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CELLULAR AND MOLECULAR BASIS OF b-AMYLOID PRECURSOR PROTEIN METABOLISM

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1. ABSTRACT

In molecular neurobiology, perhaps no molecule has been as thoroughly examined as Alzheimer's β-amyloid precursor protein (βAPP). In the years since the cDNA encoding βAPP was cloned, the protein has been the subject of unparalleled scrutiny on all levels. From molecular genetics and cellular biology to neuroanatomy and epidemiology, no scientific discipline has been left unexplored - and with good reason. β-amyloid (Aβ) is the main constituent of the amyloidogenic plaques which are a primary pathological hallmark of Alzheimer's disease, and βAPP is the protein from which Aβ is cleaved and released. Unraveling the molecular events underlying Aβ generation has been, and remains, of paramount importance to scientists in our field. In this review we will trace the progress that has been made in understanding the molecular and cellular basis of βAPP trafficking and processing, or alternatively stated, the molecular basis for Aβ generation. Imperative to a complete understanding of Aβ generation is the delineation of its subcellular localization and the identification of proteins that play either direct or accessory roles in Aβ generation. We will focus on the regulation of βAPP cleavage through diverse signal transduction mechanisms and discuss possible points of therapeutic intercession in what has been postulated to be a seminal molecular step in the cascade of events terminating in the onset of dementia, loss of neurons, and eventual death from Alzheimer's disease.

2. INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in the elderly, accounting for over 50% of the typical, late-onset cases of dementia. AD is characterized clinically by an insidious onset and an inexorable progression of dementia, and pathologically by the abnormal accumulation of neuritic plaques and neurofibrillary tangles in vulnerable brain regions. Plaques consist of deposits of variously sized peptides called β-amyloid (Aβ) (1, 2) which are derived through proteolytic processing of the β-amyloid precursor protein (βAPP). The neurofibrillary tangles are composed largely of hyperphosphorylated twisted filaments of a cytoskeletal protein, tau, (3) whose contribution to AD pathogenesis is beyond the scope of this review. Evidence causally linking βAPP to AD was first provided by the discovery of mutations within the βAPP coding sequence that segregated with disease phenotypes in autosomal dominant familial AD (FAD) (4-6). Although documented FAD is rare (<10% of all AD), the characteristic clinicopathological features, amyloid plaques, neurofibrillary tangles, synaptic and neuronal loss, neurotransmitter deficits and dementia are apparently indistinguishable when FAD is compared with typical, common, "non-familial," or sporadic AD.

In addition to mutations within βAPP, there are actually many more reported pedigrees in which early onset FAD segregates with two other genetic loci. Mutations in the *presenilin 1* gene on chromosome 14 (7) and the *presenilin 2* gene on chromosome 1 (8) cause FAD by altering βAPP metabolism. Specifically, presenilin mutations cause a selective increase in the production of the 42 amino acid form of Aβ (Aβ42) (9-10). This increase of Aβ42 occurs in the plasma, and in media from cultured

skin fibroblasts derived from patients carrying these mutations, and can be detected presymptomatically (10). This elevation of Aβ42 is significant because Aβ42, despite being produced far less abundantly than Aβ40, is more highly amyloidogenic than Aβ40 (11, 12) and is believed to form the core of the amyloidogenic plaques (13, 14). Cells tranfected with mutant PS1 cDNA secrete higher levels of Aβ42 (9, 15); similarly, transgenic mice expressing mutant PS1 show increased levels of Aβ42 levels in their brains (16). Furthermore, it was already known that patients with Down's syndrome or trisomy 21 (*bAPP* is on chromosome 21) invariably develop AD pathology by age 50 and can show Aβ42 immunoreactive plaques as early as age 12 (17). This data from humans, transgenic mice and cells transfected with mutations in *bAPP* and *PS1*, has strengthened the integrated amyloid cascade hypothesis: diverse molecular anomalies all lead to enhanced production or aggregation of Aβ peptides. It is the goal of this review to introduce the various hypotheses regarding the metabolism of βAPP and the generation of Aβ, and to suggest how interrupting or delaying this early and invariable event in AD may be therapeutically feasible.

3. DISCUSSION

3.1. b**APP Structure Gives Clues to its Function**

The partial purification of amyloid peptides from the microvasculature of AD brains by Glenner and Wong (1) began the modern AD research era; within three years, the sequence of this small peptide was used to successfully clone the full-length cDNA of βAPP (18). The deduced amino acid sequence of βAPP predicts a type 1 transmembrane protein encoded by alternatively spliced mRNA resulting in isoform diversity. Isoforms of 751 and 770 amino acids contain a protease inhibitor domain in the extracellular region of the βAPP molecule (19, 20). An isoform of 695 amino acids missing the protease inhibitor domain, however, is the main isoform expressed in neurons. A definitive cell biological role has yet to be ascribed for βAPP and its metabolites, however, physiological roles for βAPP in transmembrane signal transduction (21), calcium metabolism (22) and neuritic outgrowth (23) have been suggested. Potential functional motifs within βAPP identified by the presence of consensus sequences, suggest roles in metal ion binding (24), heparin binding (25), cell adhesion (26), as a receptor for a currently unrecognized ligand, and in regulating cell growth (27). Initial studies of βAPP knockout mice did not reveal an obvious disease phenotype (28), however, more recent examinations have suggested deficits in specific synaptic proteins (29). A family of novel βAPP-like proteins (APLPs) was discovered (30, 31) suggesting that βAPP may be a member of a larger family of related molecules. APLPs lack the Aβ domain, however, and therefore cannot serve as precursors to Aβ.

3.2. b**APP is Trafficked and Processed Through the Secretory Pathway**

βAPP is synthesized and cotranslationally inserted into membranes in the endoplasmic reticulum (ER). Although it has been recently suggested that Aβ42 can be generated within a compartment early in the secretory pathway (32), such as the ER or early Golgi, most βAPP molecules exit the ER uncleaved. Once exiting the ER, βAPP molecules are transported to the Golgi apparatus where the majority of βAPP molecules are found under steady state conditions. Within the Golgi apparatus, βAPP is significantly modified by N- and O-glycosylation, tyrosyl sulfation, and sialylation (33, 34). βAPP is also phosphorylated in both the extracellular and intracellular domains (34, 35). In addition, some βAPP molecules are chondroitin-sulfated in their ectodomains (36).

The proteolytic processing events underlying βAPP metabolism have been studied intensely. The first proteolytic cleavage product of βAPP processing to be definitively identified by purification and sequencing was a fragment which results primarily from a cleavage event within the Aβ domain. The entire amino-terminal fragment of the βAPP extracellular domain, termed s-βAPP or βAPPs, is released into the medium of cultured cells (26, 34, 37, 38) and into the cerebrospinal fluid, leaving a nonamyloidogenic carboxyl-terminal fragment associated with the cell (39). This pathway was designated the α-secretory pathway after the still unidentified enzymatic activity termed the α-secretase which cleaves the protein with that specificity. Thus one important processing event in the biology of βAPP acts to preclude Aβ formation by cleaving βAPP within the Aβ domain.

3.3. a**-Secretase Cleaves** b**APP at the Cell Surface**

An increasing number of secreted proteins are now recognized as being derived from integral plasma membrane proteins in which the secretory event is actually post-translational hydrolysis from the cell surface. This shedding or solubilization involves either an endoprotease or a phospholipase. βAPP appears to belong to this large family of proteins including membrane receptors, receptor ligands, ectoenzymes, leukocyte antigens, and cell adhesion molecules in which an enzymatic event at the cell surface may be physiologically relevant (40). It has been demonstrated that α-secretase is most active late within the secretory pathway and at the plasma membrane (41). The α-cleaved βAPP molecule is likely to have a physiological relevance that extends beyond merely preventing Aβ formation: both *in vitro* and *in vivo* effects of βAPPs on cytoprotection and cell neurotrophy have been documented (27).

Recent evidence has suggested that Tumor Necrosis Factor-α Converting Enzyme (TACE), a member of the ADAM family (A Disintegrin And Metalloprotease family) of proteases, may be involved in regulating α-cleavage of βAPP (42). Inhibiting TACE cleavage affected both βAPP secretion and Aβ generation in cultured cells suggesting that TACE or a closely related molecule, could be involved in an α-secretase-like cleavage event. Membrane-bound tumor necrosis factor-α (TNF-α) is also similar to βAPP in that it is a transmembrane protein that can be proteolytically cleaved to release its extracellular

domain as soluble TGF-α. Amino acid substitutions around the βAPP α-secretase cleavage site do not alter βAPP cleavage suggesting that α-secretase cleavage relies more upon the distance from the membrane than on the primary amino acid sequence (41). Progressive deletion of the extracellular juxtamembrane amino acids demonstrated that only 11 amino acids of the protein sequence were required to sustain βAPP cleavage at the cell surface (41). The cell regulation of the α-secretase pathway will be discussed later in this review. Simply, however, the activation of Protein Kinase C (PKC), through diverse routes, strongly stimulates α-secretase cleavage. This event can be ascribed either to indirect effects of PKC on the cellular localization of βAPP and the α-secretase or direct effects on the enzymes themselves through altering their state of phosphorylation (43-45).

3.4. Alternative bAPP Metabolism Gives Rise to the ß-Amyloid Peptide

Until 1992, Aβ was, by general consensus, an abnormal metabolite derived from βAPP. Soluble Aβ was not initially detected in brain, cerebrospinal fluid or from cell culture systems due to the limitations imposed by antibody reagent sensitivity. The conformation, aggregation and low endogenous levels of Aβ produced by cells also made Aβ detection difficult. Most early information regarding βAPP metabolism therefore, was derived from experiments studying the carboxy-terminal fragments of βAPP. High-level overexpression of human βAPP using recombinant vaccinia viruses (46), baculoviruses (47), or stable transfections in association with supraphysiological levels of protein phosphorylation (43) resulted in the accumulation of heterogeneous C-terminal βAPP fragments. These studies supported the hypothesis that an alternative cleavage of βAPP molecules could occur in which C-terminal fragments not cleaved by the α-secretase, but rather containing the complete Aβ sequence, remained as potential precursors from which Aβ could be further derived. This cleavage activity was designated the β-secretase. Sequencing of these putative β-cleaved C-terminal species confirmed them as potential intermediaries in the pathway to Aβ generation (48).

This non α-secretase-mediated cleavage immediately prompted the search for alternative intracellular routes through which βAPP could be trafficked and metabolized into Aβ-generating precursors. The existence of an alternative secretory pathway for βAPP was difficult to prove. Several groups began searching for the secretory compartments, within which βAPP was metabolized and Aβ generated. A favored hypothesis posited that Aβ was generated within an acidic intracellular compartment with a pH permissive for maximal secretase activity. Vesicle neutralizing agents such as chloroquine and ammonium chloride applied to cultured cells facilitated the recovery of full length βAPP and an array of C-terminal fragments (44, 49, 50) - a similar population of fragments as was recovered from purified lysosomes (51).

The possibility that an endosomal-mediated metabolism of βAPP was important in the generation of Aβ peptides was further indicated by the discovery of a clatherin-coated vesicle (CCV) targeting motif in the LDL receptor. This NPXY motif, required for proper internalization of the LDL receptor, was also present on the cytoplasmic tail of βAPP (52). The co-purification of βAPP with CCVs (53) suggested the possibility that βAPP may be a receptor for a yet undiscovered or recognized ligand. These experiments helped define a second normal processing pathway for βAPP that does not involve a cleavage event within the Aβ domain of βAPP. This work supported the hypothesis that βAPP can be reinternalized from the cell surface and targeted to the endosome/lysosome pathway where it may contribute, in part, to the generation of βAPP-derived fragments containing Aβ (54).

Despite progress in elucidating the subcellular compartments within which βAPP processing enzymes are active, until recently, all attempts to discover their identities have failed using traditional mammalian systems. To overcome the difficulties inherent in mammalian gene discovery, several groups attempted to reconstitute βAPP processing in *Saccharomyces cerevisiae*, an organism with a much simpler, and completely sequenced genome. Combined genetic and biochemical approaches identified several proteolytic processing enzymes in yeast which have been found to be homologous to enzymes that catalyze the same reactions in higher organisms. Using similar approaches, it was demonstrated using yeast strains transfected with human βAPP, that yeast contain enzymes that exhibit α-secretase-like activity (55). This reaction is catalyzed by the glycosyl-phosphatidylinositol-linked yeast aspartyl proteases, MKC7 and YAP3 (56, 57). A more recent study demonstrates the presence of β-secretase-like enzymatic activity in yeast as well (58). Given these findings along with the availability of mutant yeast strains and the well-characterized yeast secretory pathway, yeast may become an attractive system for studying βAPP processing and Aβ generation.

3.5. Ab Peptides may be Generated Intracellularly

Hypotheses regarding the abundance of a 4 kD Aβ species were based upon the belief that Aβ was an abnormal and toxic species - a product restricted to the brains of aged or demented humans. It was believed that a cell would need to be significantly injured for a protease to gain access to the interior of the membrane bilayer to cleave βAPP and generate Aβ. This concept was soundly refuted when several groups discovered that a soluble Aβ species was detectable in body fluids from various species (59) and in the conditioned medium of cultured cells (60) but was not detectable in the lysates of cultured cells. Cultured cells were shown to release the 4 kD Aβ peptide into medium in high picomolar to low nanomolar concentrations (60, 61). In addition to cultures of neuronal-like cell lines, cortical cultures derived from the brains of fetal mice, rats and humans all secrete Aβ peptides in quantifiable levels (59, 62) and Aβ can be similarly

detected from the cerebrospinal fluid of humans and rats (59).

The subcellular processing pathway involved in Aβ generation was also debated, with the generation of Aβ through either endocytic or exocytic pathways, each rigorously defended. One shared feature of the two pathways is the unquestioned reliance of βAPP passage through an acidic intracellular compartment. In support of this, treatment of βAPP-expressing cells with Bafilomycin A (Baf A), a vacuolar-type H^+-ATPase inhibitor, abolished Aβ generation (63). Attempts to isolate Aβ from the late endosomes/lysosomes failed, leading to the hypothesis that Aβ production requires maturation of βAPP through the Golgi and processing in an acidic compartment other than lysosomes (64).

This hypothesis was confirmed when it was demonstrated with the use of a cell-free system that Aβ can be generated from the trans-Golgi network (TGN) in the absence of vesicle formation (65). This result is consistent with previous findings because the TGN, like the endosome, provides an ideal acidic environment for maximal secretase activity and Aβ generation. β-secretase activity within the late Golgi was confirmed in cells expressing βAPP harboring a pathogenic point mutation (66). The late Golgi has many known cellular functions that support it as a likely site for Aβ generation. As previously mentioned, under steady state conditions βAPP is principally localized within the TGN. It has been appreciated that physiologically relevant events, such as prohormone processing, occur here as well (67). Finally, the TGN is a known sorting station for many lysosomal enzymes and plasma membrane proteins. These facts support the notion that if βAPP and relevant secretases are co-localized within the TGN, this late secretory compartment could be a major site of Aβ generation. Interestingly, implicating the TGN as an important location for Aβ production raises the possibility that the excess production of Aβ seen in AD may be due to a defect in the general secretory/processing apparatus of neurons which could occur after many decades of unfaltering function.

βAPP is initially synthesized and cotranslationally inserted into the endoplasmic reticulum (ER) and recent studies have demonstrated ER generation of Aβ42 (68-72). Retaining βAPP in the ER using brefeldin A or ER retention signals increases production of Aβ42, reinforcing the hypothesis that it is generated within the ER. Aβ42 generation was detected in the ER of both primary neurons and cultured human NT2N cells. Using human NT2N cells, a pool of insoluble Aβ42 making up over half of the total intracellular Aβ42 was extracted with formic acid from the ER (71). These studies, using ELISA assays with C-terminal specific antibodies, could not distinguish between the N-termini of the peptides and differentiate between Aβ42 species. A cell-free assay was used to investigate the subcellular compartments within which distinct amyloid species are produced and those from which they are secreted (72). It was determined that Aβ40 (Aβ1-40 and Aβx-40) is generated exclusively in the TGN and secreted in post-TGN secretory vesicles. It was also demonstrated that while the Aβx-42 is produced and retained in the ER in an insoluble state, both Aβ1-42 and Aβx-42 species are generated in the TGN and packaged into secretory vesicles which accounts for the secreted pool of Aβ42. The identification of the subcellular compartments in which the distinct species of Aβ are produced and from which they are secreted could aid in the search for the proteolytic enzymes responsible for Aβ formation.

Several studies have recently been conducted which support the hypothesis that Aβ is generated intracellularly. Recent studies demonstrated that an insoluble pool of intracellular Aβ was highly stable and accumulated in NT2N cells as a function of aging in culture. These studies showed a 12-fold increase in the amount of intracellular Aβ over a seven-week period in culture (71). This substantial increase in intracellular Aβ suggests that intracellular Aβ accumulation may be more important than previously thought in AD pathogenesis. A recent study showed that human neurons in AD-vulnerable brain regions contain Aβ42 immunoreactivity which appears to precede both neurofibrillary tangles and Aβ plaque formation (73), suggesting that intracellular Aβ42 accumulation may be an early pathogenic event.

3.6. FAD Mutations and the b-secretase

The early discovery of mutations within βAPP lent support to those who believed the accumulation of Aβ was a crucial event in AD pathogenesis because it linked, for the first time, mutations in βAPP, with early onset forms of AD. All βAPP mutations studied in transfected or primary cells led to an increase of Aβ secretion, particularly of Aβ42 (74, 75). Strikingly, Aβ levels within the plasma of families harboring these mutations are significantly raised well before AD symptoms manifest (10). For this reason, there is little doubt that these mutations cause AD, in these patients, by providing an enhanced cleavage site upon which the proteolytic secretases can act more quickly or efficiently. Of all the known genetic causes of FAD, however, βAPP missense mutations at or near the sites of endoproteolysis are a relatively rare cause of familial AD. Nevertheless, the belief that FAD and sporadic AD have a similar amyloid-dependent pathogenesis suggested that discovering a secretase, would provide a single therapeutic target for all forms of AD. The recent cloning and characterization of a transmembrane aspartyl protease with β-secretase activity (76, 77) named BACE (for beta-site APP-cleaving enzyme) finally achieved this goal.

Overexpression of BACE increased β-secretase cleavage products, Aβ1-42 and Aβ11-40/42 (78). BACE was found to be expressed in higher levels in neurons than in glial cells, supporting neurons as the primary source of extracellular Aβ. BACE's active site is lumenal which agrees with the topological orientation of the β-secretase cleavage site in βAPP. BACE was maximally active at an

acidic pH and was localized within acidic intracellular compartments of the secretory pathway, primarily the Golgi and the endosomes. Whether or not other enzymes with β-secretase activity exist as well is still uncertain. Inhibitors of this characterized aspartyl protease with β-secretase activity could provide a novel approach to treating against AD.

3.7. Role of Presenilin in Ab Generation and AD Pathogenesis

Although βAPP and BACE are integral to AD pathogenesis, mutations in two genes actually cause disease in the majority of known FAD pedigrees: the *presenilin 1* (PS1) gene on chromosome 14 (7) and the *presenilin 2* (PS2) gene on chromosome 1 (8). These genes encode polytopic membrane proteins with high homology to one another. The functions of these proteins are not well understood, but clues have been provided. Homologues of the PS proteins occur in *Caenorhabditis elegans*. Mutations in the *C. elegans* Spe-4 gene lead to deficits in spermatogenesis by disrupting protein trafficking in the Golgi (79), and mutations in the Sel-12 gene can disrupt a crucial intracellular Notch signaling mechanism (80). Notch, a protein involved in regulating cell-fate decisions and development within the nervous system initiates proper signaling pathways after being cleaved within a transmembrane domain. This processing event closely parallels the γ-secretase cleavage predicted to occur within the transmembrane domain of βAPP. Interestingly, wild-type PS1 can rescue sel-12 mutants (81), further underscoring their functional similarities. Finally, PS1 knockout mice exhibit developmental deficits similar to those observed in mice in which other components of the Notch signaling pathway have been mutated or knocked out (82). These genetic interactions between Notch and PS1 homologues suggested that PS1 may be involved in the Notch signaling pathways.

While examining the effects of PS1 deficiency on Notch processing, it was demonstrated that the release of the Notch intracellular domain was reduced in neurons derived from PS1 knockout mice (83). It was also determined that γ-secretase inhibitors blocked the parallel step in Notch processing, suggesting that a similar proteolytic activity was cleaving within the transmembrane domain of both Notch and βAPP. It was demonstrated that loss-of-function mutations in the Drosophila PS1 gene cause lethal Notch-like phenotypes (84). These recent discoveries that PS1 may play similar roles in both Notch and βAPP processing may lead to a more detailed understanding of the mechanisms behind PS1's involvement in βAPP processing. Unfortunately, it also suggests that targeting the γ-secretase activity for AD treatment may cause unwanted side effects by inhibiting important pathways mediated by Notch signaling.

Emphasis has been placed on studying the presenilins because by understanding their functions, we may gain insight into the role that βAPP plays, both as a normally functioning protein and as a disease protein within AD pathogenesis. While the presenilin molecules appear to influence βAPP metabolism, a direct molecular interaction between the two molecules, while proposed (85), has been difficult to convincingly prove (86), keeping elusive the answer of how that influence is achieved on a molecular level. Despite an unclear mechanism, the downstream effects of presenilin mutations on βAPP metabolism have been extensively documented. Plasma from individuals with PS1 mutations provided the first evidence of this link when it was shown that their Aβ42 levels were significantly elevated (10). Similarly, brains and fibroblasts from these patients all demonstrate this same significant elevation (10, 87). When transgenic animals, or cultured cells bearing mutated PS genes, were analyzed and compared with animals or cells bearing wild type PS genes, the mutant counterparts all produced a consistent reinforcing result. Expressing mutant PS1 effects βAPP metabolism which results in significantly elevated secretion of Aβ42 relative to Aβ40 (9, 16). These results provided powerful support for placement of the presenilin proteins within the framework of the amyloid cascade hypothesis. Mutations in the presenilin proteins could now join Down's syndrome and βAPP-linked familial AD as directly causing AD pathogenesis through an increase in Aβ generation.

The subcellular localization of PS1 regulation of βAPP processing, has been established using subcellular fractionation studies which suggested that the presence of CTFs was significantly increased in both the ER and the Golgi fractions of fibroblasts from PS1 knockout mice (88). Analysis of these fractions supported results from another study showing that Aβ42 was the major species in the ER and that a mutant PS1 strain significantly increased intracellular Aβ42 levels (69).

The hypothesis that PS1 can regulate βAPP metabolism received perhaps its most validating experimental evidence from a study examining Aβ generation in cells cultured from the hippocampus of PS1 knockout mice and infected with a recombinant Semliki Forest virus encoding human βAPP (89). These cells secreted 80% less Aβ (both Aβ40 and Aβ42) than did control cells which had been infected with the same virus. The authors speculate that PS1 may actually activate the enzyme (γ -secretase) which gives rise to the C-terminal cleavage of the Aβ peptide. This model has precedence: the SREBP-(Sterol-regulatory element binding protein) cleavage activating protein (SCAP) was demonstrated to facilitate what is suspected to be intramembranous cleavage of SREBP (90) - an event which may closely parallel γ-secretases intramembranous cleavage of βAPP.

A recent study demonstrated that mutations in PS1's two conserved transmembrane asparate residues, Asp 257 and Asp 385, significantly reduced Aβ generation and enhanced the carboxy-terminal fragments of βAPP, normal substrates of γ-secretase (91). These effects, observed in three different cell lines, indicate that these two asparate residues may be necessary for both PS1 endoproteolysis and γ-secretase activity. This led the authors to propose that PS1 is either a diaspartyl cofactor for γ-secretase or the

γ-secretase itself, making it an autoactivated intramembranous aspartyl protease.

While that possibility remains intriguing, PS1 may play an alternative role in the sorting or trafficking of βAPP. Recent studies examined the trafficking and metabolism of both βAPP and TrkB in neurons that lacked PS1 (92). It was demonstrated that these neurons did not secrete Aβ and had a significantly increased rate of sAPPα secretion. In addition, the accumulation of the carboxyl-terminal fragments (CTFs) derived from βAPP in these PS1 deficient neurons suggested that PS1 may play a role in promoting intramembrane cleavage or degradation of CTFs. It was also observed that the maturation of TrkB and the BDNF-inducible TrkB autophosphorylation are altered by PS1's absence. The combination of these results suggests that PS1 plays an essential role in regulating trafficking and metabolism of proteins involved in βAPP processing. This hypothesis suggests that mutations in PS1 may retard the normal sorting or trafficking of βAPP at an early stage within the secretory pathway. If normal PS1 is a sorting or trafficking molecule responsible for ensuring βAPP's timely and properly directed exit from the ER towards the Golgi, knocking out PS1 may cause βAPP to lag within the ER, resulting in elevated levels of Aβ42. Alternatively, this may cause βAPP to enter the anterograde bulk protein trafficking pathway through the Golgi and to the cell surface, resulting in extremely reduced levels of both Aβ42 and Aβ40 due to the limited exposure of βAPP to the secretases in the ER/Golgi. This hypothesis is exciting because it may not only account for the effects of PS1 mutations or knocking out PS1 entirely, on βAPP metabolism, but it may also account for the lethality of PS1 knockouts. In addition, the retardation of the proper sorting and trafficking of membrane proteins to cell surface during development may aid in explaining the phenotype of mutations in PS1 homologues in *C. elegans*.

3.8. Signal Transduction Regulates bAPP Trafficking

Although the evidence that the mutated presenilin proteins may cause AD by altering βAPP metabolism is relatively recent, the idea that βAPP metabolism is a highly regulated phenomenon, is not novel. For example, it has been known for a decade that adding phorbol esters to cultured cells leads to a stimulation of the α-secretase pathway, increasing βAPPs secretion and C-terminal fragment generation within the Aβ domain, and decreasing secreted Aβ. Studies demonstrating this (43-45) provided evidence that the routing of βAPP through the α-secretase pathway was enhanced by protein phosphorylation through activation of protein kinase C (PKC). It was demonstrated that PKC can phosphorylate serine 655 of βAPP, both on synthetic peptides containing this phospho-domain (35), and utilizing *in vitro* assays using permeabilized PC12 cells (93).

These results were compelling because they suggested that PKC may act to directly phosphorylate βAPP, perhaps in response to direct depolarization of the neuron itself. In support of this hypothesis, it was demonstrated that when muscarinic acetylcholine receptors were transfected into PC12 cells, receptor agonists led to the same increase in βAPPs, but could not elicit that response when staurosporine was present simultaneously (94). Alternatively, it was possible that PKC, although able to directly phosphorylate βAPP, actually exerted its effects on βAPP metabolism by phosphorylating an entirely different target such as the α-secretase or other phospho-proteins. This latter hypothesis revealed itself to be true following the demonstration that PKC can stimulate βAPPs secretion even when the phosphorylation sites on βAPP are themselves mutated, or when the entire cytoplasmic domain has been deleted (95). So although it was clear that PKC played a crucial role in βAPP metabolism, alternative hypotheses were needed to explain PKC's potent anti-amyloidogenic effects.

One mechanistic explanation is that PKC phosphorylates a TGN phosphoprotein resulting in the redistribution of βAPP from the TGN to the cell surface. The subcellular localization of βAPP within the TGN and α-secretase in the plasma membrane support this hypothesis, confirmed in studies in which the formation of βAPP-containing vesicles was reconstituted from the TGN in a cell-free system (93, 96). In these studies it was demonstrated that PKC treatment results in a redistribution of βAPP from the TGN to post-TGN compartments where it can undergo α-secretase processing. Evidence was also provided that protein kinase A (PKA) can similarly reduce Aβ formation and stimulate the release of constitutive secretory proteins from the TGN via a mechanism involving enhanced formation of post-TGN transport vesicles (96). Although PKC and PKA converge on the level of formation from the TGN of βAPP-containing vesicles, additional evidence indicates that the regulatory mechanisms involved are distinct; the effects of PKC and PKA are additive, suggestive of independent substrate sites. Recently processing events that underlie βAPP metabolism have been examined in primary rodent neuronal cultures (76). This study showed that neurons generate abundant Aβ peptide variants, especially Aβ Glut11 peptides, and demonstrated the role of protein phosphatases in regulating Aβ generation in neurons.

One alternative hypothesis, which may help explain PKC's effect on α-secretase cleavage, involves the multicatalytic complex of the proteasome. It was demonstrated that proteasome inhibitors can drastically decrease PDBu-stimulated βAPPα secretion and may play a role in the basal secretion levels of βAPPs (97). Unfortunately from a therapeutic standpoint, PKC activation can stimulate βAPP transcription, indirectly increasing Aβ formation; therefore, it remains an important goal to definitively identify phosphorylation targets for the kinases if we are to have a realistic hope of therapeutically intervening in AD pathogenesis via stimulation of this anti-amyloidogenic pathway. In addition, calcium, a cofactor for the activation of PKC and tyrosine kinases, may play an important role in the pathogenesis of AD. Calcium's effect on APP processing has been examined by increasing the ion's intracellular levels through upregulating its release from internal storage compartments or through the use of

ionophores. Calcium's role in the regulation of βAPP processing was first examined in studies demonstrating that thapsigargin, an inhibitor of calcium reuptake in the ER, increased sβAPPα release and reduced Aβ generation (98). This effect was independent of PKC activation suggesting the presence of an alternative pathway.

3.9. Hormone Regulation of bAPP Metabolism

Increasing epidemiological evidence has suggested that post-menopausal women receiving estrogen replacement therapy have both a delayed onset and reduced risk for developing AD (99, 100). The central role of Aβ in AD pathogenesis together with the indication that estrogen replacement therapy may prevent AD, suggested to us that one mechanism of action of estrogen in the brain might be to modulate βAPP metabolism and Aβ generation. A recent study reported that physiological concentrations of estrogen reduce neuronal Aβ generation (101) which is in agreement with prior data demonstrating that estrogen increased secretion of βAPPs (102). Importantly, these changes were not due to changes in βAPP transcription, or to alteration of PS1 levels. These data suggest one compelling mechanism through which estrogen may protect against AD.

The anti-degenerative effects of estrogen have received increased attention in recent years. In addition to its anti-amyloidogenic properties, estrogen may also modulate (i) basal forebrain cholinergic activity and integrity (103), (ii) dendritic plasticity (104), (iii) NMDA receptor density (105), and (iv) neurotrophin signaling (106), as well as play a role in the prevention of oxidative toxicity due to glutamate, free radicals and Aβ (107). Although it plays many roles in the brain, the Aβ-reducing effect of estrogen strongly supports the notion that one relevant neuropharmacological activity of estrogen is to reduce Aβ generation, and that such an activity contributes to the ability of estrogen replacement therapy to protect against AD.

The cell biological basis for estrogen's regulation of Aβ formation remains to be elucidated. It has been demonstrated that estrogen can affect the morphology of the trans-Golgi network by enhancing the biogenesis and trafficking of post-TGN constitutive secretory vesicles and granules (108, 109). Since formation of Aβ in the TGN and its export from that organelle are regulated by signal transduction pathways, we hypothesize that estrogen promotes the egress of βAPP from the TGN thereby reducing the local concentration of βAPP available as a substrate for Aβ production.

The finding that estrogen reduced the generation of Aβ peptides raised the question of whether these effects were specific for estrogen or if other estrogen-like steroid molecules would have similar effects on neuronal Aβ secretion. It appears that neuronal cultures treated with testosterone show a similar increase in the secretion of the non-amyloidogenic βAPP fragment, sAPPα and decrease in the secretion of Aβ (110). Although clinical studies on testosterone supplementation have not been conducted to date, these results may suggest that testosterone may have a therapeutic benefit in protecting against the development of AD in elderly men, similar to estrogen's efficacy in protecting post-menopausal women against AD.

Levels of adrenal glucocorticoid hormones are known to increase with age, especially in individuals with AD (111, 112). However, treating neurons with corticosterone does not effect sβAPPα or Aβ secretion (110) suggesting that estrogen's and testosterone's ability to reduce Aβ secretion is not characteristic of all steroid hormones. In addition, cholesterol's influence on βAPP metabolism has been studied by several groups. Increasing cholesterol levels in cellular membranes resulted in a decreased secretion of sβAPPα (113, 114). Interestingly, reducing cellular cholesterol using mevalonate can decrease Aβ generation in cultured hippocampal neurons (115), while increasing cholesterol levels by direct treatment of cultured neurons leads to an increase in Aβ secretion (110). These results, demonstrating the complex influences of estrogen, testosterone and cholesterol on Aβ generation, highlight the inherent difficulty in studying the molecular and cellular basis of βAPP metabolism and underscore the challenge we face in trying to intercede in this pathologically crucial event.

4. PERSPECTIVE

The last decade has provided tremendous insight into the molecular mechanisms that are responsible for βAPP metabolism. In particular, several signal transduction pathways have been identified which can regulate βAPP metabolism to reduce Aβ generation. A major goal, however, remains the elucidation of compounds that, while effective at reducing Aβ generation, are therapeutically safe. Reducing the amyloid burden in the brain, and therefore the progression of AD, may eventually prove analogous to lowering one's cholesterol to prevent heart disease. As scientists, we are close to fully understanding the molecular basis for βAPP metabolism and Aβ generation. However, that will fulfill only half our mission; as healers we have much work to do before we can offer AD patients realistic hope that their disease will become anything other than an irreversible decline into dementia.

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