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Therapeutic Targeting of the Endoplasmic Reticulum in Alzheimer's Disease

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Abstract

The extensive prevalence of Alzheimer's disease (AD) places a tremendous burden physiologically, socially and economically upon those directly suffering and those caring for sufferers themselves. Considering the steady increases in numbers of patients diagnosed with Alzheimer's, the number of effective pharmacotherapeutic strategies to tackle the disease is still relatively few. As with many other neurodegenerative mechanisms, AD, is characterized by the continued presence and accumulation of cytotoxic protein aggregates, *i.e.* of beta-amyloid and the microtubule associated protein, tau. Therefore, one novel therapeutic avenue for the treatment of AD may be the actual targeting of factors that control protein synthesis, packaging and degradation. One of the prime cellular targets that, if effectively modulated, could accomplish this is the endoplasmic reticulum (ER). The ER can not only control cellular protein synthesis, trafficking and degradation but it is also closely associated with cytoprotective mechanisms, including calcium ion regulation and unfolded protein responses. This review will delineate some of the most important functional physiological features of the ER that, if effectively modulated, could result in beneficial amelioration or remediation of the negative cellular aspects of AD initiation and progression. While not a classical drug target, even with minimal levels of beneficial modulation, its multifactorial efficacy may amplify small effects resulting in significant therapeutic efficacy.

Keywords

Endoplasmic; reticulum; Alzheimer's disease; amyloid; stress; calcium; protein synthesis; folding

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CONFLICT OF INTEREST

None

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INTRODUCTION

Approximately 35 million people worldwide suffer from Alzheimer's disease (AD), the most common form of dementia, often leading to death within 3 to 9 years of diagnosis. There are 1275 new cases per year per 100,000 persons over the age of 65, signifying a doubling of the disease incidence every five years [1, 2]. The prevailing hypothesis for the cause of cognitive decline is thought to stem from an increase in soluble amyloid beta (A β) oligomers [3] and hyperphosphorylated tau, which coalesces into soluble, paired, helical filaments [4]. A β production is regulated by beta and gamma secretase proteolytic processing of the amyloid precursor protein (APP). Over the past decade several therapeutic strategies, borne out of the amyloidogenic hypothesis of Alzheimer's, have been developed that directly address A β aggregation [5], production [6] or clearance [7]. Although several of these strategies demonstrably reduced A β -linked pathology, a more complete and effective strategy for treating Alzheimer's disease remains to be identified.

Alzheimer's disease is a highly complex multifactorial disorder that impacts numerous biochemical pathways, cellular functions and neurological circuits. Therefore, each step of the pathology-physiology interface represents a new potential therapeutic target [8, 9]. Furthermore, investigation of such pathology-physiology interfaces might also promote identification of novel targets for ameliorating other long-term, aging-related, disease progressions, *e.g.* Parkinson's or Huntington's disease. In this review we will consider the neuronal endoplasmic reticulum (ER), a typically overlooked cellular organelle (in this context), as a possible drug target for disrupting the development of an A β -ER pathophysiology interface.

The neuronal endoplasmic reticular system is a unique and potentially profitable drug design target, because it is integral to many cellular systems disrupted in AD. Disruptions including aberrancies in stress responses, calcium buffering, protein synthesis and transport, and apoptotic signaling have been identified. Accordingly, a therapeutic targeted at ER function could exert diverse effects that would synergistically benefit cell function. In this review, we delineate the role of the endoplasmic reticular system in normal and pathological physiology of the CNS. We also discuss various strategies for therapeutically disrupting the formation of an A β -driven pathology-physiology interface.

ER AND NEURONAL FUNCTION

The endoplasmic reticulum (ER) is an intracellular organelle common to both neuronal and non-neuronal cells alike. Like other organelles, the ER represents a compartmentalized microenvironment. Proper function of the ER is essential to protein synthesis and folding, subcellular protein trafficking and dynamic calcium homeostasis in all cells. However, ER regulation of calcium concentrations within neurons is even more critical, as it is required for synaptic transmission. Whereas reduced pre-synaptic calcium levels impair neurotransmitter release, reduced post-synaptic calcium levels hinder intracellular signaling. Conversely, excess calcium could facilitate greater release of neurotransmitters, which, at glutamatergic synapses, would prompt excitotoxic initiation of apoptotic pathways. Although the aforementioned detrimental consequences of unregulated calcium

concentrations are somewhat obvious, it is also worth noting that the ER compartment promotes signal transduction efficiency, and offers additional control over gene transcription [10] and mRNA translation. In fact, the importance of the ER to neuronal cell function is so crucial that the ER can be found not only in the soma, but also in dendritic spines.

CALCIUM HOMEOSTASIS

As described earlier, controlling intracellular concentrations of calcium is required for proper synaptic function and vital for neurological health. The ER represents a calcium store that sequesters up to 250 μ M calcium, while the cytosol maintains a calcium concentration in the nanomolar range. Calcium derived from ER stores is critically involved in the close regulation of gene transcription [10], activation of calcium sensitive currents [11–13] and synaptic plasticity modulation [14–16].

Moderation of ER calcium is regulated by three receptors; the inositol triphosphate receptors (IP₃R) and ryanodine receptors (RyaR) which control calcium release from the ER, while the sarcoplasmic/endoplasmic reticulum calcium AT-Pase (SERCA) channels facilitate calcium re-uptake into the ER. The IP₃R is activated by extracellular ligand binding to and activating G α q-, and to a lesser extent [17] G α i-protein coupled receptors located on the plasma membrane. These G α q proteins in turn activate phospholipase C which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) into two second messengers: IP₃ and diacylglycerol (DAG). IP₃ then activates the IP₃R, facilitating calcium release from the ER. The IP₃R is also modulated by cytosolic calcium concentrations [Ca²⁺_c]. Low [Ca²⁺_c] promotes IP₃R channel opening, while higher [Ca²⁺_c] suppresses IP₃R channel opening, thus representing a local negative feedback loop. Whereas the IP₃R is modulated by both cytosolic calcium and the second messengers IP₃ and DAG, the RyaR is considered a calciumgated ion channel, as its activity is mediated solely by [Ca²⁺_c]. The effect of [Ca²⁺_c] on the RyaR is the same as that of [Ca²⁺_c] on the IP₃R (*i.e.* opening at low [Ca²⁺_c] and closing at high [Ca²⁺_c]). Calcium released from the ER, by IP₃R and RyaR, is recaptured by SERCA channels, which continuously pump calcium, in an ATP-dependent manner, from the cytosol back into the ER. Thus, SERCA channel activity is essential to maintaining the calcium concentration gradient that exists across the lipid bi-layer that serves as the ER microenvironment perimeter.

PROTEIN SYNTHESIS

In addition to its role in calcium homeostasis, the ER (via resident ribosomes) functions in the translation of mRNA into proteins and provides an environment that fosters proper folding of proteins, through chaperone-protein interactions. To facilitate protein folding, the ER maintains an oxidizing environment that promotes formation of secondary and tertiary protein structures. When proteins are misfolded, ER resident chaperones are enlisted to assist in refolding.

The ER contains a host of chaperone factors. The most common (or most abundant) of which are: protein disulfide isomerase (PDI), glucose regulated protein (grp) 78 (also known as Bip), grp 94, calnexin and calreticulin. The role of folding chaperones is considerable, given that approximately 30% of newly synthesized proteins are misfolded. Such misfolding

events equate to alterations in protein-protein interactions, structural flexibility or ablation of catalytic activity. Without remediation of protein folding by chaperones, protein function would be compromised. Delivery of these proteins to their intended target location would result in multiple and systemic reductions in cellular signal transduction efficiency. Eventually, these local malfunctions in pathway signaling would manifest into a pathophysiological state. To avoid this homeostatic catastrophe, the ER follows a stringent quality control policy that targets proteins, incapable of being properly folded, for lysosomal degradation. This process has been appropriately termed ER associated protein degradation (ERAD) [18]. ERAD is a multistep process that identifies and transports misfolded proteins, in a retrograde fashion, back to the cytosol for lysosomal degradation. Although ERAD is designed to prevent pathways from incorporating malformed proteins into their functional framework, and therefore interrupt cellular functions, routine misfolding and inadequate or delayed delivery of functional proteins can clog ER machinery. Overloading ER machinery can have both local and distal consequences. It impairs the synthesis and trafficking of new proteins, while simultaneously disrupting normal homeostatic turnover of resident and nonresident ER proteins. In short order this causes both ER and cellular stress. The obvious consequence of this stress is manifestation of a pathophysiological state. Not surprisingly, many neurological disorders are believed to take root in a homeostatically imbalanced ER, in which the ER stress response is sufficient to disrupt cell function so severely as to prompt apoptosis. Naturally, when this paradigm becomes prevalent in multiple neurons and neural circuits, neurological degeneration will result.

ER RESPONSES TO PATHOPHYSIOLOGY

ER Stress Response Systems

The ER stress response is governed by the unfolded protein response (UPR) signaling cascade, which is triggered by binding of grp78/BiP to misfolded proteins. Grp78/BiP natively binds and inhibits the action of three ER transmembrane proteins: double-stranded RNA activated protein kinase-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) α and β and inositol requiring kinase 1 (IRE1) α and β [19, 20]. However, when misfolded proteins cause grp78/BiP to dissociate from PERK, ATF6 and IRE1, each of them is free to activate independent signaling pathways. The UPR signaling cascades culminate in the production of transcription factors for a variety of stress response genes. However, the intrinsic signaling cascade for each of the UPR pathways are unique and have been summarized below. During acute ER stress these pathways are activated to ensure cell survival, however, persistent stress initiates apoptotic cascades.

PERK Pathway

Under normal homeostatic conditions Bip (grp78) binds to the PERK luminal domain where it is maintained in a monomeric, inactive state. One of the first responses to ER stress is an attenuation of protein synthesis. ER stress initiates Bip dissociation from PERK, allowing PERK to homodimerize and undergo autophosphorylated activation. Active PERK phosphorylates Serine⁵¹ of eukaryotic initiation factor 2 α (eIF2 α), thus hampering its ability to initiate protein translation. Activated eIF2 α , however, initiates translation of the transcription factor ATF4. ATF4 then in turn upregulates several other ER stress target

genes, including the stress induced phosphatase growth arrest and DNA damage inducible gene 34 (GADD34). Lastly, to recover translation following cessation of acute ER stress, GADD34 dephosphorylates eIF2 α .

IRE1 Pathway

ER stress activates IRE1 via Bip-dissociation induced autophosphorylation. Once activated, IRE1 serves as both a serine/threonine kinase and an endoribonuclease. IRE endoribonuclease activity initiates an intron splicing event of the mRNA encoding X-Box-binding protein 1 (XBP-1). Removal of the 26 nucleotide intron advances expression of XBP-1. XBP-1 is a stable and potent transcription factor for many UPR genes. IRE1 has also been shown to mediate the cleavage of additional mRNAs targeted to the ER, as well as cleavage of the 28S ribosomal subunit. These concerted activities allow IRE1 to attenuate protein translation through degradation of ER targeted mRNA transcripts or ribosomal subunits that mediate translation.

ATF6 Pathway

ATF6 activity is regulated not only by physically associating with Bip, but also by intra-protein disulfide bonds that occur at the luminal interface. Unlike PERK and IRE1 α , dissociation of Bip does not facilitate autophosphorylation of ATF6 but rather reveals two Golgi localization signals. These signals facilitate ATF6 transfer to COPII vesicles and eventual translocation to the Golgi compartment for proteolytic processing by site-1 (S1P) and site-2 (S2P) proteases (S1P). Proteolytic cleavage of ATF6 releases a 50kDa leucine zipper transcription factor, which then translocates to the nucleus and binds to several ER stress response gene promoter elements.

PRO-APOPTOTIC SIGNALING IN THE ER

Unfolded Protein Response Pathway Induced Apoptosis

The unfolded protein response (UPR) is initiated by accumulation of unfolded/misfolded proteins in the ER lumen. The UPR process attempts to ameliorate stress-induced damage by cessation of protein translation and activation of protein chaperoning activity signaling pathways, the latter of which leads to increased production of molecular chaperones that are involved in protein folding. If these remediating efforts are unsuccessful at restoring ER homeostasis, the UPR reorients to initiate programmed cell death (apoptosis).

Chronic ER stress promotes apoptosis through upregulation of the C/EBP homologous protein transcription factor (CHOP), which is also known as growth arrest and DNA damage inducible gene 153 (GADD153). Importantly, upregulation of CHOP, in and of itself, is sufficient to promote expression of the Bcl-2 interacting mediator of cell death (BIM). All three ER stress-response pathways (PERK, IRE1, ATF6) are capable of CHOP activation, although, the PERK pathway seems the most widely used. Potentiation of CHOP can subsequently downregulate Bcl2 expression, depleting intracellular glutathione which results in excessive production of reactive oxygen species (ROS) [21]. CHOP also controls expression of target ER stress genes, such as GADD34 and ERO1 α .

Oligomerized IRE1 is believed to associate with TRAF2, through interactions facilitated by the c-jun NH2-terminal inhibitory kinase (JIK). This complex can recruit and activate apoptosis regulating kinase 1 (ASK1) which subsequently activates the downstream JNK pathway leading to a pro-apoptotic state. TRAF2 itself has been linked to procaspase activation, which serves to initiate apoptosis. In fact, this specific pathway appears to be partly responsible for the apoptotic-based neuronal loss associated with AD. As evidence of this, A β peptide activates caspase 12 directly. This pathway is likely specific for the A β peptide, since cortical neurons deficient in caspase 12 are resistant to A β -induced apoptosis; however, they remain susceptible to apoptosis induced by staurosporine or trophic factor deprivation [22].

MITOCHONDRIA-INDUCED APOPTOSIS IN CHRONIC ER STRESS

ER stress-induced apoptotic signaling can be further exacerbated by mitochondrial membrane permeabilization. Mitochondrial potentiation of the pro-apoptotic response is derived from activation of caspases by the Bcl-2 family of proteins. Bcl-2 proteins are divided into three groups: anti-apoptotic, pro-apoptotic and pro-death. The proteins are categorized according to the presence or absence of four sequence homology domains, BH1, BH2, BH3 and BH4. Whereas anti-apoptotic Bcl-2 family members contain all four BH domains, pro-apoptotic members lack the BH4 domain, and the pro-death proteins contain only the BH3 domain, in a single α helical loop. Bcl-2 anti-apoptotic proteins bind to and sequester pro-apoptotic proteins at the ER membrane thereby reducing cell death signaling [23, 24]. Ng *et al.* [25] reported that Bcl-2 and Bcl-XL associate with the pro-apoptotic protein Bap31 and pro-caspase 8 at the ER membrane, preventing caspase cleavage and Bap31 activation.

PUMA – p53-Upregulated Modulator of Apoptosis

PUMA and NOXA are thought to be the first pro-apoptotic family members to be transcriptionally-activated during prolonged ER stress. Repression of these genes engenders partial resistance to ER-induced apoptosis [26–28]. Cells lacking BIM have also been shown to be resistant to prolonged ER stress. Under chronic ER stress, BIM activates ER membrane caspases following its translocation there via the dynein motor complex of the microtubule cytoskeleton. Prolonged ER stress also promotes the dephosphorylation of BIM by phosphatase 2A which prevents its ubiquitination and degradation [29, 30].

Although the exact signal responsible for initiating mitochondrial-related apoptosis is unknown, downstream signaling events have been studied in great detail. Chronic ER stress either activates, transcriptionally upregulates or post translationally-modifies BH3-only proteins which in turn binds one of the multi-domain pro-apoptotic proteins BAX or BAK to induce its homo-oligomerization in the outer mitochondrial membrane [31, 32]. This mitochondrial membrane alteration allows for the release of pro-apoptotic mitochondrial matrix proteins (cytochrome c, SMAC/Diablo) which can bind downstream effector proteins, leading to caspase 3 activation and subsequent cell death [33]. BH3-only proteins can be grouped according to their functional roles in apoptosis as either, *i*) sensitizers which facilitate apoptosis by binding to and antagonizing Bcl-2 anti-apoptotic proteins thereby

releasing BH3-only proteins, or *ii*) activators that directly activate BAX or BAK to release mitochondrial matrix proteins.

CALCIUM SIGNALING-INDUCED APOPTOSIS

Calcium acts as a functional cofactor for multiple cytoplasmic proteases. Therefore, perturbations in Ca^{2+} homeostasis are strongly associated with initiation of the ER stress-response. Activation of calpain, a calcium-dependent protease, highlights this causal interaction through its cleavage and activation of several caspases, most notably caspase 12. Additional pro-apoptotic factors are also tightly regulated by ambient calcium levels. For example, dephosphorylation of calcineurin, which is a calcium/calmodulin dependent phosphatase [22], promotes activation of BAD.

It is also worth noting that, when stressed, the ER extends its stress response to other aspects of cellular function by secreting excess calcium into the cytosol. In fact, pathologically-elevated calcium influxes are known, in a phospholipase-A2-dependent manner, to raise ROS levels and prompt calcium release from the ER. This ER-mediated calcium release then drives calcium into the mitochondria, elevating calcium concentrations within that compartment, thus demonstrating a close functional relationship between the ER and the mitochondria. The mitochondria try to restore homeostasis, and prevent calcium toxicity, by taking up excess calcium. Unfortunately, mitochondria are also inherently sensitive to calcium. Thus excessive calcium within mitochondria increases expression of permeability transition pores (PTP); that attempt to alleviate excess calcium concentrations within the mitochondrial matrix. Despite the objective of PTP expression, to relieve toxic calcium levels, their insertion into mitochondrial membranes facilitates release of cytochrome c, which induces apoptosis.

In summary, dysregulation of calcium homeostasis, whether locally within the ER or throughout the cell, negatively impacts cell function and potentially triggers apoptosis. Consequently, it seems plausible that buffering ER calcium, pharmacologically or genetically, could prove beneficial to cells subjected to chronic ER stress, *i.e.* specifically for the form of chronic stress that is coincident with neurodegenerative disorders like Alzheimer's, Parkinson's and Huntington's disease.

ALZHEIMER'S DISEASE AND ER FUNCTION

Many of the age-related cognitive deficits affiliated with Alzheimer's disease (AD) are believed to stem from increased levels of soluble A β oligomers [3] and soluble paired helical filaments, comprised of hyperphosphorylated tau [4]. A β is produced by proteolytic cleavage of amyloid precursor protein (APP) by beta and gamma secretase, which are transmembrane proteases. A third APP-processing enzyme, alpha secretase, cleaves within the A β sequence- yielding a non-amyloidogenic product of APP processing. Nonetheless, beta and gamma secretase production of A β monomers (*in vivo*) favors the formation of A β dimers, which subsequently oligomerize and forward the neurodegenerative effects that are concomitant with AD.

The essential role of beta and gamma secretases in A β synthesis qualifies them, most appropriately, as targets for pharmacological intervention. However, design of therapeutics aimed at interfering with beta secretase function has been hampered by difficulty in accessing the proteolytic domain of the enzyme. On the other hand, gamma secretase is a multifaceted enzyme complex that consists of four proteins: presenilin (PS), nicastrin, anterior pharynx defective-1 (APH-1) and PEN2; that cooperate to cleave APP. Thus gamma secretase represents not only one, but numerous therapeutic targets for interfering with A β synthesis. However, recent studies have shown that gamma secretase inhibitors actually aggravate the cognitive impairment exhibited by AD patients [34]. Further diminishing hopes that disruption of A β synthesis might represent a viable treatment for AD.

The majority of AD cases reported are the result of late-onset sporadic AD (SAD), brought about by a variety of environmental factors and perhaps, as of yet unknown, genetic predispositions. SAD is typified by the presentation of symptoms beyond age 65. Conversely, individuals diagnosed with familial AD (FAD) exhibit AD symptoms well before age 65. This “early onset” form of AD is commonly the result of inherited genetic factors that demonstrate an autosomal pattern of inheritance. Mutations within 3 genes; amyloid precursor protein (APP), presenilin-1 (PS-1) and presenilin-2 (PS-2); are the most common risk factors for developing FAD. However, apolipoprotein E4 (ApoE4) is another genetic risk factor strongly associated with AD. In fact, individuals carrying more than one ApoE4 allele have a 30-times higher risk of developing AD later in life. Although, unlike APP/PS-1/2 mediated FAD, ApoE4-associated AD usually occurs late in life, *i.e.* over the age of 65 [35, 36].

ER STRESS AND FAMILIAL ALZHEIMER’S DISEASE

Presenilins in ER Stress

PS-1 is an integral ER membrane protein that contributes to the gamma secretase complex. PS-1 has also been shown to interact with the unfolded protein response (UPR) factors PERK, IRE1 α and ATF6. Surprisingly though, alterations to PS-1 expression does not preclude these proteins from participating in the ER stress response system. Indeed, PS-1/2 knockout experiments have shown (*in vitro*) that there is no perturbation in the intrinsic ER stress response system. In contrast, whereas PS-1 protein expression levels do not impact the ER stress response system, mutations to the PS-1 gene (found in AD) disrupt the actions of PERK, IRE1 α and ATF6. Several groups have characterized such interactions. Ye and Connor [37] demonstrated that IRE1 α is unable to undergo autophosphorylation-mediated activation during ER stress in cells expressing mutant PS-1. Additionally, Katayama *et al.* [38] discovered that, during ER stress, ATF6 translocation from the ER to the Golgi is retarded by the presence of mutant PS-1. Mutant PS-1-expressing cells also demonstrated reduced Bip expression and were unable to survive an ER-induced stress response [39].

Mutant PS-1 has also been shown to perturb PERK function by reducing Bip transcription [39]. An interesting correlate is that Bip expression is severely attenuated in the brains of AD patients, as compared to healthy control subjects, suggesting that decreased Bip expression levels are involved in the etiology of AD. This raises the possibility that therapeutically elevating Bip expression would alleviate or retard the development of AD.

Support for such an approach comes from a recent investigation by Kudo [40] that revealed a decrease in ER-stress derived apoptosis, following upregulation of Bip, gpr94, calreticulin and CHOP mRNA. The discovery was made with Bip inducer x (BIX)-treated mice. Thus BIX has been identified as a potential ER-targeted therapeutic for AD-related apoptosis.

The PS-1 mutation can also affect ER calcium homeostasis by increasing calcium release through an unknown mechanism. It seems plausible, however, that this action involves co-localization (and interaction) of PS-1 with SERCA, IP₃R, RyaR or calreticulin within the ER. Supporting this posit, increases in RyaR expression have been observed in primary hippocampal neurons expressing mutant PS-1 [41]. Increased RyaR expression was coincident with enhanced ER calcium release, which was measured during blockade of both IP₃Rs and SERCAs. These results provide strong evidence for a direct link between PS-1 mutations and RyaRs that would contribute to an imbalance of ER-calcium homeostasis [41].

However, several reports have offered less convincing evidence for a direct connection between the PS-1 mutation and dysregulation of ER calcium homeostasis. Chan *et al.* [41] observed that enhanced calcium release from the ER of PS-1 mutation hippocampal cells was due to increased activity of SERCA pumps. Chan *et al.* [41] proposed that the increased ER calcium load allowed for enhanced calcium release upon activation of IP₃R, and subsequently RyaRs. Similar results were garnered from the experiments of Ferreiro *et al.* [42], who demonstrated that cortical neuronal cells exposed to A β peptides exhibit a lower threshold for apoptosis. Triggering of apoptosis appeared to be initiated by elevated cytosolic calcium that was derived from ER calcium stores, which were forwarded to the cytosol, following increased RyaR and IP₃R activity [42]. As a whole, it seems that disruption of ER calcium homeostasis is more attributable to increased A β production, than it is to a direct effect of mutant PS-1 on ER calcium channels [41].

PS-1 activity can also be modulated by ER ATF4 activity. ATF4 can bind to the regulatory region of human PS-1 gene and ATF4 knockdown experiments have demonstrated that the A β ₄₂ isoform and gamma secretase cofactors are modulated by this transcription factor [43]. The ATF4 component of the ER stress response system may therefore be a potential target for Alzheimer's disease.

AMYLOID PRECURSOR PROTEIN IN ER STRESS

Eight isoforms of APP, ranging from 677 to 770 amino acids, are produced through alternate splicing of APP exons 7, 8 and 15. APP₆₉₅, APP₇₅₁ and APP₇₇₀ are the most abundant isoforms; with variant 695 being the most prevalent in neurons [44–46]. Although the presence of 750 and 751 isoforms is less abundant, secretase enzyme specificity appears to be comparable for all variants and all have the potential to be amyloidogenic. APP is transported to the ER, following protein synthesis, where it undergoes N-glycosylation before being transported to the Golgi for both N- and O-glycosylation to eventually form mature APP [47–49].

Altered energy metabolism has become recognized as a hallmark of AD. Not surprisingly, AD-related metabolic changes are often associated with mitochondrial degeneration.

Distressed energy metabolism has been shown to pathologically impact APP processing within the ER and Golgi complex [50]. Experimental disruption of mitochondrial metabolism leads to generation of an 11.5kDa carboxyl-terminal derivative of APP, which contains the full-length A β sequence. This 11.5kDa peptide is produced either in the Golgi or the ER under normal homeostatic conditions, however disruption of cellular metabolism significantly promotes its production. Cells experiencing irregular mitochondrial metabolism will retain both APP₆₉₅ and APP₇₅₁ species within the ER and prevent their translocation to the plasma membrane [51]. Retention of APP within the ER or Golgi facilitates the generation of the A β -like 11.5kDa amyloidogenic peptide by gamma and beta secretase enzymes. Decreased concentrations of neurotrophic sAPP α fragments have also been reported when cells are subjected to metabolic disruption; presumably due to decreased availability of full-length mature APP at the plasma membrane, which serves as a substrate for alpha secretase cleavage.

Contrasting evidence of the effects of ER stress upon APP processing have also been presented, underscoring the complexity of the ER stress response system in AD. For example, tunicamycin-induced ER stress actually reduces A β production [40]. This action has been attributed to the retrograde transport of APP from the Golgi to the ER, by Bip. There, in the ER, APP is presumably protected from amyloidogenic processing [52]. Interestingly, the majority of APP that was immunoprecipitated with Bip was immature, indicating that retrograde transport of APP may be part of the UPR system. Further interest in the role of Bip stems from the realization that significant reductions in Bip expression have been observed in the brains of AD patients; suggesting that loss of the UPR complex marks the initiation of neuronal degeneration. This raises the possibility that A β accumulation is merely a symptom of ER disruption- and not the root cause of the disease.

Like all proteins trafficked through the ER, both APP and tau proteins interact with ER chaperone proteins. To emphasize the importance of molecular chaperones in ER stability and the development of AD symptomology, Bcl-2 overexpression has been employed, in a murine AD model (3xTgAD), to attenuate APP and tau processing. The resultant phenotype exhibited a reduction in both the number of neurofibrillary tangles (NFTs) and extracellular A β plaques [53]. Moreover, the Bcl-2 overexpressing mice also demonstrated reduced caspase-9 activity, which normally promotes NFTs formation by processing tau into components that are more prone to aggregation. Such findings support the potential utility of therapeutics aimed at treating AD through targeting ER protein trafficking.

Therapeutic targeting of the ER could certainly come in the form of ER chaperone molecules. Such molecules would be employed to fulfill critical protein folding functions in an ER overwhelmed by the APP and tau processing workload. The prescribed facilitation of forward trafficking would, in this case, represent an effective strategy for maintaining the UPR complex in AD. Phenylbutyric acid (PBA) has demonstrated efficacy as a small molecular chaperone by protecting cells against ER stress induced apoptosis as well as promoting the non-amyloidogenic processing of APP [54]. Importantly, PBA also offers good bioavailability and readily crosses the blood-brain-barrier. These features perhaps make PBA an excellent lead candidate therapeutic for neurological disorders such as AD [55].

APP is known to modulate calcium-regulating ion channels, including L-type voltage-gated calcium channels, and exert profound effects on neuronal excitability. Overexpression of APP and mutations of APP that promote amyloidogenesis, *e.g.* APP^{swe}, effectively inhibit calcium oscillations due to a decrease in APP translocation to the plasma membrane [56]. Conversely, acute inhibition of endogenous APP expression reduces the amplitude of calcium oscillations and increases their frequency. It seems reasonable- if not obvious- then, that perturbations in energy metabolism, that alter APP processing, have a collateral effect of dampening neuronal excitability. Development of this situation, however, is dependent entirely on full length APP as APP metabolites seem to have no bearing on excitability [56].

Amongst a host of other calcium related effects, altered calcium homeostasis has also been shown to affect proteolytic processing of APP. High cytosolic calcium concentrations, present in AD, inhibit α -secretase cleavage of APP, leading to accumulation of highly toxic A β ₄₂. This particular processing mechanism of APP requires extracellular calcium entry through L-type calcium channels. However, ER calcium stores have not been implicated in this highly detrimental version of APP processing [57]. Nonetheless, abnormally high ER calcium stores, as can be found in PS-1 mutant neuronal cells, could aggravate cytosolic calcium levels and consequentially modify APP processing.

Neuroblastoma cells (B103) stably expressing APP_{Lon} (London mutation) exhibit a greater sensitivity to apoptosis following ER stress than cells expressing wild type APP [58]. Yet, when A β ₄₂ production is inhibited through M596I mutation (blocks β -secretase cleavage of APP) or by treatment with γ -secretase inhibitors, APP_{Lon} cells are less susceptible to ER stress-induced apoptosis. Exogenous treatment of B103 cells with A β ₄₂-conditioned media, which does not cross the plasma membrane, had no bearing upon apoptotic resistance. Therefore, intracellular A β ₄₂ is the primary mediator of ER-stress induced apoptosis. Esposito *et al.* [58] predicted that intracellular A β ₄₂ interacts with elements of the p53 pathway to bring about apoptosis. Another possible mechanism of A β ₄₂ and A β ₄₀ provoked apoptosis is direct disruption of microtubule-ER interactions. The microtubular network interacts with the ER through the cytoskeleton-linking membrane protein (CLIMP-63), a rough ER integral membrane protein, which anchors the ER to microtubules and hence stabilizing the compartment structure. Intracellular, low molecular weight, A β has been shown to disrupt this interaction, de-stabilizing the ER and causing apoptosis-related autophagosomes and lysosomes to form and proliferate [59].

ER STRESS AND SPORADIC ALZHEIMER'S DISEASE

mRNA Splicing

mRNA splicing defects are associated with diseases such as spinal muscle atrophy [60], fronto-temporal dementia, Parkinsonism [61], cystic fibrosis [62] and sporadic Alzheimer's disease [SAD: 63]. PS2V is a splice variant of PS2 that is associated with AD pathology. The variant is characterized by a base sequence that lacks exon 5 of the PS2 pre-mRNA. The ablation results in a frameshift that produces a premature stop codon in exon 6. The PS2V mRNA yields a single transmembrane protein with an additional 5 amino acids on its carboxyl-terminus. The protein responsible for excluding the fifth exon is an 18kDa PS2 pre-mRNA binding factor called high mobility group protein A1a (HMGA1a). The

HMGA1a protein operates in exon 5 splicing of PS2 *in vitro* when cells are under extremely hypoxic conditions. Overexpression of the protein can also induce PS2V production, in the absence of hypoxic conditions [64]. Cells expressing PS2V demonstrate a vulnerability to ER stress-induced apoptosis that is similar to cells expressing FAD PS-1 mutants. PS2V has been found to bind IRE1 and inhibit its autophosphorylation, reducing Bip expression and eventually leading to apoptosis under chronic ER stress. PS2V can also inhibit APP translocation from the ER to the Golgi, which can effectively promote increased A β ₄₂ production through amyloidogenic processing of APP within the ER [65]. PS2V has been found in the brains of many SAD patients, but it is not clear whether this splice variant is a direct cause of the disease or if it is attributable to general cellular pathophysiology, brought on by AD.

CHOLESTEROL METABOLISM

The cholesterol (CH) content of the plasma membrane directly influences the structural integrity and functional specificity of the cell by regulating membrane fluidity. Cholesterol homeostasis in the brain is controlled through *de novo* synthesis; peripheral cholesterol is prevented from passing through the blood brain barrier (BBB). Oligodendrocytes and astrocytes, not neurons, are primarily responsible for CH synthesis in the brain. Whereas oligodendrocyte-derived CH is devoted to the formation of myelin sheaths, astrocyte-derived CH is exported using the ATP-binding cassette A1 and G1 (ABCA1/G1) transporters, so that it can form a complex with ApoE4 and be taken up by neurons. Despite BBB-mediated exclusion of peripheral CH from the central nervous system, experimental animals fed a high cholesterol diet exhibit accumulation of A β plaques in the brain [66–68]. These animals also display increased cortical iron levels which may also contribute to cellular damage through ROS generation. The A β plaques appear to initiate apoptosis through activation of the intrinsic ER stress pathway. This dietary induced stress on the ER causes a down-regulation of ER chaperone proteins (calreticulin, grp78, grp94), activation of the growth and arrest DNA damage protein, GADD153 and subsequently disintegration of the BBB grants peripheral CH access to the brain [69].

Excessive CH within the central nervous system could promote or modify the formation and function of lipid rafts, as well as enzymes contained therein. Lipid rafts are membrane microdomains that can concentrate APP amyloidogenic proteolytic machinery. For instance, beta secretase activity significantly increases when the enzyme is associated within lipid rafts [70]. Although a definitive and causal link between CH and AD has yet to be established, CH-lowering drugs such as statins, have a proven ability to lower A β levels and reduce the risk of AD [71].

The CH synthetic enzymes, HMG-CoA reductase and acyl co-enzyme A, and the enzyme responsible for CH catabolism, cholesterol acetyltransferase (ACAT), all reside in the ER. Disruption in CH homeostasis may therefore affect normal ER functioning. ACAT is responsible for the formation of CH-esters from cholesterol and acyl coenzyme A for storage in cytoplasmic lipid droplets. ACAT also facilitates the production of A β ₄₂ through the PS-1/gamma secretase processing of APP₇₅₁ [72]. Inhibition of ACAT with the experimental compound, CP-113,818, can reduce amyloid pathology in mice expressing

APP_{lon} and APP_{swe} mutations [73,74]. Mice treated with CP-113,818 demonstrated reduced amyloid plaque and membrane/insoluble A β levels, suggesting that ACAT inhibition may represent a potential, ER-related, therapeutic strategy for AD.

OXIDATIVE STRESS

High CH levels can lead to accelerated degradation of CH, which can profoundly elevate local levels of ROS. Oxidative stresses likely affect ER functioning, stress responses and generation of CH derivatives that interact with amyloidogenic secretase enzymes. Cholesterol derivatives containing aldehyde groups, such as 4-hydroxy-nonenal, may react with secretase enzymes and increase their activity through chemical cross-linking. A β also contributes heavily to the generation of ROS [75]. In addition, mitochondria, in their creation of reactive metabolites from oxidative phosphorylation, represent a primary source of cellular oxidative stress.

How does oxidative stress negatively impact ER function? Acute exposure to oxidative stress has been shown to diminish grp94 and grp78 mRNA levels in primary neuronal cells, rendering them more vulnerable to thapsigargin-induced ER stress [76]. Notably, elevation in free divalent transition metal ion (iron, copper, zinc, aluminum) concentrations have been shown to aggravate and participate in ROS mediated damage and neurodegeneration. These metals also promote the aggregation of tau and A β .

In addition to classical ROS, such as peroxides and superoxides, endogenously produced nitrous oxides can exert significant effects in ER-related AD pathophysiology. Increases in nitric oxide (NO) production lead to increases in the S-nitrosoation of proteins. This post-translational protein modification can mimic the negative effects of genetic mutations by contributing to protein misfolding. Protein disulfide isomerase (PDI) has been reported to be S-nitrosoated in AD which would likely result in a functional disruption of the UPR complex [77]. Pathological production of NO in the central nervous system often entails activation of nitric oxide synthase (nNOS), in a calcium/calmodulin and NMDA receptor activation-dependent manner. S-nitrosoation is also thought to affect molecular chaperones, such as HSP90 in the cytoplasm and GRP in the ER. HSP90 stabilizes misfolded proteins and can also synergistically control the activity of related signaling proteins such as nNOS and calreticulin. Intramembrane levels of HSP90 are found to be significantly increased in AD where it is thought to interact with A β and tau, thereby preventing aggregation by maintaining them in their soluble state. S-nitrosoation of HSP90 abolishes its ATPase activity which reduces its ability to bind A β and tau, thereby allowing an increase in intracellular accumulation of these pathological proteins.

One possible therapeutic mechanism to reduce NO levels would be through a reduction of nNOS activity by reducing calcium influx via inhibition of the NMDA receptor. Memantine, an adamantane derivative NMDA receptor antagonist, has a low micromolar affinity for the receptor. Memantine acts by blocking the NMDA receptor associated ion channel when it is excessively open. A second generation of memantine derivatives, termed NitroMemantines have also been developed which are thought to enhance neuroprotective activity without sacrificing safety. These drugs function by generating NO-related species which bind to and

nitrosoate critical cysteine residues on the amino-terminus of the NMDA receptor which result in its accelerated downregulation. Memantine is currently approved for moderate to severe AD while the second generation NitroMemantines, which have improved efficacy, also offer promise in the future with respect to NO-mediated ER stress effects.

CONCLUSIONS

AD is a complex multifaceted disease that results in neurodegeneration and cognitive decline through cellular atrophy and/or apoptosis. The functionality of the ER system seems to be at the center of the apoptotic trigger through chronic stimulation of the UPR pathway. While not a classical target, the many protein/gene factors that control the intrinsic ER stress response system may allow for the creation of highly synergistic AD pharmacotherapeutics. By pharmacologically supplementing the ER stress response system, we may be able to ameliorate AD-related pathological systems that include, calcium overload, protein misfolding, amyloidogenic APP processing, microtubular disruption and ROS protection. As such a proposed agent would, in theory, act simultaneously in multiple cell processes, even poorly efficacious or bioavailable agents (or series of agents) would have their acute actions amplified by the UPR system. Molecular screening of small molecule chaperones or even unrelated molecule libraries against a functional ER assay could be the best approach to identify compounds of this nature. Given the relative convenience of high-throughput analysis, multiple aspects of ER function could be assayed simultaneously to generate important lead compounds.

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ABBREVIATIONS

Aβ	Amyloid beta
ABCA1	ATP-binding cassette subfamily A1
ABCG1	ATP-binding cassette subfamily G1
ACAT	Cholesterol acetyltransferase
AD	Alzheimer's disease
APH1	Anterior pharynx defective-1
APP	Amyloid precursor protein
BBB	Blood-brain barrier
BIX	Bip-inducer x
Ca²⁺	Calcium ion
CH	Cholesterol
CHOP	C/EBP homologous protein transcription factor

CNS	Central nervous system
DAG	Diacylglycerol
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
FAD	Familial Alzheimer's disease
GADD153	Growth arrest and DNA-damage inducible gene 153
GRP	Glucose-regulated protein
HMG-CoA Reductase	3-Hydroxy-3-methyl-glutaryl-CoA reductase
HSP90	Heat-shock protein 90
IP₃R	Inositol triphosphate receptor
mRNA	Messenger ribonucleic acid
NFT	Neurofibrillary tangle
NMDA	N-methyl-D-Aspartate
NO	Nitric oxide
PDI	Protein disulphide isomerase
PEN2	Presenilin enhancer 2
PS	Presenilin
PTP	Permeability transition pore
ROS	Reactive oxygen species
RyaR	Ryanodine receptor
SAD	Sporadic Alzheimer's disease
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
TRAF2	Tumor necrosis factor receptor-associated factor 2
UPR	Unfolded protein response

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