aggregation of CT_{100} of β-APP within vesicular structure in the cytoplasm and in abnormal-appearing neurites in the CA2/3 region of the hippocampus in transgenic mice (Table 2).

ii. Toxicity of Carboxyl-Terminal Fragments. Neurotoxicity of CTF in cultured mammalian cells. CTF induced a significant lactate dehydrogenase release from cultured rat cortical and hippocampal neurons, PC12 cells and SHSY5Y cells in a concentration- and time-dependent manner, but did not affect the viability of U251 cells originating from human glioblastoma. Moreover, when PC12 cells were induced to differentiate into neurons by pretreatment with nerve growth factor, the cells were much more sensitive against CTF. In contrast to CTF, Aβ increased lactate dehydrogenase release only slightly at 50 μM (Kim and Suh, 1996; Suh, 1997). In addition, C-terminal fragments of APP without Aβ and transmembrane or NPTY domain significantly induced the death of nerve growth factor-differentiated PC12 cells and rat cortical neurons. Thus, these findings suggest that C-terminal end of APP without Aβ and transmembrane itself may be involved in the neuronal degeneration, which is associated with AD (Lee et al., 2000a).

Carboxyl terminal fragment-induced membrane currents. CTF induced strong nonselective inward currents in *Xenopus* oocytes, whereas Aβ did not show significant effects (Fraser et al., 1996, 1997). Regarding

channel formation in planar lipid bilayer, CTF-induced channels are more selective for Ca^{2+} and Na^{+} with the permeability sequence $P_{Ca}{}^{2+} > P_{Na+} > P_{Ca}{}^{2+} > P_{Cs+} > P_{Mg}{}^{2+}$, whereas A$\beta$-induced channels are more selective for Cs^{+} and Li^{+} with the selective sequence $P_{Cs+} > P_{Li+} > P_{Ca}{}^{2+} > P_{K+} > P_{Na}{}^{+}$ (Arispe et al., 1993; Kim et al., 1999). CT-induced pores have higher selectivity to Ca^{2+} and Na^{+}, the two primary ions involved in various neurodegenerative processes, than Aβ-induced pores. Transient inward currents associated with calcium influx were induced by localized applications of CTF to discrete dendritic regions of intact Purkinje cells. Inward currents were also observed after applications of CTF to isolated patches of somatic Purkinje cell membrane. Aβ peptides and CTF induced a great depression of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor-mediated synaptic transmission between parallel fiber and Purkinje cells through a combination of pre- and postsynaptic effects (Hartell and Suh, 2000). CTF increased membrane conductance in a concentration-dependent fashion, and CTF was more effective than Aβ in forming channels (Fraser et al., 1996, 1997; Kim et al., 1999; Hartell and Suh, 2000) (Table 3). This nonselective channel or pore-forming effect seems to the reminiscent of the properties of pore-forming proteins, e.g., Perforin, the membrane attack complex of complement, and eosinophilic cation protein, known to cause membrane damage and cytotoxicity (Young et al., 1986; Bhakdi and Tranum-Jensen, 1987; Drake et al., 1994). These results suggest that the neuronal ionic homeostasis in brain with AD can be more easily disturbed by CTF than by Aβ.

Effects of carboxyl-terminal fragments on the calcium homeostasis in the neuronal cells. CT_{100} expression significantly increased cellular vulnerability to the calcium ionophore A23187 and decreased vulnerability to H_2O_2 (McKeon-O'Malley et al., 1999). CTF significantly inhibited calcium uptake into rat brain microsomes. In contrast, Aβ peptides had no effect on the microsomal calcium uptake at 50 μM concentration (Kim et al., 1998) (Table 3).

CTF increased intracellular calcium concentration by approximately 2-fold in SK-N-SH and PC12 cells, but not in U251 cells (Kim et al., 1999, 2000). Cholesterol and MK801 in SK-N-SH and PC12 cells reduced this calcium increase and toxicity induced by CTF, whereas the toxicity of Aβ peptide was attenuated by nifedipine and verapamil. CTF rendered SK-N-SH cells and rat primary cortical neurons more vulnerable to glutamate-induced excitotoxicity. Full-length CTF was found to block IP3-sensitive Ca^{2+} release in *Xenopus* oocytes by altering IP3-induced receptor-signaling pathway rather than by simply blocking the IP3 receptor directly (Kim et al., 2002). Kim et al. reported that YENPTY sequence in the cytoplasmic tail had a more significant role in inhibiting IP3 signaling than the Aβ or transmembrane domain (Fig. 7). This and other results (DeGiorgio et al., 2000; Cao and Sudhof, 2001; Kimberly et al., 2001; Kim et al., 2002) indicate that YENPTY sequence plays a crucial role in inhibiting IP3 signaling and inducing nuclear translocation to affect the expression of some genes. Also, conformational studies using circular dichroism experiments showed that CTF had approximately 15% of β-sheet content in phosphate buffer and aqueous 2,2,2-trifluoroethanol solutions. However, the content of β-sheet conformation in dodecylphosphocholine micelle or in the negatively charged vesicles is increased to 22 to 23%. The results showed that CTF disrupted calcium homeostasis and rendered neuronal cells more vulnerable to glutamate-induced excitotoxicity, and that some portion of CTF had partial β-sheet conformation in various environments, which might be related to the self-aggregation and toxicity. This may be significantly involved in inducing the neurotoxicity characteristic of AD (Kim et al., 1998, 1999, 2000).

Learning and memory impairment and neuropathological changes induced by central injection of carboxyl-terminal fragments in mice. CTF caused significant impairments in cued, spatial, and working memory performances in a dose-dependent manner after a single intracerebroventricular injection of CTF to mice (Song et al., 1998; Choi et al., 2001). The acetylcholine (ACh) level in the cerebral cortex and hippocampus was significantly decreased, accompanied by a reduction of mitochondrial pyruvate dehydrogenase activity (Choi et al., 2001). Also, CTF induced reactive gliosis in neocortex and hippocampus and neurodegeneration in neocortex (Song et al., 1998) (Table 3). These results indicate that centrally administered CTF induces behavioral impairment and neuropathologic changes, suggesting a direct toxic effect of CTF, per se.

Carboxyl terminal fragment-induced inflammatory reaction through mitogen-activaed protein kinase- and nuclear factor κB-dependent astrocytosis and inducible nitric-oxide synthase induction. Extensive neuronal loss with an accompanying increase in reactive astrocytes around the senile plaques is one of the well-established pathological findings in brains with AD (Duffy et al., 1980; Wisniewski and Wegiel, 1991; Potter, 1992; Pike et al., 1995). To explore the direct role of CTF in the inflammatory processes, the effects of the CTF of APP on the production of TNF-α and matrix metalloproteinase-9 (MMP-9) were examined in a human monocytic THP-1 cell line. CTF elicited a marked increase in TNF-α and MMP-9 production in the presence of interferon-γ in a dose- and time-dependent manner. Genistein, a specific inhibitor of tyrosine kinase, dramatically diminished both TNF-α secretion and subsequent MMP-9 release in response to CTF through MAPK pathway (Chong et al., 2001). CTF (100 nM) induced astrocytosis morphologically and immunologically. CTF exposure resulted in activation of MAPK pathways as well as transcription factor NF-κB (Bach et al., 2001) (Table 3 and Fig. 7). These suggest that CTF may participate in Alzheimer's

pathogenesis through MAPK- and NF-κB-dependent astrocytosis and iNOS induction. CTF induced IL-1β and TNF-α expression, and the IL-1β induced by CTF upregulated iNOS gene expression through NF-κB activation in astrocytes and microglial cells. In addition, CTF induced astroglial and microglial chemokines such as MIP-1α, MCP-1, and RANTES, which play roles in accumulation of microglial cells around amyloid plaques (Rah et al., 2001) (Table 3).

b. The Involvement of Carboxyl-Terminal Fragments of Amyloid Precursor Protein in Gene Transactivation. DeGiorgio et al. (2000) first reported the presence of APP C-terminal fragment in the nucleus of substantia nigra pars reticulata neurons after neurotoxic striatal lesion, suggesting that intranuclear APP C-terminal fragments may play a role in genomic events contributing to delayed neuronal degeneration (Table 3).

Cao and Sudhof (2001) provide evidence that when the cytoplasmic tail is cleaved from APP, it binds to Fe65 and moves to the nucleus in which they bind a protein called Tip60, which is part of a large complex of proteins involved in gene transcription. This is the first time anyone has linked APP or carboxyl-terminal fragment to the control of gene expression in AD. That could be important for understanding AD.

It has further been confirmed that AICD, γ-secretase-cleaved fragment is stabilized by Fe65 and translocates to the nucleus in a Notch-like manner (Kimberly et al., 2001; Gao and Pimplikar, 2001; Cupers et al., 2001b). In addition we found various carboxyl-terminal fragments that could form ternary complex with Fe65 and CP2/LSF/LBP1 transcription factor in the nucleus, which induces glycogen synthase kinase-3β and tau phosphorylation (H. S. Kim, E. M. Kim, K. A. Chang, E. A. Yu, C. H. Park, S. J. Jeong, and Y. H. Suh, unpublished data). In addition, the CT-Fe65 partners foster the cell movements needed in wound healing (Sabo et al., 2001). These suggest that APP or CTF of APP may turn out to have more talents than anyone suspected.

D. Amyloid and Tau

AD is pathologically characterized by neuritic plaques, largely composed of extracellular deposits of Aβ peptide and NFTs, which are composed of intracellular filamentous aggregates of hyperphosphorylated tau protein. There has been controversy over how these lesions and their constituent molecules are pathogenically related to each other and to the neuronal and synaptic losses that characterize the disease (Hardy and Allsop, 1991; Roses, 1994; Selkoe, 1994). The important problem is that mouse models of AD do not accurately recapitulate the dual pathology of the disease. Some transgenic mouse models for AD, overexpressing mutant human APP alone or with mutant PS1, develop senile plaques; however, these mice lack NFTs and exhibit little neuronal loss (Games et al., 1995; Duff et al., 1996; Hsiao et al., 1996; Borchelt et al., 1997; Holcomb et al., 1998; Lewis et al., 2000).

The presence of the tau mutation was necessary for NFT formation because bigenic mice generated by crossing APP transgenic with amyloid deposits and wild-type tau transgenic mice with tau inclusions do not show classic AD pathology in which amyloid plaques are surrounded by a corona of dystrophic neuritis containing intracytoplasmic tau tangles (Duff et al., 2000). Homozygous transgenic mice expressing human wild-type tau failed to develop NFTs in response to $A\beta_{42}$ either at 6 or 12 months of age (Gotz, 2001; Gotz et al., 2001). Exposure to Aβ alone is not sufficient to induce tangle formation. Why Aβ should so resolutely fail to stimulate tau pathology with wild-type tau, despite its neurotoxicity and its pathology-enhancing effect on mutant tau, remains a mystery. The double mutant tau (P301L)-mutant APP (APPsw) developed Aβ deposits and neurofibrillary tangle pathology that was substantially enhanced in the limbic system and olfactory cortex, indicating that either APP or Aβ influences the formation of neurofibrillary tangles (Lewis et al., 2001) (Fig. 5).

Gotz and coworkers (2001) injected $A\beta_{42}$ fibrils into the brains of P301L mutant tau transgenic mice and noted a factor of five increases in the number of NFTs in the amygdala from where neurons project to the injection sites. These findings further support the hypothesis that there is an interaction between the Aβ and tau pathologies in AD.

In summary, $A\beta_{42}$ fibrils can significantly accelerate NFT formation in P301L mice and provide further support for the hypothesis that Aβ can be a causative pathogenic factor. The fact that mutations in tau give rise to τ-inclusion tangles but not plaques and mutations in APP or in the probable APP proteases give rise to both plaques and tangles almost proves that amyloid pathology occurs upstream of tau pathology.

Their data do not exclude the possibility that other factors can also induce NFT formation in brain, in view of the many tauopathies associated with NFT formation in the absence of β-amyloid plaques (Buee et al., 2001; Gotz, 2001a,b,c). Although NFTs were morphologically similar in the double mutant and JNPL3 (mutant tau) mice, older female mice (9 to 11 months) had a marked increase in NFTs in limbic areas. Female mice develop NFT pathology significantly earlier than do males (Lewis et al., 2001). Male TgAPP mice did not develop similar enhanced NFT pathology in limbic regions.

This likely reflects gender differences in the development of NFT pathology. However, the difference between female and male TgAPP mice could also be caused by significant sex differences in amyloid burden or could reflect hormonal changes in aging female TgAPP mice. The latter possibility is interesting given the higher incidence of AD in women (Jorm et al., 1987; Rocca et al., 1991; Kawas et al., 1999).

The discovery of possible interactions between amyloid-β deposits and tau tangles and the availability of transgenic mouse models containing both pathologies will facilitate efforts to develop more effective AD therapies. These double-mutant models should allow therapies to be developed and tested that address not only amyloid deposition but also NFT formation and neuronal loss, features of AD that previous transgenic mice have failed to recapitulate. Thus, eliminating amyloid-β deposits by administering an amyloid-β vaccine may improve cognition in AD patients who have few tau tangles, but may have little or no effect on late-stage AD patients who have already developed significant tau pathology. The double-mutant mice may provide an in vivo assay to determine whether amyloid-lowering therapies such as Aβ vaccination are effective in preventing NFT formation in vivo. It would be wise for future AD therapies to combine the targeting of amyloid-β deposits with strategies for eliminating tau tangles (Lee, 2001).

E. Transgenic Models of Amyloidogenesis

Transgenic mice are important tools that provide insight into the function of a gene in vivo and can provide models of disease states to test hypotheses for potential therapeutic intervention. There are multiple lines of Tg mice that show Aβ deposits and neuritic plaques, which are main characters in AD brain. Selected APP Tg models are reviewed in the following sections.

1. APPswe Transgenic Mice (Tg2576). Human APP_{695} containing the double mutation ($Lys^{670} \rightarrow Asn$, $Met^{671} \rightarrow Leu$) was inserted using a hamster prion protein promoter for overexpressing human APP695swe and was first described by Hsiao et al. (1995). The brains in this model, with increasing age, showed elevated levels of $A\beta_{40}$ and $A\beta_{42}$, leading to robust Aβ deposits in cortex, hippocampus, and amygdala (Hsiao et al., 1996). These mice exhibited a heightened frequency of spontaneous death accompanied by behavioral changes including neophobia and impairment of several memory tests (e.g., spatial reference memory task in the Morris water maze, alteration task in the Y-maze, and spatial working-memory task in forced-choice T-maze alternation test). They also showed evidence of oxidative stress, including enhanced expression of superoxide dismutase 1 and hemoxygenase-1 and increased amounts of 4-hydroxynonenal (Pappolla et al., 1998; Smith et al., 1998). In addition to these characters, the mice showed mild tau pathology (hyperphosphorylation) and inflammatory changes. However, no overt AD-like pathology was evident. They did not develop neurofibrillary tangles, did not show neuronal loss in CA1, and the conspicuous brain atrophy present in AD was not seen. Thus, these mice are incomplete models of AD.

2. Amyloid Precursor Protein V717F Transgenic Mice (PDAPP Mice). The platelet-derived growth factor β-chain promoter was used to drive the expression of a human APP minigene that encodes the FAD-linked APP-V717F in an outbred strain of mice (Games et al., 1995). Human APP exhibits a number of splice variants of 695-, 751-, and 775-amino acid residues, reflecting the presence or absence of exon 7 and 8 encompassing the so-called Kuniz inhibitor domain. These mice were constructed to encompass APP of these spliced forms. The brains of these mice showed both Aβ diffuse and mature neuritic plaques. There were synaptic loss and an increased number of astrocytes and microglia, clustered in and around plaques. These mice also showed deficits in object-recognition memory and in alteration spatial reference and working memory (Dodart et al., 2000), and abnormalities of synaptic transmission that precedes the deposition of Aβ (Hsia et al., 1999). Although hyperphosphorylation of tau is detected in this line, neurofibrillary tangles were absent (Irizarry et al., 1997). Unlike other transgenic mice, premature death is not reported to be associated with this line (Johnson-Wood et al., 1997).

3. APP-751swe/V717I Transgenic Mice. The Thy-1 expression cassette was used in this model to drive HuAPP-751 with either the Swedish mutation or the Swedish mutation and the V717I mutation (Sturchler-Pierrat et al., 1997; Calhoun et al., 1999; Phinney et al., 1999). These mice showed Aβ plaques and neurites associated with some hyperphosphorylated tau immunoreactivity with increasing age. Behavioral abnormalities have not been described.

4. TgAPP23. APP_{751} with optimized Kozak sequence as an APP cDNA and murine Thy-1 as a transgene promoter were used in this model to drive Swedish mutation (Sturchler-Pierrat et al., 1997). These mice showed approximately seven times APP overexpression and diffusible plaques in neocortex and hippocampus. Astrocytosis and microgliosis were also detected in these mice. At 14 to 18 months of age, neuronal loss was observed up to 25% in hippocampus CA1 region. However, neurofibrillary tangles were not detected.

5. C_{100}/C_{104} Transgenic Mice. C_{100} Tg mice expressed the carboxyl terminal 100-amino acid peptides of APP under the control of the brain dystrophin promoter (Kammesheidt et al., 1992). These Tg mice showed profound degeneration of neurons and synapses in Ammon's horn and in the dentate gyrus of the hippocampus (Oster-Granite et al., 1996). Also, intracellular Aβ, proliferation of microglia, and thickened basement membrane were detected in the mice. Furthermore, accumulations of amyloid in the cerebrovasculature with the highest expression of the C_{100} transgene as well as spatial memory deficiency were observed.

Tg mice expressing carboxyl terminal 104-amino acid peptides of APP (C_{104} Tg mice) under the transcriptional control of the long neurofilaments regulatory sequence developed extracellular Aβ immunoreactivity, increased gliosis, glial fibrillary acidic protein-positive reactive astrocytes, microglial reactivity, and cell loss in the CA1

region in the hippocampus (Nalbantoglu et al., 1997). Adult C104 Tg mice demonstrated spatial learning deficiency in the Morris water maze task. In addition, these mice showed electrophysiological alterations in terms of deficits in NMDA-dependent long-term potentiation in the hippocampal CA1 region. The overall pathology of APP Tg mice is summarized in Table 4.

III. Presenilin

A. Preliminary Remarks

Relatively recently, the identification of PS1 and PS2 as the chromosomes 14- and 1-encoded proteins, respectively, has profoundly modified our approach of the disease (Levy-Lahad et al., 1995a; Rogaev et al., 1995; Sherrington et al., 1995). That mutations on these proteins seem responsible for most of the FAD has triggered a huge amount of work aimed at better understanding PS1 and PS2 physiologies. The delineation of dysfunctions triggered by FAD-linked mutations should be evidence of the neurodegenerative process taking place in AD.

B. Cell Biology of Presenilins

Presenilin 1 was first identified by positional cloning (Li et al., 2000b). The PS1 gene spans more than 50 to 75 kb and is organized into 12 exons, the first two corresponding to 5′ untranslated regions (Alzheimer's Disease Collaborative Group, 1996; Mitsuda et al., 1997). The longest open reading frame, encoded by exons 3 to 12, leads to a 467-amino acid protein. Northern blot analysis of PS1 mRNA revealed two mRNA, a major species of approximately 3 kb, and a minor message of approximately 7 kb (Sherrington et al., 1995) expressed ubiquitously within human brain nuclei and peripheral tissues (Sherrington et al., 1995). By in situ hybridization, the localization of PS1 mRNA seemed similar in human healthy and sporadic Alzheimer's disease-affected brains (Nishiyama et al., 1996).

Several splice variants of PS1 have been identified. One lacks the sequence encoded by exon 8 (and therefore seems to have deleted one of the putative transmembrane domains of presenilin 1, as discussed below), whereas another derives from the alternative splicing at exon 3, leading to the deletion of a tetrapeptide (Val-Arg-Ser-Gln) corresponding to a consensus phosphorylation site (Sherrington et al., 1995), the functionality of which remains to be established.

The number of mutations identified on the PS1 sequence still increases. Today, more than 70 missense to about 100 missense mutations have been identified (Hardy, 1997), some of them causing AD before age 30 years (Campion et al., 1996; Wisniewski et al., 1998). In addition, a mutation abolishing the splice acceptor site of exon 10 (formerly identified as exon 9) results in the deletion of the sequence encoded by this exon (referred to as ΔE10-PS1 below) (Perez-Tur et al., 1995).

The gene organization of PS2 is very similar to that of PS1, with 12 exons corresponding to a 24-kb genomic region. Exons 3 to 12 give rise to an open reading frame encoding a 448-amino acid protein (Levy-Lahad et al., 1996; Prihar et al., 1996). Two messenger RNAs of 2.4 and 2.8 kb can be detected, the former being distributed within brain, lung, liver, and placenta, whereas the longer form appearing more ubiquitously expressed (Levy-Lahad et al., 1995b; Rogaev et al., 1995). To date, 6 mutations on PS2 have been characterized.

PSs are transmembrane proteins, the exact structure of which remains discussed. Several groups have postulated an even number of hydrophobic domains (6 or 8) for PS1 ((Doan et al., 1996; Lehmann et al., 1997b) or SEL-12 ((Li and Greenwald, 1996), its *Caenorhabditis elegans* counterpart (see below) implying that both the N- and C-terminal end of PS1 would be located in the same cell compartment believed to be the cytoplasm (Doan et al., 1996; Li and Greenwald, 1996; De Strooper et al., 1997; Lehmann et al., 1997). Conversely, Dewji and Singer (1997b) suggested that PS1 would be a more classic seven-transmembrane domain protein.

PS1 and PS2 display a 67% amino acid identity but diverge mainly at their N termini and at the sixth hydrophilic loop. Most of the missense mutations detected in PS1 occur at amino acids conserved between the two proteins. Although mutations appear all along the PS, they seem particularly clustered within transmembrane domains.

Cellular and subcellular localizations of proteins often give clues about their putative functions. By means of various monoclonal, polyclonal or affinity-purified antibodies against PS1, it was shown that the labeling seems to be neuronal (Moussaoui et al., 1996; Culvenor et al., 1997; Huynh et al., 1997) whereas oligodendrocytes, microglia, and astrocytes remain immunonegative (Kim et al., 1997a). PS1-like immunoreactivity is mainly associated with perikarya and dendrites with very weak axonal label (Elder et al., 1996) in mouse as well as human temporal cortex (Kim et al., 1997a). These features are found in various cell lines such as NT2N, rat hippocampal neurons (Cook et al., 1996; Capell et al., 1997), and SH-SY5Y neuroblastoma cells (Busciglio et al., 1997).

Very limited information concerns PS2 expression. By in situ hybridization, similar patterns of PS2 and PS1 expression in human brain were observed, mainly in the neuronal cell population (Kovacs et al., 1996; Huynh et al., 1997). However, a recent study indicated that, although PS2 colocalizes with PS1 in mouse brain, PS2-like immunoreactivity seemed exclusively present within neuronal cell bodies (Blanchard et al., 1997).

Several studies have reported the subcellular localization of PS1 (Cook et al., 1996; Kovacs et al., 1996; Takashima et al., 1996; Walter et al., 1996; Culvenor et al., 1997; De Strooper et al., 1997; Huynh et al., 1997; Cupers et al., 2001a) and PS2 (Kovacs et al., 1996;

Walter et al., 1996; Culvenor et al., 1997; Huynh et al., 1997) in the endoplasmic reticulum (Cook et al., 1996; Kovacs et al., 1996; Takashima et al., 1996; Walter et al., 1996; De Strooper et al., 1997; Cupers et al., 2001) and Golgi apparatus (Kovacs et al., 1996; De Strooper et al., 1997). This distribution does not seem to be affected by FAD-linked mutations (Cook et al., 1996; Kovacs et al., 1996). Other studies have documented the association of PS1 (Takashima et al., 1996; Dewji and Singer, 1997a; Georgakopoulos et al., 1999; Schwarzman et al., 1999) and PS2 (Dewji and Singer, 1997a) at the plasma membrane, suggesting the possibility that PS expression at the cell surface could mediate cell-to-cell contacts responsible for adhesion processes (Takashima et al., 1996; Georgakopoulos et al., 1999; Schwarzman et al., 1999) or transcellular binding (Dewji and Singer, 1997a, 1998). Finally a study that is not yet confirmed suggested a nuclear localization of PS at the inner membrane of kinetochores (Li et al., 1997).

These controversial results could be caused by the specificity of the various immunological probes used in these studies. Furthermore, because PS are maturated proteins (discussed below), PS-like immunoreactivity could correspond to either holoproteins or some of their derived fragments. In this context, possible variable recognition of these products by the antibodies together with putative cell-specific maturation processes and/or distinct localization of holoproteins and processed fragments could explain distinct phenotypic immunolabeling on intact tissues or cells.

Presenilins are neither sulfated, acylated, nor glycosylated (Walter et al., 1996; De Strooper et al., 1997). PS2 holoprotein can be highly phosphorylated in COS and CHO cells (Walter et al., 1996; De Strooper et al., 1997; Walter et al., 1999) by kinases other than protein kinase C (Walter et al., 1996; De Strooper et al., 1997) and protein kinase A (De Strooper et al., 1997). In vitro experiments performed with various purified kinases corroborated these data (De Strooper et al., 1997) and suggested the possible involvement of casein kinases 1 and 2 (Walter et al., 1996). PS2 incorporates phosphate on serine residues (Walter et al., 1996; De Strooper et al., 1997) at positions 7, 9, and 19 (Walter et al., 1996) that are not conserved in the PS1 sequence, thereby explaining the lower susceptibilty to phosphorylation displayed by PS1 (Walter et al., 1996; De Strooper et al., 1997). Another explanation could be that PS1-phosphorylated holoprotein is more prone to dephosphorylation than PS2, as was suggested by the drastic enhancement of the phosphorylated state of PS1 but not PS2 upon treatment with the phosphatase inhibitor okadaic acid in transfected COS cells (De Strooper et al., 1997). In vivo, amino acids of PS2-incorporating phosphate were recently mapped and identified as aspartyl residues in positions 327 and 330 (Walter et al., 1999).

Presenilins are efficiently maturated proteins. N-terminal product of 28 to 30 kDa and its 18 kDa C-terminal counterpart are endogenously produced by a "presenilinase" activity. These fragments are recovered in high amounts and accumulate in various mammalian cell lines as well as in transgenic mice (Thinakaran et al., 1996; Ward et al., 1996; Baumann et al., 1997; Kim et al., 1997c; Shirotani et al., 1997). PS1-N-terminal fragment and C-terminal fragment could physically interact (Capell et al., 1998) and form high-molecular-mass heterodimer complexes. The accumulation of processed PS fragments concomitant to low levels of holoproteins is likely related with longer lifetimes and metabolic stability of the former species (Ratovitski et al., 1997).

The presenilinase cleavage clearly occurs inside the sequence encoded by exon 10 because the ΔE10-PS1 construction is never processed (Thinakaran et al., 1996). Whether FAD-linked mutations affect PS maturation is unclear. Okochi and colleagues (1997) reported a lack of association between a series of PS1 mutations and their susceptibility to processing, which was in agreement with a recent work indicating that PS1 fragments accumulate in the brain of patients harboring the I143T and G384A mutations to an extent similar to that observed in control or sporadic brains (Hendriks et al., 1997). Conversely, Murayama et al. (1997) showed that Cys410 > Tyr PS1 resisted proteolysis, whereas Gly384 → Ala or Leu392 → Val did not. Mutations Met146 → Val and Ala246 → Glu also impair PS processing in PC12 cells (Mercken et al., 1996). The latter data are in apparent contradiction with those obtained with the same mutations in transgenic mice because in this system, processed fragments accumulate to a higher extent than those generated from the wild-type PS1 (Lee et al., 1997).

Unlike its parent holoprotein, CTF-PS1 behaves as a protein kinase C (Seeger et al., 1997; Walter et al., 1997) and protein kinase A (Walter et al., 1997) substrate. The selective phosphorylation of CTF-PS1 could be seen as a clue to postulate that it corresponds to the functionally occurring PS1-related species.

PS1 and PS2 undergo additional alternative cleavages at Asp345/Ser346 and Asp329/Ser330, located in consensus sequences reminiscent of those usually targeted by caspase-like proteases (Kim et al., 1997b; Loetscher et al., 1997; Grünberg et al., 1998). Van de Craen et al. (1999) reported on the susceptibility of PS1 and PS2 to cleavages by various caspase activities, but they clearly have the highest activities for caspases 8- and 3-mediated hydrolysis. Caspase-mediated cleavages seem to be exacerbated by Asn141 → Ile PS2 mutation in H4 cells (Kim et al., 1997b), whereas a series of FAD-linked PS1 mutations do not influence susceptibility to caspase cleavage (Van de Craen et al., 1999). The C-terminal PS1 fragment also behaves as a caspase substrate (Grünberg et al., 1998) that, from the use of specific caspase inhibitors, mutational analysis and hydrolysis of PS by recombinant caspase could be caspase 3 or a close congener (Loetscher et al., 1997; Grünberg et al.,

1998). Recently, Tekirian and colleagues (2001) demonstrated that C-terminal presenilinase- and caspase-derived PS2 fragments exhibit distinct subcellular localization during their constitutive productions (Tekirian et al., 2001). Furthermore, the caspase-derived fragment distribution within cellular organelles varies according to PS2 expression (Tekirian et al., 2001) suggesting a complex and distinct regulation of these various proteolytic events in cells.

Downstream to the above-described post-translational events, both PS1 (Marambaud et al., 1998b; Fraser et al., 1998; Steiner et al., 1998; Honda et al., 1999) and PS2 (Kim et al., 1997c; Marambaud et al., 1998a) are ultimately catabolized by the proteasome, in agreement with our demonstration that both proteins undergo ubiquitination (Marambaud et al., 1998a,b). FAD-linked mutations on PS1 (Marambaud et al., 1998b) and PS2 (Marambaud et al., 1998a) do not modify their susceptibility to proteasomal degradation. This multicatalytic complex also controls the cellular concentrations of the C-terminal PS1/PS2 fragments (Marambaud et al., 1998a; Alves da Costa et al., 1999).

C. Presenilins and Their Molecular Partners

PSs are remarkable chaperoning molecules that were shown to interact with a series of molecular partners. Not surprisingly, with respect to the PS function in APP physiopathological maturation described below, a physical interaction of presenilins with β-APP and nicastrin, a recently discovered endogenous physiological regulator of APP processing (Yu et al., 2000), was revealed by coimmunoprecipitation and affinity purification experiments (Waragai et al., 1997; Weidemann et al., 1997; Xia et al., 1997b; Pradier et al., 1999; Yu et al., 2000). Several independent studies demonstrated that presenilins belong to a protein complex that includes β catenin (Tesco et al., 1998; Yu et al., 1998; Zhang et al., 1998b; Nishimura et al., 1999) and could bind several members of the Bcl-X_L/bcl-2 family (Alberici et al., 1999; Passer et al., 1999), indicating a possible implication of these proteins in the control of cell death. PSs also interact with epithelial cadherin (Baki et al., 2001), telencephalin (Annaert et al., 2001), and Notch (Ray et al., 1999b), which is in line with a putative role in both adhesion processes and embryogenesis.

Other studies that remain to be confirmed indicate that the presenilin 1 interacts with a brain Go-protein (Smine et al., 1998) and participates in a proteic complex associating glycogen synthase kinase-3β and its substrate tau protein (Takashima et al., 1998a). Both presenilins 1 and 2 seem to bind calsenilin, a calcium-binding protein (Buxbaum et al., 1998a), and rab11, a GTPase activity involved in vesicular membrane (Dumanchin et al., 1999). Finally, PS1 and PS2 interact with PAMP and PARL, two novel putative metalloproteases (Pellegrini et al., 2001), and with two subunits of the proteasome (Van Gassen et al., 1999). Whether all of these interactions are of physiological relevance is discussed (Van Gassen et al., 2000).

D. Physiological and Pathological Roles of Presenilins.

1. Presenilins and the γ-Secretase Cleavage of β-Amyloid Precursor Protein. That presenilins directly or indirectly control the physiopathological maturation of β-APP has been suggested by a network of evidenced from immunohistochemical, genetic, and cell biology approaches. All show that when a PS is harboring a "pathogenic" mutation, the phenotypic alteration of β-APP processing corresponds to an exacerbation of $A\beta_{42}$ production. Thus, this Aβ species was immunohistochemically detected in the brains of patients bearing several FAD-linked missense mutations or the deletion of exon 10 of presenilin 1 (Ishii et al., 1997; Mann et al., 1997a), as well as in the frontal cortex of six cases exhibiting the Asn141 → Ile PS2 mutation (Mann et al., 1997b). Other studies indicated that total Aβ (Martins et al., 1995) and more particularly $A\beta_{42}$ (Scheuner et al., 1996) were recovered in higher amount in the plasma or in the secreted medium of fibroblasts derived from patients bearing FAD-linked PS1 and PS2 mutations. This favored overproduction of $A\beta_{42}$ can also be observed in all biological systems in which mutated PS are overexpressed, such as transfected cells (Ancolio et al., 1997; Tomita et al., 1997; Xia et al., 1997a) or transgenic mice overexpressing mutated PS1 either alone (Borchelt et al., 1996; Duff et al., 1996; Citron et al., 1997) or in combination with mutated β-APP (Borchelt et al., 1997; Holcomb et al., 1998). The physiological secreted product sβ-APP seems drastically reduced by PS1 (Ancolio et al., 1997) and PS2 (Marambaud et al., 1998a) FAD-linked mutations.

The mechanism by which PS could control β-APP processing is not yet clear. Presenilins could act as genuine γ-secretases(s) or upstream as a chaperoning protein bringing β-APP to a cell compartment containing γ-secretase activities, both functions being affected by pathogenic PS mutations. In agreement with the former hypothesis was the demonstration that β-APP-Semliki Forest virus-infected cultured neurons from the hippocampus of PS1 knockout mice brain produce drastically lower amounts of both $A\beta_{40}$ and $A\beta_{42}$ (De Strooper et al., 1998). The remaining Aβ formation seemed to be caused by PS2 because a double knockout of PS1 and PS2 was reported to fully prevent the Aβ formation derived from overexpressed Swedish mutated β-APP (Herreman et al., 2000; Zhang et al., 2000). Whether knocking out the PS1 gene has abolished γ-secretase activity by directly "removing" endogenous γ-secretase or by misrouting β-APP from a cell compartment containing γ-secretase remains unknown.

The most direct lines of evidence supporting this possibility came from biochemical, pharmacological, and mutational approaches. Thus, presenilins seem to copurify with γ-secretase-like activity in a high-molecular-

weight complex (Li et al., 2000b), and peptide-based transition-state aldehyde inhibitors were shown to prevent Aβ production and interact with presenilins after cross-linking (Esler et al., 2000; Li et al., 2000a; Seiffer et al., 2000). Presenilins were proposed to behave as a novel type of autocatalytically activated aspartyl protease because mutations of two critical aspartyl residues prevented presenilinase-like maturating endoproteolysis of PS and abolished Aβ (Ray et al., 1999a; Berezovska et al., 2000; Capell et al., 2000).

This hypothesis is still discussed infrequently (Checler, 2001; Wolfe, 2001). First, it has been reported that mutation of the aspartate 257 residue, unlike that of Asp385 of presenilin 1, did not affect Aβ recovery (Capell et al., 2000). A recent article even showed that the mutation of both aspartyl residues does not modify Aβ recovery (Kim et al., 2001). These observations are hard to reconcile with the hypothesis of a diaspartyl group directly involved in the catalytic events responsible for Aβ production because in all acidic proteases, the two aspartyl residues behave as an acid-base couple that requires one protonated and one deprotonated aspartyl moiety, and therefore, the mutation of one of the aspartyl residues is always sufficient to fully abolish catalysis.

Another important result came from data indicating that the mutation of a glycine residue adjacent to aspartate 385 (glycine 384, using the presenilin 1 numbering) affects Aβ recovery and therefore mimics the phenotype associated with Asp385 (Steiner et al., 2000). However, glycine residues are the only amino acids lacking a side chain, an obligate structural element needed to contribute to the polarization of the scissile bond in all catalytic processes. Therefore, this glycine residue can be seen as biologically inert as far as the direct catalytic mechanism is concerned without ruling out the possibility that this residue could contribute to the substrate binding/recognition site. It remains clear that mutating a residue that cannot be directly involved in catalysis can mimic the effect of the aspartyl residue mutations. As a corollary, the phenotype ascribed to Asp385 mutation could be related to molecular mechanisms others than direct catalysis.

Wolfe and colleagues (Wolfe et al., 1998) have described peptide-based transition-state analog inhibitors [one of which is difluoroketone peptidomimetic 1 (MW167)] as potent inhibitors of Aβ production (Wolfe et al., 1998; De Strooper et al., 1999). If presenilins are autocatalytically activated secretases, these agents should be expected to also prevent presenilin endoproteolysis. In disagreement with this statement, we recently established that MW167 and newly designed isocoumarin inhibitors (Petit et al., 2001) were unable to block endoproteolysis of endogenous or overexpressed presenilin (Petit et al., 2001). Conversely, our data agree well with those of another study showing that an engineered uncleavable presenilin, in which an FAD-linked mutation had been introduced, still exacerbated $A\beta_{42}$ production (Steiner et al., 1999). Thus, by both pharmacological and mutagenesis approaches, it is clearly possible to discriminate between presenilinase activity and γ-secretase.

More recently, we established that endogenous secreted and intracellular $A\beta_{40}$ and $A\beta_{42}$ could be still produced in cells devoid of PS1 and PS2 (Armogida et al., 2001). The fact that a γ-secretase-like activity was still present in PS-deficient fibroblasts was in agreement with a recent article (Berechid et al., 2002) showing that in PS−/− cells, there still existed γ-secretase inhibitor-sensitive phenotypes (see *Presenilins and Notch Signaling*). Also interesting was the observation that in fibroblasts, there exists a γ-secretase activity liberating the 42 end of $A\beta_{2-42}$ that remains unaffected by PS1−/− depletion.

As an alternative hypothesis, several recent elegant studies indicated that PS1 deficiency drastically affected the intracellular trafficking of a series of membrane proteins, including β-APP and one of its homolog APLP1 (Naruse et al., 1998) but also unrelated other proteins including TrkB (Naruse et al., 1998) and β-catenin (Nishimura et al., 1999). The latter study furthermore established that this defective intracellular routing was exacerbated by FAD-linked PS1 mutations (Nishimura et al., 1999). Recently, by mutagenesis, Kim and coworkers (2001) demonstrated the drastic influence of the "catalytic" aspartyl residues on β-APP trafficking. The fact that PS1 could physically interact with β-APP, β-catenin, and Rab11, a small GTPase activity involved in vesicular transport (see above) argues in favor of a PS1-dependent chaperoning of β-APP. This would likely occur early in the routing of β-APP through the constitutive secretory pathway, as suggested by the fact that PS displays favored interaction with immature β-APP (Weidemann et al., 1997). The fact that the proteasome controls the degradation of wild-type and mutated PS1 and PS2 (Marambaud et al., 1996, 1998a,b) and modulates the production of both Aβ and β-APP (Marambaud et al., 1998a,b) also suggests that β-APP/PS interaction likely occurs upstream of a putative misrouting afforded by pathogenic PS mutations.

2. Presenilins and Notch Signaling. PSs display high homology with SEL-12, a *C. elegans* protein that facilitates the signaling mediated by the LIN12 receptor. Wild-type PS1 behaves as a functional analog of SEL-12 since it can rescue an egg-laying phenotype abolished by *sel*-12 mutations in *C. elegans* (Levitan and Greenwald, 1995; Baumeister et al., 1997), whereas FAD-linked PS1 and PS2 only poorly complement such SEL-12 function (Levitan et al., 1996; Baumeister et al., 1997). LIN12 is responsible for cell fate during embryogenesis and is the *C. elegans* analog of the Notch receptor, a transmembrane protein believed to mediate signal transduction between the cell surface and the nucleus. PS1 gene disruption is lethal and null embryos exhibit alterations in somitic development (Shen et al., 1997; Wong et al.,

1997) and massive neuronal loss (Shen et al., 1997) in agreement with a study showing that PS1−/− homozygous mice display growth retardation and die at the late embryogenic stages (De Strooper et al., 1998). Interestingly, PS1 null mice can be rescued for embryonic lethality by both wild-type and FAD-linked PS1 (Davis et al., 1998; Qian et al., 1998), indicating that pathogenic mutations responsible for FAD did not affect PS1 function at the embryonic stage. It should be noted that further clues to the putative function of PS1 in embryogenesis came from a recent study (Zhang et al., 1998a) showing that PS1 interacts with cytoskeletal-associated ABP280/Fh1, two proteins that were previously proposed to regulate cell shape, polarity, and motility by interacting with actin filaments network.

The above-mentioned studies suggested that PS could "control" a Notch-mediated cell signaling important for normal embryologic development in the mouse. Two recent studies further document an intimate link between PS and Notch signaling in the *Drosophila* (Struhl and Greenwald, 1999; Ye et al., 1999). Thus, *Drosophila* PS mutants display an altered development caused by Notch processing impairment. Indeed, null mutations abolish the cleavage, giving rise to NICD, the C-terminal fragment of Notch that corresponds to the active cytoplasmic Notch species translocated into the nucleus to mediate Notch signaling (Struhl and Greenwald, 1999; Ye et al., 1999). This agrees with a previous study showing that the invalidation of PS1 gene also impairs the production of NICD in mammalian cells (De Strooper et al., 1999). However, it should be noted that Ye et al. (1999) reported that constitutively activated N-terminally truncated Notch species triggering cell signaling and bearing the potential C-terminal cleavage site retained their functionality even when the PS gene was inactivated. This seems to indicate that the absence of PS did not fully prevent the cleavage generating the C-terminal Notch fragment and raised the possibility that PS acts upstream to Notch processing.

Because Notch breakdown leading to NICD occurs in the transmembrane domain and because both Aβ and NICD-forming enzymes seemed abolished by PS gene invalidation/knockout (De Strooper et al., 1998, 1999; Struhl and Greenwald, 1999; Ye et al., 1999), it was proposed that PS could be the enzyme responsible for both γ-secretase-like cleavages. However, several works clearly indicated that the β-APP and Notch γ-secretase-like cleavages could be discriminated by pharmacological and mutagenesis approaches. First, the mutation of the aspartate 257 residue of presenilin 1 abolishes NICD production without affecting Aβ recovery (Capell et al., 2000). The ability to discriminate between presenilin-mediated Aβ and NICD production was further demonstrated by Kulic and colleagues (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 $A\beta_{42}$ recovery was greatly enhanced, whereas NICD production was impaired (Kulic et al., 2000).

Peptide-based transition-state analog inhibitors designed by Wolfe and colleagues (1998) also block Notch cleavage (De Strooper et al., 1999). Conversely, our isocoumarin JLK inhibitors that prevent Aβ formation did not affect NICD production (Petit et al., 2001). These data are in firm opposition with the fact that the same enzyme would be responsible for both NICD- and Aβ-forming activities because an enzyme/inhibitor complex is characterized by a unique K_i value, and this constant does not vary according to the type of substrate, even if a single enzyme displays various affinities for distinct substrates. Therefore, mutational (aspartate 257 of presenilin1) or pharmacological discrimination (our JLK inhibitors) approaches both converge to cast a shadow over the hypothesis of a presenilin enzymatic entity.

Most acidic proteases display a maximal activity at a pH close to the p*K* value of the aspartyl carboxyl, i.e., around pH 4. This allows the occurrence of one ionized and one protonated lateral chain of the two aspartyl residues (Pearl, 1987), an absolute requirement for functional acidic proteases (as described above). In the environment of the plasma membrane, in which NICD is generated, the neutral pH would lead COO-deprotonated forms of the two aspartyl residues. This inactive enzymatic form of acidic protease located where NICD is formed is a spatial paradox not yet resolved.

3. Presenilins and Programmed Cell Death. The first clue that PS could influence apoptotic processes came from the observation that the C-terminal 103 amino acids of PS2 displayed 98% identity with ALG3, a mouse protein able to rescue a T-cell hybridoma from Fas-induced apoptosis (Vito et al., 1996a). Wolozin et al. (1996) showed that PS2 can trigger pertussis toxin-sensitive apoptosis in PC12 cells. Araki and colleagues (2001) demonstrated that PS2-mediated apoptosis could be linked to a down-regulation of the antiapoptotic oncogene *bcl-2* in primary cultured neurons. Recently, we delineated the intracellular pathway involved in wild-type and mutated PS2-mediated cell death in human cells and in neurons (Alves da Costa et al., 2002). We showed that both parent proteins triggered a caspase-3-like activation that was accompanied by an increase of both expression and transcriptional activity of the tumor suppressor apoptotic effector p53 (Alves da Costa et al., 2002). In agreement, we established that fibroblasts derived from PS2-knockout mice display drastically lower p53 transcriptional activities (Alves da Costa et al., 2002).

It is interesting to note that ALG3 antagonizes the PS2-induced cell death in PC12 (Vito et al., 1996b). In keeping with this observation, the same group establishes that C-terminal PS2 fragments derived from alternative transcription or caspase-3 cleavage (referred to as Ccas-PS2) mimic the antiapoptotic effect of their mouse homolog ALG3 (Vito et al., 1997). Thus, this

Ccas-PS2 would act as a dominant-negative of PS2-mediated apoptosis. In opposition to this hypothesis, Walter et al. (1999) showed that slowing down the caspase cleavage of the presenilinase-derived CTF-PS2 by phosphorylation or mutagenesis slowed down Ccas-PS2 formation and delayed apoptosis in HeLa cells. In this case, this implies that the caspase cleavage leading to Ccas-PS2 relies on a proapoptotic phenomenon that is in disagreement with Vito et al. (1997).

The idea that the caspase-mediated cleavage of PS2 could modulate cell death came from the observation that mutated PS2 were more apoptotic than wild-type PS2 in H4 (Kim et al., 1997b), HeLa (Janicki and Monteiro, 1997), and PC12 cells (Deng et al., 1996). Is this proapoptotic function of FAD-linked PS2 related to their tendency to exacerbate $A\beta_{42}$ production? Several lines of evidence indicate that $A\beta_{42}$-mediated toxicity could be caused by enhanced apoptosis via disruption of calcium homeostasis and free radical overproduction (Loo et al., 1993; Behl et al., 1994; La Ferla et al., 1995; Mark et al., 1995; Li and Greenwald, 1996). We recently confirmed that the presenilinase-derived CTF-PS2 fragment activates p53-dependent caspase-3-like activity and increased Aβ production (C. Alves da Costa and F. Checler, unpublished data). Both phenotypes were antagonized by a selective caspase-3 inhibitor that increases the CTF-PS2-like immunoreactivity (C. Alves da Costa and F. Checler, unpublished data). This first confirms that CTF-PS2 is targeted by caspase 3, leading to Ccas-PS2. That Ccas-PS2 formation is associated with increased Aβ recovery also argues in favor of a proapoptotic function of Ccas-PS2 instead of a dominant-negative antiapoptotic function.

The involvement of PS1 in the control of apoptotic processes is more puzzling. PC12 cells overexpressing Leu286 → Val PS1 exhibit increased susceptibility to various apoptotic stimuli, including serum deprivation and exposure to Aβ (Guo et al., 1997) through a *bcl-2*-sensitive modification of calcium homeostasis and overproduction of free radicals (Guo et al., 1997). This potentiation of apoptosis by FAD-linked mutations was recently shown to be related to a destabilization of the PS-β-catenin complex (Zhang et al., 1998b), likely through caspase activation (Tesco et al., 1998) (Bursztajn et al., 1998). Conversely, Bursztajn et al. (1998) recently established that herpes simplex virus expression of either PS1 or its A246E mutant did not reveal any signs of apoptosis in infected neurons (Bursztajn et al., 1998) but rather protects them against etoposide and staurosporine-induced apoptosis (Bursztajn et al., 1998). This antiapoptotic function of PS1 agrees well with a recent work showing that NT2 cells expressing PS1 antisense cDNA display increased apoptosis and can be rescued by the antiapoptotic oncogene *bcl-2* (Hong et al., 1999). Furthermore by an antisense approach, Roperch et al. (1998) showed that the down-regulation of PS1 expression reduced cell growth and increased apoptosis. Overall, these studies shed light on the fact that PS1 and PS2 could have distinct and even opposite functions in the control of cell death and/or other functions. This hypothesis was further substantiated by our recent work showing that p53-dependent caspase 3 activation elicited by PS2 overexpression is accompanied by a drastic reduction of PS1 expression (Alves da Costa et al., 2002). This cross-talk between the two parent proteins, likely through the control of p53, is strengthened by the observation that PS1 expression is reduced in several p53-dependent systems (Roperch et al., 1998).

4. Presenilins and the Unfolded-Protein Response. The unfolded-protein response (UPR) is provoked by several natural or artificial stresses that trigger an endoplasmic-reticulum response characterized by increased synthesis of chaperoning proteins including BiP/GRP78 and CHOP. This molecular mechanism is indeed a cell defense against the misfolding of proteins, the accumulation of which is extremely detrimental for the cell (Mori, 2000; Imaizumi et al., 2001). Several stress transducers involved in the UPR include ATF6 and the kinases Ire1 and PERK. Niwa and colleagues (1999) reported on the participation of PS1 in the UPR through the nuclear accumulation of cleaved fragments of Ire1. This Ire1 processing, which is an activation mechanism, is strongly reduced in PS1 knockout cells. Katayama et al. (1999) showed that FAD-PS1 mutations down-regulated the UPR by decreasing the expression of GRP78 and perturbating IRE1 function. In agreement, the same group later suggested that the mutated PS1-induced disturbance of the UPR also affected other cellular actors of this cell response, namely PERK and ATF6 (Katayama et al., 2001). It is interesting to note that the inhibition of the catalytic activation of Ire1 by PS1 mutations again implies a PS-mediated control of proteolytic events. However, although this phenotype is altered by FAD-PS1 mutations, it seems that mutations at the catalytic aspartyl residues 257 and 385 did not affect the UPR response (Katayama et al., 2001). The contribution of PS1 in the UPR is discussed. Thus, Sato and colleagues (2000) indicated that the up-regulation of chaperoning proteins occurring during the UPR appeared independent of the expression of presenilins. By another approach, Siman and colleagues (2001) showed that the knock-in of mutated PS1 does not modify the endoplasmic reticulum stress-induced cleavages of several caspase activities, does not alter the stress-induced increase of Grp78 and Grp94 expressions, and does not modify the activation pathway of PERK. Finally, ATF6 processing still occurs when dominant-negative mutants of PS1 are overexpressed (Steiner et al., 2001). Therefore, the implication of PS in the UPR is, as is often with PS, still a matter of discussion.

5. Other Putative Functions of Presenilins.

a. Presenilin As a Receptor/Channel. First, the transmembrane structure could call for structural, receptor, or channel functions. The latter function seems

unlikely because it has been demonstrated that cRNA of wild type and several forms of mutated PS1 did not modify endogenous currents in *Xenopus laevis* oocytes, either injected alone or in combination with the cRNA of FAD-linked β-APP (Dauch et al., 1997).

b. Presenilin in Cell Adhesion. PS appears to bind directly E-cadherin and thereby stabilizes the cadherin/catenin complex that is probably responsible for cell-cell interactions, at least in epithelial cells (Georgakopoulos et al., 1999; Baki et al., 2001). These observations strongly suggest a plasma membrane localization of a pool of presenilins involved in epithelial cell adhesion. Whether this function takes place in the central nervous system is obviously an important issue to be determined.

c. Other Putative Functions. Briefly, other possible functions of PS1 would be to bring the glycogen-synthase kinase-3β (GSK-3β) to the proximity of tau proteins that behave as one of the kinase substrates (Takashima et al., 1998a). Interestingly, GSK-3β displays a higher affinity for FAD-linked PS1 (Takashima et al., 1998b). Therefore, it is tempting to speculate that in this case, this higher affinity leads to a higher recruitment/phosphorylation of endogenous tau. This is obviously of interest when considering that hyperphosphorylation of tau proteins likely underlies the development of neurofibrillary tangles, a major histopathological hallmark in Alzheimer's disease (Goedert, 1993). Presenilins were also recently suggested to be involved in the Erb-4 tyrosine kinase-mediated intranuclear signaling (Ni et al., 2001; Lee et al., 2002b).

PSs are localized on the centrosome-kinetochore microtubules in M-phase-arrested HEK293 cells (Jeong et al., 2000) and in centrosome at interphase of the human fibroblast cell and COS cell (Li et al., 2000), suggesting presenilin's role as a bridge between centrosome and kinetochore during cell division. In addition, both PS1 and PS2 antisense oligonucleotides arrest cell division, but either PS1 or PS2 antisense oligonucleotide has no effect on the cell division (Jeong et al., 2000). These observations suggest that both PS1 and 2 may be essential for the cell division coordinately and may compensate each other during cell division.

E. Concluding Remarks on Presenilin Physiology

The discovery of presenilins has triggered a large number of studies in a relatively short time period that opened fields that are often still discussed. Undoubtedly, the link between the presence of PS missense mutations and the occurrence of aggressive early-onset familial forms of Alzheimer's disease to FAD has been confirmed and consensually linked with an overproduction of $A\beta_{42}$. One of the unsolved questions is when and how these mutations affect β-APP processing and/or routing? PS1 and PS2 are multifunctional proteins and likely display distinct functions. This likely contributes to an explanation of various phenotypic forms of AD having distinct onset and evolution.

Several other tracks concerning the additional functions of PS will be investigated. Some of them have been discussed in this review, which could be related to embryogenesis, development, and cell signaling. This is not in contradiction with the suggestion of a role for PS in the control of apoptosis, because programmed cell death participates in cell fate and tissue organization at early stages of development. PS could well participate in other unknown physiological functions and/or contribute to pathologies unrelated to Alzheimer's disease.

IV. α-Synuclein

A. Molecular and Cell Biology of α-Synuclein

α-Synuclein is a 140-amino acid peptide of 18 to 20 kDa that is encoded by a single gene consisting of seven exons borne by chromosome 4 (Chen et al., 1995; Spillantini et al., 1995). This protein, cloned in 1993 from human brain (Uéda et al., 1993), is the mammalian counterpart of a synuclein-like sequence first identified in the electric organ of *Torpedo californica* (Maroteaux et al., 1988). Several members of the synucleins family have been described, including β- and γ-synucleins, two parents proteins encoded by chromosomes 5 (Spillantini et al., 1995) and 10, respectively (Lavedan et al., 1998). The β-synuclein gene encodes a 134-amino acid protein that is highly homologous to bovine phosphoneuroprotein, 14 kDa, and displays a 61% homology with α-synuclein (Jakes et al., 1994). The human γ-synuclein protein of 127 residues is the less conserved member of this small family, with divergence of sequences mainly located at the C terminus. γ-Synuclein was first identified as a breast-cancer marker (Ji et al., 1997). This protein potentiates breast-tumor growth when applied exogenously (Jia et al., 1999). This proinvasive phenotype related with γ-synuclein is not discussed further in this review.

All synucleins are highly enriched in the central nervous system of mammals, whereas other tissues seem relatively poor in synuclein-like immunoreactivities, except perhaps for skeletal muscles and spleen (Hong et al., 1998). In the brain, synucleins are present in various nuclei, but their presence in high concentrations in the neocortex, hippocampus, and substantia nigra, three brain zones reminiscent of those affected early in Alzheimer's and Parkinson's diseases, is of particular interest and likely underlies the participation of these proteins in neurodegenerative disorders (as discussed below). The subcellular localization of synucleins indicates that these proteins are mainly at the synapse. That synucleins are confined to the synaptic vesicles in nerve terminals agrees very well with previous works showing that in the electric organ of *T. californica*, α-synuclein was essentially located in presynaptic nerve endings (Maroteaux et al., 1988).

α-Synuclein undergoes few post-translational events, but several reports consistently documented the suscep-

tibility of this protein to phosphorylation. Thus, α-synuclein is constitutively phosphorylated in PC12 and HEK293 cells mainly at serine 129 and, to a lesser extent, at serine 87 (Okochi et al., 2000). Both in vitro phosphorylation experiments and cell biology approaches indicated that casein kinase 1 and 2 could be the kinase candidates (Okochi et al., 2000). Another study showed that all synucleins behave as substrates of G protein-coupled receptor kinases, a family of kinases that was believed to be mainly involved in the regulation of numerous multipass transmembrane receptors that are coupled to G proteins (Pronin et al., 2000). This study shows that regulating the phosphorylated state of this additional substrate modulates its function. Thus, α-synuclein was recently shown to interact with acidic phospholipids (Davidson et al., 1998) and inhibit phospholipase D2 (Jenco et al., 1998), a protein involved in vesicle trafficking. G protein-coupled receptor kinase-mediated α-synuclein phosphorylation abolishes its ability to bind phospholipids and blocks phospholipase D2 (Pronin et al., 2000).

Kinases of the Src family are abundant in the central nervous system and were examined as natural candidates for α-synuclein phosphorylation. Through a pharmacological approach, it was shown that the Src kinase inhibitor PP2 inhibits the pervanadate-induced phosphorylation of α-synuclein (Ellis et al., 2001). Both in vitro kinase assays and cell-transfection analyses indicated that α-synuclein could be targeted by Src protein tyrosine kinases Fyn and c-Src (Ellis et al., 2001). These data were in agreement with another study by Nakamura et al. (2001) who confirmed the involvement of Fyn and showed that other kinases such as Lyn, PYK2, focal adhesion kinase, MAPK, JNK, and cdk-5 were inactive on α-synuclein (Nakamura et al., 2001). α-Synucleins in which Parkinson disease-related mutations have been incorporated (see below) undergo identical Fyn-mediated phosphorylation (Nakamura et al., 2001). It should be noted that the apparent state of α-synuclein phosphorylation is increased in various synucleinopathies that include PD, dementia with Lewy bodies, and multiple-system atrophy (Fujiwara et al., 2002), indicating that phosphorylation could alter the biophysical properties or physiological function of α-synuclein.

α-Synuclein is remarkably stable in cell cultures (Okochi et al., 2000). We found that both wild-type and mutated α-synuclein do not undergo ubiquitination and therefore do not seem to be targeted by the 26S proteasomal activity (Ancolio et al., 2000). That α-synuclein could undergo ubiquitin-independent proteasome degradation indicates that only the 20S proteasome contributes to the inactivation of α-synuclein. It seems that proteasome inhibitors lead, in vivo, to the accumulation of α-synuclein without any ubiquitination process (Tofaris et al., 2001). It remains possible that α-synuclein monomers are not degraded by the proteasome but that, upon phosphorylation-dependent aggregation process (see below), accumulated proteins could enter a proteasome-dependent degradation process. It also seems possible that a small proportion of "abnormal" α-synuclein variants undergo ubiquitination by parkin, an E3 ubiquitin ligase protein with which αSp22 interacts (Shimura et al., 2001). Thus, a new glycosylated form of α-synuclein (αSp22) incorporates ubiquitin but accumulates in a nonubiquitinated state in parkin-deficient brains (Shimura et al., 2001). It is interesting to note that some parkin mutations are responsible for few autosomal-recessive cases of PD (Shimura et al., 2001). This mutation abolishes the ability of parkin to bind αSp22 (Shimura et al., 2001). These data suggest that even in a more general context, the parkin-α-synuclein interaction could play a central role in Parkinson's disease pathology (see below).

α-Synuclein is a protein that is extremely prone to aggregation. The first evidence of such biochemical feature came from the observation that in Lewy bodies (these intracytoplasmic inclusions that occur in both sporadic PD disease and dementia with Lewy bodies), α-synuclein, but apparently not β-synuclein, accumulates as full-length and truncated aggregated species (Baba et al., 1998). The aggregation of α-synucleins can be potentiated by several genetic and biochemical factors. Thus, Narhi et al. (1999) showed that wild-type α-synuclein displays the propensity to form β-sheet at physiological temperature and that the two α-synuclein mutations responsible for the familial cases of PD both drastically accelerate this process. This agrees well with a subsequent study showing that pathogenic mutations increase α-synuclein to self-interact and aggregate (Li et al., 2001). This exacerbated aggregation of wild-type (Uversky et al., 2001) and mutated (Ostrerova-Golts et al., 2000) α-synucleins is also triggered by heavy metals, suggesting a possible link between environmental features such as metal exposure and sporadic or familial PD.

In relation to the possible influence of subcellular localization of α-synuclein, it was shown that there exists a membrane-bound counterpart of α-synuclein that could seed the subsequent aggregation of the cytosolic counterpart (Lee et al., 2002a). In cell-free system, this process occurs in a time-dependent and antioxidant-sensitive manner (Lee et al., 2002a). This seems also to stand in intact neurons in which McLean and colleagues (2000) showed that transient transfection of α-synuclein in pure cultured neurons led to a close association of α-synuclein with the membrane, probably through both N- and C-terminal moieties. These data suggest that the occurrence of a membrane-bound pool of α-synuclein is important to trigger aggregation. Conversely, Narayanan and Scarlata (2001) suggested that the interaction of α-, β-, phosphorylated, and mutated α-synucleins is physiological and that pathological conditions correspond to a disruption of the α-synuclein/membrane interaction. A recent work identified a 12-amino acid do-

main in the middle of the sequence (from 71 to 82) of α-synuclein that seems to bear the potential of triggering aggregation (Giasson et al., 2001). Thus, introduction of a polarized residue in the sequence or its deletion drastically reduced aggregation propensity of the modified α-synuclein (Giasson et al., 2001). This peptide resists degradation and self-polymerization and triggers α-synuclein fibrillization in vitro. It is interesting to note that β-synuclein, which is much less aggregable than its α-counterpart, lacks this hydrophobic peptide stretch (Giasson et al., 2001). This work, therefore, suggests a possible therapeutic target to intervene in Parkinson's disease as far as the aggregation process is concerned and represents a causative factor of this disease (Rajagopalan and Andersen, 2001).

Synucleins display chaperoning properties. By two distinct assays classically used to monitor chaperoning properties, Souza and coworkers (2000) established that α-, β-, and γ-synucleins all abrogate thermal-induced aggregation of alcohol dehydrogenase and chemically induced insulin aggregation. This property was also exhibited by A53T-α-synuclein, one of the mutated forms that leads to Parkinson's disease (Henning-Jensen et al., 1999). α-Synuclein displays a 40% homology with 14.3.3, a typical member of a chaperone protein family (Ostrerova et al., 1999). This empiric chaperoning potential borne by α-synuclein seems to have functional implications. Thus, α-synuclein interacts with BAD, protein kinase C (particularly the α- and γ- isoforms) and with Erk but not Arf kinase (Ostrerova et al., 1999). The interaction of α-synuclein with proteins involved in the Erk cascade and PKC suggests a role in cell death (Ostrerova et al., 1999).

Several articles have identified putative cellular interacting proteins. Thus α-synuclein binds to Tau, a microtubule-associated protein that undergoes pathogenic phosphorylation in Alzheimer's disease and other neurodegenerative diseases (Delacourte and Defossez, 1986). This α-synuclein/Tau association seems functional because it leads to enhanced protein kinase A-mediated Tau phosphorylation (Henning-Jensen et al., 1999). It seems that tubulin can compete for such an interaction, thereby inhibiting α-synuclein/Tau binding (Henning-Jensen et al., 1999). It was recently suggested that tubulin could seed α-synuclein fibrillation in vitro and colocalizes with it in Lewy bodies (Alim et al., 2002). The role of tubulin as a modulator of α-synuclein/Tau interaction in vivo remains to be explored.

Synphilin-1 was recently identified as a novel protein that binds α-synuclein after yeast two-hybrid screening (Engelender et al., 1999). This interaction seemed to be potentiated by the A53T-pathogenic mutation (Engelender et al., 1999; Kawamata et al., 2001) and was confirmed in vitro as well as in various human cell lines (Engelender et al., 1999; Kawamata et al., 2001). The cotransfection of full-length α-synuclein and synphilin does not trigger morphological changes in HEK293 (Engelender et al., 1999). However, the same type of experiments performed with synphilin and the nonamyloid component (NAC) domain of α-synuclein led to the formation of eosinophilic inclusions (Engelender et al., 1999). The fact that NAC is the main synuclein fragment detectable in senile plaques of Alzheimer's disease-affected brains may suggest a role for synuclein at the interface of Alzheimer's and Parkinson's disease pathologies (see below). Finally, two articles indicated that α-synuclein could interact with the transcription factor Elk-1 (Iwata et al., 2001b) and with Tat binding protein 1 (Ghee et al., 2000), suggesting a role in the modulation of MAP kinase pathway and proteasomal activity.

B. Putative Functions of α-Synuclein in Cell Death

Although the physiological function of α-synuclein remains to be definitely established, it seems that this protein controls cell death. Data on this subject are sometimes contradictory, but close examination of the various studies can reconcile the divergent interpretations. The first unifying conclusion are data showing that Parkinson's disease-related mutations all lead to an exacerbated proapoptotic response or, alternatively, increase the cell responsiveness to toxic insults. Thus, Kanda and colleagues (2000) established that human dopaminergic SH-SY5Y neuroblastoma cells expressing mutated α-synucleins (particularly the one bearing the A53T mutation) display enhanced vulnerability to oxidative stress triggered by hydrogen peroxide. The fact that mutated α-synuclein triggered exacerbated susceptibility to oxidative insult has been further documented in other human neuroblastoma cells (Ko et al., 2000). The loss of viability induced by mutated α-synuclein was accompanied by increased levels of lipid peroxides and accelerated cell death (Lee et al., 2001). Is the proapoptotic phenotype elicited by α-synuclein mutations an exacerbation of a normal proapoptotic function of wild-type α-synuclein, or is it from the abolishment of a normal antiapoptoptic function of the parent protein. Junn and Mouradian (2002) indicated that human α-synuclein overexpression increases free-radical levels and dopamine susceptibility in SH-SY5Y. This agrees with results from two other studies showing that the overexpression of α-synuclein could lead to the death of rat (Zhou et al., 2000) and human (Zhou et al., 2002) primary-cultured mesencephalic neurons. This could occur through the Rab-5-dependent endocytosis of α-synuclein (Sung et al., 2001) or through the down-regulation of the MAPK pathway (Iwata et al., 2001a). Conversely, two studies indicated that wild-type α-synuclein could trigger an anti-apoptotic phenotype. Thus, α-synuclein delays the serum withdrawal and hydrogen peroxide-induced cell death in human NT-2 cell line (Lee et al., 2001) and the serum deprivation, hydrogen peroxide-, and glutamate-induced cell death in rat primary-cultured cortical neurons and SHSY-5Y, GT1–1, and PC12 cells (Seo et al., 2002). This protective

role was also documented by Alves da Costa and colleagues (2000)) who showed that wild-type α-synuclein could protect TSM1 neurons and HEK293 cells from staurosporine-induced cell death. This phenotype was associated with a drastic lowering of p53-dependent caspase activation (Alves da Costa et al., manuscript in preparation) and an increase in Akt expression (Seo et al., manuscript submitted for publication). In the two latter independent studies, α-synuclein-mediated antiapoptotic phenotypes were abolished by pathogenic mutations (Alves da Costa et al., 2000; Lee et al., 2001). It is interesting to note here three studies arguing in favor of an antiapoptotic physiological function of endogenous wild-type synuclein. First, Kholodilov et al. (1999) showed that in a rat model of dopaminergic neurons in cell death, α-synuclein was only expressed at the cellular level in neurons devoid of any apoptotic stigmata. Furthermore, these authors showed that 6-hydroxydopamine-induced lesions in rat striatum led, as expected, to the induction of apoptosis at the efferent brain zone, i.e., rat substantia nigra (Kholodinov et al., 1999). These authors demonstrated that cells exhibiting apoptosis did not display α-synuclein immunoreactivity, whereas cells bearing α-synuclein never undergo cell death (Kholodinov et al., 1999). This inverse relationship between the occurrence of wild-type synuclein and apoptotic phenotype argues for α-synuclein as a dopaminergic-neuron endogenous protector.

More indirectly, 14.3.3 and one of its isoforms (14.3.3σ) that exhibits both structural and functional homologies with α-synuclein (see above) prevents apoptosis (Samuel et al., 2001) and promotes cell survival (Masters and Fu, 2001) through direct interaction/sequestration of proapoptotic oncogenes such as BAD (Masters and Fu, 2001) and *bax* (Samuel et al., 2001; Seo et al., manuscript submitted for publication). The apparent discrepancies between the studies dealing with wild-type α-synuclein could be solved by envisioning as a key feature the level of α-synuclein expression. It is possible that at endogenous levels and physiological conditions, α-synuclein exerts an antiapoptotic control and that its overexpression triggers aggregation and subsequent abolishment of this function. This hypothesis is reinforced by the observation that both wild-type and mutated α-synucleins, after aging and aggregation in vitro, induce apoptotic cell death in human neuroblastoma cells (El-Agnaf et al., 1998). Furthermore, it is noteworthy that in all neuropathologies in which α-synuclein has been implicated, this protein is always found in an aggregated state in proteic inclusions such as Lewy bodies and senile plaques (Rajagopalan and Andersen, 2001).

C. α-Synucleinopathies

Recent works have indicated that α-synuclein could be the key molecule of several neurodegenerative diseases including Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Both PD and DLB are characterized by the occurrence of intracytoplasmic inclusions called Lewy bodies (LB). Until recently, these cerebral lesions were relatively poorly characterized according to their biochemical content except that these LB display filamentous structure and appeared enriched in ubiquitin-like immunoreactivity (Goldman et al., 1983). Of most interest was the demonstration at the immunohistochemical level, that α-synuclein could be the common major constituent of LB in both PD and DLB (Spillantini et al., 1997). Later, these authors show by biochemical extraction and immunoelectron microscopy examination that LB filaments display high α-synuclein-like immunoreactivity (Spillantini et al., 1998). These observations were confirmed by others (Arima et al., 1998, 1999; Galvin et al., 1999; Campbell et al., 2000; Wirdefeldt et al., 2001). It is interesting to note that the biochemical nature of α-synuclein found in PD with Lewy bodies and DLB is distinct from α-synuclein accumulating in the oligodendroglial inclusions observed in multiple-system atrophy (Campbell et al., 2001), as if the highly aggregated state of α-synuclein was the signature of all neurodegenerative diseases with Lewy bodies.

Of greatest importance was the observation that rare inherited cases of Parkinson's disease seem to be linked with mutations taking place on the α-synuclein gene (Polymeropoulos et al., 1997; Krüger et al., 1998). These are the only mutations reported to date that are responsible for autosomal-dominant forms of PD. Parkin, when mutated, accounts for some cases of autosomal-recessive juvenile parkinsonism (Kitada et al., 1998; Abbas et al., 1999), whereas genetic analysis of synphilin indicated no pathogenic-associated mutations in familial PD (Bandopadhyay et al., 2001; Farrer et al., 2001).

As usual, further insights on the involvement of α-synuclein in PD came from transgenic and knockout mice analysis. Transgenic mice overexpressing human α-synuclein exhibit a neuronal accumulation of this protein that is accompanied by the presence of ubiquitin-positive intracellular inclusions in brain zones selectively affected by PD and particularly in the substantia nigra (Masliah et al., 2000). This results in a loss of dopaminergic terminals that probably explains a clear motor impairment in the transgenic animals (Masliah et al., 2000). Transgenic mice overexpressing the A53T-mutant α-synuclein exhibit a cerebral α-synucleinopathy which resembles that observed in PD-affected brains (Van der Putten et al., 2000). Of greatest interest was the observation that β-synuclein could reverse some of the α-synuclein-associated phenotypes (Hashimoto et al., 2001a). Thus, in mice overexpressing both α- and β-synucleins, the motor deficit observed for α-synuclein mice was improved. In line with these data, cell lines overexpressing β-synuclein seem to block the propensity of overexpressed α-synuclein to aggregate (Hashimoto et al., 2001a), suggesting the possibility that β-synuclein acts as an endogenous regulator of α-synuclein func-

tions. Deletion of α-synuclein also triggers alterations of the dopaminergic system. Thus, α-synuclein$^{-/-}$ mice display increased dopamine release and attenuated typical dopamine-dependent paradigm, namely the amphetamine-induced locomotor response (Abeliovich et al., 2000).

To summarize, the α-synuclein-related molecular dysfunction occurring during Parkinson's disease could be drawn schematically. α-Synuclein would have a normal physiological antiapoptotic function. This control would be abolished by all mechanisms triggering exacerbated aggregation and leading to Lewy bodies, the intracellular inclusions that correspond to the typical PD lesions. These aggregating promoting events could be caused by phosphorylation or altered structural features associated with PD-related α-synuclein mutations. These aggregates could be ubiquitinated and subsequently degraded by the proteasomal machinery. When aggregated α-synuclein occurs in excess, the clearing process could be insufficient to avoid accumulation. In support of this view, a recent report indicated that protein aggregates could per se act as inhibitors of the proteasome (Bence et al., 2001). Furthermore, the fact that parkin mutations responsible for recessive juvenile Parkinsonism are characterized by an impairment of the intrinsic E3-ligase activity argues in favor of a deleterious influence of an altered degradation process. Then, aggregated α-synuclein species could trigger cell death and neurodegenerescence occurring in PD. What molecular mechanisms could underlie the fact that PD pathology is associated with selective neurodegenerescence of the dopaminergic pathway while α-synuclein displays a wider distribution in the brain? A recent work shed light on the fact that both dopamine and L-dopa inhibit α-synuclein fibril formation but stabilize the potentially toxic intermediate protofibril α-synuclein species in vitro. This process is reversed by antioxidants (Conway et al., 2001). These data give a possible molecular explanation to the dopaminergic region-specific α-synuclein-associated dysfunction occurring in PD.

D. α-Synuclein: A Bridge between Parkinson's and Alzheimer's Pathologies

Besides the presence of amyloid β-peptides, the major component of senile plaques corresponds to a formerly unrecognized component referred to as NAC (Masliah et al., 1996). The molecular cloning of NAC indicated that it derives from the proteolytic cleavage of a precursor of 19 kDa (Uéda et al., 1993) that, in fact, corresponds to α-synuclein. The brain expression of α-synuclein seems inversely correlated with the severity of AD cases, suggesting that in AD, deleterious effects of α-synuclein accumulation could occur at an early stage of the disease (Iwai et al., 1996). However, it should be noted that Culvenor and colleagues (1999), by means of antibodies directed toward the NAC sequence, did not correlate amyloid deposits with NAC-like immunoreactivity. These apparently distinct results are probably related to the use of antibodies displaying various specificities. It remains that aged transgenic mice overexpressing the β-amyloid precursor protein mimic some of the anatomical stigmata of human AD and particularly show typical senile plaques to which α-synuclein-like immunoreactivity seemed associated (Yang et al., 2000). Masliah and coworkers (2001) showed that transgenic mice overexpressing α-synuclein display typical motor deficits that are drastically exacerbated by the concomitant expression of the β-amyloid precursor protein (Masliah et al., 2001). These data raise the possibility that in some cases, a deleterious cross-talk between Aβ and α-synuclein could lead to a mixed pathology corresponding to Alzheimer's disease with Lewy bodies. It is interesting that this hypothesis is not only empirical, because several human cases with clinical signs of AD and PD have been reported (Ditter and Mirra, 1987; Hansen et al., 1990; Galasko et al., 1994). Whether the dialogue and cross-influence between the two proteins is more frequent than previously reported remains to be demonstrated. This would add other putative therapeutic possibilities to intervene for slowing down or preventing these two devastating diseases.

V. Therapeutic Targets for Alzheimer's Disease

Although much has been learned about AD, the only FDA-approved drug therapy is the use of acetyl-cholinesterase inhibitors (e.g., tacrine, donepezil, rivastigmine, and galantamine). Other therapies are undergoing clinical trials. These include monoamine oxidase inhibitors (e.g., selegiline), antioxidants (e.g., vitamins E and C), estrogen replacement therapy, and anti-inflammatory agents (e.g., nonsteroidal anti-inflammatory drugs) (Cutler and Sramek, 2001). Currently, such treatments are more palliative than preventative or curative.

Treatment strategies from the molecular knowledge of AD are currently in development. They include protein kinase cdk-5 inhibitors to limit tau phosphorylation, other tau phosphorylation inhibitors (cathepsin inhibitors, lithium, MAP kinase inhibitors, and protein phosphatase 2A), β- and/or γ-secretase inhibitors to limit Aβ production, and immunization with Aβ peptide to prevent Aβ plaque formation (Schenk et al., 1999; Janus et al., 2000; Morgan et al., 2000; Cutler and Sramek, 2001), modulators of inflammation (Lim et al., 2000; Wyss-Coray et al., 2001), and cholesterol-lowering drugs (Fassbender et al., 2001). The development of β-secretase inhibitors seems particularly promising because β-secretase knockout mice show a normal phenotype but no Aβ generation (Cai et al., 2001; Luo et al., 2001). A potential concern about γ-secretase inhibition was that it could inhibit Notch signaling, which could affect the bone marrow. However, recent evidence has shown that the two effects can be dissociated with inhibitors (Petit

et al., 2001). In the reduction of Aβ peptide formation, one area of research interest is in using antisense RNA to APP to reduce APP expression and subsequent production of Aβ peptides. Recent evidence indicates that effective doses of Aβ antisense phosphorothiolate oligonucleotides can cross the blood-brain barrier (BBB) and serve to reduce learning and memory deficits in aged SAMP8 mice (a mouse model for AD characterized by the overexpression of APP with age) (Banks et al., 2001). Another direction focuses on the idea of hydrolyzing the Aβ peptides with neprolysin and IDE to prevent their deposition into amyloid plaques (Mukherjee et al., 2000). Other researchers have focused therapeutic efforts on preventing the toxic effects of the Aβ and CTF peptides. One area of interest is in the prevention of Aβ peptide-triggered complement proteins in the innate immune system response (Akiyama et al., 2000). Because β-amyloid peptides and antibodies bind to different regions of C1q complement protein to initiate the inflammatory response, an ideal drug would inhibit β-amyloid binding (preventing autotoxicity and thus preventing neurodegeneration) while leaving the brain capable of responding to antibodies via the adaptive immune system (Akiyama et al., 2000). Other research efforts have focused on the development of "decoy peptides" that coaggregate with Aβ peptides and thus serve as antagonists/inhibitors of Aβ peptide toxicity (Blanchard et al., 1997). Another research focus has been on identifying endogenous rescue factors. Recently, humanin has been identified as one such rescue factor (Hashimoto et al., 2001b). Another new and exciting research idea involves the possible destruction of plaques. Preliminary work with Clioquinol indicates that it can prevent new plaque formation and also clear existing plaques by solubilizing Aβ aggregates (Helmuth, 2000; Cherny et al., 2001). Although these treatment strategies look exciting and promising, much work remains to be done to either prevent or affect a cure for AD.

A. Agents Affecting Secretary Amyloid Precursor Protein-α

sAPP is the soluble N-terminal fragments derived from the α-secretase cleavage, and these fragments can be produced from APLP as well as APP. sβ-APP was reported to protect neurons from ischemic injury or enhance recovery from ischemic injury in adult animals (Bowes et al., 1994; Smith-Swintosky et al., 1994) and showed the effects on ion fluxes (Fraser et al., 1997). In addition, sα-APP can stimulate neurite outgrowth in PC12 cells, promote the proliferation of fibroblasts, and protect cultured neurons from metabolic and excitotoxic insults (Mattson et al., 1993). Regarding signal transudation pathway, sα-APP is implicated to play a role in activation of NF-κB (Barger and Mattson, 1996), phospholipase C/protein kinase C, inositol trisphosphate (Ishiguro et al., 1998), and extracellular signal-regulated protein kinase (Greenberg et al., 1994) (Table 5). Many studies have shown that acetylcholine receptor agonists affect Aβ accumulation by modulating APP metabolism. The cholinergic stimulation elicits an increase in sα-APP release, which is known to have potent neurotrophic and neuroprotective effects (Mattson et al., 1993) and thus leads to the reduction of toxic Aβ generation (Rossner et al., 1998). In HEK cells transfected with human gene for M1 and M3 muscarinic acetylcholine receptor, it was proved first that amyloidogenic or nonamyloidogenic release of APP is mediated by stimulation of muscarinic acetylcholine receptor and coupled with protein kinase (Buxbaum et al., 1992; Nitsch et al., 1992; Marambaud et al., 1998b, 1999). In subsequent studies, the evidence that APP secretion and processing is regulated by cholinergic stimulation has been clarified further. Cholinergic agonists such as acetylcholine, nicotine, or carbacol stimulated the release of soluble sAPP in primary-cultured chromaffin cells (Efthimiopoulos et al., 1996). Cortical nonamyloidogenic APP processing under basal forebrain cholinergic control is mediated through muscarinic receptor (Rossner et al., 1997). Therefore, proper stimulation of cholinergic receptor in the brain can increase secretion of the soluble form of APP and thus lead to a reduction in production and toxicity of Aβ (Table 5).

The role of nicotinic acetylcholine receptor (nAChR) in APP secretary pathway has also been determined to develop a novel nAChR antagonist that is able to reduce the Aβ burden. There is accumulating in vitro evidence that stimulation of the nicotinic receptor might play an important role in neuroprotection against Aβ-induced cytotoxicity and might exert AD pathogenesis to slow onset (Kihara et al., 1997, 1998; Zamani et al., 1997; Shimohama and Kihara, 2001).

We confirmed that treatment of PC12 cells with nicotine increases the release of a secreted form of APPα, and the effect is attenuated by the modulation of calcium entry through neuronal nAChR (Kim et al., 1997). Moreover, our recent study showed protective effects of nicotine on cytotoxicity induced by toxic carboxyl-terminal fragments of APP (Seo et al., 2001). Further extensive study is needed to elucidate which pathway is involved in nicotine-mediated APP metabolism in AD, although two controversial findings indicate that the protective role of nicotine is correlated with β-sheet conformational change of Aβ (Salomon et al., 1996; Kihara et al., 1999).

A pilot clinical finding showed that short-term administration of nicotine increased perceptual and visual attention in patients with AD, indicating that nicotine may have some beneficial effect for acquiring the information in AD despite no improvement of short-term memory function (Jones et al., 1992). To assess the short-term effects of ABT-418, a novel nicotinic receptor, behavioral changes were investigated in patients with early AD (Potter et al., 1999). Verbal and nonverbal learning and memory ability were significantly improved after three doses of ABT-418 (6, 12, and 23 mg/

TABLE 5
Functions of sAPPα

Cell Type	Function	Reference
Cortical neurons	Cell survival	Araki et al., 1991
Hippocampal neurons	Protection against excitotoxic, metabolic, and oxidative insults	Barger et al., 1995, 1996; Furukawa et al., 1996; Goodman and Mattson, 1994; Mattson et al., 1993
Cortical and hippocampal neurons	Protect neurons against Aβ or CT toxicity	Kihara et al., 1997; Zamani et al., 1997; Seo et al., 2001
CA1 neurons	Protection against ischemic injury or enhance recovery from ischemic injury	Bowes et al., 1994; Smith-Swintosky et al., 1994
PC12 cells	Neurite outgrowth promotion	Milward et al., 1992
Hippocampal neurons	Modulation of excitability	Furukawa et al., 1996
Neocortical cells	Activate phospholipase C/protein kinase C	Ishiguro et al., 1998
Neuroblastoma cells, hippocampal neurons	Induction of neuroprotective κB-dependent transcription	Barger and Mattson, 1996
Hippocampal neurons	Activated K^+ channels and reduced $[Ca^{2+}]_i$	Furukawa et al., 1996
Fibroblasts	Induces proliferation	Saitoh et al., 1989
Platelets	Modulates blood coagulation	Smith et al., 1990

kg) on each of 4 days. These results strongly implicate that nicotine or nicotinic receptor agonist might have therapeutic potential for AD by reducing amyloidogenic processing of APP.

Because it has been reported that acetylcholinesterase (AChE) may possess nonamyloidogenic α-secretase activity cleaving APP from the membrane releasing the soluble ectodomain (Small et al., 1991), several kinds of AChEIs have also been examined to determine whether they can affect APP metabolism. Mori et al. (1995) demonstrated that AChEIs including physostigmine, heptylphysostigmine, and 2,2-dichlroro-vinyldimethylphosphate significantly enhance the release of sα-APP (Mori et al., 1995) in brain slice of rat. Short- and long-term treatment of metrifonate and dichlorvos, another active cholinesterase inhibitor, induced an increased secretion of the soluble fragment of APP, sα-APP, via activation of PKC-coupled muscarinic receptors without changes in APP expression (Pakaski et al., 2000; Racchi et al., 2001) (Table 6).

On the contrary, the FDA-approved AD drug tacrine might inhibit nonamyloidogenic processing of APP and thus contribute to Aβ deposition in brain with AD (Lahiri et al., 1994; Chong and Suh, 1996). Subsequent study showed that tacrine could reduce the release of sα-APP and total Aβ in the conditioned medium of human neuroblastoma cell, not accompanying by increase in β-APP synthesis and cell death (Lahiri et al., 1998; Lahiri et al., 2000). They demonstrated that tacrine might affect the trafficking of β-APP and/or increase intracellular proteolysis. However, the detailed influence of tacrine on APP secretion remains to be studied further.

The first clinical study investigated whether another available acetylcholinesterase inhibitor, donepezil (5 mg/day for 30 days), can affect the ratio of platelet APP forms in patients with AD (Borroni et al., 2001). The ratio of APP forms is restored to control level, and cognitive ability is improved in the donepezil-treated patient with AD. Dehydroevodiamine HCl (DHED), a new potential drug for AD and vascular dementia (Park et al., 1996, 2000), has been found to increase sα-APP release from PC12 cells and to protect neurons against Aβ or carboxyl terminal peptide (CT)-induced toxicity (unpublished data). Thus, the administration of cholinergic agonist or AChE inhibitor to patients with AD may produce the favorable clinical results in ameliorating not only ACh-dependent cognitive function but also AD pathogenesis by the modulation of APP metabolism.

B. Acetylcholinesterase Inhibitors

A primary clinical symptom of Alzheimer's dementia is the progressive deterioration in learning and memory ability. A body of evidence suggests that profound losses in the cholinergic system of brain, including dramatic loss of cholineacetyltransferase level, choline uptake,

TABLE 6
Factors affecting α-secretase activity

Factor	Cells	α-Secretase Activity	Reference
Acetylcholine	PC12 cells	↑	Buxbaum et al., 1992; Haring et al., 1995; Nitsch et al., 1992
Electrical stimulation	Hippocampus	↑	Nitsch et al., 1993
Metabotropic glutamate	Hippocampus	↑	Lee et al., 1995; Lee and Wurtman, 1997
Agonist	HEK293 cells		Nitsch et al., 1997
5-HT_{2a} and 5-HT_{2c} agonist	3T3 cells	↑	Nitsch et al., 1996
NGF and EGF	PC12 cells	↑	Refolo et al., 1989
NGF	Hippocampus	↑	Clarris et al., 1994
Phorbol esters	PC12 cells	↑	Buxbaum et al., 1990, 1992; Caporaso et al., 1992
cAMP	C6 glioma cells	↓	Efthimiopoulos et al., 1996
	HEK293 cells	↑	Marambaud et al., 1998b, 1999

EGF, epidermal growth factor; 5-HT, 5-hydroxytryptamine.

and ACh level in the neocortex and hippocampus and reduced number of the cholinergic neurons in basal forebrain and nucleus basalis of Meynert, are closely associated with cognitive deficits observed in the disease (Bartus et al., 1982; Coyle et al., 1983; Gaykema et al., 1992; Cummings and Kaufer, 1996). Furthermore, pharmacological modulations enhancing or blocking cholinergic neurotransmission produces some improvement or impairment in learning and memory (Giacobini, 1997). ACh, a neurotransmitter in the brain that plays a critical role in learning and memory function, is synthesized from acetyl-CoA and choline by cholineacetyltransferase, and released ACh into synaptic cleft is hydrolyzed by AChE into choline and acetic acid. Choline is taken up again into the presynaptic neurons for use in ACh synthesis. AChE, which is widely distributed in central nervous system (CNS) and peripheral nervous system, has been the focus of much attention because of its relationship to ACh hydrolysis and cognitive impairment in AD. Although overall AChE activity is reduced (Fishman et al., 1986), it is increased in neuritic plaque and neurofibrillary tangle at early stages in the brain of patients with AD (Martzen et al., 1993). It has also been suggested that AChE may promote aggregation of Aβ into a more toxic amyloid form (Inestrosa et al., 1996; Opazo and Inestrosa, 1998; Munoz and Inestrosa, 1999). Therefore, the blocking of AChE activity might increase ACh neurotransmission in the synaptic cleft of the brain and diminish Aβ burden, resulting in improving cognitive function and alleviating the process of amyloid deposition (Table 7).

Because the main cause of AD is still not known clearly, many therapeutic approaches have focused on improving cognitive deficits by augmenting cholinergic neurotransmission in the brain. There are four possible strategies aimed at increasing cholinergic neurotransmission: 1) supplementing the acetylcholine precursor; 2) blocking acetylcholine hydrolysis with AChEI; 3) directing the stimulation of cholinergic receptor, such as nicotinic and muscarinic receptor; and 4) using agents with other cholinomimetic activities. Accumulating animal and human data have suggested that the AChEI, among various cholinomimetics, would be the most valuable therapeutic drug for maintaining ACh levels in the brain and improving cognitive ability. After the FDA approved tacrine in 1993, several kinds of AChEIs such as donepezil, galantamine, and rivastigmine have become available for the symptomatic treatment of patient with mild-to-moderate AD. However, weakness of such AChEIs caused by limitations related to short biological half-life, transient and weak effects, narrow therapeutic range, low BBB, and frequent adverse effects block their way to treating cognitive deficits in AD. Although pharmaceutical companies have tried to find new therapeutic drugs for AD that are dominant in safety and efficacy, cholinergic-based therapy using AChEI is currently

TABLE 7
Acetylcholinesterase inhibitors for Alzheimer's dementia

	Approved by FDA and Available				Not Approved by FDA or not Available	
	Tacrine (Cognex)	Donepezil (Aricept)	Galantamine (Reminyl)	Rivastigmine (Exelon)	Metrifonate (ProMem)[a]	Huperzine A
Mechanism of action	Reversible AChE inhibitor	Reversible AChE inhibitor	Reversible AChE inhibitor, nicotinic receptor agonist	Reversible AChE inhibitor	Reversible and pseudoirreversible AChE inhibitor	Reversible AChE inhibitor
Half-life	24 h	60 h in young adults, 104 h in elderly patients	5–6 h	2 h	2.5 h	4.8 h
Dosage frequency	4 times daily	Once daily	Twice daily	Twice daily	Once daily	Twice daily
Starting dose	40 mg/day	5 mg/day	8 mg/day	6–12 mg/day	30–60 mg	0.1–0.2 mg
Maximum daily dose	120 mg	10 mg	16 mg/day	12 mg/day		0.2 mg/day
Recommended titration	Increase to 80 mg/day after 6 weeks	Increase to 10 mg/day after 2 weeks	Increase to 24 mg/day at 4 weeks after treatment of 16 mg/day	Increase to 12 mg/day at 4 weeks		
Side effects	Severe hepatotoxicity, nausea, vomiting	Anorexia, nausea, diarrhea, vomiting at higher dose	Neausia, diarrhea, abdominal cramps, anorexia at higher dose	GI upset, vomiting, anorexia, dyspepsia, asthenia, weight loss	Leg cramps, GI upset, diarrhea, vomiting, muscle weakness at higher dose	Rarely mild sleeping, nausea, vomiting

GI, gastrointestinal.
[a] ProMem is manufactured by Bayer AG (Wuppertal, Germany.

known to be the best clinical approach for improving cognitive deficits in AD (Table 7).

1. Tacrine Hydrochloride (Cognex). Tacrine (Cognex; Parke-Davis Pharmaceuticals), which was initially synthesized more than 40 years ago, was the first drug approved by the FDA in 1993. This agent inhibits AChE reversibly in a noncompetitive manner (Heilbronn, 1961). The severe side effects and short half-life limit its use in the treatment of AD. In approximately 27% of the patients tested, serious hepatotoxicity showing alanine aminotransferase levels greater than three times the upper limit of normal occurred in clinical testing (Watkins et al., 1994). The serum half-life is approximately 1.6 to 3.0 h after intravenous dose of 25 mg, 50 mg, and continuous treatment. Low bioavailability—that tacrine is rapidly cleared by the liver—is one of the restrictions to its clinical usefulness (Forsyth et al., 1989).

In a 30-week randomized, controlled trial of tacrine (40–160 mg/day) in patients with AD (Knapp et al., 1994), significant group differences were shown between high dose (160 mg/day) and low dose (80 and 120 mg/day). In this study, tacrine-treated patients showed frequently hepatotoxicity such as elevated alanine aminotransferase level exceeding 10 times the upper limit of normal and cholinergic adverse effects such as nausea, vomiting, diarrhea, anorexia, dyspepsia, and abdominal pain. Inevitable defects such as severe hepatotoxicity and short biological half-life and its low clinical effects limit its clinical usefulness in AD.

2. Donepezil Hydrochloride (Aricept). Donepezil hydrochloride (Aricept, Eisai Inc., Teaneck, NJ), approved by the FDA in 1999, is a piperidine-based reversible inhibitor of acetylcholinesterase with long plasma half-life of approximately 70 h and higher brain selectivity (Snape et al., 1999; Kosasa et al., 2000). Unlike tacrine, donepezil does not induce hepatotoxicity. The most common cholinergic side effects were observed at dosages of 5 and 10 mg/day, but the frequency and extent were comparable with those of the placebo-treated group and thus may be controlled by the rate of titration. AD patients were randomly assigned to receive a single daily dose of placebo or 5 or 10 mg/day donepezil hydrochloride in a clinical study of 15 or 30 weeks' duration (Rogers et al., 1998). The cognitive ability was significantly improved by treatment with two dose levels. Although the effect was shown slightly greater in treatment with 10 mg/day, there were no significant differences between the two active treatments. Long half-life and lower adverse effects of this drug have provided some convenience for daily dosing.

3. Galantamine (Reminyl). Galantamine (Reminyl, Janssen Pharmaceutica, New Brunswick, NJ) is a selective competitive acetylcholinesterase inhibitor that is approximately 50 times more effective against human AChE than against human butyrylcholinesterase at therapeutic doses. In addition, galantamine may also act as a nicotinic receptor agonist in the brain (Schrattenholz et al., 1996; Coyle and Kershaw, 2001). AChE inhibition and nicotinic receptor-modulating activity may maximize cholinergic function. Galantamine is well-absorbed with absolute oral bioavailability of approximately 90% and has a serum half-life of 4 to 6 h. In a study of 21 weeks' duration, galantamine (8, 16, or 24 mg/day) or placebo was administered to 978 patients. At 21 weeks of treatment, the cognitive ability was significantly improved at dose levels of 16 or 24 mg/day compared with the placebo group. There was no significant difference between 16 and 24 mg/day. In a study of 26 weeks' duration, 636 patient received 24 or 32 mg/day of galantamine. Both treatments significantly improved cognitive function compared with placebo group. The most frequent adverse effects in the dose-escalation phase included nausea, vomiting, diarrhea, and headache (Rainer, 1997; Raskind et al., 2000; Tariot et al., 2000).

4. Rivastigmine Tartrate (Exelon). Rivastigmine tartrate (Exelon, Novartis, Basel, Switzerland) is a reversible acetylcholinesterase inhibitor with higher brain selectivity (Enz et al., 1993). The drug has already been approved in more than 40 countries. Like the other drugs already approved for the treatment of AD, this drug also affects only acetylcholine in the brain, preventing its breakdown, which in turn helps memory and other brain functions. It is well-absorbed with absolute bioavailability of approximately 40% (3-mg dose) and has a plasmatic half-life of 2 h, and its effect persists much longer than its presence in plasma (Spencer and Noble, 1998). In a study of 26 weeks' duration, cognitive ability of both treatment groups (1–4 or 6–12 mg/day of Exelon) was significantly increased compared with the placebo group (Sramek et al., 1996; Rosler et al., 1999). Exelon shows severe gastrointestinal adverse reaction, including nausea, vomiting, anorexia, and weight loss. Therefore, it is recommended that patients should be always started at a dose of 1.5 mg taken twice daily and then have the dosage titrated to their maintenance dose.

C. Agents Inhibiting Aggregation of Amyloid Precursor Protein Metabolites

1. Metal Chelators. Several reports provide evidence that the pathogenesis of AD is linked to the characteristic neocortical amyloid-β deposition, which may be mediated by abnormal interaction with Aβ as well as metal-mediated oxidative stress. There is increasing evidence that some biometals such as aluminum, iron, zinc, and copper promote Aβ aggregation and neurotoxicity in the AD brain (Bush et al., 1994a,b; Lovell et al., 1998). Although discrepancies exist in aluminum-related hypothesis (Landsberg et al., 1992), structural analysis supports evidence of a direct biochemical interaction between aluminum and Aβ and a potential role for aluminum in the formation of neurofibrillary tangle and neuritic plaque in AD (Exley et al., 1993; Hollosi et al.,

1994). An in vitro study also suggested that aluminum, iron, and zinc strongly induced Aβ aggregation (Mantyh et al., 1993; Chong and Suh, 1995; Murayama et al., 1999). Epidemiological studies of aluminum in drinking water and AD showed some positive association between aluminum intake and AD progression, although the relative risks were generally not high and unreliable results are inevitable (Flaten, 2001). Understanding the role of aluminum in AD, therapeutic approaches with highly binding ligands to aluminum (silicates) or the metal chelator desferrioxamine (DFO) have been attempted to reverse and slow the progression of Aβ accumulation (Murayama et al., 1999).

The level of iron and zinc is abnormally elevated in various regions of the brain in patients with AD, implicating that transition metals have the potential to accelerate neurodegeneration through oxidative damage (Cornett et al., 1998). These previous data suggest that inhibition of Aβ accumulation by metal chelator in the brain may be one possible therapeutic approach for AD treatment.

In the first clinical trial to use metal chelator as a therapeutic method for AD, DFO (125 mg, an ion-specific binding agent isolated from *Streptomyces pilosus*, was administered intramuscularly daily to 48 patients with AD for 24 months. The clinical progression of dementia associated with AD was retarded by sustained administration of desferrioxamine (Crapper-McLachlan, 1991). After 2 years, McLachlan and coworkers (1993) reported that neocortical aluminum concentration in DFO-treated groups was decreased significantly to near control concentrations in autopsied brain of patients with AD, accompanied by behavioral improvement. Although DFO has the chelating effect of aluminum, chelating effect of zinc or copper could not be excluded.

Cherny et al. (1999) showed that Aβ can bind copper and zinc. Copper and zinc are elevated in the neocortex in AD and particularly concentrated in neuritic plaque and potentiate Aβ aggregation and neurotoxicity in vitro. They also reported the possibility that the copper/zinc metal chelator Clioquinol solubilized Aβ from postmortem AD brain (Cherny et al., 1999), and a significant decrease in Aβ accumulation in the brain was observed in a blinded study of APP Tg mice treated with Clioquinol for 9 weeks without any serious adverse effects (Cherny et al., 2001), but the levels of soluble Aβ increased by 52%. This result suggests that some metal chelators such as Clioquinol can inhibit Aβ accumulation and thus have therapeutic use for the treatment of AD. However, several reports indicate that synaptic, physiological, and behavioral abnormalities precede Aβ plaque deposition in AD transgenic mice, supporting the possibility that plaque may not be the critical pathogenic entity, and soluble Aβ levels are the Aβ correlates of cognitive dysfunction in AD (McLean et al., 1999; Wilson et al., 1999; Selkoe, 2001b). It may therefore be more crucial to block the accumulation of both soluble and extracellular plaque Aβ than that of plaque Aβ. Therefore, careful clinical investigations are needed to develop metal chelator as a novel therapeutic drug for AD.

2. β-Sheet Breakers. The secondary structure determines several important properties of peptides that may be relevant to the pathogenesis of neurodegenerative diseases. In the case of Aβ, it has been demonstrated that Aβ-induced neurotoxicity is associated with the formation of β-sheet or amyloid fibrils (Pike et al., 1993; Buchet et al., 1996). The ability of Aβ to form fibrils is directly correlated with the content of β-sheet structures adopted by the peptide (Soto et al., 1995). Transition to the β-sheet conformation proceeds faster at a pH level lower than 6.5 and at an increased Aβ concentration (Barrow and Zagorski, 1991; Burdick et al., 1992). Furthermore, fibril formation accelerates upon nucleation when initial fibrils have formed. Because Aβ fibril disassembler or β-sheet breaker could inhibit amyloid plaque deposition in the brain, this is one of possible therapeutic approaches for AD. On the basis of these theories, a small number of study has suggested the possibility that β-sheet breaker has some therapeutic potential for the treatment of AD through in vitro and in vivo study. Soto et al. designed a peptide that is able to bind Aβ and block the interaction between monomers and oligomers, resulting in blocking the formation of amyloid fibrils (Soto et al., 1996). This peptide ($iA\beta_{11}$) containing 11 amino acids was designed from the N-terminal domain of Aβ (LVFFA) that mainly contributes to Aβ fibrillogenesis (Barrow et al., 1992; Soto et al., 1995). It was also shown in this study that $iA\beta_{11}$ highly interacted with Aβ and inhibited in vitro amyloid formation in a concentration-dependent manner.

The same group also reported that a shorter β-sheet breaker peptide, $iA\beta_5$, was synthesized, and they examined its antiamyloid activity lines (Soto et al., 1998). This peptide inhibited amyloid formation by $A\beta_{1-40}$ and $A\beta_{1-42}$ in in vitro systems and reduced Aβ-induced toxicity in human neuroblastoma cell. In addition, the size of the Aβ deposits in the rats injected with the mixture of $A\beta_{1-42}$ and iAb5 was decreased to 51% compared with those in rats injected with $A\beta_{1-42}$ alone. A subsequent in vivo observation that the $iA\beta_5$-induced disassembly of fibrillar amyloid deposit reversed neuronal shrinkage and inflammatory reaction by Aβ has intensified further the prediction that β-sheet breakers may diminish Aβ-related neuropathogenesis (Sigurdsson et al., 2000).

Peptide inhibitors such as β-sheet breakers have some barriers to go across before they are used as therapeutic drugs for AD: 1) degradation by endogenous enzymes before reaching the affected region or absorbing into body; 2) poor blood-brain barrier permeability; and 3) putative side effects induced by conformational change of AD-unrelated peptide. If an effective β-sheet breaker is able to minimize these defects in the near future, it may decrease Aβ formation and deposition and thus be useful for preventing AD.

D. Antioxidants

Accumulating evidence suggests that oxidative damage to neurons plays an important role in the AD pathogenesis. Thus, therapeutic strategies to reduce oxidative injury and increase antioxidant protection might retard and prevent the onset of the disease. Preclinical studies suggest that some antioxidants may have therapeutic potential for AD. Ginko biloba extract protects neurons from hydrogen peroxide-induced oxidative stress (Oyama et al., 1996). Egb761, a particular extract of Ginko biloba, was examined to assess the efficacy and safety in patient with AD and multi-infarct dementia (Le Bars et al., 1997). Patients who were treated with Egb761 (120 mg/day) for 52 weeks showed the improvement on the Alzheimer's Disease Assessment Scale-Cognitive subscale and the Geriatric Evaluation of Relative's Rating Instrument. Melatonin can reduce neuronal damage induced by oxygen-based reactive species in experimental models of AD. In addition to its antioxidant effect, melatonin also has antiamyloidogenic activities (Pappolla et al., 2000). Studies with cultured cells or animal models revealed that Aβ-induced neurotoxicity and cognitive impairments can be attenuated by either vitamin E or Idebenone, which is an antioxidant and free-radical scavenger (Behl et al., 1992; Yamada et al., 1999; Huang et al., 2000). The efficacy and safety of Idebenone, a coenzyme Q10 analog, was examined in 450 patients with mild-to-moderate AD. The beneficial effects of Idebenone in the patient were maintained until 2 years after treatment, and there were no troubles in safety and tolerability (Gutzmann and Hadler, 1998) (Table 8).

In the double-blind, controlled, clinical 2-year study of patient with moderately advanced AD, the primary outcome of the disease progression was delayed with treatments of selegiline (10 mg/day, monoamine oxidase B inhibitor) or vitamin E (2000 IU/day, antioxidant), or both (Sano et al., 1997). Although there were no significant effects on cognitive ability, these results implicate that the use of selegiline or vitamin E might play some helpful roles in delaying clinical deterioration related to AD.

Recent in vitro studies showed that several kinds of anticholinesterase such as tacrine and Huperzine A and DHED can attenuate Aβ-induced oxidative damage and thus may enhance their therapeutic efficacy for AD more (Xiao et al., 2000a,b,c; unpublished observation). Our recent study showed that DHED protected neurons against hydrogen peroxide and glutamate and decrease reactive oxygen species production (C. H. Park, S. H. Choi, J. H. Seo, J. C. Rah, H. S. Kim, and Y. H. Suh, manuscript submitted for publication). In conclusion, it is believed that antioxidant or free-radical scavengers might have some beneficial activities for the prevention and treatment of AD.

TABLE 8
Aβ-deposit antagonist, antioxidant, and cholinomimetics

Mechanism of Action	Agents	Effects	Reference
Metal chelator	Desferrioxamine, Clioquinol	Reduction of Aβ accumulation	Murayama et al., 1999; Crapper-McLachlan, 1991; Cherny et al., 1999, 2001
Antioxidants	Vitamin E, ginkgo biloba, melatonin, idebenone	Reduction of neuronal damage, retarding AD progression	Le Bars et al., 1997; Behl et al., 1992; Yamada et al., 1999; Gutzmann and Hadler, 1998; Sano et al., 1997; Xiao et al., 2000a–c
sβ-APP–releasing agent	Nicotine, acetylcholine, carbacol	Stimulation of sAPPβ release; reduction of Aβ burden	Efthimiopoulos et al., 1996; Rossner et al., 1997; Jones et al., 1992
Nicotinic receptor agonist	Galantamine, ABT-418	Increase in attention and memory function	Potter et al., 1999; Samochocki et al., 2000; Woodruff-Pak et al., 2001
Muscarinic receptor agonist	Xanomeline, YM-796	Increase in cognitive function	Wei et al., 1992; Bodic et al., 1997; Frederick et al., 2002
ACh precursor	Lecithin, acetyl-L-carnitine	Increase in ChAT activity, ACh synthesis and cognitive function	Piovesan et al., 1994; Pettegrew et al., 1995; Ratnakumari et al., 1995; Levy et al., 1982

E. Anti-Inflammatory Agents

Retrospective studies that compared the frequency of use of nonsteroidal anti-inflammatory drugs (NSAIDs) and the progression of AD suggested a possible therapeutic benefit with conventional anti-inflammatory medications in delaying the onset of AD (Broe et al., 1990; McGeer et al., 1990; Li et al., 1992; Breitner et al., 1994). In the study by McGeer et al. (1990), it was proposed that sustained use of NSAIDs, corticosteroids, methotrexate, or other anti-inflammatory drugs might alleviate the neurodegenerative process of AD. Supporting this hypothesis, the occurrence of AD was reported to be significantly less frequent in patients with rheumatoid arthritis than in the rest of the population (Myllykangas-Luosujarvi and Isomaki, 1994). In addition, it was reported that patients with AD who took NSAIDs were less seriously affected than those who did not (Rich et al., 1995). Chui et al. (1994) showed that Aβ deposition was significantly lower in the brain of aged patients with leprosy. A previous epidemiological study suggests that antileprosy drugs (which have anti-inflammatory activity) are beneficial in preventing senile dementia (McGeer et al., 1992), and the low level of Aβ deposition may be caused by the effects of antileprosy drugs. Ibuprofen exerted a beneficial effect by reducing Aβ deposition and senile plaque formation (Lim et al., 2000). The relative risk of developing AD was also lower for NSAID users and was inversely related to the duration of drug use (Carrada et al., 1996; Stewart et al., 1997). Furthermore, it was suggested that NSAIDs might also prevent or delay the initial onset of AD (Breitner et al., 1994). Results from animal studies provide further evidence that some NSAIDs may protect against AD by suppressing the inflammatory process associated with senile plaque (Netland et al., 1998; Lim et al., 2000). Such studies now suggest as inverse association between AD and prior use of NSAIDs or other anti-inflammatory drugs (Table 9).

Data from homozygous twins also support this concept (Breitner et al., 1994). Similar inverse reactions has been reported between AD and sustained prior use of histamine H_2 antagonists (Breitner, 1991; Anthony et al., 2000) and glucocorticoid steroids (Graves et al., 1990). Breitner's sibling study (1991) showed that there was a trend suggesting that NSAIDs were effective mainly in subjects who lacked the pathogenic Apo ϵ4 allele. Postmortem studies also show that premortem use of NSAIDs reduces the numbers of activated microglia in brain (Mackenzie and Munoz, 1998). NSAIDs also are known to antagonize some of the effects of interleukin-1, presumably mediated by suppression of prostaglandin synthesis.

However, compared with the observations described above, in a case-control study, Kukull et al. (1994) found that prior exposure to NSAIDs was not associated with a decreased risk of AD. It was also reported that there was no relation between NSAID use and risk and/or frequency of AD (Heyman et al., 1984; Graves et al., 1990). No association was found between AD risk and use of acetaminophen, and there was no trend of decreasing risk with increasing duration of use (Stewart et al., 1997). Some authors even reported a decrease in cognitive skills in users of the NSAIDs naproxen and ibuprofen (Goodwin and Regan, 1982). A trial of low-dose prednisone administration also failed (Asien et al., 2000). Because steroids are known to have toxic and side effects on neurons, only low doses can be used over long periods of time. Either of these factors could be responsible for the negative result. Moreover, epidemiological studies suggest a much more modest effects of steroids compared with NSAIDs (Mackenzie, 2000).

The anti-inflammatory mechanism of NSAIDs has not been clearly understood. However, it is well known that its mechanism is caused by their inhibiting effect on cyclooxygenase (COX), which oxidizes arachidonic acid to prostaglandins. There are two COX isoforms: COX-1 and COX-2. Although these two isoforms have some overlapping physiological functions and share structural and enzymatic similarities, they are differently regulated at the molecular level and may have distinct functions. COX-1 is responsible for physiological prostanoid synthesis, and selective COX-1 inhibitors reduce the production of the prostaglandins. A trial using the COX-1 inhibitor indomethacin seemed significantly

TABLE 9
NSAIDs and AD

Association between NSAIDs and AD	Reference
Positive association	Breitner et al., 1991, 1994 (Anti-inflammatory drugs prevent or delay the initial onset of AD)
	Broe et al., 1990 (Positive association in a case-control study of AD in Australia)
	Li et al., 1992 (Positive association in a case-control study of AD in China)
	Lim et al., 2000 (Ibuprofen decreases IL-1β, GFAP levels, and amyloid pathology in brains of Tg2576)
	McGeer et al., 1990, 1992 (Positive association between rheumatoid arthritis and AD)
	Rich et al., 1995 (Decline among NSAIDs patients than among non-NSAIDs patients on measures of verbal fluency, spatial recognition, and orientation)
No association	Beard et al., 1991 (No association between rheumatoid arthritis and AD)
	Kukull et al., 1994 (No association between NSAIDs and a decreased risk of AD)
	Graves et al., 1990; Heyman et al., 1984 (No association between NSAIDs and risk/frequency of AD)
	Stewart et al., 1997 (No trend of decreasing risk with increasing use of acetaminophen)
Negative association	Goodwin and Regan, 1982 (Decrease in cognitive skills in users of naproxen and ibuprofen)

GFAP, glial fibrillary acid protein.

more effective than placebo in stabilizing cognitive skills when it was tested in patients with AD (Rogers et al., 1993). On the other hand, COX-2 usually occurs as a result of tissue injury by cytokines, growth factors, or mitogens. Thus, selective COX-2 inhibitors will suppress the prostanoid biosynthesis triggered by pathological events and be devoid of the side effects associated with the inhibition of the constitutive prostanoid biosynthesis. There is evidence that NMDA-mediated neuronal death is diminished in a dose-dependent manner by COX-2 inhibitors in primary neuronal cultures (Hewett and Hewett, 1997). New evidence that COX is involved in neurodegeneration along with the development of selective COX inhibitors has led to renewed interest in the therapeutic potential of NSAIDs in AD (Pasinetti, 1998).

However, a recently reported clinical trial of a COX-2 inhibitor in AD indicated that neither was it protective nor did it accelerate the decline. For example, a large trial using the COX-2 inhibitor celecoxib has failed. Wallace et al. (1999) pointed out to the fact that in some studies, the anti-inflammatory efficacy was not observed unless doses of the COX-2 inhibitors were used that were greater than those required for COX-2 inhibition. Because COX-2 in brain is highly expressed in neurons but not in the microglia target cells, it seems probable that COX-2 is the wrong COX target for anti-inflammatory action in brain. Inhibition of COX limits inflammation, but it may also alter neural metabolic pathways, resulting in cell death from excitotoxicity or oxidative stress. These new findings stress the point that further refinement in the development of successful anti-inflammatory therapeutics is necessary.

Du and Li (1999) suggested another mechanism of action for NSAIDs by interference with NO production. Aspirin inhibits iNOS by influencing both the synthesis of the iNOS protein and the catalysis of the enzyme, but indomethacin and sodium salicylate have no effects (Amin et al., 1996). NSAIDs did not seem to inhibit iNOS directly because no inhibitory effects were observed after cell activation with lipopolysaccharide, although six NSAIDs (salicylate, aspirin, indomethacin, ibuprofen, diclofenac, naproxen) inhibit or abolishes $NO_2^- + NO_3^-$ accumulation in cell-free media. Thus it is indicated that NSAIDs affect early steps in iNOS expression (Ignarro et al., 1996). The mechanism of action of NSAIDs in inhibiting iNOS mRNA expression is suggested to be attributed to interference with NF-κB activation (Ignarro et al., 1996).

F. Estrogens

Clinically, AD is characterized mainly by a progressive loss of memory. Although the severity of this dementia is similar in both sexes, women with AD exhibit a greater difficulty in semantic memory tasks (Henderson et al., 1994). In men, testosterone decrease is gradual, and neurons have the ability to convert this hormone into estradiol, which may explain a lesser incidence of cognitive disorders in elderly male patients.

Estrogen is one of a family of sex hormones. Besides its classic function as a sex steroid (Evans et al., 1988; Beato, 1989), estrogen modulates transmembrane receptor function (Wetzel et al., 1998; Gu et al., 1999), affects intracellular signal transduction cascades (Curtis et al., 1996; Migliaccio et al., 1996; Marino et al., 1998; Watters and Dorsa, 1998), and is regarded as a molecule that is important in providing neuroprotective and neurotrophic actions in mammal brain tissue (Wickelgren, 1997). Estrogen also participates in the process of synaptic morphogenesis and function (Matsumoto, 1991) and shows a variety of other biological actions (Moss et al., 1997; Revelli et al., 1998). Furthermore, many clinical studies indicate that estrogen is closely associated with neurodegenerative diseases such as Parkinson's disease (Marder et al., 1998) and AD (Tang et al., 1996), both receptor- and nonreceptor-mediated. Recently, it was shown that the ascendant cholinergic pathways and the hippocampus, seriously affected in AD, possess nuclear receptors for estrogen (Shughrue et al., 1998), which exert a modulatory action on cholinergic transmission (Kritzer and Kohama, 1999). Effects of estrogen are known to be mediated by NMDA receptors for glutamate (Foy et al., 1999) and by mechanisms dependent on ApoE, a protein modulated by estrogen.

After menopause in women, plasma levels of the two principal estrogens, estradiol and estrone, plummet. It has been reported that the risk of developing AD and cognitive decline is lower in women with a history of estrogen-replacement therapy (ERT) during the postmenopausal period (Henderson et al., 1994; Paganini-Hill and Henderson, 1994; Tang et al., 1996; Kawas et al., 1997). Even though this has not been universally accepted (Brenner et al., 1994), the most recent prospective studies, which include a fairly large population, strongly suggest that ERT may decrease the risk of AD (Kawas et al., 1997) and the severity of cognitive loss associated with AD (Henderson et al., 1996).

Although several mechanisms of estrogen have been proposed, including antioxidative action (Behl et al., 1994; Smalheiser and Swanson, 1996), blockade of Aβ-induced neurotoxicity (Green et al., 1996), and stimulation of α-secretory pathway of APP processing (Jaffe et al., 1994), it is not clear at present which cellular processes mediate beneficial effects of estrogen on AD. First, several investigations have demonstrated that physiological levels of estradiol reduce the endogenous production of Aβ, prevent Aβ aggregation in vitro, and increase the secretion of the nonpathological fragment of sβ-APP in neural and non-neural cells (Paganini-Hill and Henderson, 1994; Xu et al., 1998; Mattila et al., 2000). In estrogen-receptor-containing cell-culture system, the administration of 17β-estradiol at physiologic concentration increases the secretory metabolism of sβ-APP without increasing APP intracellular levels (Jaffe

et al., 1994). Therefore, estrogen may favorably modify APP metabolism, thereby reducing the accumulation of the neurotoxic Aβ fragment.

Second, estrogen has antioxidant effects that maybe related to its phenolic structure. Supporting this pathway, Goodman et al. (1996) have shown that estradiol protects neurons in primary and organotypic slice cultures against Aβ-induced cell degeneration. The antioxidant effect of estradiol is an intrinsic property of the molecule, and it seems not to be estrogen receptor-mediated, because both 17β-estradiol (active at estrogen receptors) and 17α-estradiol (inactive at estrogen receptors) show equal protective capacity. Estrogen is now known to interact with and increase the anti-oxidative activity of glutathione (Gridley et al., 1998) and decrease free-radical generation at the mitochondrial level. Because estrogen shows potent antioxidant effect and crosses the blood-brain barrier easily, it may have good therapeutic value as a free-radical scavenger in neurodegenerative diseases.

Recently, it was reported that estrogen could regulate the transcription of various genes, including apoptosis-related genes such as the bcl-2 family (Dena et al., 1999), and could modulate the expression of antiapoptotic protein Bcl-xL (Pike, 1999). It was observed that the administration of estradiol attenuated the activation of NF-κB induced by Aβ (Dodel et al., 1999). Moreover, estrogen is also reported to be able to cross-talk within the cytoplasm and interact with various intracellular signaling pathways such as MAPK, cAMP response-element binding protein, and extracellular signal-regulated protein kinase.

Inflammation procedure is modified by estrogen by acting on IL-6, a cytokine that contributes to the formation of senile plaques. Thus, anti-inflammatory estrogen action has a protective AD role, given that inflammatory mechanisms are implicated in senile plaque formation (Jorm et al., 1987; Paganini-Hill and Henderson, 1996; Paganini-Hill, 1998).

Estradiol has the capacity to induce tau expression in the rat hypophysis by a nuclear receptor-dependent mechanism (Matsuno et al., 1997). The phosphorylation of the Thr212 residue of tau by GSK-3β is prevented by protein kinase A, involved in the neuroprotective effects of estrogens (Imahori and Uchida, 1997; Pap and Cooper, 1998).

Estrogen also reduces plasma levels of ApoE (Applebaum-Bowden et al., 1989; Kushwaha et al., 1991; Muesing et al., 1992). The expression of ApoE is modulated by estrogen in rodent tissues (Stone et al., 1997), and estradiol promotes synaptic sprouting in response to injury through an ApoE-dependent mechanism (Stone et al., 1998). Finally, estrogen is known to increase cerebral blood flow (Belfort et al., 1995), modulate cerebral glucose use (Bishop and Simpkins, 1992), and modulate lipid-stimulated kinase in the corpus luteum.

Although some studies have failed to demonstrate a significant association between estrogen use and AD (Heyman et al., 1984; Amaducci et al., 1986; Broe et al., 1990), in general, the studies have shown that women receiving ERT presented AD in a smaller percentage than women not receiving estrogen therapy (Brenner et al., 1994; Paganini-Hill and Henderson, 1994, 1996; Green et al., 1997). Almost every recent study supports the clear protective action of estrogen against the risk of AD in a percentage of at least 35% in the women receiving ERT (Paganini-Hill and Henderson, 1996; Paganini-Hill, 1997). No important differences were found among the kind of estrogen and routes of administration (Brenner et al., 1994; Paganini-Hill and Henderson, 1996). However, estrogen did not show a beneficial effect on severe AD patients, suggesting that ERT has a place in the prevention of AD but not in the treatment of severe AD (Paganini-Hill and Henderson, 1994).

G. Vaccines

The simple immunization with Aβ_{1-42} can reduce Aβ levels, inhibit the deposition of amyloid onto existing plaques, and clear established senile plaques that are present in the brain of transgenic mice. This has raised hope for a potentially important new therapeutic approach to the treatment of AD (Schenk et al., 1999). Schenk et al. (1999) injected mice carrying a mutant human APP gene with human Aβ_{1-42} (AN-1792) and found that the mice produced antibodies against the peptide to great effect. Plaques were largely prevented from forming, and some of the pre-existing plaques in older mice were even dissolved. The same group also reported that antibodies which recognize Aβ_{1-42} injected into the peritoneum work as well as immunization with the Aβ_{1-42} peptide.

The logic behind this scheme is that immunization will stimulate the immune system to fight the abnormal pathologies associated with Aβ and thereby accelerate removal of the amyloid plaques. Schenk et al. (1999) described the appearance of Aβ-containing microglial cells around remaining plaques, indicating that antibody-mediated phagocytosis may be important for clearance. Supporting this logic, Bard et al. (2000) showed that anti-Aβ antibodies cross the BBB, enter the CNS, bind to amyloid plaques, activate microglial cells, and induce the clearance of pre-existing amyloid. These authors also demonstrated that, in an ex vivo assay using brain sections from PDAPP mice or AD cases, exogenously added anti-Aβ antibodies triggered exogenously added microglial cells to clear plaques through Fc receptor-mediated phagocytosis and subsequent peptide degradation. Evidence in favor of this possibility includes the observation that of the few plaques remaining in the immunized animals, a percentage of them were labeled with IgG.

Another alternative logic, a peripheral Aβ sink, was suggested by DeMattos et al. (2001). These authors

found that a monoclonal antibody (m266) directed against the central domain of Aβ was able to bind and completely sequester plasma Aβ. Peripheral administration of m266 to PDAPP mice, in which Aβ is generated specifically within the CNS, results in a rapid 1000-fold increase in plasma Aβ, caused, in part, by a change in Aβ equilibrium between the CNS and plasma. Although peripheral administration of m266 to PDAPP mice markedly reduces Aβ deposition, m266 did not bind to Aβ deposits in the brain. The authors conclude that the likely mechanism to explain why peripherally administered m266 can remove Aβ deposits from brain is by altering the dynamic equilibrium of Aβ between brain, CSF, and plasma such that a reduction of plasma Aβ can lead to an efflux of brain Aβ to the CSF and into the circulation.

Two separate research teams have replicated and extended these results, showing that vaccination was also accompanied by a corresponding improvement in cognitive and memory functions (Helmuth, 2000; Janus et al., 2000; Morgan et al., 2000). However, in some transgenic mice that carried presenilin 1 as well as APP transgenes, cognitive protection caused by vaccination was obtained despite no significant decrease in amyloid burden (Morgan et al., 2000). To explain this disconnect, Lambert et al. (2001) suggested that vaccination might target-soluble, nonfibrillar toxins derived from Aβ. It is known that Aβ toxicity resides not only in fibrils, but also in soluble oligomers and protofibrils (Walsh et al., 1997, 1999; Hartley et al., 1999; Hsia et al., 1999; Mucke et al., 2000; Klein et al., 2001).

The study by Weiner and coworkers (2000) provides the first confirmation of the Schenk study (1999), despite some differences in methodology, and it extends the concept of immune-based therapies as a treatment of AD. Weiner et al. used oral or nasally administered $A\beta_{1-40}$ peptide to immunize the PDAPP mice. The major finding was a 50 to 60% reduction in amyloid burden in the brains of mice immunized intranasally with high doses (25 μg) of Aβ. As in the Schenk study, there were also fewer dystrophic neurites and cellular (astroglial and microglial) infiltrates in the immunized animals. There were no significant benefits seen in mice immunized intranasally with a lower dose of Aβ (5 μg) or in mice immunized orally. These results confirm that immunization Aβ, through either a parental or an intranasal route, can reduce amyloid plaque load in brain. These authors also detected the presence of T cells, albeit in limited numbers, which expressed IL-4, IL-10, and TGF-β, suggesting that a cellular immune response with release of anti-inflammatory cytokines is another possible immune mechanism.

Concerns have been raised that immunization of AD patients with Aβ might initially accelerate the brain's amyloid deposition process, because the peptide crosses the BBB and could send further fibril formation and further neuronal death. However, a recent study used immunization with an alternative peptide $K6A\beta_{1-30}$-NH_2 that is similar to Aβ, highly soluble in water (unlike natural Aβ), includes the immunogenic $A\beta_{1-11}$ and $A\beta_{22-28}$ regions, yet is entirely nontoxic and nonfibrillar. Repeated immunization with this Aβ-like peptide also reduced amyloid deposition in brains of transgenic Tg2576 mice, another AD animal model (Sigurdsson et al., 2001). Recently Schenk's group announced initial results from clinical trials showing that the $A\beta_{1-42}$ vaccine is safe and well-tolerated in humans. However, the drug's manufacturer, Elan (Dublin, Ireland) and its collaborating company, Wyeth-Ayerst (Princeton, NJ) announced on 18 January 2002 that they were temporarily suspending a 360-patient trial of the vaccine (AN-1792) being carried out in the United States and Europe because the 4 patients showed clinical signs consistent with inflammation in the CNS (Check, 2002). Some scientists warn that AN-1792 could cause a dangerous autoimmune response in humans, which might target APP. However, it is premature to draw conclusions on this issue, given the small number of patients involved.

Together, these studies seem to be promising as a new and powerful strategy for the prevention or treatment of AD, but there are also important issues raised about the ultimate efficacy and safety in humans. The most important question is whether or not it will work in patients. It is not certain that humans mount as vigorous an immune response to the human $A\beta_{1-42}$ peptide as do mice. If they do not, passive immunization may be the way forward. Preventing and reducing plaques in AD-model transgenic mice and reversing the course of AD in humans are two very different problems. The mice used for the transgenic mice only partially mimic the human disease. It is important to remember that a causative role for amyloid has not been proved definitely, and there are other important pathological changes in the AD brain. Mice show neither the loss of nerve cells nor the behavioral abnormities associated with the human condition. If amyloid is not deposited, these other pathologies will still occur (Levey, 2000). As discussed above, safety is also a concern for human studies, especially considering the potential for autoimmunity. Antibodies reactive with either Aβ or APP could potentially induce immune-mediated tissue damage. Antibodies could also bind to the Aβ sequence within APP and produce an autoimmune response in tissues expressing this protein in brain and in the periphery. However, this possibility is probably limited, because the relevant portion of APP is transmembranous, and such antibodies were not detected in either study.

The current debate is whether the Aβ-clearing effects of active and passive Aβ immunization are caused by the clearance of Aβ antibody complexes by local microglial (Bard et al., 2000) and/or the transport of such complexes out the brain and into CSF and blood (DeMattos et al., 2001). If such immunization could be shown to be effective and without harmful side effects in humans,

this could provide a powerful intervention to help deal with the rapid rising tide of AD. If successful, this would represent a novel therapeutic use of the immune response. Passive immunization might be able to circumvent any ineffectiveness of active immunization of elderly people whose immune responses are frequently blunted.

H. β-Secretase Inhibitors

The prediction that inhibition of CTF-β production should prevent amyloid plaque formation suggests that β-secretase would be an extremely attractive therapeutic target for the treatment of AD. Because BACE has been known as a secretase enzyme converting APP into sβ-APP and CTF-β (Sinha et al., 1999; Vassar et al., 1999, Yan et al., 1999), the blocking of this enzyme would be expected to slow down the production of amyloid β peptides. The optimal way to prevent the production of the toxic fragments of APP such as Aβ and CTF might be the inhibition of BACE activity rather than γ-secretase or other factor that are able to use CTFβ as a substrate, because BACE can also produce toxic fragments of APP even though γ-secretase activity is blocked. Another related secretase, BACE-2, has been identified (Hussain et al., 1999; Acquati et al., 2000), but BACE-2 is not highly expressed in mammalian brain tissue. Furthermore, cortical cells isolated from BACE−/− mice failed to produce detectable amount of Aβ peptides (Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001). The observation that BACE−/− animals were viable and displayed no gross anatomical or physiological abnormalities suggests that BACE inhibition would be free of major side effects. Taken together, these results indicate that BACE-1 is indeed the major neural β-secretase. Recently, several kinds of BACE inhibitors have been found. Sinha et al. (1999) first found β-secretase inhibitor on the base of APP substrate. It was designed on the observations that BACE activity was predominant in acidic conditions and β-cleavage was being carried out by a pepstatin-insensitive aspartic proteinase. They achieved improvement in the inhibitory potency by substituting valine for aspartic acid at the P1′ position and (*S*)-statine at the P′ position. The IC_{50} of this substituted substrate (KTEEISEVN-Stat-VAEF) was approximately 30 nM.

Two inhibitors of BACE, OM99–1 and OM99–2, were recently synthesized by Ghosh et al. (2000). These inhibitors were designed from a β-secretase cleavage site in APP and are changed from Asp into Ala at the P1′ site and at the peptide bond between P1 and P1′ sites into hydroxyethylene transition-state isotere, which is a highly effective transition state analog for the inhibition of aspartic proteases. These inhibitors showed strong inhibitory activity against recombinant BACE (K_i values for OM99–1 and OM99–2 were 6.84×10^{-8} M ± 2.72×10^{-9} M and 9.58×10^{-9} M ± 2.86×10^{-10} M, respectively).

Abbenante et al. (2000) described two kinds of β-secretase inhibitors that are also based on the β-secretase cleavage site (VNL-DA) of APPsw. This simple tripeptide aldehyde showed an inhibitory effect on the β formation by inhibiting β-secretase activity (IC_{50} = 700 nM). Although this inhibitor showed lower inhibitory potency than other previous inhibitors, it also acts on the γ-secretase sites of APP. Reduced molecular size and polarity would make BBB penetration more readily. They are attempting to design more potent as well as specific analogs of the synthesized candidate compounds using antibodies specific to sβ-APP, P3, and sα-APP in a cell-based assay.

The structural analysis of BACE complexed to BACE inhibitor provided some clues for designing more selective and strong BACE inhibitors (Hong et al., 2000). They confirmed that BACE has the conserved general folding of aspartic protease, and active site of BACE is more open and less hydrophobic than that of other aspartic protease.

Last year, Turner et al. reported that the complete subsite preference of BACE were from results from both substrate kinetics and the binding study of a combinatorial inhibitor library to BACE (Turner et al., 2001). This investigation gave further information for being able to facilitate the design of smaller potent BACE inhibitor and synthesize a new BACE inhibitor successfully, OM00–3 (ELDLAVEF), which has more improved potency (K_i = 0.3 nM) than OM99–2 (K_i = 1.6 nM) with structural modulation.

There are some considerable factors in the seeking and rational design of BACE inhibitors. BACE-2 is highly expressed in heart, kidney, and placenta, suggesting it may be important in highly vacuolarized systemic tissues (Farzan et al., 2000). If so, it would be critical to develop an AD drug that selectively inhibits BACE-1 but not BACE-2. Although BACE knockout mice did not show any phenotypic defects (Roberds et al., 2001), probable side effects that would be caused by inhibition of BACE should be considered in development of BACE inhibitor. Even though BACE is effectively inhibited, other enzymes such as caspase still may generate toxic C-terminal fragments of APP such as CT_{31}. Additionally, for the enhancement in clinical efficacy, molecular size, blood-brain barrier permeability, and activity in the neuron should be also considered. It is also believed that inhibitors that originate from natural products may be more superior in safety and stability than other peptide-based inhibitors.

I. γ-Secretase Inhibitors

γ-Secretase may be an important enzyme for the processing of APP and other proteins (e.g., Notch). Notch is involved in the regulation of neuronal differentiation, spermatogenesis, oogenesis, and myogenesis. The membrane-associated C terminus is cut by a γ-secretase-like activity within the postulated transmembrane domain

to release the NICD, which then translocates to the nucleus where it interacts with and activates transcription factors. The presenilins are also critical for the processing of the Notch receptor (Artavanis-Tsakonas et al., 1999).

A potential concern about γ-secretase inhibition was that it could inhibit Notch signaling, which could affect bone marrow. However, Petit et al. (2001) suggested that the two effects can be dissociated with novel inhibitors.

1. Peptidic Inhibitors.

Difluoroketone peptidomimetic 1; MW167 ($C_{33}H_{57}F_2N_5O_9$). This compound is a reversible and selective peptidomimetic inhibitor of γ-secretase with an IC_{50} of 13 μM for the inhibition of Aβ production in β-APP-transfected Chinese hamster ovary cells. It displays only weak inhibitory activity against calpain 2 (IC_{50} = 100 μM in a purified enzyme assay). The alcohol counterparts of selected difluoroketones retained inhibitory effects on Aβ production, indicating that the ketone carbonyl is not essential for activity and suggesting that these compounds inhibit an aspartyl protease (Wolfe et al., 1999b). Also, this compound inhibits NICD production with an IC_{50} of 10 to 30 μM (De Strooper et al., 1999; Berezovska et al., 2000).

2-Naphthoyl-VF-CHO; *N*-(2-Naphthoyl)-Val-phenylalaninal ($C_{25}H_{26}N_2O_3$). This cell-permeable, reversible inhibitor of γ-secretase inhibits the release of $Aβ_{x-40}$ (ED_{50} = 2.6 μM) and $Aβ_{x-42}$(ED_{50} = 2.7 μM) in HEK293 cells stably transfected with amyloid precursor protein Swedish mutants (Sinha and Lieberburg, 1999).

Z-LF-CHO; N-Benzyloxycarbonyl-Leu-phenylalaninal ($C_{23}H_{28}N_2O_4$). This compound is a cell-permeable, reversible inhibitor of γ-secretase that inhibits the release of $Aβ_{x-40}$ (ED_{50} = 5.0 μM) from HEK293 cells stably transfected with amyloid precursor protein Swedish mutants (Sinha and Lieberburg, 1999).

1-(*S*)-endo-*N*-(1,3,3)-Trimethylbicyclo[2.2.1]hept-2-yl)-4-fluorophenyl sulfonamide ($C_{16}H_{22}FNO_2S$). This is a potent, cell-permeable inhibitor of γ-secretase that blocks the formation of $Aβ_{42}$ (IC_{50} = 1.8 μM). Treatment of HEK293 cells with this compound results in an increase in β-secretase-cleaved APP fragments and secreted $APP_{Sα}$, but no change in secreted APPsβ, indicating a specific inhibition of γ-secretase activity (Rishton et al., 2000).

t-3,5-DMC-IL-CHO; *N-trans*-(3,5-dimethoxycinnamoyl)-ile-leucinal ($C_{23}H_{34}N_2O_5$). This compound is a potent cell-permeable, reversible γ-secretase inhibitor that preferentially seems to inhibit the secretion of $Aβ_{1-40}$ (>90%) versus $Aβ_{1-42}$ (~15%). $Aβ_{total}$ IC_{50} ~ 15 μM; $Aβ_{1-40}$ IC_{50} ~ 22 μM; $Aβ_{1-42}$ IC_{50} >50 μM in CHO cells stable transfected with the cDNA encoding $β\text{-}APP_{695}$. It is reported to be approximately 10-fold more potent than Z-Val-Phe-CHO (MDL28170) (Higaki et al., 1999).

Boc-GVV-CHO; *N-tert*-utyloxycarbonyl-Gly-Val-Valinal ($C_{17}H_{31}N_3O_5$). This compound is a cell-permeable, substrate-based ($γ_{40}$-site) γ-secretase inhibitor that is reported to preferentially (>90%) inhibit $Aβ_{40}$ cleavage in transiently transfected 293T cells overexpressing APP695NL. It does not have any significant effect on the production of $Aβ_{42}$ (Murphy et al., 2000).

Z-LLL; carbobenzoxyl-Leu-Leu-leucinal; MG132. MG132 decreased the amount of $Aβ_{1-40}$ by approximately 39% at a concentration of 5 μM and by approximately 70 to 87% at a concentration of 25 μM and almost completely blocked secretion at 50 μM, whereas $Aβ_{1-42}$ secretion was not affected (Klafki et al., 1996; Skovronsky et al., 2000). Also this inhibits NICD production (De Strooper et al., 1999).

Z-LL-CHO; *N*-benzyloxycarbonyl-leu-leucinal ($C_{20}H_{30}N_2O_4$). This is a cell-permeable, reversible inhibitor of γ-secretase that reduces the formation of both $Aβ_{total}$ (IC_{50} ~ 35 μM) and $Aβ_{1-42}$ using CHO cells stably transfected with amyloid precursor protein 751. A 3-h treatment of these cultures with 100 μM led to an almost complete inhibition of total Aβ. It is reported to be nontoxic and specific for γ-secretase (Figueiredo-Pereira et al., 1999).

Z-VF-CHO; *N*-benzyloxycarbonyl-Val-phenylalaninal; MDL28170. MDL inhibitor, an amino terminal-blocked dipeptide aldehyde, is a calpain proteinase inhibitor. MDL28170 (>50 μM) strongly and significantly decreased the production of both total Aβ and total p3 in CHO cultures stably transfected with the 695-amino acid isoform of β-APP. In contrast, $Aβ_{42}$ and $p3_{42}$ had only a small and insignificant decrease at 200 μM (Higaki et al., 1995; Citron et al., 1996). Also this inhibits NICD production (De Strooper et al., 1999).

2. Nonpeptidic Inhibitors.

a. JLK Inhibitors. Nonpeptidic potential inhibitors of γ-secretase, JLK2, JLK6, and JLK7, decreased the amount of total Aβ secreted from HEK293 cells overexpressing wild-type β-APP by approximately 70 to 80% at a concentration of 100 μM, whereas JLK4, JLK5, and JLK8 were apparently ineffective. These compounds are not toxic, do not affect constitutive secretory pathway, and are biologically inert on β-secretase (Petit et al., 2002). Aβ recovery from cells overexpressing Swedish-mutant APP was also inhibited by JLK2 and JLK6, but JLK4 and JLK8 were inactive. These inhibitors are totally unable to affect the mΔEnotch-1 cleavage that leads to generation of the NICD. These represent the first nonpeptidic inhibitors that are able to prevent γ-secretase cleavage of β-APP without affecting the processing of mΔEnotch-1 or endoproteolysis of presenilins (Petit et al., 2001).

N-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenglycine *t*-butyl ester. This is an inhibitor of γ-secretase activity that reduces the formation of $Aβ_{total}$ (IC_{50} ~ 0.02 μM) using HEK293 cells overexpressing human APP_{751}. Also in human primary neuronal cultures, both measures of Aβ production are similarly inhibited with potencies (Aβ total IC_{50} = 115 nM, $Aβ_{42}$ IC_{50} = 200 nM) of 5- to 10-fold lower than is observed in HEK293 cells. Oral administration of

this compound to mice transgenic for human APPV717F reduces brain levels of Aβ in a dose-dependent manner within 3 h. This compound is used in the first study demonstrated a reduction of brain Aβ in vivo (Dovey et al., 2001).

L-685,458. A potent inhibitor of γ-secretase with an IC_{50} of 17 μM is reported to inhibit $A\beta_{40}$ and $A\beta_{42}$ production with a similar potency in human neuroblastoma SHSY5Y cells overexpressing the construct spβA4CTF, which serves as a direct substrate for the β-APP γ-secretase (Shearman et al., 2000).

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Correction to "Amyloid Precursor Protein, Presenilins, and α-Synuclein: Molecular Pathogenesis and Pharmacological Applications in Alzheimer's Disease"

As discussed in the "Notice of Concern" published in this issue, several paragraphs in the above article [Suh Y-H and Checler F (2002) *Pharmacol Rev* **54:**469–526] were republished from an earlier work [Selkoe DJ (2001) Clearing the brain's amyloid cobwebs. *Neuron* **32:**177–180] without attribution. Drs. Suh and Checler have provided the following comments and corrections.

Dr. Suh would like to clearly state that the material used from the Selkoe publication without attribution was an inappropriate and unacceptable action and that Dr. Checler was absolutely not involved in the written part taken from Selkoe's article.

While editing the text and references, citations of Selkoe's *Neuron* article at some places in the text and the entry for the *Neuron* article in the references were deleted as a result of the authors' mistakes and carelessness.

Dr. Suh sincerely wants to correct the text with appropriate citations as follows.

On page 477, in the left column, after "*a. Insulin-Degrading Enzyme*," quotation marks should enclose the text following the heading until the end of the next paragraph, and the citation "(Selkoe, 2001b)" should be added at the end of the quotation.

On page 477, in the first paragraph of the right column, the text beginning with "The intracerebral injection of synthetic Aβ peptides" to the end of the next paragraph should be in quotation marks.

On page 478, in the left column, after "*d. Endothelin-Converting Enzyme*," quotation marks should enclose the text following the heading until the end of the paragraph, and the citation "(Selkoe, 2001b)" should be added at the end of the quotation.

On page 522, the following reference should be added: "Selkoe DJ (2001b) Clearing the brain's amyloid cobwebs. *Neuron* **32:**177–180."