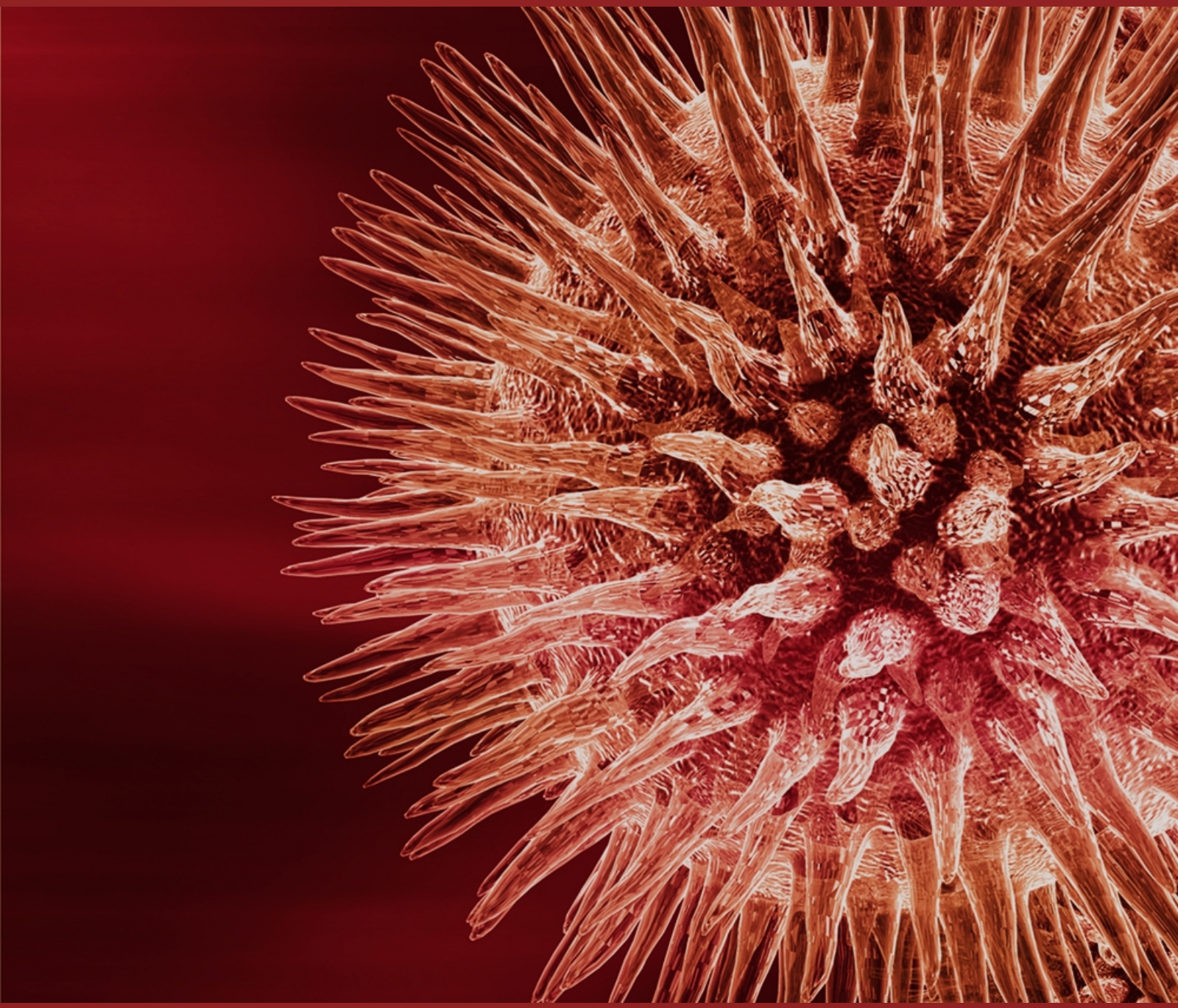
Journal of Biomedicine and Biotechnology

Neurodegenerative Diseases: Mechanisms and Therapies

Guest Editors: Mark A. Smith, George Perry, Xiongwei Zhu, and Abdelali Haoudi



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Editorial

Neurodegenerative Diseases: Mechanisms and Therapies

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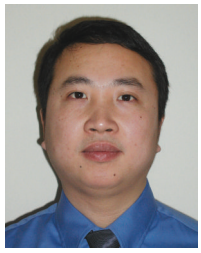
Neurodegenerative diseases are amongst the most costly and devastating diseases, both to patients as well as to their families. This year will mark the 100th anniversary of the first case description of what we now call Alzheimer's disease, the most common neurodegenerative disease. This special time provides a unique opportunity for us to reflect on how much we have learned and yet to also appreciate how much more is left to do to solve this enormous health and social problem. Indeed, there has been an exponential increase in our knowledge of disease mechanisms especially during the past decade: the old doctrine was rejected while new ones arose from its debris. This is a fascinating and, we believe, critical period for novel findings and new ideas to quickly and globally change our understanding of Alzheimer's disease and other neurodegenerative diseases. At this historical time, in this issue of the Journal of Biomedicine and Biotechnology, we have been fortunate to gather some of the foremost thinkers in the field to discuss up-to-date information concerning new developments in this exciting area of research as well as their therapeutic implications. This area of research has attracted some of the most innovative research groups in the field and, as Editors, we are truly privileged that many of these investigators have contributed to this issue. We express our sincerest gratitude to the contributing authors as well as to the vision of the Editor-in-Chief, Dr Abdelali Haoudi, for the opportunity provided by the Journal of Biomedicine and Biotechnology.

Mark A. Smith
George Perry
Xiongwei Zhu

Mark A. Smith is a Professor of Pathology at Case Western Reserve University, Cleveland, Ohio. He received a B.S. degree in molecular biology and biochemistry from Hatfield College, Durham University, England (1986), and a PhD degree in biochemistry from Nottingham University, England (1990). Following a fellowship at Sandoz Forschungsinstitut, Vienna, Austria, he joined CWRU in 1992. He serves on the Editorial Boards of many aging-related journals including Age, Aging Cell, and Neurobiology of Aging, and on Review Boards for the NIH and Alzheimer's Association. His research focuses on the pathological mechanism(s) underlying selective neuronal death in neurodegenerative diseases such as Alzheimer's disease. His current work is directed towards elucidating triggers of damaging oxidative stress including fundamental metabolic alterations, homeostatic dysregulation of transition metals, signal transduction alterations, and inappropriate re-entry into the cell cycle. He has authored over 550 peer-reviewed manuscripts and chapters. He is the recipient of several awards including the Ruth Salta Junior Investigator Achievement Award from AHAF, Young Scientist Lectureship Award from the International Society for Neurochemistry, the Nathan Shock New Investigator Award from The Gerontological Society of America, the Jordi Folch-Pi Award from the American Society of Neurochemistry, and the Esterbauer Award from the HNE Society.

George Perry obtained his bachelor of arts in zoology with high honors from the University of California at Santa Barbara in 1974 and his PhD degree in marine biology from the Scripps Institution of Oceanography, University of California, San Diego, in 1979. After postdoctoral studies in cell biology at Baylor College of Medicine, he joined the Faculty of Case Western Reserve University in 1982 as Assistant Professor and rose to become Professor in the Departments of Pathology and

Neurosciences in 1994. In 2006 he was appointed Dean of the College of Sciences, University of Texas at San Antonio. Additionally, he was named Fellow by the American Association for the Advancement of Science in 1998 and is an internationally known Invited Lecturer and has numerous papers, presentations, and publications to his credit. His studies focus on the mechanism of formation and physiological consequences of the cytopathology of Alzheimer's disease. His group has shown that oxidative damage is the initial cytopathological abnormality. His current studies focus on three issues: (i) the metabolic basis for the mitochondrial damage restricted to vulnerable neurons; (ii) the consequences of RNA oxidation on protein synthesis rate and fidelity; and (iii) role of phosphorylation in controlling oxidative adduction.



Xiongwei Zhu is an Assistant Professor of Pathology at Case Western Reserve University, Cleveland, Ohio. He received his BS degree in 1995 and his MS degree in 1998 from the Department of Biochemistry at Wuhan University in China. He received his PhD degree in 2002 from the Department of Pathology at Case Western Reserve University and became Assistant Professor in 2004. He is the recipient of several awards including the International Junior Investigator Award from the International College of Geriatric Psychoneuropharmacology and the Vector Laboratories Young Investigator Award from the International Congress of Histochemistry and Cytochemistry. The focus of his research is on the neurodegenerative mechanisms underlying Alzheimer's disease and other neurodegenerative diseases. Alzheimer's disease is a major public health problem because it has a huge impact on individuals, families, and society and it has attracted increasing public attention as the population ages, which highlights the urgency to understand and treat this disease effectively. This group demonstrated that both oxidative stress and cell cycle-related abnormalities are among the earliest contributors to the disease. The major hypothesis being pursued is that while either oxidative stress or abnormalities in mitotic signaling can independently serve as initiators, both processes are necessary to propagate disease pathogenesis and progression.

Abdelali Haoudi received his PhD degree in cellular and molecular genetics jointly from Pierre & Marie Curie University and Orsay University in Paris, France. He then joined the National Institutes of Health (NIEHS, NIH) for a period of four years after winning the competitive and prestigious NIH Fogarty International Award. He then joined the Myles Thaler Center for AIDS and Human Retroviruses at the University of Virginia Medical School, Charlottesville, then shortly after joining the Faculty in the Department of Microbiology and Molecular Cell Biology at Eastern Virginia Medical School in Norfolk, Va, in 2001. He is interested in uncovering mechanisms by which mobile genetic retroelements, both retroviruses and retrotransposons, induce genetic instability and apoptosis in human cells and the molecular basis of cancer including cell cycle checkpoints and DNA repair mechanisms. He is also the Codirector of the Cancer Biology and Virology Focal Group. He has founded the Journal of Biomedicine and Biotechnology (http://www.hindawi.com/GetJournal.aspx?journal=JBB) and is also the Founder and President of the International Council of Biomedicine and Biotechnology (http://www.i-council-biomed-biotech.org).

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Review Article

β-Amyloid Degradation and Alzheimer's Disease

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Extensive β-amyloid (Aβ) deposits in brain parenchyma in the form of senile plaques and in blood vessels in the form of amyloid angiopathy are pathological hallmarks of Alzheimer's disease (AD). The mechanisms underlying Aβ deposition remain unclear. Major efforts have focused on Aβ production, but there is little to suggest that increased production of Aβ plays a role in Aβ deposition, except for rare familial forms of AD. Thus, other mechanisms must be involved in the accumulation of Aβ in AD. Recent data shows that impaired clearance may play an important role in Aβ accumulation in the pathogenesis of AD. This review focuses on our current knowledge of Aβ-degrading enzymes, including neprilysin (NEP), endothelin-converting enzyme (ECE), insulin-degrading enzyme (IDE), angiotensin-converting enzyme (ACE), and the plasmin/uPA/tPA system as they relate to amyloid deposition in AD.

INTRODUCTION

Alzheimer's disease (AD) is the commonest cause of senile dementia and increases in frequency with age. Clinically, AD is characterized by early and progressive memory loss due to neuronal and synaptic loss in the cortex and limbic structures, including the hippocampus and amygdala. In later stages of the disease process, the extensive involvement of cortical and subcortical regions results in loss of higher cognitive abilities, including speech and praxis, and in impaired motor abilities. Grossly, AD brains show global atrophy and reduced weight and volume. Histologically, AD is characterized by amyloid plaques, neurofibrillary tangles, dystrophic neurites, extensive neuronal loss, and gliosis.

Although beta-amyloid (Aβ) accumulation and senile/neuritic plaque formation are striking morphological hallmarks of AD and widely used in the neuropathologic diagnosis of AD, it is clearly recognized that amyloid deposition in the brain parenchyma and in vessels also is common for nondemented individuals in advanced age. Many possible explanations for excessive Aβ deposition have been put forward, including increased production, decreased degradation, and abnormal transport between brain parenchyma and plasma or CSF [1–3]. Although overproduction of Aβ is critical to the pathogenesis of some forms of familial AD, there is still little evidence to suggest that increased Aβ production is important in amyloid deposition in aging and sporadic AD. Recently, the role of degradation has been increasingly recognized in Aβ homeostasis. Several enzymes have been described with a range of abilities to degrade Aβ. This review will focus on enzymes capable of degrading Aβ and their potential significance to the pathogenesis of AD.

THE AMYLOID CASCADE HYPOTHESIS

The mechanisms underlying the pathogenesis of AD remain unclear and are hotly debated. One proposal focuses on Aβ production and deposition, the so-called amyloid cascade hypothesis (Figure 1) [4–6]. This hypothesis posits that increased Aβ production and deposition plays the key role in triggering neuronal dysfunction and death in AD. Evidence, including Aβ deposition in AD brain, the toxic properties of Aβ to neurons in vitro, and the identification of mutations in amyloid precursor protein (APP) in familial early onset AD have supported the amyloid cascade hypothesis. Based on this theory, tremendous efforts had been made during the last decade to uncover the mechanisms underlying the production of Aβ. From these studies it has been shown that sequential cleavage of APP by β-secretase and γ-secretase generates Aβ peptides (Figure 2) [7, 8]. Indeed, pharmacologic intervention targeted at Aβ generation through inhibitors of β-site cleaving enzyme (BACE) and γ-secretase is being

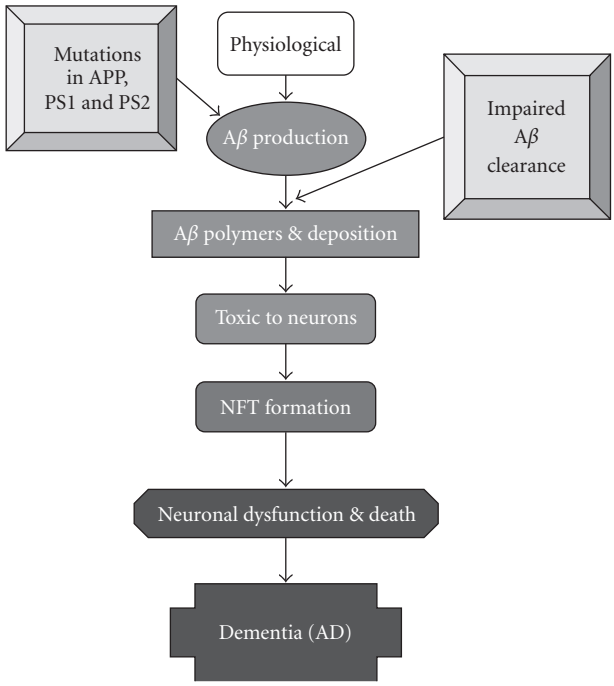

Figure 1: Amyloid cascade hypothesis. Aβ is a normal metabolite which, under physiological conditions, is constantly produced and quickly degraded. Due to genetic defects such as mutations in APP, PS1, or PS2, Aβ production is increased, resulting in familial AD. A similar phenotype can occur with reduction in the Aβ catabolic pathways. Accumulating Aβ will initially oligomerize, gradually form fibrils, and culminate in microscopically visible amyloid plaques. Soluble and fibrillar Aβ and associated plaque proteins are toxic to neurons, resulting in synaptic loss, the formation of neurofibrillary tangles, and eventual neuronal death and AD [5].

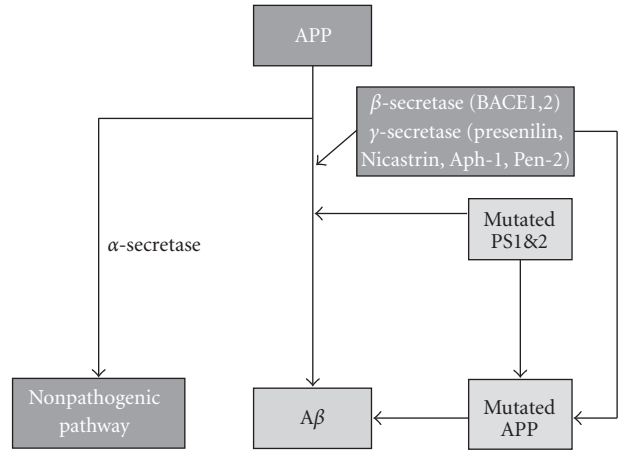

Figure 2: Aβ biogenesis. Normally, Aβ is derived from the transmembrane region of amyloid precursor protein (APP) through the sequential cleavage by BACE and γ-secretase. Under physiological conditions, Aβ maintains a steady-state level and is necessary for multiple physiological functions [168]. In AD, Aβ production is increased due to mutations in APP and/or in presenilin (PS1 and PS2) genes. There is an α-secretase cleavage site located between β- and γ-secretase cleavage sites that generates soluble, nonpathogenic peptides [8].

widely pursued [9, 10]. Attempts to block or regulate those two enzymes together with immunotherapy aimed at reducing brain Aβ have been or will soon be tried in AD patients [9, 11–13].

CANDIDATE ENZYMES FOR Aβ DEGRADATION

Altering catabolism is another way to reduce Aβ levels in the brains of AD. Many proteases or peptidases have been reported with the capability of cleaving Aβ either in vitro or in vivo. These include neprilysin (NEP) [14–16], endothelin-converting enzyme (ECE)-1 [17], insulin-degrading enzyme (IDE) [18–20], angiotensin-converting enzyme (ACE) [21], uPA/tPA-plasmin system [22, 23], cathepsin D [24, 25], gelatinase A [26], gelatinase B [27], matrix metalloendopeptidase-9 [28], coagulation factor XIa [29], antibody light chain c23.5 and hk14 [30], and α2-macroglobulin complexes [31]. Many of them have more than one cleavage site in the Aβ peptide (Figure 3). The basic biological features of these enzymes are summarized in Table 1. There are probably other proteases with potential to cleave Aβ if all peptide bonds are taken into consideration, but only those physiologically or pathologically relevant are discussed. Among them, NEP, IDE, ECE, ACE and plasmin, tissue plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA) system are the most promising Aβ-degrading candidates.

NEPRILYSIN (NEP)

NEP is also known as neutral endopeptidase-24.11, EC 3.4.24.11, enkephalinase, neutrophil cluster-differentiation antigen 10 (CD10), or common acute lymphoblastic leukemia antigen (CALLA) [32–38]. In humans, the NEP gene is located on chromosome 3q21–q27 and contains 24 exons [39, 40]. NEP is composed of 750 amino acids with a calculated molecular weight of approximately 86 kDa [41]. Because of abundant posttranslational modifications, especially glycosylation [42], NEP from human brain tissues migrates between 97–110 kDa on denaturing gel electrophoresis. As a plasma membrane-bound glycoprotein, NEP is composed of a short N-terminal cytoplasmic tail, a membrane-spanning domain, and a large C-terminal extracellular catalytic domain. The latter contains a HExxH zinc-binding motif [43, 44], which facilitates the hydrolysis of extracellular oligopeptides (< 5 kDa) on the amino side of hydrophobic residues, such as the small, hydrophobic Aβ40 and Aβ42 peptides.

NEP is widely expressed in many normal tissues including the brush-border of intestinal and kidney epithelial cells, neutrophils, thymocytes, lung, prostate, testes, and brain [45–49]. In the brain, it is expressed on neuronal plasma membranes, both pre- and postsynaptically [50, 51], and is most abundant in the nigrostriatal pathway, as well as in brain areas vulnerable to amyloid plaque deposition, such as the hippocampus [43, 52].

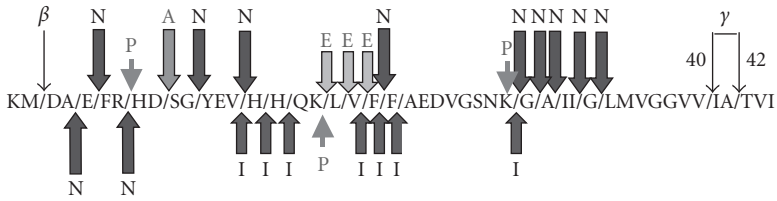

Figure 3: Cleavage sites of Aβ by various enzymes including NEP (N) [14, 169, 170], ECE (E) [17, 169], IDE (I) [170, 171], plasmin (P) [172], and ACE (A) [21]. β: γ-secretase; γ: γ-secretase; 40: Aβ40; 42: Aβ42. Modified from [173].

Table 1: Features of selected Aβ-degrading enzymes.

Protease	a.k.a.	Class	Subcellular location	Substrates in addition to Aβ
Neprilysin	CD10, CALLA, EC 3.4.24.15	M	Cellular & intracellular membrane including presynaptic membrane	Enkephalin, cholecystokinin, neuropeptide Y, substance P, opiod peptides, atrial natriuretic peptides, bombesin-like peptides, chemotatic peptides, adrenocorticotropin homone (ACTH)
Insulin-degrading enzyme	EC 3.4.24.56 Insulysin, IDE	M	Cytosol, cellular, and intracellular membrane extracellular space	Insulin, glucagon, atrial natriuretic factor, β-endophin amylin, APP intracellular domain TGFα
Endothelin-converting enzyme	EC 3.4.24.71 ECE	M	Trans-Golgi network cell surface	Big endothelin, substance P, bradykinin, oxidized insulin B chain
Angiotensin-converting enzyme	EC 3.4.15.1; ACE; dipeptidyl carboxypeptidase	M	plasma membranes perinuclear region	Angitensin-I, enkaphalins, bradykinin
Plasmin/uPA/tPA	EC 3.4.21.31 tissue plasminogen activator for tPA; urokinase-type plasminogen activator for uPA	S	Extracellular space	fibrin, plasminogon, & other matrix proteins

Note: a.k.a. is also known as M: metalloproteases and S: serine.

The first clue that NEP was involved in Aβ degradation was provided by Howell et al [14]. Using high-performance liquid chromatography (HPLC) combined with mass spectroscopic analysis, they found that NEP cleaved Aβ between residues Glu3-Phe4, Gly9-Trp10, Phe19-Phe20, Ala30-Ile31, and Gly33-Leu34. The true breakthrough demonstrating the importance of NEP was demonstration that NEP was the rate-limiting enzyme for Aβ degradation in vivo made by Iwata et al in 2000 [15]. After injecting radio-labeled Aβ peptides into rat hippocampus in the presence or absence of various protease inhibitors, the resultant Aβ fragments were analyzed by HPLC equipped with flow scintillation. Iwata and coworkers found that Aβ42 was degraded in the hippocampus, with a half-life of 17.5 minutes and with Aβ10–37 as the major catabolic intermediate. Infusion of thiorphan, a specific NEP inhibitor [53], directly into rat hippocampus for 3 days elevated endogenous Aβ levels, and infusion for 30 days resulted in further endogenous Aβ accumulation and accumulation of extracellular Aβ deposits resembling amyloid plaques [15, 54]. They also found that almost all radio-labeled Aβ42 could be recovered from the hippocampus 1 hour after the injection, which suggested that Aβ clearance depends predominantly on local proteolysis, rather than transport across the blood brain barrier into the blood or into the cerebrospinal fluid [15]. Interestingly, in another independent study, it was found that radio-labeled Aβ40 injected into mouse brain was more readily transferred to blood, compared with Aβ42, suggesting that the relative contributions of degradation and transport to brain Aβ clearance might be different for these two peptides [55]. Furthermore, it had been found that NEP was able to degrade not only monomeric, but also oligomeric forms of both

Aβ40 and Aβ42 [56], both intracellularly and extracellularly [57].

The role of NEP in Aβ degradation was solidified by studies in transgenic mice. In partially NEP deficient animals, the degradation of both endogenous and exogenous Aβ peptides was tightly correlated with gene dose, suggesting that even partial down-regulation of NEP activity could contribute to Aβ accumulation. These studies also established that NEP is a physiologically relevant Aβ degrading enzyme [16]. On the other hand, overexpression of NEP by gene transfer in amyloid-depositing transgenic mice slowed, and in some cases reversed Aβ deposition [54, 58–60].

Studies in human subjects have also supported the notion that NEP plays a key role in brain Aβ metabolism and AD pathogenesis. As mentioned above, aging is one of the most important risk factors for AD [61] and is associated with the accumulation of Aβ even in cognitively normal elderly [62, 63]. Although systematic study of the relationship between NEP and aging in humans remains to be done, aging mice show region selective decreases in NEP mRNA expression [52, 64, 65]. These changes occurred despite maintenance of synaptic and neuronal numbers suggesting gene specificity. Immunohistochemical studies on AD brains have revealed NEP immunoreactivity in senile plaques [49]. Quantitative analysis showed that both NEP mRNA and protein were significantly lower in AD than in age-matched normal control brains [65–68]. Reductions occurred selectively in the regions most vulnerable to AD pathology, but not in other brain areas such as cerebellum or in peripheral organs [65, 66]. NEP was also decreased in the cerebrospinal fluid (CSF) of prodromal Alzheimer's disease patients [69], consistent with cause and effect. Interestingly, an inverse relationship between NEP and Aβ levels in AD brain vasculature has been reported. These data suggested that NEP may play a role in cerebral amyloid angiopathy (CAA), another very common pathological change found in AD brains [70]. Consistent with these findings, Aβ mutations identified in familial AD found in Dutch, Flemish, Italian, and Arctic families do not increase Aβ production, but rather cause presenile parenchymal amyloidosis and CAA [71].

Recent data from our study showed that NEP decreased in AD brains, but not in pathological aging (PA), a term to describe neurologically normal individuals with high brain amyloid burden (sufficient to diagnose AD with the Khachaturian criteria), but minimal or no neurofibrillary degeneration (Braak neurofibrillary tangle stages of three or less) [63, 72]. Interestingly, NEP levels were inversely correlated with a range of amyloid measures including senile plaque counts and levels of Aβ40 and Aβ42 in cortical homogenates. The NEP levels were also correlated with clinical cognitive scores, with highest levels of NEP in those with best performance on clinical measures, regardless of whether or not there were cortical amyloid deposits [72]. These results suggest that the deposition of Aβ in AD and PA brains differs in some way, either quantitatively or qualitatively. The results were not merely due to synaptic loss in AD, but also not in PA as measured by synaptic markers since NEP was not decreased in frontal dementia with decreased synaptic markers. These data support the hypothesis that decreased NEP contributes to Aβ deposition in AD, but perhaps in means that are not entirely linked to visible amyloid deposition [72], perhaps implicating failed degradation of toxic soluble intermediates in AD.

Taken together, these data indicate that NEP is an important enzyme that contributes to the normal metabolism, accumulation, and perhaps toxicity Aβ in AD.

ENDOTHELIN-CONVERTING ENZYME (ECE)

Endothelin (ET) is a potent vasoconstrictive peptide produced in vascular endothelial cells [73]. In addition, ET also plays an important role in early development of the neural crest and, thus, organogenesis [74]. Endothelin-converting enzyme (ECE) is a transmembrane metalloprotease that catalyzes the conversion of pro-ET (also referred to big-ET) into vasoactive endothelin. So far, two different isoforms of ECE—ECE-1 and ECE-2—have been cloned in humans [75–77]. It has been estimated that expression of ECE-2 is only 1–2% as much as the more abundant ECE-1 based on comparative mRNA transcript levels in endothelial cells [78]. Studies have suggested that ECE-1, but not ECE-2, is a possible brain Aβ-degrading enzyme [17].

ECE-1 consists of 758 amino acids [79] and is the major enzyme responsible for specific cleavage of biologically inactive pro-ET-1 to active ET-1 in vascular endothelial cells. It is a membrane-bound type II metalloprotease and shares significant sequence identity (about 38% homologue at the amino acid level) with NEP. ECE-1 is abundantly expressed in the vascular endothelial cells of all organs and is also widely expressed in nonvascular cells of many tissues, including lung, pancreas, testis, ovary, adrenal gland, and kidney [75, 80–83]. Recent systematic immunohistochemical analyses have shown ECE-1 widely expressed in human brain, including neurons in the diencephalon, brainstem, basal nuclei, cerebral cortex, cerebellar hemisphere, amygdala, and hippocampus [84, 85]. Four isoforms of ECE-1 have been identified to date [75, 86–90]. All of them are encoded by a single gene located on chromosome 1 (1p36), and they differ in their cytoplasmic tail domains through alternative promoter usage. The four isoforms cleave pro-ET with similar efficiency, but they differ in their tissue distribution and subcellular localization [87, 90]. Human ECE-1a is localized predominantly in plasma membrane. Human ECE-1c and ECE-1d have also been reported to be localized in plasma membrane, but also in intracellular compartments. In contrast, human ECE-1b is expressed exclusively intracellularly, particularly in Golgi-like structures and the cytoplasmic face of the plasma membrane [90–92].

Although both ECE-1 and NEP are metalloendopeptidases and thus subject to competitive inhibition by the metalloprotease inhibitors nanomolar concentrations of thiorphan and phosphoramidon can inhibit NEP, whereas ECE-1 is inhibited only at micromolar concentrations of phosphoramidon, and it is insensitive to thiorphan [53]. Another difference is that ECE-1 is active only at neutral pH, while

NEP is active over a slightly wider pH range (pH 6.5–7.5) [72, 93].

By using HPLC, mass spectrometry, and N-terminal sequence analysis, Eckman and her colleagues provided the first evidence that ECE-1 may be involved in the metabolism of Aβ. They found that ECE-1 expressed in cultured Chinese hamster ovary cells that lack endogenous ECE activity, reduced the concentration of extracellular Aβ by up to 90%. In vitro, recombinant ECE-1 cleaves synthetic Aβ40 in at least three sites, resulting in formation of Aβ fragments Aβ1–16, Aβ1–17, Aβ1–19, and Aβ20–40 [17]. In mice deficient for ECE-1 and the closely related ECE-2, both Aβ40 and Aβ42 levels were significantly higher when compared with age-matched wild-type littermate controls. Taken together, the results suggest that ECE activity might be an important factor involved in Aβ clearance in vivo [94]. How important is ECE-2 in this process is yet to be determined, and direct evidence that ECE contributes to Aβ deposition in human AD brains remains to be determined.

INSULIN-DEGRADING ENZYME (IDE)

IDE is also known as EC 3.4.24.56, insulin protease, insulysin, or insulinase [95, 96]. Cloned human IDE consists of 1019 amino acids [97]. The IDE gene was mapped to chromosome 10q23–q25, which made it a candidate gene for the Alzheimer disease-6 locus (known as AD6) [98, 99]. It is a zinc metalloendopeptidase that hydrolyzes multiple peptides, including insulin, glucagon, atrial natriuretic factor, transforming growth factor-α, β-endorphin, amylin, and the APP intracellular domain (AICD) in addition to Aβ [100, 101]. Purified IDE from several mammalian tissues, including blood cells, skeletal muscle, liver, and brain, migrates as a 110 kDa band on denaturing gel electrophoresis, but it migrates as a 300 kDa band under nondenaturing conditions. These results suggest that native IDE exists as a mixture of dimers and tetramers [100, 102]. IDE is active at neutral pH and dimers have greater activity than monomers [96, 103, 104]. Subcellularly, IDE is primarily located in the cytosol, although it also had been found in peroxisomes [105], plasma membrane [106, 107], and in a secreted form [20].

Kurochkin and Goto reported the first evidence that IDE might involved in Aβ degradation [18]. They found that ^{125}I-labeled synthetic Aβ specifically cross-linked to a single 110 kDa protein, which was shown to be IDE, in cytosol fractions from rat brain and liver. Purified rat IDE effectively degraded synthetic Aβ in vitro. Subsequently, it was shown that an IDE-like activity from soluble and synaptic membrane fractions from postmortem human and fresh rat brain also degraded Aβ peptides [19, 108]. Studies in the cultured cells also proved that IDE could degrade both endogenous and synthetic Aβ in vitro [20, 109]. The overexpression of IDE in Chinese hamster ovary cells resulted in a marked reduction in levels of intracellular detergent-soluble Aβ, as well as reduced levels of extracellular Aβ40 and A42 [110].

Transgenic mice overexpressing IDE showed significant reductions of total amyloid burden and improved survival rates [58], while IDE knockout mice demonstrated a clear elevation of brain Aβ and the APP intracellular domain. Additionally, heterozygous mice exhibited Aβ levels that were intermediate between wild-type controls and knock-out mice, indicating that IDE affected Aβ level in a gene-dose dependent manner [111, 112].

Immunohistochemical studies showed that IDE was primarily expressed in neurons, but was also located in senile plaques, in AD brain [113]. The finding that IDE mRNA and protein were reduced in the hippocampus of AD patients, especially in APOE e4 carriers, suggested that APOE e4 might be sensitive to IDE expression levels with downstream effects on Aβ metabolism [114]. Like NEP, IDE also showed progressively decreased expression that was age- and region-dependent [65]. Thus, strong evidence exists that IDE is another important Aβ-degrading enzyme that may play a role in the amyloid pathology of AD.

ANGIOTENSIN-CONVERTING ENZYME (ACE)

Angiotensin-converting enzyme, also known as EC 3.4.15.1, dipeptidyl carboxypeptidase, or ACE, is a membrane-bound zinc metalloprotease. At least two ACE isotypes (ACE1 and ACE2) had been cloned in humans, thus far [115]. ACE is composed of 732 amino acids [116] and contains two proteolytically active domains that are located at N- and C-termini, respectively [117]. The major function of ACE is to catalyze the conversion of angiotensin I (AngI) to angiotensin II (AngII), which plays an important role in maintaining blood pressure, body fluid, and sodium homeostasis [118].

ACE is also widely expressed both outside and within the CNS. In the brain, ACE was found at highest levels in circumventricular organs such as the subfornical organ, area postrema, and the median eminence [119]. It was detected in other areas as well, including the caudate nucleus, putamen, substantia nigra pars reticularis, nucleus of the solitary tract (NTS), dorsal motor nucleus, median preoptic nucleus, and choroid plexus in rat, human, rabbit, sheep, monkey [120].

Most of the evidence for the potential relationship between ACE and AD has come from human genetic studies [121–127]. Patients at higher AD risk had an insertion (I) polymorphism within intron 16 of the ACE gene, which was associated with AD [121]. Interestingly, patients with a deletion polymorphism had a lower risk of AD [123, 124]. Genetic analysis of postmortem AD brains showed homozygous I/I was associated with higher brain Aβ levels compared to D/D allele carriers [128]. Results from earlier preclinical and clinical studies suggested that ACE might have a role in the modulation of cognitive memory processes in the rat and in humans [129].

Hu and coworkers provided the first evidence that ACE could significantly inhibit the aggregation, deposition, and cytotoxicity of Aβ in vitro by cleavage of Aβ at Asp7-Ser8. This was a surprising finding given the known specificity of ACE [130] and the failure of ACE inhibitors to alter Aβ degradation in vivo [15, 16]. Whether this discrepancy was due to different experimental systems (eg, in vitro versus in

vivo) is not clear. Further work in other experimental systems such as ACE-deficient or knockout animals is needed to clarify the role ACE might have in amyloid pathology in AD.

A very recent report by Hemming and Selkoe, showed that ACE expression promoted the degradation of endogenous Aβ40 and Aβ42 [131]. Using site-directed mutagenesis, they also showed that both N- and C-terminal proteolytically active domains contributed to Aβ degradation. Captopril, a widely prescribed ACE inhibitor blocked Aβ cleavage in culture medium. This is potentially very important observation because it suggests widely used ACE inhibitors could increase cerebral Aβ levels in patients with hypertension.

Unlike other candidate Aβ-degrading enzymes discussed above, the levels of both ACE protein and activity were elevated in postmortem brains [132–134]. Given that other Aβ-degrading enzymes such as NEP and IDE are decreased in AD brains compared to age-matched healthy controls [65–68, 72], ACE may show compensatory up-regulation in response to accumulating Aβ. Along with concurrent evaluation of NEP, ECE, IDE, ACE, and possibly others in the same panel of postmortem human brains with the spectrum of pathology from normal aging, early and advanced AD will be helpful in clarifying respective functions of these proteases.

PLASMIN, TISSUE PLASMINOGEN ACTIVATOR (tPA), AND UROKINASE-TYPE PLASMINOGEN ACTIVATOR (uPA)

Plasmin is a serine protease important in the degradation of many extracellular matrix components [135]. The principal components of this system include plasminogen/plasmin, tissue plasminogen activator (tPA), urokinase-type plasminogen activator (uPA) [136]. tPA and uPA cleave plasminogen to yield the active serine protease, plasmin. In the nervous system, plasminogen and uPA are expressed in neurons, while tPA is synthesized by neurons and microglial cells [137]. The plasmin system is involved in many normal neural functions, such as neuronal plasticity [138], learning, and memory [139].

Several studies showed that Aβ aggregates could substitute for fibrin aggregates in activating tPA, and suggested that tPA may be activated by Aβ in AD [140, 141]. Later, it was reported that brain plasmin enhanced Aβ degradation [142, 143], while plasmin and its activity were decreased in AD brains [142, 144]. In cultured cells purified plasmin significantly decreased the level of neuronal injury induced by aggregated Aβ, presumably by degrading Aβ [143, 145]. However, the in vivo effect of plasmin could be very different given that serum amyloid P, that is associated with amyloid pathology in AD brain, is able to prevent proteolysis of purified cerebral Aβ [146]. Indeed, plasminogen deficient mice did not show increased Aβ in the brain or in the plasma and suggested that plasmin does not regulate steady-state Aβ levels in nonpathologic conditions [147], although it might be involved in the degradation of pathological Aβ aggregates.

OXIDATIVE DAMAGE TO Aβ-DEGRADING ENZYMES

Some studies have indicated that genetic polymorphisms of Aβ-degrading enzymes including NEP, IDE, ACE might be associated with AD [122, 125, 127, 148–155], although these results remain controversial [128, 156–161]. Further clinical and pathologic studies of large numbers of individuals carrying various mutations in possible Aβ-degrading enzymes are needed to clarify this issue.

In addition to genetics, many environmental factors such as oxidative stress can potentially impair the activity of Aβ-degrading enzymes [162–164]. Recent data showed that NEP and IDE might be substrates for oxidative damage during aging and in AD [65, 68]. Both NEP and IDE from AD brain tissues could be modified by 4-hydroxy-nonenal (HNE), a by-product of lipid peroxidation [165]. The ratio of oxidized NEP from frontal cortex [68] and IDE from hippocampus [65] was greater in AD brains than in age-matched controls. Studies reported by Russo et al failed to confirm these findings [166]. In their study, they found that NEP mRNA from AD brains was significantly lower than in controls, but not NEP protein [166], which was contradictory to several previous reports [65–68]. One possible reason for such a discrepancy might be purely technical, reflecting different immunoprecipitation protocols and incomplete antigenic recovery [166]. Although very recent data confirmed that both recombinant IDE and the extracellular domain of NEP were modified by HNE in vitro [167], additional, in vivo studies of neuronal proteases are needed to clarify this potentially very important mechanism for Aβ deposition in AD development.

SUMMARY

Since the majority of AD cases are sporadic without clear genetic causes, and that even a large percentage of familial cases cannot be explained by the overproduction of Aβ, multiple factors are likely involved in the pathogenic metabolism of Aβ in AD (Figure 4). Exploration for possible mechanisms underlying Aβ accumulation in AD is crucial to resolve these issues. There are growing and compelling data now available to implicate Aβ degradation in AD pathogenesis. Aβ is a substrate of a wide range of proteases, which are likely contribute to the accumulation of Aβ in AD. Both enzymatic loss through genetic mutations and nongenetic factors, such as direct oxidative damage or enhanced production of inhibitors, may contribute to aberrant Aβ catabolism. Current results from in vitro and animal models support NEP, IDE, ECE, and ACE as probable enzymes for Aβ degradation, but data from humans remain largely missing. Due to clear limitations of animal models, validation in human subjects with AD will be critical to establish the physiologic significance of these proteases. Measurement of brain Aβ levels, amyloid pathology and clinical cognitive performance with enzyme activity, location and expression will help to clarify which of these many enzymes that are capable of cleaving Aβ are actually key players in human disease.

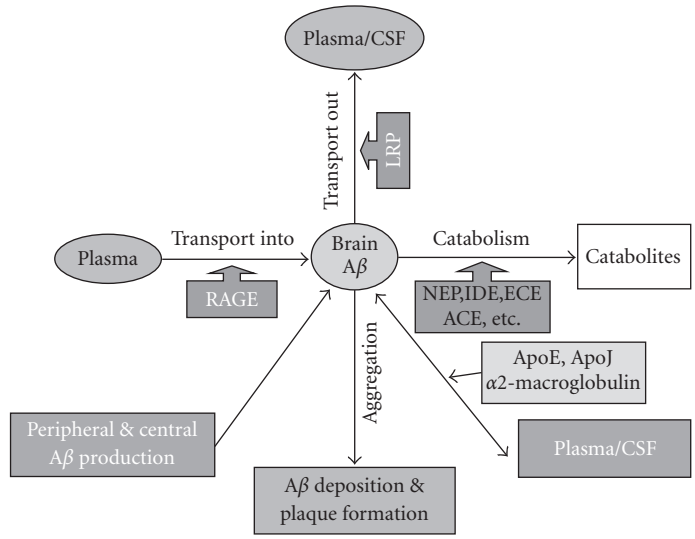

Figure 4: Possible brain Aβ clearance mechanisms. Aβ peptides may be removed by enzymatic degradation within brain parenchyma [38, 173] or they can be transported through the blood-brain-barrier into the blood or CSF by receptor for advanced glycation endproducts (RAGE), ApoE, β-2-macroglobulin, and the low-density lipoprotein receptor (LRP) [3, 174–176]. The steady-state level of brain Aβ depends upon a balance between production and catabolism. Increased production (like in familial AD) and/or decreased clearance (for most sporadic AD) will result in elevated brain Aβ levels and potentially trigger or accelerate the pathogenesis of AD. RAGE: receptor for advanced glycation end products; LRP: low-density lipoprotein receptor-related protein.

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Review Article

Tau Phosphorylation, Aggregation, and Cell Toxicity

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Protein aggregation takes place in many neurodegenerative disorders. However, there is a controversy about the possible toxicity of these protein aggregates. In this review, this controversy is discussed, focussing on the tau aggregation that takes place in those disorders known as tauopathies.

INTRODUCTION

Many neurodegenerative disorders are characterized by their presence in neural tissue of aberrant protein aggregates (see Table 1). In general, these aggregates arise after the modification of a native protein. That modification could result in a conformational change of the native protein that promotes the aberrant aggregation.

The most studied model of this mechanism has been the prion protein where a change from an alpha helix to beta sheet structure facilitates the polymerization of a protein with a different conformation that appears to have a cytotoxic effect [1].

In a similar way, conformational changes between a native protein and its aberrant protein counterpart with capacity for self-assembly have been studied in many neurodegenerative diseases. Among the most common techniques used for these analyses are X-ray diffraction, nuclear magnetic resonance, circular dichroism, or Fourier-transformed infrared spectroscopy. Similarly, to the case of prion protein, in some disorders a change from alpha helix to beta sheet conformation has been suggested to cause protein aggregation (Table 1), probably because in alpha helix, intramolecular hydrogen bonds could occur whereas intramolecular hydrogen bonds are facilitated in beta sheet conformation, facilitating protein aggregation. However, there is one case, the formation of aberrant aggregates of tau, where the aggregated protein contains also a high proportion of alpha helix structure [2].

Although, in some cases, like that of prion protein, the formation of aberrant aggregates of protein could result in a toxic effect in the affected neurons [1], in other cases, like huntingtin aggregates, the formation of the aberrant aggregates could be a survival response of the affected neurons [3]. In other neurodegenerative diseases, it will be of interest to know if protein aggregation is synonymous of cell toxicity or not.

Protein conformation could also play a role in a possible toxic mechanism. In this way, a protein with a high proportion of alpha helix and hydrophobic regions could be inserted in cell membrane promoting toxic effects [4]. Additionally, the presence of aberrant polymers could affect the protein degradation cell machinery (the proteasome complex), decreasing its activity and promoting a toxic effect [5].

Recently, some good reviews have been published on protein aggregation and neurodegenerative disorders [6, 7]. In this review we will mainly focus on those aggregates assembled from tau protein, aggregates that could be present in the neurological disorders known as tauopathies (for a review see [8]) (Table 1).

TAU AGGREGATION

It has been described that large amounts of native or unmodified tau protein were enough to promote tau assembly into fibrillar polymers resembling those found in AD [9–12]. Thus, obviously, an increase in tau concentration will favour the formation of tau polymers. Recently, it has been reported that not all the brain areas have a similar amount of tau protein [13]. Thus, it suggests a different probability in the formation of tau polymers in different brain regions.

The amount of tau will be the consequence of its synthesis and its degradation. Changes in transcription have

Table 1: Examples of neurological diseases where aberrant protein aggregates are found, and the suggested conformations in the aberrant aggregates are indicated.

		Proteinopathies (aberrant aggregation)	
Disease	Protein	Pathological finding	Protein conformation
Prion diseases	PrP^{Sc}	PrP amyloid plaques	β-sheet
Alzheimer's disease	Aβ	Aβ amyloid plaques	β-sheet
	Tau	Paired helical filaments in neurofibrillary tangles	β-sheet + α-helix
Parkinson's disease	α-synuclein	Lewy bodies	β-sheet + α-helix
Frontotemporal dementia	Tau	Straight filaments and paired helical filaments	——
Pick's disease	Tau	Pick bodies	——
Progressive supranuclear palsy	Tau	Straight filaments is neurfibrillary tangles	——
Amyotrophic lateral sclerosis	Neurofilament	Neural aggregates	——
Huntington's disease	Huntingtin	Nuclear inclusions	β-sheet
Spinocerebellar ataxia			
Type 1	Ataxin 1	Nuclear inclusions	β-sheet
Type 2	Ataxin 2	Cytoplasmic inclusions	β-sheet
Machado-Joseph disease	Ataxin 3	Nuclear inclusions	β-sheet

been indicated for other proteins related to neurodegenerative disorders [14], where a TATA binding protein may play a role. Tau degradation may take place through the proteasome complex [15, 16] and it has been suggested that such degradation could be regulated by posttranslational modifications occurring in tau molecule, like its phosphorylation [17]. Also, tau degradation by other proteases could be regulated by its level of phosphorylation [18]. It should be also indicated that in some cases like Parkinson's disease or Lafora disease, mutations in the E3 ubiquitin ligases like parkin [19] or malin [20] will result in the appearance of aberrant protein aggregates.

A conformational change, that could be followed by antibodies that react with tau molecule after that conformational change [21–24] has been also suggested to be required for the transition tau monomer-tau polymer.

Also, it has been suggested that different posttranslational modifications like phosphorylation [25], glycation [26], or truncation [27], may play a role in the formation of tau polymers.

Due to the alternative splicing of its heterogenous (or nuclear) RNA, different tau isoforms could be expressed and, therefore, different tau aggregates with different tau isoforms in different tauopathies could occur, but we will not discuss this point here. For further information see [8].

Mainly, studies on phosphorylation and truncation have been done. About truncation, it has been suggested that removal of the amino and/or carboxy terminal regions, leaving the tubulin binding region will facilitate tau polymerization [21, 22, 28].

Some work has been done in vitro [29] and in vivo [30, 31] about a possible role of tau phosphorylation on tau assembly, suggesting that in some conditions tau phosphorylation may increase the capacity of tau for its self-assembly. Not only an increase in serine/threonine phosphorylation of tau could regulate its aggregation but also an increase in tau tyrosine phosphorylation may increase the formation of tau aggregates [32]. This assembly process may involve the formation of oligomers [33], filaments, and aggregates of filaments (tangle-like structures). In the formation of these aggregates of filaments, glycation may play a role [26].

The possible relation between phosphorylation and tau aggregation has been studied in transgenic mice expressing human tau bearing some mutations found in human fronto-temporal dementia linked to chromosome 17 (FTDP-17) [31]. In this mouse, tau phosphorylation mainly occurs by GSK3 [34]. When a specific inhibitor of this kinase, lithium, was given to the transgenic mice no tau phosphorylation was found, and in addition no aggregation of the protein was detected [31] suggesting a correlation between tau phosphorylation and aggregation in this model. This result was supported by an additional experiment using another mouse also expressing human tau with a FTDP-17 mutation [30].

Alternatively, protein chaperones, acting on tau or in phosphotau, could modify the level of tau aggregation, examples could be the protein 14-3-3 [35], musashi-1 [36], or Pin-1 [37, 38]. The chaperone associated ubiquitin ligase CHIP could be able to target phosphotau for proteasomal degradation [16, 18].

TOXICITY OF PHOSPHOTAU

It has been described that tau binding to microtubules is regulated by the level of tau phosphorylation at some specific sites [39]. It is known that hyperphosphorylated tau binds with less affinity to microtubules resulting in the decrease in the interaction with microtubules, in a decrease of microtubule stability [8], and probably in a microtubule dysfunction inside the cell that could result in a toxic effect [40]. Also, tau phosphorylation could result, as indicated above, in a decrease of its proteolysis [17]. More recently, it has been indicated that expression of a tau mutant (P301L) could result in an increase of its phosphorylation, since once it is phosphorylated, that mutation can prevent the binding of those phosphatases involved in its dephosphorylation [41]. This phosphotau could have a decreased capacity for microtubule binding and it could be toxic for the cell. Additionally, it has been indicated that hyperphosphorylated tau can cause neurodegeneration, in the absence of large tau aggregates [42]. On the other hand, only the overexpression of wild-type human tau in a mouse is sufficient to cause tau phosphorylation, aggregation, and neural toxicity [43]. On the other hand, it has been suggested that tau phosphorylation may represent a protective function in AD [44].

TOXICITY OF AGGREGATED TAU

Sometime ago, the development of tau pathology, related with dementia in AD [45], was clearly described by Braak and Braak [46] by following the development of neurofibrillary lesions at different stages of the disease. Also, the formation of neurofibrillary (tau aggregates) pathology within those neurons of hippocampus and cerebral cortex affected at different stages was found. These neurons could degenerate yielding extracellular ghost tangles (eNFT) [47]. In the hippocampus, an inverse relation has been found between the number of eNFT and the number of surviving neurons [48–51]. It suggests that neurons that degenerate, have previously developed tau aggregates. On the other hand, it has been indicated that neurons bearing neurofibrillary lesions could survive for a long period of time [52], and, by comparing with other neurodegenerative disorders, like Huntington disease [3], it can be suggested that tau aggregates could protect against neurodegeneration by sequestering toxic (phospho?) monomeric tau that could be present in a high amount inside a cell in pathological conditions. Also, it has been suggested, using a transgenic mouse model [53], that behavioural (memory) deficits could be unrelated to the formation of tau polymers, although, more recently, the discussion of those experiments suggested that hyperphosphorylated, aggregated tau intermediates could be the ones that cause neurodegeneration [33]. In this way, the implication of different types of protein aggregates in neurodegeneration has been extensively discussed [19, 54]. A possibility about the existence of neurotoxic tau intermediate aggregates in human tauopathies is based in the fact that patients with FTDP-17 show an extensive neurodegeneration with a high level of tau phosphorylation but with a low number of tangles [55].

In any case, even if the formation of tau aggregates has a protective function for the neurons, that function is not working well, as described by Braak and Braak [46], and afterwards by Delacourte et al [56], indicating a correlation between progression of tau pathology and progression of the disease. This idea is supported by those experiments indicating that neural loss and neurofibrillary tangle number increase in parallel with the progression of the disease [57]. Similar results have been described in other neurological disorders like brain encelphalopathies, where the formation of aberrant polymers are related to the onset of neurodegeneration [1]; whereas this is far from clear in other disorders like Huntington disease.

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Review Article

Dysregulation of Protein Phosphorylation/Dephosphorylation in Alzheimer's Disease: A Therapeutic Target

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Studies during the last two decades have provided new insights into the molecular mechanism of Alzheimer's disease (AD). One of the milestone findings in AD research was the demonstration that neurofibrillary degeneration characterized by tau pathology is central to the pathogenesis of AD and other tauopathies and that abnormal hyperphosphorylation of tau is pivotal to neurofibrillary degeneration. This article reviews the recent research advances in tau pathology and the underlying dysregulation of the protein phosphorylation/dephosphorylation system. An updated model of the mechanism of neurofibrillary degeneration is also presented, and a promising therapeutic target to treat AD by correcting dysregulation of protein phosphorylation/dephosphorylation is discussed.

INTRODUCTION

Although Alzheimer's disease (AD) and its main brain histopathology, that is, senile plaques and neurofibrillary tangles (NFTs), were described a century ago, significant research advances in the disease began only a few decades ago. The discoveries of the major protein components of senile plaques as amyloid β-peptide [1, 2] and of NFTs as abnormally hyperphosphorylated tau [3, 4] in the 1980s initiated a new era of AD research. Since then, much research has focused on the molecular mechanisms of initiation and formation of the senile plaques and NFTs and their roles in the pathogenesis of AD. Evidence accumulated in the last two decades indicates that malprocessing of both tau and β-amyloid precursor protein, which produces β-peptide, is pivotal, if not central, to the molecular mechanism of AD. The severity of dementia symptoms in AD strongly correlates to the number of NFTs, but not of senile plaques, in AD brains [5–9], suggesting that tau pathology might be associated with the disease mechanism more directly. Abnormal hyperphosphorylation of tau and its deposits in the brain is also seen in several other neurodegenerative diseases that are collectively named tauopathies (for review, see [10, 11]). The discovery of tau mutations that cause hereditary frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) [12–14] further indicates that tau abnormality alone is sufficient to produce dementia. Therefore, for developing rational therapeutic treatment of AD, it is essential to understand the molecular mechanism by which tau abnormalities lead to neurofibrillary degeneration.

Because tau aggregated in the brain of AD and all other tauopathies is always abnormally hyperphosphorylated, numerous studies have focused on the roles of the abnormal hyperphosphorylation and the mechanism leading to tau hyperphosphorylation. Recent studies demonstrate that it is the abnormal hyperphosphorylation that makes tau lose its normal function to stimulate microtubule assembly, gain toxic activity, and aggregate into NFTs [15–23]. In addition to tau, several other brain proteins such as neurofilaments, microtubule-associated protein (MAP) 1 B, β-tubulin, and β-catenin are also found to be hyperphosphorylated [24–27], suggesting that the protein phosphorylation/dephosphorylation system might be dysregulated in AD brain. This article attempts to review the recent advances in this respect. Because abnormally hyperphosphorylated tau is pivotal to AD and has been extensively studied, this review focuses on tau hyperphosphorylation. Prevention and reversal of abnormal hyperphosphorylation of tau as a potential promising therapeutic strategy is also discussed.

TAU PROTEIN

Tau was first discovered by Weingarten et al [28] as a microtubule-associated protein that stimulates microtubule

assembly. There was not much research interest in tau protein until a decade later, when it was found to make up the paired helical filaments (PHFs) that form NFTs in AD brain [3, 4, 29]. Human tau gene was found on the long arm of chromosome 17 (position 17q21) and was found to contain 16 exons [30]. This single tau gene encodes six tau isoforms in adult human brain as a result of alternative splicing of its mRNA [31]. The six isoforms of tau differ from each other by the presence or absence of one or two inserts (29 or 58 amino acids) in the *N*-terminal part and by the presence of either three or four repeats in the *C*-terminal half. The *N*-terminal inserts are highly acidic. The repeats in the *C*-terminal half of tau are the domains that bind to microtubules [32–34]. The region upstream of the microtubule-binding domains contains many proline residues and, hence, is called the proline-rich region.

The best-known biological functions of tau are to stimulate microtubule assembly and to stabilize microtubule structure. Tau binds to microtubules via its microtubule-binding domains located at the *C*-terminal half of the molecule [32–34]. The *N*-terminal part projects from the microtubule surface, where it may interact with other cytoskeletal elements and the plasma membrane [35, 36]. Each of the six tau isoforms possibly has its particular physiological roles and differential biological activities, because they are differentially expressed during development and have different activities to stimulate microtubule assembly [37, 38]. Only the shortest isoform of tau is expressed in fetal brain, whereas all six isoforms are seen in adult brain [39, 40]. In addition to stimulating microtubule assembly, several studies have suggested that tau may have other physiological functions. It appears to interfere with binding of kinesin and kinesin-like motors to microtubules, leading to a preferential inhibition of plus-end-directed axonal transport [41]. Overexpression of tau inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum [42]. This may explain the symptoms of amyotrophic lateral sclerosis with neurofilament accumulation in motor neurons of several transgenic models of tau overexpression [43–46]. Tau has been found to interact with mitochondria [47], plasma membrane [36], and nucleic acids [48, 49], suggesting that it may act as a mediator between microtubules and these organelles. Tau also appears to interact with src-family nonreceptor tyrosine kinases such as fyn [50, 51] and phospholipase *C*-γ [52, 53] via its proline-rich region. These data suggest that tau may also play a role in the signal transduction pathways involving src-family tyrosine kinases and phospholipase *C*-γ. However, the physiological significance of these interactions remains to be elucidated.

As early as 1977, tau was found to be a phosphoprotein [54]. In 1984, it was demonstrated that phosphorylation of tau negatively regulates its activity in promoting microtubule assembly [55]. Because tau is abnormally hyperphosphorylated in AD and other tauopathies, tau phosphorylation has been studied extensively. Normal brain tau contains 2 or 3 moles of phosphates per mole of tau [56–58]. Studies on human brain biopsy tissue indicated that several serine and threonine residues of tau are normally phosphorylated at substoichiometrical levels [59, 60]. A normal level of phosphorylation appears to be required for tau's optimal function, whereas the hyperphosphorylated tau loses its biological activity [15, 16, 61–69].

ABNORMAL HYPERPHOSPHORYLATION OF TAU IN AD BRAIN

The discovery that tau aggregated in AD brain is abnormally hyperphosphorylated has stimulated many studies on the extent and sites of tau hyperphosphorylation and their role in the pathogenesis of AD. The phosphorylation level of tau isolated from autopsied AD brains is 3- to 4-fold higher than that from normal human brains [56–58]. In addition, the hyperphosphorylated tau is accumulated in both brains [70, 71] and cerebral spinal fluid [72–80] of individuals with AD. All six isoforms of tau are aggregated into PHFs in the abnormally hyperphosphorylated forms in AD brains [3, 4, 31, 81]. To date, at least 37 serine and threonine residues have been found to be phosphorylated in PHF-tau (for review, see [82]). These residues include Thr39, Ser46, Thr69, Thr123, Ser137, Thr153, Thr175, Thr181, Ser198, Ser199, Ser202, Thr205, Ser208, Ser210, Thr212, Ser214, Thr217, Thr231, Ser235, Ser237, Ser238, Ser241, Ser262, Ser285, Ser305, Ser324, Ser352, Ser356, Ser396, Ser400, Thr403, Ser404, Ser409, Ser412, Ser413, Ser416, and Ser422. Many of these residues are also phosphorylated in normal human brains without NFTs at smaller extents, but they are rapidly dephosphorylated during postmortem delay and tissue processing [59, 60]. However, the phosphate groups at these sites are not readily dephosphorylated during the postmortem period and tissue processing of AD brain, probably because of the deficient protein phosphatase activities [83–89]. Some of the phosphorylation sites seen in PHF-tau are not phosphorylated at all in normal brains. These sites include Thr212/Ser214, Thr231/Ser235 [90], and Ser422 [91, 92].

Because all of the previously identified phosphorylation sites of normal tau and PHF-tau are at either serine or threonine residues, it was thought that tau was phosphorylated only at serine and threonine residues. However, recent studies suggest that tau in developing brain and in AD brain is also phosphorylated at tyrosine residues. The src-family nonreceptor tyrosine kinase fyn can bind to and phosphorylate tau in vitro and in transfected cells [50, 51, 93]. The phosphorylation site of tau was mapped as Tyr18. Tyrosine phosphorylated tau at this position is also seen immunohistochemically in the brain of transgenic mice that express mutated human $\text{tau}_{\text{P301L}}$ [51]. Williamson et al [94] demonstrated that in primary human and rat brain cortical cultures tau is phosphorylated at Tyr 29 upon treatment with Aβ. The tyrosine phosphorylation of tau appears rapid and transient. Interestingly, antibodies specific to tyrosine phosphorylated tau labeled purified PHF-tau, but not normal tau, suggesting that PHF-tau is phosphorylated at the tyrosine residues [93, 94]. In addition, Tyr394 was also found to be phosphorylated in PHF-tau and in tau from fetal brains, and the phosphorylation at this site is catalyzed by another nonreceptor

tyrosine kinase *c*-Abl [95]. It is not clear if the phosphorylation at any of the above tyrosine residues is stoichiometrically significant. Therefore, whether the tyrosine phosphorylation of tau has any pathophysiological relevance remains to be elucidated.

Numerous studies have demonstrated the important role of abnormal hyperphosphorylation of tau in its aggregation into NFTs and in Alzheimer's neurofibrillary degeneration. In cultured cells, hyperphosphorylation of tau after treatment with phosphatase inhibitors impairs its activity to bind to microtubules and induces filamentous aggregation of tau [21]. Pseudohyperphosphorylated tau that simulates abnormally hyperphosphorylated tau by mutation of serine or threonine residues into glutamate at selected AD-related sites exerts a cytotoxic effect, whereas wild-type tau is neutral [22]. In contrast, neurons from tau-knockout mice are resistant to Aβ-induced neurotoxicity [96]. Overexpression of human tau in combination with phosphorylation by Drosophila GSK-3β homologue Shaggy, but not tau overexpression alone, exacerbates tau-induced neurodegeneration and results in the formation of NFT-like filamentous tau aggregates [23]. This study shows a causal relationship between tau hyperphosphorylation and neurofibrillary degeneration in vivo. A study in Disabled-1 (an adapter protein) knockout mice further demonstrates that tau hyperphosphorylation causes early death of the animals [97]. Most importantly, tau in inclusions of all tauopathies in human and animal models is always hyperphosphorylated (for reviews, see [11, 98]). Abnormal hyperphosphorylation of tau appears to precede its aggregation into NFTs in AD brain [57, 99–101]. Taken together, these studies suggest that the abnormal hyperphosphorylation of tau is crucial to neurofibrillary degeneration in AD and other tauopathies.

The largest isoform of human brain tau (441 amino acids) contains 80 serine and threonine residues and five tyrosine residues [31]. Phosphorylation at nearly half of these residues has been reported in PHF-tau (see [82] for review). Many studies have demonstrated that phosphorylation of tau at different sites has different impacts on its biological function and on its pathogenic role. For instance, a quantitative in vitro study demonstrated that phosphorylation of tau at Ser262, Thr231, and Ser235 inhibits its binding to microtubules by ~35%, ~25%, and 10%, respectively [102]. In cultured cells, phosphorylation of tau at Ser214 and Ser262 decreases its binding to microtubules and appears to inhibit its assembly to filaments [103]. In vitro kinetic studies of the binding between hyperphosphorylated tau and normal tau suggest that phosphorylation of tau at Ser199/Ser202/Thr205, Thr212, Thr231/Ser235, Ser262/Ser356, and Ser422 are among the critical phosphorylation sites that convert tau to a toxic molecule to sequester normal MAPs from microtubules [19]. Further phosphorylation at Thr231, Ser396, and Ser422 promotes self-assembly of tau into filaments. Similarly, tau mutated at Ser396 and Ser404 (changing Ser into Glu) to mimic phosphoserine is more fibrillogenic than wild-type tau [104], and a tau construct in which Ser422 is mutated to Glu shows a significantly increased propensity to aggregate [105]. Consistent with these observations is that mutation of Ser422 to Ala prevents Aβ-induced tau aggregation [106]. These results suggest that phosphorylation of Ser422 may play a key role in tau filament formation in vivo.

An important question is, by what mechanism is the tau abnormality involved in the pathological cascades that lead to neurodegeneration in AD and other tauopathies. Does a hyperphosphorylation-induced defect in its activity to stimulate microtubule-assembly contribute to cell dysfunction? Is it the formation of insoluble tau aggregates that is pathogenic? Although tau loses its activity to stimulate microtubules, lack of overt phenotype of tau knockout transgenic mice [107] suggests that it is very unlikely that tau abnormality contributes to neurodegeneration via loss of normal function due to its hyperphosphorylation. By a series of studies, we have found that both the abnormally hyperphosphorylated tau isolated from AD brain and in vitro hyperphosphorylation tau gain a toxic activity to sequester normal tau and other MAPs, such as MAP1 and MAP2, and cause microtubule disassembly [16, 18, 66, 108]. Upon dephosphorylation, they lose this toxic activity. Polymerization of the hyperphosphorylated tau into PHFs also abolishes this toxic activity (Alonso A et al, unpublished observation). Hence, we speculate that the abnormal hyperphosphorylation of tau causes neurodegeneration by gain of toxic activity rather than by loss of normal activity that can be compensated for by other MAPs and that formation of PHFs/NFTs from the hyperphosphorylated tau in neurons is a defense mechanism by which neurons aim to reduce the toxic activity of the abnormally hyperphosphorylated tau. This speculation is supported by recent in vivo studies. Conditional overexpression of GSK-3β in the transgenic mouse brains induces tau hyperphosphorylation and neurodegeneration, but no tau aggregation [109]. In contrast, there are NFTs but no memory loss in several lines of tau transgenic mice (for review, see [110]). This phenomenon is probably common to other diseases characterized by abnormal protein aggregates such as Huntington disease and cardiomyopathy, in which the abnormal, nonfibrillar protein oligomers, rather than the aggregates themselves, appear to be pathogenic [111, 112].

IMBALANCE OF PHOSPHORYLATION/ DEPHOSPHORYLATION IN AD BRAIN

To understand the mechanism leading to abnormal hyperphosphorylation of tau in AD, protein kinases and phosphatases that regulate tau phosphorylation level must be identified first. In the last two decades, numerous studies aimed to the identification of tau kinases and phosphatases have been carried out. It was found that in vitro, dozens of phosphoseryl/phosphothroenyl protein kinases and most of the major protein phosphatases could act on tau protein at various phosphorylation sites (for reviews in detail, see [82, 113, 114]). Tau appears to be a universal substrate for protein kinases and phosphatases in vitro. This may not be surprising, because nearly 20% of the amino acid residues of tau molecule are serines and threonines, and nearly 50% of these residues are phosphorylated to certain degrees in AD

brain (see [82] for review). However, it is unlikely that all these enzymes that act on tau in vitro catalyze tau phosphorylation/dephosphorylation in vivo. Immunohistochemical studies also have shown a colocalization of more than a dozen protein kinases and several protein phosphatases with NFTs of AD brain. As we now know that NFTs are very "sticky" structures that can be stained immunohistochemically by antibodies to numerous antigens, immunohistochemical colocalization with NFTs can only support other data that indicate a role of the specific protein or enzyme in the formation of NFTs, but itself cannot indicate such a role.

Further studies in cultured cells, in situ, and especially in vivo suggest that a few protein kinases and phosphatases may be involved in regulation of tau phosphorylation in the brain. The kinases that most likely play a role in phosphorylation of tau in the brain include glycogen synthase kanase-3β (GSK-3β), cyclin-dependent kinase 5 (cdk5), cAMP-dependent protein kinase (PKA), stress-activated protein kinases, and calcium/calmodulin-dependent kinase II (CaMK-II). Johnson and Stoothoff [115] have critically discussed this issue. The sites of tau phosphorylation by these kinases, except stress-activity protein kinases, have been summarized in our recent review [82]. Among protein phosphatases, PP2A has been shown to be the major tau phosphatase in the brain [69, 116–120]. In a recent study, we compared the catalytic kinetics of tau dephosphorylation by various major brain protein phosphatases and determined the relative contributions of these phosphatases to the regulation of tau phosphorylation quantitatively. We found that PP2A accounts for ~70% of the total tau phosphatase activity, whereas PP1, PP2B, and PP5 each accounts for only ~10% of the total tau phosphatase activity [88]. Because PP2B activity is upregulated rather than downregulated in AD brain, it is unlikely that it regulates tau phosphorylation in vivo [121].

Accumulated evidence indicates that tau phosphorylation is regulated by several protein kinases and that more than one kinase might be involved in abnormal hyperphosphorylation of tau in AD brain. Interestingly, GSK-3β phosphorylates tau at both prime sites (ie, tau needs to be primed by phosphorylation with other kinases at other sites) and unprimed sites [122–126]. In a cotransfection study, Cho and Johnson [125] found that a GSK-3β mutant (GSK-3β-R96A) that only phosphorylates unprimed sites has no negative impact on tau's ability to bind to microtubules, in contrast to wild-type GSK-3β, which significantly impairs tau's ability to bind to microtubules. Further studies demonstrate that primed phosphorylation of tau at Thr231 by GSK-3β plays a critical role in decreasing tau's ability to both bind to and stabilize microtubules [126]. In rat brains, activation of PKA not only induces primed phosphorylation of tau by GSK-3β, but also impairs the spatial memory of rats [124, 127]. GSK-3β appears to be regulated by both phosphoinositol-3 kinase and protein kinase *C* pathways [128–131].

An obvious approach to understanding how tau becomes abnormally hyperphosphorylated in AD is to study whether tau kinase(s) or tau phosphatase(s) are dysregulated in AD brain. Several studies have focused on whether the activities and expression of these enzymes are altered in AD brain. Among protein kinases, cdk5 was reported to be upregulated in AD brain by one laboratory [132], but this result was challenged by others [133–136]. On the other hand, both the activity and the expression of PP2A as well as the activities of PP1 and PP5 are decreased in the selected areas of AD brain [83–89]. Consistent with this finding, several other neuronal proteins such as neurofilaments, MAP1B, β-tubulin, and β-catenin are also hyperphosphorylated in AD brain [24–27]. Hence, it appears that downregulation of the phosphatases, especially of PP2A, might underlie the abnormal hyperphosphorylation of tau and other proteins in AD brain. Studies of metabolically active rat brain slices and transgenic mice suggest that the downregulation of PP2A may produce hyperphosphorylation of tau, not only by the deficient dephosphorylation of tau, but also through the activation of several PP2A-regulated protein kinases, including PKA [137], CaMK-II [138], MAP kinases, and stress-activated protein kinases [139–141]. Nevertheless, inhibition of PP2A activity in animal brain could only induce hyperphosphorylation of tau at some of the hyperphosphorylation sites seen in PHF-tau, but does not result in NFTs. Attempts to produce massive tangles of PHFs in animal models merely via alteration of tau phosphatase and/or kinase activities have not yet been successful. These observations suggest that the downregulation of tau phosphatases in AD brain may be only partially responsible for the abnormal hyperphosphorylation of tau.

The causes leading to decreased PP2A activity in AD brain are not well understood. Downregulation of PP2A expression [85] and upregulation of PP2A endogenous inhibitor proteins I_1^{PP2A} and I_2^{PP2A} [142] in AD brain may both contribute to the downregulation of PP2A activity. Because the activities of PP1 [83, 88] and PP5 [88, 89], which contribute to regulation of tau phosphorylation to a much smaller extent than PP2A [88], are also decreased in AD brain, there might be a common factor that downregulates the activities of the major brain protein phosphatases in AD brain.

In addition to tau kinases and phosphatases, alterations of tau itself, the substrate of these enzymes, may also play an important role in its abnormal hyperphosphorylation and conversion into PHFs. Tau is also modified post-translationally by β-*N*-acetylglucosamine (GlcNAc) via a glycosidic bond at the hydroxyl groups of serine and/or threonine residues, and this modification is called *O*-GlcNAcylation [143–145]. Because *O*-GlcNAc could modify the same serine or threonine residues of tau as phosphate does and a reciprocal relationship between *O*-GlcNAcylation and phosphorylation has been seen in many proteins (for review, see [146]), *O*-GlcNAcylation could affect phosphorylation of tau. Recent studies in various systems found that tau phosphorylation is indeed regulated by *O*-GlcNAcylation inversely [144, 145, 147]. Most interestingly, fasting of mice induces downregulation of tau *O*-GlcNAcylation, which relies on glucose metabolism to supply UDP-GlcNAc as a donor for protein *O*-GlcNAcylation, and in turn leads to hyperphosphorylation of tau [145]. These findings led to the novel hypothesis that impaired glucose uptake/metabolism in AD brain, which was well established decades ago, contributes

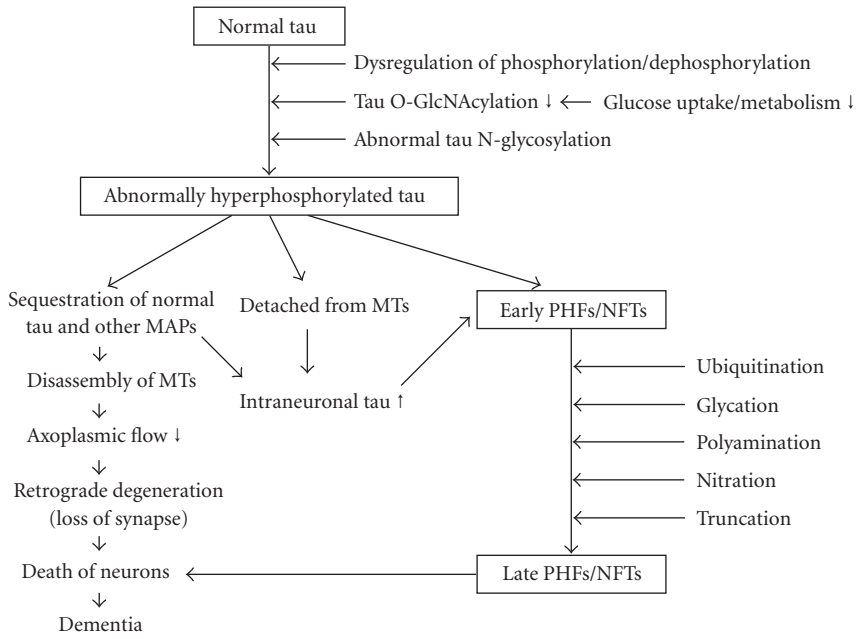

Figure 1: Proposed mechanism of neurofibrillary degeneration. MAPs, microtubule-associated proteins; MTs, microtubules; PHFs, paired helical filaments; NFTs, neurofibrillary tangles.

to the disease pathogenesis via downregulation of tau *O*-GlcNAcylation and, consequently, upregulation of tau phosphorylation that leads to neurofibrillary degeneration [148].

Classical *N*-linked glycosylation is a modification of protein at asparagine residues by oligosaccharides, which normally modifies only membrane proteins and secreted proteins. Tau in AD brain, but not in normal human brain, was found to be modified by *N*-glycosylation [68, 149, 150], and this aberrant tau modification appears to precede and facilitate abnormal hyperphosphorylation of tau [150–152]. This modification has been reviewed in detail in a recent article [82].

MECHANISM OF NEUROFIBRILLARY DEGENERATION

There is no doubt that the abnormality of tau plays a central role in neurofibrillary degeneration in AD and other tauopathies. A critical review of the literature accumulated in the last two decades sheds light onto the probable mechanism of neurofibrillary degeneration of AD (Figure 1).

Tau is the major microtubule-associated protein of mature neurons where it stimulates microtubule assembly and stabilizes microtubule structure. Tau is normally modified by both phosphorylation and *O*-GlcNAcylation. The phosphorylation level of tau is regulated by tau kinases and tau phosphatases, as well as by the alteration of tau itself. In AD and probably also in other tauopathies, metabolic and genetic abnormalities lead to dysregulation of signal transduction pathways, which in turn causes an imbalance of the phosphorylation/dephosphorylation system, that is, downregulation of PP2A in the brain. This imbalance results in increased phosphorylation (ie, hyperphosphorylation) of tau. The impaired brain glucose uptake/metabolism that precedes AD also facilitates hyperphosphorylation of tau via downregulation of tau *O*-GlcNAcylation [148]. Aberrant *N*-glycosylation of tau in AD brain also makes tau a more favorable substrate for major tau kinases and less favorable for tau phosphatases [151, 152], thereby facilitating tau hyperphosphorylation.

The abnormally hyperphosphorylated tau resulting from any of the above causes not only loses its biological activity to stimulate microtubule assembly, but also becomes a toxic molecule, sequesters normal tau, MAP1, and MAP2, and causes disassembly of microtubules. The breakdown of the microtubule network in the affected neurons compromises axonal transport and leads to retrograde degeneration, which in turn results in neuronal death and dementia. On the other hand, the abnormally hyperphosphorylated tau detached from microtubules is not only easier to polymerize into PHFs as a result of hyperphosphorylation, but it also causes increased intraneuronal soluble tau concentration due to sequestration of normal tau from microtubules, which further facilitates tau aggregation into PHFs. The polymerized abnormal tau is further modified by ubiquitination, glycation, polyamination, nitration, and truncation (for review, see [82]), and forms mature PHFs/NFTs. Unlike the unpolymerized hyperphosphorylated tau that is toxic, PHFs/NFTs appears to be inert (Alonso A et al unpublished observation), but these lesions grow in size with disease progression

and eventually might physically choke the affected neuron to death.

THERAPEUTIC TARGET TO TREAT AD BY CORRECTING DYSREGULATION OF PROTEIN PHOSPHORYLATION/ DEPHOSPHORYLATION

Because neurofibrillary degeneration plays a central role in the pathogenesis of AD, one of the most attractive therapeutic targets of AD is to inhibit neurofibrillary degeneration. As outlined in Figure 1, the most promising approaches to achieve this goal are to inhibit the abnormal hyperphosphorylation of tau and to inhibit its sequestration of normal MAPs. The former approach is more effective since it should both rescue the disruption of microtubule and axoplasmic flow and prevent further deposition of NFTs. Several academic groups and pharmaceutical companies have been investigating this approach by restoring PP2A activity or inhibiting tau kinase activity in the brain. Memantine, a low-to-moderate-affinity antagonist of NMDA receptor, which improves mental function and the quality of daily life of individuals with moderate to severe AD [153, 154], reverses the okadaic-acid-induced inhibition of PP2A activity and prevents tau hyperphosphorylation in hippocampal slice cultures from adult rats [155]. The restoration of PP2A activity to normal level by memantine also leads to restoration of the expression of MAP2 in the neuropil and a reversal of hyperphosphorylation and accumulation of neurofilaments. Wang's group has demonstrated that treatment of brain slices and rats with melatonin can restore PP2A activity that is inhibited by okadaic acid or calyculin A and reverse hyperphosphorylation of tau and neurofilament proteins as well as cytotoxicities [156–158]. Melatonin also prevents tau hyperphosphorylation and aggregation induced by overactivation of GSK-3 or PKA [131, 159]. These are examples showing that inhibition of dysregulation of protein phosphorylation/dephosphorylation is a promising target to treat AD. Further investigation of new compounds that can inhibit abnormal hyperphosphorylation of tau will likely provide new treatments for AD.

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Review Article

Oxidative Damage to RNA in Neurodegenerative Diseases

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Since 1999, oxidative damage to RNA molecules has been described in several neurological diseases including Alzheimer's disease, Parkinson's disease, Down syndrome, dementia with Lewy bodies, prion disease, subacute sclerosing panencephalitis, and xeroderma pigmentosum. An early involvement of RNA oxidation of vulnerable neuronal population in the neurodegenerative diseases has been demonstrated, which is strongly supported by a recent observation of increased RNA oxidation in brains of subjects with mild cognitive impairment. Until recently, little is known about consequences and cellular handling of the RNA damage. However, increasing body of evidence suggests detrimental effects of the RNA damage in protein synthesis and the existence of several coping mechanisms including direct repair and avoiding the incorporation of the damaged ribonucleotides into translational machinery. Further investigations toward understanding of the consequences and cellular handling mechanisms of the oxidative RNA damage may provide significant insights into the pathogenesis and therapeutic strategies of the neurodegenerative diseases.

INTRODUCTION

Growing body of evidence has indicated that oxidative damage is involved in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) [1, 2]. Although RNA in a cell should be subject to the same oxidative insults as DNA and other cellular macromolecules, oxidative damage to RNA has not been the major focus in investigating the magnitude and the biological consequence. Because RNA is mostly single-stranded and its bases are not protected by hydrogen bonding and probably less protected by specific proteins, RNA may be more susceptible to oxidative insults than DNA [3]. Indeed, greater oxidation in cellular RNA than that in DNA was demonstrated in several experimental studies on nonneuronal cell lines and tissues, where oxidized nucleosides, 8-hydroxydeoxyguanosine (8 OHdG) and 8-hydroxyguanosine (8 OHG), were measured as markers for oxidative damage to DNA and RNA, respectively [4–6]. It is now becoming evident that RNA molecules are not only intermediates in the transfer of genetic information from DNA to proteins but also key players in many mechanisms controlling expression of genetic information [7]. Therefore, RNA damage is detrimental to cells and may be involved in the pathogenesis of neurodegenerative diseases [3]. Here we review recent studies demonstrating RNA oxidation in vulnerable neuronal population in several neurological diseases and discuss the biological significance of the damage to RNA.

RNA OXIDATION IN VARIOUS NEUROLOGICAL DISEASES AND EXPERIMENTAL CONDITIONS

Among multiple adducts of nucleoside oxidation, 8 OHdG and 8 OHG are two of the best characterized and studied forms of DNA and RNA oxidation, respectively [4, 5]. The availability of highly specific antibodies with 8 OHdG and 8 OHG has enabled us to perform in situ approaches to nucleoside oxidation in postmortem brain samples taken from patients with neurological diseases [29, 30]. In 1999, prominent 8 OHdG/8 OHG immunoreactions were demonstrated in the vulnerable neuronal populations in postmortem brains of patients with AD and PD [8, 9]. In both AD and PD, the neuronal 8 OHdG/8O HG immunoreactions showed cytoplasmic predominance, which indicated

Table 1: Summary of studies on RNA oxidation in the central nervous system. (ELISA: enzyme-linked immunosorbent assay; HPLC: high-performance liquid chromatography; IB: immunoblot; ICC: immunocytochemistry; IEM: immunoelectronmicroscopy; RT-PCR: reverse transcription and polymerase chain reaction.)

Year	Human neurological diseases	Materials/Procedures	Authors
1999	Alzheimer's disease	Brain (hippocampus/cerebral cortex)/ICC	Nunomura et al [8]
	Parkinson's disease	Brain (substantia nigra)/ICC	Zhang et al [9]
2000	Down syndrome	Brain (cerebral cortex)/ICC	Nunomura et al [10]
2001	Alzheimer's disease	Brain (hippocampus/cerebral cortex)/IEM	Nunomura et al [11]
2002	Dementia with Lewy bodies	Brain (hippocampus/cerebral cortex)/ICC	Nunomura et al [12, 13]
	Familial/sporadic Creutzfeldt-Jakob disease	Brain (cerebral cortex)/ICC	Guentchev et al [14]
	Subacute sclerosing panencephalitis	Brain (cerebral cortex)/ICC	Hayashi et al [15]
	Alzheimer's disease	Cerebrospinal fluid/HPLC	Abe et al [16]
	Parkinson's disease/multiple-system atrophy	Cerebrospinal fluid/ELISA	Kikuchi et al [17]
2003	Alzheimer's disease	Brain (hippocampus/cerebral cortex)/IB and RT-PCR (mRNA)	Shan et al [18, 19]
	Parkinson's disease	Cerebrospinal fluid/HPLC	Abe et al [20]
2004	Familial Alzheimer's disease	Brain (cerebral cortex)/ICC	Nunomura et al [21]
2005	Alzheimer's disease	Brain (hippocampus)/IB and RT-PCR (rRNA)	Honda et al [22]
	Xeroderma pigmentosum (group A)	Brain (globus pallidus)/ICC	Hayashi et al [23]
	Gerstmann-Straussler-Scheinker disease	Brain (hippocampus/cerebral cortex)/ICC	Petersen et al [24]
	Alzheimer's disease/mild cognitive impairment	Brain (cerebral cortex)/IB (rRNA)	Ding et al [25]
Year	Experimental conditions	Materials/Procedures	Authors
2002	Old rat	Brain (hippocampus)/ICC	Liu et al [26]
2003	Adult rat with intermittent hypoxia	Brain (hippocampus)/ICC	Row et al [27]
2004	Culture neuron under proteasome inhibition	Mixed astrocyte and neuron cultures/ICC and IB	Ding et al [28]

that mitochondrial DNA and cytoplasmic RNA in neurons were major targets of oxidative damage. Because the neuronal 8 OHdG/8 OHG immunoreactions in AD brain were diminished greatly by RNase pretreatment but not by DNase pretreatment, the oxidized nucleoside was predominantly associated with RNA rather than DNA [8]. This notion was further supported by the immunoelectron microscopic observation that most of the oxidized nucleoside was localized to the ribosomal structures [11].

Similar RNA oxidation in neuronal cytoplasm was observed in brain samples of patients with Down syndrome [10], dementia with Lewy bodies [12, 13], Creutzfeldt-Jakob disease [14], and subacute sclerosing panencephalitis [15], as summarized in Table 1. The oxidative damage to RNA was demonstrated not only in sporadic form of the diseases but also in familial form of AD [21] and prion disease, that is, familial Creutzfeldt-Jakob disease and Gerstmann-Strausler-Scheinker disease [14, 24]. Furthermore, nuclear DNA oxidation and cytoplasmic RNA oxidation were observed in brains of patients with a genetic defect of nucleotide excision repair mechanism, xeroderma pigmentosum, showing cutaneous hypersensitivity to sunlight and progressive neurological disturbances [23].

These immunocytochemical studies demonstrating neuronal RNA oxidation in the neurological diseases were followed by biochemical detection of the oxidized nucleoside in AD brain with immunoblot approaches [18, 19, 22, 25]. Shan et al [18, 19] used Northwestern blotting with a monoclonal anti-8 OHG antibody, to isolate and identify oxidized RNA species and showed that significant amount of poly $(A)^+$ mRNA species were oxidized in AD brain. The oxidation to mRNA was further confirmed by cDNA synthesis and Southern blotting of the immunoprecipitated mRNA species. Densitometric analysis of the Southern blot results revealed that 30–70% of the mRNAs from AD frontal cortices were oxidized, while only 2% of the mRNAs were oxidized in age-matched normal controls [19]. Interestingly, reverse transcription-PCR and filter array analyses of the identified oxidized mRNAs revealed that some species were more susceptible to oxidative damage in AD, while no common motifs or structures were found in the oxidatively susceptible mRNA species. Some of the identified known oxidized transcripts were related to AD, which included p21ras, mitogen-activated protein kinase (MAPK) kinase 1, carbonyl reductase, Cu/Zn superoxide dismutase, apolipoprotein D, calpains, but not amyloid β protein precursor or tau [18]. Although these studies by Shan et al [18, 19] focused on mRNA species that account for only a few percent of total cellular RNA, Honda et al [22] and Ding et al [25] reported that rRNA, extremely abundant in neurons, contained 8 OHG in AD brain. rRNA showed higher binding capacity to redox-active iron than tRNA, and consequently oxidation of rRNA

by the Fenton reaction formed 13 times more 8 OHG than tRNA [22].

Of note, both immunocytochemical studies [8, 9, 12, 13] and biochemical studies [18, 25] revealed that the regional distribution of the RNA oxidation in the brain was consistent with the selective neuronal vulnerability in each neurological disease. There were increased levels of 8 OHG in the hippocampus and cerebral neocortex in AD as well as in the substantia nigra in PD, while no alteration in the 8 OHG level was found in the cerebellum in both AD and PD compared with controls [8, 9, 18, 25].

Significantly increased levels of the oxidized RNA nucleoside, 8 OHG, have been identified not only in brain tissue but also in cerebrospinal fluid collected from patients with AD and PD [16, 17, 20] as well as in serum of PD patients [17], which indicates that 8 OHG is a possible biomarker of the diseases. As we describe in the next section, 8 OHG is a potent candidate of an early-stage marker of the diseases or a marker predicting conversion from the prodromal stage into an early stage of the diseases.

Experimental studies with rodent have shown that neuronal RNA oxidation and spatial memory deficit are observed in old animals [26] as well as animals with intermittent hypoxia [27]. In both aging and hypoxia models, antioxidants or mitochondrial metabolites can reduce the oxidative damage and the spatial memory deficit. In another experimental model using mixed astrocyte and neuron cultures [28], DNA oxidation and RNA oxidation have been observed following proteasome inhibition that is associated with several neurodegenerative features such as protein aggregation, activated apoptotic pathways, and induction of mitochondrial disturbances. Interestingly, in this proteasome inhibition model, neuron underwent larger increases in nucleic acid oxidation compared to astrocyte cultures, and RNA appeared to undergo a greater degree of oxidation than DNA, which was exactly identical in AD brain [8].

RNA OXIDATION: AN EARLY-STAGE EVENT IN THE PROCESS OF NEURODEGENERATION

Because RNA oxidation is involved with a wide variety of neurological diseases (Table 1), it may be considered an event in common neurodegenerative pathway that occurs in a late stage of the diseases. However, that is not the case in AD and PD. There is a considerable amount of evidence supporting an early involvement of RNA oxidation in the pathological cascade of neurodegeneraton, especially in AD (Table 2). Namely, RNA oxidation has been observed in postmortem brains of cases with early-stage AD [11], a presymptmatic case with familial AD mutation [21], Down syndrome cases with early-stage AD pathology [10], and subjects with mild cognitive impairment (MCI) who possibly represent podromal stage of AD [25]. Furthermore, the increased level of RNA oxidation in cerebrospinal fluid is more prominent in cases with shorter duration of AD and PD [16, 20]. Recent studies of MCI subjects have demonstrated also increased oxidation to protein and lipid in postmortem brain [31], increased lipid peroxidation in cerebrospinal fluid, plasma, and urine [32], increased DNA oxidation in peripheral leukocytes [33] as well as decreased plasma antioxidant vitamins and enzymes [34], and decreased plasma total antioxidant capacity [35]. From clinical points of view, the notion of an early involvement of oxidative damage in the pathogenesis of these degenerative diseases should have a great importance to establish a diagnostic tool and a therapeutic target, as we have reviewed recently [36, 37].

Of note, an early-stage involvement of neuronal RNA oxidation is identified not only in age-associated neurodegenerative diseases, but also in cases with subacute sclerosing panencephalitis that is caused by persistent measles virus infection in the central nervous system and is pathologically accompanied with brain atrophy and neurofibrillary tangles [15].

SOURCES OF REACTIVE OXYGEN SPECIES (ROS) RESPONSIBLE FOR RNA OXIDATION

The brain is especially vulnerable to oxidative damage because of its high content of easily peroxidizable unsaturated fatty acids, high oxygen consumption rate (accounting for 20–25% of the total body oxygen consumption, but for less than 2% of the total body weight), and relative paucity of antioxidant enzymes compared with other organs (e.g., the content of catalase in brain is only 10–20% of liver and heart) [37]. Therefore, neurons are continuously exposed to ROS such as superoxide, H_2O_2, and hydroxyl radical that are produced from the mitochondrial electron transport chain through normal cellular metabolism. Hydroxyl radical can diffuse through tissue only in the order of several nanometers and superoxide is hardly permeable through cell membrane. In consideration of widespread damage to cytoplasmic RNA in the neurodegenerative diseases, RNA species are likely attacked by hydroxyl radical, which is formed from the reaction of highly diffusible H_2O_2 with redox-active metals through Fenton reaction [8, 22]. In AD brain, disrupted mitochondria likely play a central role in producing abundant ROS as well as supplying redox-active iron into the cytosol [38, 39]. Indeed, ribosomes purified from AD hippocampus contain significantly higher levels of redox-active iron compared to controls, and the iron is bound to rRNA [22]. Therefore, mitochondrial abnormality and metal dysregulation are key features closely associated with ROS formation responsible for the RNA oxidation in AD. Interestingly, both of the features are found in the substantia nigra of PD also [36].

RNA OXIDATION AND THE BIOLOGICAL CONSEQUENCE

Although more than 20 different types of oxidatively altered purine and pyrimidine bases have been detected in nucleic acids [40], guanine is the most reactive of the nucleic acid base [41]. Therefore, the oxidized base, 8 OHG, is the most abundant among the oxidized bases [3]. The 8 OHG can be formed in RNA by direct oxidation of the base and also by the incorporation of the oxidized base from the cytosolic pool

Table 2: Summary of evidence suggesting temporal primacy of RNA oxidation in the process of neurodegeneration.

Materials/subjects	Findings
Postmortem brains of patients with Alzheimer's disease	RNA oxidation is more prominent in cases with lesser amounts of $A\beta$ plaque deposition or shorter disease duration [11].
	RNA oxidation is more prominent in hippocampal neurons free of neurofibrillary tangles compared to neurons with neurofibrillary tangles [11].
	RNA oxidation is increased in a presymptomatic case with presenilin-1 gene mutation [21].
Postmortem brains of subjects with mild cognitive impairment	RNA oxidation is increased in brains of subjects with mild cognitive impairment, who, at least in part, represent a prodromal stage of dementia [25].
Postmortem brains of patients with Down syndrome	RNA oxidation precedes $A\beta$ plaque deposition in a series of Down syndrome brains, a model of Alzheimer's-type neuropathology [10].
Postmortem brains of patients with subacute sclerosing panencephalitis	RNA oxidation is observed in cases with shorter disease duration, while lipid peroxidation is observed in cases with longer disease duration [15].
Cerebrospinal fluid of patients with Alzheimer's disease	RNA oxidation is more prominent in cases with shorter disease duration or higher scores in mini-mental state examination [16].
Cerebrospinal fluid of patients with Parkinson's disease	RNA oxidation is more prominent in cases with shorter disease duration [20].

into RNA through the normal action of RNA polymerase [40, 41]. Not only 8 OHG, but also 8-hydroxyadenosine, 5-hydroxycytidine, and 5-hydroxyuridine have been identified in oxidized RNA [41], which may have altered pairing capacity and thus may be at the origin of erroneous protein production. Indeed, the 8 OHG can pair with both adenine and cytosine, and thus the oxidized RNA compromises the accuracy of translation [40].

The biological consequence of oxidatively damaged mRNA species has been investigated in vitro by expressing them in cell lines. Oxidized mRNAs lead to loss of normal protein level and protein function, and potentially produce defective proteins leading to protein aggregation, a common feature of neurodegenerative diseases [18]. Also the biological consequence of ribosomal oxidation has been investigated in vitro by translation assay with oxidized ribosomes from rabbit reticulocyte, which shows a significant reduction of protein synthesis [22]. Recently, a study on brains of subjects with AD and MCI has demonstrated ribosomal dysfunction associated with oxidative RNA damage [25]. Isolated polyribosome complexes from AD and MCI brains show decreased rate and capability for protein synthesis without alteration in the polyribosome content. Decreased rRNA and tRNA levels and increased 8 OHG in total RNA pool, especially in rRNA, are accompanied with the ribosomal dysfunction, while there is no alteration in the level of initiation factors.

These findings have indicated that RNA oxidation has detrimental effects on cellular function whether the damaged RNA species are coding for proteins (mRNA) or performing translation (rRNA and tRNA). It is noteworthy that studies on some anticancer agents have shown that RNA damage can lead to cell-cycle arrest and cell death, as much as DNA damage does [42]. RNA damage may cause cell death via pathway involving either p53-dependent mechanism associated with inhibition of protein synthesis or p53-independent mechanism different from inhibition of protein synthesis.

COPING WITH RNA DAMAGE

Until recently, it has been considered that damaged RNA may be only degraded rather than repaired. However, Aas et al [43] has suggested that the cells have at least one specific mechanism to repair RNA damage, indicating that cells may have a greater investment in the protection of RNA than previously suspected [3, 42]. Indeed, alkylation damage in RNA is repaired by the same mechanism as a DNA-repair, catalyzed in the bacterium *Escherichia coli* by the enzyme Alk B, and in humans by the related protein [43]. Alk B and its homologue hABH3 cause hydroxylation of the methyl group on damaged DNA and RNA bases, and thus directly reverse alkylation damage. Alk B and hABH3, but not hABH2, repair RNA, since Alk B and hABH3 prefer single-stranded nucleic acids while hABH2 acts more efficiently on double-stranded DNA.

DNA damage can be repaired not only by the mechanism of direct reversal of the modified bases but also by base excision repair mechanism. Specific DNA glycosylases excise the damaged base and DNA polymerases replace the nucleotide [42, 44]. Furthermore, cells have mechanism of dealing with nucleotide damage other than direct repair, which seems to be useful for defense against oxidative damage to both DNA and RNA. Because oxidation of nucleotides can occur in the cellular nucleotide pool and the oxidized nucleotide can be incorporated into DNA and RNA, the mechanism avoiding such incorporation of the oxidized nucleotide is involved in coping with nucleic acid damage [40, 42, 44]. MutT protein

in *E coli* and its mammalian homologues MTH1 and NUDT5 participate in this error-avoiding mechanism by hydrolyzing the oxidized nucleoside diphosphates and/or triphosphates to the monophosphates [3, 40, 44]. Indeed, the increase in the production of erroneous proteins by oxidative damage is 28-fold over the wild-type cells in *E coli mutT* deficient cells, which is reduced to 1.2- or 1.4-fold by the expression of MTH1 or NUDT5, respectively [40].

Then, one important question is whether cells have machineries against oxidatively damaged nucleotides that are contained in RNA. Recently, proteins that bind specifically to 8 OHG-containing RNA have been reported, namely, *E coli* polynucleotide phosphorylase protein (Pnp) and human Y box-binding protein 1 (YB-1) [45, 46]. The binding of the specific protein likely makes the 8 OHG-containing RNA resistant to nuclease degradation [45]. However, it has been proposed that these proteins may recognize and discriminate the oxidized RNA molecule from normal ones, thus contributing to the fidelity of translation in cells by sequestrating the damaged RNA from the translational machinery [45, 46].

It is possible that the RNA quality control mechanisms are defective or inefficient in cancer cells as well as cells of neurodegenerative diseases. Further elucidation of the mechanisms of repair or avoidance of RNA damage and their potential role in preventing human diseases might provide new approaches to a number of unresolved issues of life science, while it has not been the major focus in investigation for a long period [3].

CONCLUSION

An early involvement of RNA oxidation of vulnerable neuronal population in neurodegenerative diseases such as AD and PD has been demonstrated in immunocytochemical and biochemical studies. Indeed, oxidized RNA is associated with a disturbance in protein synthesis in vitro and in vivo. Although there are only a small number of studies suggesting the existence of coping mechanisms for RNA damage at present, the known mechanisms may be the tip of iceberg of cellular investment in counteracting the RNA damage. Understanding of the consequences and cellular handling mechanisms of the oxidative RNA damage may provide clues to both basic research and the treatment of the neurodegenerative diseases.

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Review Article

Mitochondrial Oxidative Damage in Aging and Alzheimer's Disease: Implications for Mitochondrially Targeted Antioxidant Therapeutics

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The overall aim of this article is to review current therapeutic strategies for treating AD, with a focus on mitochondrially targeted antioxidant treatments. Recent advances in molecular, cellular, and animal model studies of AD have revealed that amyloid precursor protein derivatives, including amyloid beta ($A\beta$) monomers and oligomers, are likely key factors in tau hyperphosphorylation, mitochondrial oxidative damage, inflammatory changes, and synaptic failure in the brain tissue of AD patients. Several therapeutic strategies have been developed to treat AD, including anti-inflammatory, antioxidant, and antiamyloid approaches. Among these, mitochondrial antioxidant therapy has been found to be the most efficacious in reducing pathological changes and in not producing adverse effects; thus, mitochondrial antioxidant therapy is promising as a treatment for AD patients. However, a major limitation in applying mitochondrial antioxidants to AD treatment has been the inability of researchers to enhance antioxidant levels in mitochondria. Recently, however, there has been a breakthrough. Researchers have recently been able to promote the entry of certain antioxidants—including MitoQ, MitoVitE, MitoPBN, MitoPeroxidase, and amino acid and peptide-based SS tetrapeptides—into mitochondria, several hundred-fold more than do natural antioxidants. Once in the mitochondria, they rapidly neutralize free radicals and decrease mitochondrial toxicity. Thus, mitochondrially targeted antioxidants are promising candidates for treating AD patients.

INTRODUCTION

Alzheimer's disease (AD) is a complex, multifactorial, heterogeneous mental illness, which is characterized by an age-dependent loss of memory and an impairment of multiple cognitive functions. AD is associated with the presence of intracellular neurofibrillary tangles (NFTs) and extracellular amyloid beta ($A\beta$) plaques, the loss of neuronal subpopulations, mitochondrial oxidative damage, synaptic loss, and the proliferation of reactive astrocytes and microglia [1]. With the life span of humans increasing and with decreasing cognitive function in elderly individuals with AD-related dementia, AD has become a major health problem in society. Therapeutic interventions are urgently needed to minimize the ill effects of this devastating disease. Genetic mutations are responsible for causing early onset "familial" AD (constituting only 2% of AD cases), but the causal factor(s) for the vast majority of late-onset "sporadic" AD cases is still unknown. In addition, cellular changes in AD neurons that occur in late-onset AD are also unknown. This article describes cellular changes in AD progression and AD therapeutic strategies, and then focuses on mitochondrially targeted antioxidants as potential therapies for AD.

HISTOPATHOLOGICAL AND CELLULAR CHANGES IN AD PROGRESSION

Histological, pathological, molecular, cellular, and gene expression studies of AD have revealed that multiple cellular pathways are involved in AD progression [2, 3]. Pathologically, there are no differences between early- and late-onset AD [4]. In patients with late-onset AD, pathological changes—including $A\beta$ production and deposits, NFTs, synaptic damage, and neuronal loss—occur later than in patients with early-onset AD [1, 4–6]. In contrast to early-onset AD in which genetic mutations accelerate the disease process, in late-onset AD, in the absence of genetic mutation, age-related cellular changes control AD progression [6]. Therefore, late-onset AD takes more time to develop [4]. Much

research has been done on early-onset AD in terms of pathophysiology and cellular changes that regulate AD progression, but we still need more research to understand causal factors, pathophysiology, and cellular changes that are responsible for disease development and progression in late-onset AD.

Several factors are known to be involved in the development of late-onset AD, with two of the major ones being aging [1] and mitochondrial abnormalities [5–9]. Other contributing factors are the ApoE genotype [10, 11], insulin-dependent diabetes [12], and environmental conditions, including diet [13].

In early-onset AD, recent molecular, cellular, and animal-model studies have provided evidence that a 4 kd peptide, a cleavage product of amyloid precursor proteins (APP) due to cleavage of β and γ secretases, is a key factor in AD development and progression [4]. The formation of the 4 kd $A\beta$ peptide in the brains of AD patients is a progressive and sequential process. Initially, soluble monomeric and oligomeric forms of 40–42 amino acid residues accumulate and later become insoluble fibrils and $A\beta$ deposits. Recent cellular and molecular studies of triple AD transgenic mice suggested that $A\beta$ production in early-onset AD may facilitate tau pathology [14–16]. The $A\beta$ plaques in the transgenic mice were also found to be associated with activated microglia and astrocytes and to trigger inflammatory responses [17]. In addition, recent molecular, cellular, and animal model studies have revealed that mutant APP and $A\beta$ enter mitochondria and interact with an $A\beta$-induced alcohol dehydrogenase (ABAD) protein, disrupt the electron transport chain (ETC), generate reactive oxygen species (ROS), free radicals derived from molecular oxygen in the mitochondria, and inhibit the generation of cellular adenosine triphosphate [5, 6, 18, 19]. These results suggest that mutant APP and mutant $A\beta$ interacting with mitochondrial proteins cause mitochondrial dysfunction in early-onset AD [5, 6, 18].

THERAPEUTIC STRATEGIES

Recent cellular and animal model studies revealed that AD progression involves such cellular changes as inflammatory responses, mitochondrial oxidative damage, synaptic failure, and hyperphosphorylation of tau, all of which are directly related to aging and $A\beta$ production [13–16, 20–24]. Based on cellular, histopathological, and behavioral changes observed in postmortem brains of late-onset AD patients and in AD transgenic mouse models, several therapeutic strategies have been developed to treat AD patients, including immunotherapy [15, 25–28], anti-inflammatory therapy [29–32], antioxidant therapy [33–43], cholinergic therapy [44–51], cell cycle therapy [8, 52–54], and hormonal therapy [55–57]. This article briefly discusses four of these major therapeutic approaches, with special emphasis on mitochondrially targeted antioxidants.

IMMUNOTHERAPY

Current immunotherapeutic strategies are aimed at decreasing $A\beta$ levels in late-onset AD patients by inhibiting $A\beta$ generation [58], reducing soluble $A\beta$ levels [59, 60], and enhancing $A\beta$ clearance from the brain [15, 26–28].

Immunization of $A\beta$ in AD transgenic mouse models has shown that $A\beta$ levels can be reduced in the brains of AD mice [14, 25–27]. With encouraging results from in vivo studies that have aimed at abolishing $A\beta$ deposits in cellular and animal models of AD, Elan Pharmaceuticals moved quickly to investigate, in phase II clinical trials, immunotherapy to reduce $A\beta$ [25]. However, this research was stopped because AD subjects developed symptoms of aseptic meningoencephalitis [25]. The following critical issues need to be addressed before resuming immunotherapy clinical trials using AD subjects: (1) the long-term consequences of $A\beta$ immunization for the AD brain need to be identified, (2) even though immunotherapy has been found to clear $A\beta$ deposits in the AD brain, the downstream effects of AD progression still need to be determined, and (3) the relationship between the clearing of $A\beta$ and the improvement of cognitive function in AD patients needs to be clarified. Currently, several laboratories are actively involved in immunotherapy research to clear both soluble and insoluble $A\beta$ from the brain and to improve behavioral changes in AD transgenic mice.

ANTI-INFLAMMATORY THERAPY

Inflammation of brain tissue is an important component in the pathogenesis of AD, involving the activation of both microglia and astrocytes. Recent histological studies have revealed the presence of activated microglia and reactive astrocytes in and around extraneuronal $A\beta$ plaques in brains from AD patients. These activated microglia and reactive astrocytes are believed to facilitate the clearing of $A\beta$ deposits from the brain parenchyma [13]. However, now there is increasing evidence to suggest that the chronic activation of microglia, presumably via the secretion of cytokines and reactive molecules [61, 62], may exacerbate $A\beta$ plaque pathology as well as enhance the hyperphosphorylation of tau and the formation of NFTs [14–16]. Thus, the suppression of microglial activity in the AD brain has been considered a possible therapeutic strategy to treat AD patients [13, 30]. Suppressive anti-inflammatory drugs, particularly nonsteroidal anti-inflammatory drugs, have been found to lessen the effects of $A\beta$ in transgenic mice [29–32].

CHOLINERGIC THERAPY

AD affects cholinergic neurotransmission in the neurons of the basal forebrain [63]. There is increasing evidence that the enzymes involved in the synthesis of choline acetyltransferase (ChAT) and the degradation of acetylcholine (ACh) may be responsible for deficits in cholinergic neurotransmission. Strategies to boost the levels of ACh in the AD brain are being developed to treat AD patients, to reduce NFTs and $A\beta$ levels, and to improve cognition. Similar to ChAT, butylcholinesterase (BChE) inactivates the neurotransmitter ACh and, thus, is a viable therapeutic target for AD. Greig et al [47] tested potent, reversible, and brain-targeted BChE inhibitors (cymserine analogs). In rats, cymserine

analogs caused long-term inhibition of brain BChE and elevated extracellular ACh levels, without inhibiting ChAT [47]. In slices from rat brains, selective BChE inhibition augmented long-term potentiation. These compounds (cymserine analogs) improved the cognitive performance (maze navigation) of aged rats. In cultured human SK-N-SH neuroblastoma (N2a) cells, BChE inhibitors reduced intra- and extracellular $A\beta$ precursor proteins and secreted $A\beta$ peptides without affecting cell viability. Cholinergic treatment of transgenic mice overexpressing human mutant APP also resulted in lower levels of $A\beta$ peptides in their brains than the levels of $A\beta$ peptides in the brains from healthy (control) rats. Selective, reversible inhibition of brain BChE may be a viable treatment for AD to improve cognition and to modulate neuropathological markers of the disease. Discoveries in cholinergic therapeutics have already led to the development of several cholinesterase inhibitors [44–51]. Four cholinesterase-inhibiting drugs are currently being prescribed for patients with mild to moderate AD: Donepezil, Rivastigmine, Galantamine, and Tacrine. However, these drugs provide only temporary relief of AD symptoms, and there is no evidence as yet to suggest that they reduce AD pathology. As a result, cholinergic therapy is considered a short-term intervention for certain symptoms of AD, since it is still unknown whether cholinesterase inhibitors modify $A\beta$ pathology in AD mouse models and in AD patients. This issue has been under intense investigation in many laboratories across the world.

OXIDATIVE STRESS AND ANTIOXIDANT THERAPY

Oxidative stress is a major factor associated with the development and progression of AD and other forms of dementia. A large body of data suggests that free radical oxidative damage—particularly of neuronal lipids [64, 65], nucleic acids [23, 24, 66–68], and proteins [66, 67, 69, 70]—is extensive in the brains of AD patients. Increased oxidative stress is thought to result in the generation of free radicals and ROS, which is reported to be released by microglia activated by $A\beta$ [71, 72]. Compared to other organs, the brain has been found to be more vulnerable to oxidative stress due to its high lipid content, its relatively high oxygen metabolism, and its low level of antioxidant defenses [70, 73, 74]. Markers of oxidative stress, such as 8-hydroxyguanosine and hemeoxygenase, have been localized to pathologic lesions in the brains of AD patients [75–78].

Using PC 12 cells and $A\beta$ (25–35) peptide, Bozner et al studied the connection between $A\beta$ and mitochondrial DNA damage. They exposed PC 12 cells to an $A\beta$ (25–35) in frame and scrambled at 50 mM concentration for 24 hours to 50 hours. Oxidative damage of mitochondrial DNA was assessed using a Southern blot technique and a mitochondrial DNA-specific probe recognizing a 13.5-kilobase restriction fragment. PC 12 cells exposed to $A\beta$ exhibited marked oxidative damage of mitochondrial DNA as evidenced by characteristic changes on Southern blots, but not in cells exposed to the scrambled $A\beta$ peptide, suggesting that $A\beta$ peptide is responsible for mitochondrial DNA damage, and ultimately leading to mitochondrial dysfunction in AD [68]. Further, evidence from a recent gene expression study [21] suggests that mutant APP or $A\beta$ may generate free radicals and promote mitochondrial dysfunction, one or both of which may lead to oxidative damage. Altered levels of mitochondrial enzymes have been found to be directly responsible for a decrease in energy production in the brains of late-stage AD patients [5]. Soluble or insoluble forms of $A\beta$ have been suggested to impair ATP production by generating defects in mitochondrial energy metabolism and oxidative stress [79]. Taken together, these results suggest that oxidative stress is a key event in AD pathogenesis.

FREE RADICAL PRODUCTION AND MITOCHONDRIAL OXIDATIVE DAMAGE

In the literature on AD, the terms "oxidative stress" or "oxidative damage" are commonly used to explain the balance between the production of oxidants and the endogenous antioxidant defenses in neuronal cells. In general, cells undergo apoptotic death when there is an imbalance between oxidants and antioxidants (more oxidants than antioxidant defenses). This oxidative damage mainly occurs via the mitochondrial ETC [5, 6].

Mitochondria, which are cytoplasmic organelles, are responsible for the production of cellular ATP. Mitochondria are involved in 3 important cell functions: (1) producing ATP and regulating intracellular Ca^{2+}; (2) releasing proteins that activate the caspase family of proteases; and (3) altering the reduction-oxidation potential of cells [5]. Disruption of the ETC has been recognized as an early characteristic of apoptotic cell death. ETC involves the reduction of hydrogen peroxide (H_2O_2) to H_2O and O_2 by catalase or glutathione peroxidase accepting electrons donated by NADH and $FADH_2$, and then yielding energy for the generation of ATP from adenosine diphosphate and inorganic phosphate [6, 80–82].

The production of mitochondrial superoxide radicals ($O_2^{\bullet -}$) occurs primarily at discrete points in the ETC at complexes 1 and 3 [83], and in components of tricarboxylic acid (TCA), including α-ketoglutarate dehydrogenase [84] (see Figure 1). In addition, mitochondrial $O_2^{\bullet -}$ are generated in the outer mitochondrial membrane. Monoamine oxidase (flavoprotein), localized on the outer mitochondrial membrane, catalyzes the oxidative deamination of primary aromatic amines. This deamination is a quantitatively large source of H_2O_2 that contributes to an increase in the steady-state concentrations of ROS within both the mitochondrial matrix and the cytosol [80]. These released H_2O_2 and $O_2^{\bullet -}$ are carried to the cytoplasm via voltage-dependent anion channels and, ultimately, lead to the oxidation of cytoplasmic proteins (see Figure 1). The chronic exposure of ROS to cells can result in oxidative damage to mitochondrial and cellular proteins, lipids, and nucleic acids, and acute exposure to ROS can inactivate the TCA-cycle aconitase and the iron-sulfur centers of ETC at complexes 1, 2, and 3, resulting in a shutdown of mitochondrial energy production [5, 6, 85].

The generation of free radicals can occur via several cellular insults, including ultraviolet irradiation [86], redox-cycling of quinones [86], the metabolism of xenobiotics [86],

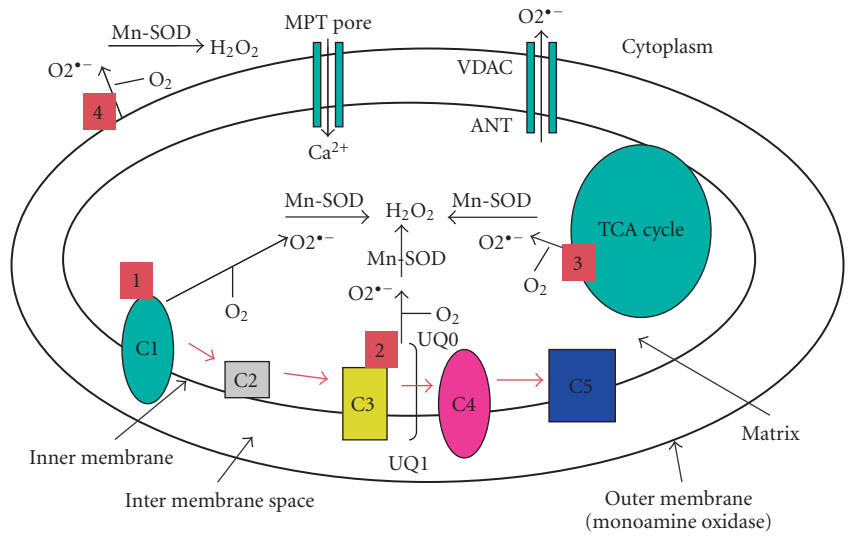

Figure 1: Illustration of sites of free radical generation in the mitochondria. In the respiratory chain, complexes 1 and 3 leak electrons to oxygen, producing primarily superoxide radicals (or $O_2^{\bullet -}$). The $O_2^{\bullet -}$ are dismutated by manganese superoxide dismutase to generate H_2O_2 and oxygen. Complex 1 generates $O_2^{\bullet -}$ only toward the matrix. Complex 3, on the other hand, generates $O_2^{\bullet -}$ toward both the intermembrane space and the matrix. The components of tricarboxylic acid, including α-ketoglutarate dehydrogenase, also generate $O_2^{\bullet -}$ in the matrix. Free radicals are generated in the outer mitochondrial membrane (monoamine oxidase) and catalyze the oxidative deamination of primary aromatic amines, leading to the generation of H_2O_2.

aging [87], environmental mitochondrial toxins [88–90], and mutant toxic proteins (eg, $A\beta$ in AD, mutant huntingtin in Huntington's disease, alpha-synuclein in Parkinson's disease, mutant SOD1 in amyotrophic lateral sclerosis) [5, 8, 87].

FREE RADICALS, ABNORMAL PROCESSING OF APP, AND $A\beta$ METABOLISM

There is mounting evidence to suggest that in late-onset AD, age-related free radicals, which are generated in the mitochondria, are carried to the cytoplasm where they activate beta secretase and facilitate the cleavage of the APP molecule [6]. The cleaved APP molecule (ie, $A\beta$) further generates free radicals, leading to the disruption of the ETC and enzyme activities, the inhibition of ATP, and the subsequent oxidation of both nuclear and mitochondrial DNA proteins. The damage caused by mitochondria ultimately leads to neuronal damage, neurodegeneration, and cognitive decline in AD patients [6].

AGE-DEPENDENT MITOCHONDRIAL Ca^{2+} AND OXIDATIVE DAMAGE

The dysregulation of age-related Ca^{2+} and an increased production of ROS may contribute to late-onset neurodegenerative disorders such as AD. These alterations are often attributed to impaired mitochondrial function, yet few studies have directly examined isolated mitochondria from various regions of the aged brain. Recently, Brown et al [91] examined Ca^{2+} influx and ROS production in isolated mitochondria from Fischer 344 rats ranging in age from 4 to 25 months. Isolated mitochondria from the cortex of the 25-month-old rat brain exhibited greater rates of ROS production and mitochondrial swelling in response to increasing Ca^{2+} loads compared to mitochondria isolated from younger (4- and 13-month-old) rats. This increased swelling is indicative of the opening of a mitochondrial permeability transition pore, suggesting an impaired Ca^{2+} influx in aged animals (see Figure 1). These age-related differences were not observed in isolated mitochondria from the cerebellum of these rats. Together, these results suggest region-specific, age-related alterations in mitochondrial responses to Ca^{2+} [91]. In addition to aging, in age-related diseases such as AD, $A\beta$ promotes the opening of the mitochondrial permeability transition pore, through which free cytosolic Ca^{2+} enter the mitochondria (see Figure 1). The mitochondrial Ca^{2+} may disrupt the ETC, which would elevate the production of free radicals in the mitochondria [92]. To determine the extent of free radical production and oxidative damage in the spinal cord and neocortex of the rat brain, Sullivan et al [93] studied several parameters of mitochondrial physiology in the normal neocortex and spinal cord. In situ measurements revealed significantly higher levels of $O_2^{\bullet -}$ production, lipid peroxidation, and mitochondrial DNA oxidation in the spinal cord than these levels in neocortical neurons. Real-time PCR analysis of mitochondrial genes demonstrated differences in mitochondrial transcripts coupled with decreases in complex 1 enzyme activity and in respiration, in the spinal cord mitochondria. The threshold for calcium-induced mitochondrial permeability transition was substantially reduced in the spinal cord relative to the neocortex and was modulated by lipid peroxidation. These findings suggest that studying mitochondrial damage in the spinal cord may

be productive in learning about causes of age-related neurodegenerative diseases [93].

Aβ AND MITOCHONDRIAL FUNCTIONAL ASSOCIATION

To determine whether mitochondria are critical for cellular toxicity induced by *Aβ*, Cardoso et al [94] investigated the effects of *Aβ* peptides in NT2 cells with mitochondria (NT2 -*P*+) and without mitochondria (NT2 -*P*0) [94]. In NT2 -*P*+ cells, they observed a decrease in cell viability, mitochondrial membrane potential, enzyme activities, and ATP levels, but they did not find such decreases in NT2 -*P*0 cells, suggesting that *Aβ* peptides require functional mitochondria for the induction of cell toxicity. Further, several studies reported the association of mutant APP derivatives with mitochondria [18, 19, 95]. Anandatheerthavarada et al [95] observed that in cortical neurons, the accumulation of full-length APP in the mitochondrial compartment in a transmembrane-arrested form of APP is responsible for mitochondrial dysfunction and impaired energy metabolism [95]. Lustbader et al [18] demonstrated that *Aβ* is localized to mitochondria in the neurons of AD transgenic mice. Further, they also found that *Aβ* directly interacts with an *Aβ*-binding ABAD protein in the mitochondria, leading to oxidative damage and mitochondrial dysfunction [18]. Crouch et al [19] studied the inhibitory potential of synthetic *Aβ* (1–42) on the activity of ETC enzyme complexes in human mitochondria. They found that synthetic *Aβ* (1–42) inhibits the terminal complex cytochrome *c* oxidase in a manner that is dependent on the presence of Cu^{2+}. In the Crouch study, maximal cytochrome *c* oxidase inhibition occurred when synthetic *Aβ* (1–42) solutions were used after they aged from 3 to 6 hours at 30°C. The level of *Aβ* (1–42)-mediated cytochrome *c* oxidase inhibition increased up to 6 hours after the *Aβ* (1–42) solution aged, and then the level declined progressively as the *Aβ* (1–42) solutions aged to 48 hours. These data strongly suggest that endogenous *Aβ* is associated with brain mitochondria and that synthetic *Aβ* (1–42) is a potent inhibitor of cytochrome *c* oxidase [19]. All of these studies combine to suggest that mitochondria are vulnerable to *Aβ* and/or age-related oxidative damage.

ANTIOXIDANT TREATMENT

Using a Tg2576 mouse model of AD and treating the Tg2576 mice with a vitamin E-supplemented diet, researchers in in vivo studies reported decreased *Aβ* (1–40) and *Aβ* (1–42) levels [34, 35]. In another study, researchers using a transgenic mouse model of tau pathology found that the administration of vitamin E ameliorated tau aggregates [33], suggesting that vitamin E may have a direct effect on AD pathology [34, 35]. The administration of curcumin (an antioxidant) to Tg2576 mice also showed encouraging results when both oxidative damage and *Aβ* deposits were reduced [38]. Further, melatonin reduced brain levels of *Aβ* and abnormal protein nitration, and increased the life span of Tg2576 mice [36]. A synthetic superoxide (a dismutase catalase mimetic) prevented cataracts in AD transgenic mice [96].

Several recent antioxidant studies using AD patients revealed beneficial effects of diets supplemented with vitamin E [39, 40, 42]. The combined administration of vitamin E and vitamin C supplements was associated with a reduced prevalence and incidence of AD in an elderly population [43]. Morris et al [41] examined whether food intake of vitamin E, alpha-tocopherol equivalents (a measure of the relative biologic activity of tocopherols and tocotrienols), or individual tocopherols protects against AD symptoms and cognitive decline. They found that higher intake of vitamin E and alpha-tocopherol equivalents was associated with a reduced incidence of AD in an elderly population, suggesting that antioxidant treatment at the early onset of disease may be effective in delaying AD progression [41]. However, the pathological effects of oxidative stress are yet to be assessed in AD patients or elderly individuals treated with antioxidants. In another clinical study, to determine the neuroprotective effects of cholinesterase inhibition and oxidative stress in AD patients, huperzine A (an antioxidant) was administered to AD patients in doses of 300 mg/d for the first 2–3 weeks of drug administration and then 400 mg/d for the next 4–12 weeks. At the end of 12 weeks, the AD patients exhibited significant improvement in their cognitive, noncognitive, and ADL functions [97]. These initial clinical trials may eventually become precursors for antioxidant clinical trials for AD patients.

MITOCHONDRIALLY TARGETED ANTIOXIDANT THERAPIES IN AGING AND AD

Krzepilko et al [98] studied exogenous mitochondrial antioxidants in yeast (*Saccharomyces cerevisiae*) mutants. They found that yeast mutants lacking CuZn-superoxide dismutase are hypersensitive to oxygen and have a significantly decreased replicative life span [98]. These defects can be ameliorated by the low-molecular weight exogenous antioxidant ascorbate. The effect of ascorbate on life span is complicated by its auto-oxidation in yeast cell culture media. Krzepilko and colleagues found that if negative effects of auto-oxidation are prevented by exchange of the medium, ascorbate prolongs not only the mean but also the maximal replicative life span of the yeast in an atmosphere of air and pure oxygen. The findings from this study suggest that the shortening of a healthy life span due to the lack of an antioxidant enzyme may be ameliorated by ascorbate [98].

In studies of the transgenic fruit fly *Drosophila* that overexpresses antioxidant enzymes targeted to mitochondria, Ruan et al [99] found that the antioxidant enzymes Mn-SOD (manganesesuperoxide dismutase) and methionine sulfoxide reductase extended the life span of *Drosophila,* suggesting that mitochondrially targeted antioxidants may reduce ROS and contribute to life span extension [99]. However, recently, Magwere et al [100] tested the effects of SOD mimetic drugs Euk-8 and -134, and the mitochondrially targeted mitoquinone (MitoQ) on life span and oxidative stress resistance of wild-type and SOD-deficient flies. They confirmed findings from other researchers that exogenous antioxidants rescue pathology associated with compromised deficiencies

to oxidative stress, but do not extend the life span of normal, wild-type flies treated with exogenous antioxidants. All three exogenous antioxidants (Mn-SOD, MitoQ, and Euk-8 and -134) led to a dose-dependent increase in toxicity in wild-type flies, an effect that was exacerbated in the presence of the redox-cycling drug, paraquat. However, important findings from this study were that in SOD-deficient flies, the antioxidant drugs increased life span. Further, the effects of these antioxidant drugs were sex-specific, and for either sex, the effects were also variable depending on (1) the stage of development at which the drugs were given, and (2) the magnitude of the dose [100].

Schriner et al [101] recently demonstrated that mitochondrially targeted catalase decreases H_2O_2, leads to reduced mitochondrial oxidative damage, and increases the life span of catalase transgenic mice. To determine the role of catalase in mitochondrial function, they created mouse lines that overexpress human catalase localized to peroxisomes, nuclei, and mitochondria, to elucidate the effects of catalase on aging from birth to death. Catalase, found mainly in peroxisomes, rapidly converts toxic H_2O_2 into H_2O and O_2 [6]. In two independent lines of mitochondrial catalase (MCAT) mice, Schriner et al [101] found that the transgenic mice treated with MCAT expressers showed about a 20% increase in median and maximal life span (on average, 5.5 months) compared to the life span of nontransgenic, age-matched wild-type littermates. The ability of catalase to increase longevity was most apparent when the enzyme was targeted to mitochondria. Schriner et al found that the transgenic mice, which express catalase in peroxisome, had a slightly longer median life span, but showed no increase in maximal life. Nuclear catalase (NCAT) expression (in NCAT mice) had no effect on either the median life span or the maximal life span of the mice [101].

In the Schriner study [101], MCAT transgenic mice appeared to age more slowly than their age-matched littermates by several measures. While histological comparisons showed little difference between wild-type and MCAT lines in young mice (9 to 11 months old), aged transgenic mice (20 to 25 months old) had significantly less arteriosclerosis and cardiomyopathy than their wild-type littermates. Biochemical studies have shown that the slower aging found in the MCAT mice is associated with a lower level of oxidative stress and DNA damage [101]. In the Schriner study [101], H_2O_2 production by cardiac mitochondria from MCAT mice decreased 25%, and mitochondria containing catalase were protected from the toxic effects of H_2O_2. Age-related increases in oxidative damage to total DNA and fragmentation of mitochondrial DNA were also slowed in the skeletal muscle of MCAT mice [101].

Overall, findings from these aging studies suggest that mitochondrially targeted antioxidants reduce ROS production and oxidative damage in that the flies were diseased and also contribute to healthy aging at least in laboratory mice. However, the effects of mitochondrial antioxidants in healthy humans and humans with neurodegenerative disease still need to be determined.

From these aging studies, it is clear that mitochondrially generated H_2O_2 is a critical factor in determining life span. If aging is a key to the generation of H_2O_2 in aged neurons, then mitochondrially targeted catalase, glutathione peroxidase, MitoQ [102], MitoVitE [103], MitoPBN [104], and several mitochondrially targeted peptides [105, 106] may likely, rapidly convert toxic H_2O_2 into H_2O and O_2. This continuous conversion of H_2O_2 into H_2O and O_2 may reduce oxidative damage in aged neurons and may maintain mitochondrial function in the neurons of aged individuals. It has been established that an overload of Ca^{2+} induces ROS and disrupts the ETC in mitochondria. It is also possible that mitochondrially targeted antioxidants reduce ROS induced by an age-related overload of Ca^{2+} in mitochondria [91, 92]. Since age is a major factor involved in the development of late-onset AD, mitochondrially targeted antioxidants may be able to reduce oxidative damage in AD, increase O_2 consumption, help increase the life span of elderly individuals, and decrease or prevent AD progression in elderly individuals [6].

A major limitation in using antioxidant therapy to treat the age-related diseases, such as AD, has been the inability of investigators to enhance the antioxidant levels in mitochondria [107]. However, in the last 5 years, considerable progress has been made in developing mitochondrially targeted antioxidants. To increase the delivery of antioxidants to mitochondria, three types of antioxidants are being studied: triphenylphosphonium-based antioxidants, and amino acid- and peptide-based antioxidants.

HOW DO MITOCHONDRIALLY TARGETED ANTIOXIDANTS ENTER MITOCHONDRIA?

The mitochondrial ETC participates in the transfer of electrons to O_2 [6]. During this transfer, a proton gradient is generated, which drives the production of ATP by ATP-synthase [86]. ATP production in turn generates a negative potential from 150 to 180 mV across the inner mitochondrial membrane. This negative potential gradient is used to deliver liphophilic cations to mitochondria. The liphophilic cations easily permeate through the lipid bilayers and subsequently accumulate by several hundred-fold within the mitochondria, given their large mitochondrial membrane potential (see Figure 2). Using this known transfer of electrons to O_2, Murphy and colleagues recently developed several mitochondrially targeted antioxidants: MitoQ (a derivative of mitochondrial quinoline), MitoVitE (a derivative of mitochondrially targeted vitamin E) [102, 107], and MitoPBN (a derivative of α-phenyl-N-*tert*-butyl nitrone) [104]. These liphophilic cations-based antioxidants were covalently coupled to a triphenylphosphonium cation and were preferentially taken up by mitochondria [102–104]. These antioxidants initially accumulated in the cytoplasm of cells, due to a negative plasma membrane potential (see Figure 2) [102, 107, 108].

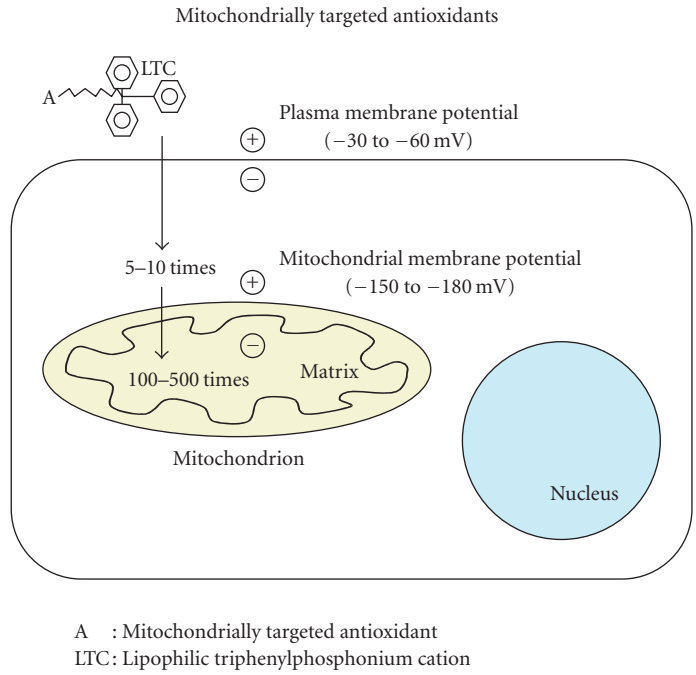

Figure 2: Illustration of mitochondrially targeted antioxidants. A generic mitochondria-targeted antioxidant is shown constructed by the covalent attachment of an antioxidant molecule to the lipophilic triphenylphosphonium cation. Antioxidant molecules accumulate 5- to 10-fold in the cytoplasm, which is driven by the plasma membrane potential, and then further accumulate several hundred-fold in the mitochondria.

MitoQ

MitoQ is an antioxidant consisting of two redox forms of mitochondrially targeted ubiquinone derivatives: reduced mitoquinol and oxidized mitoquinone [102]. MitoQ is attached to a phosphonium cation [10-(6-ubiquinonyl) decyltriphenylphosphonium bromide]. The molecular formula of MitoQ is $C_{37}H_{46}O_4PBr$, and its molecular weight is 665.65. MitoQ is a red, oily solid and can be stored at −20°C in the dark. It can be converted into a fully oxidized form by incubation in 95% ethanol and can be dissolved in DMSO.

MitoQ is a promising therapeutic antioxidant that has been successfully targeted to mitochondria [102]. MitoQ is a respiratory chain component buried within the lipid core of the inner membrane of mitochondria where it accepts 2 electrons from complex 1 or 2, to form the reduction product ubiquinol, which donates electrons to complex 3 [109]. The ubiquinone pool in vivo exists largely in a reduced ubiquinol form, acting as an antioxidant and a mobile electron transfer. Ubiquinol has been reported to function as an antioxidant by donating a hydrogen atom from one of its hydroxyl groups to a lipid peroxyl radical, thereby decreasing lipid peroxidation within the mitochondrial inner membrane [110–112]. The semiubiquinone radical formed during this process disproportionates into ubiquinone and ubiquinol [113]. The respiratory chain subsequently recycles ubiquinone back to ubiquinol, restoring its antioxidant function. MitoQ excessively accumulates in the mitochondria and converts H_2O_2 to H_2O and O_2, and reduces toxic insults from free radicals in the mitochondria. This reduction ultimately leads to the protection of neurons from age-related and/or disease-related mitochondrial insults in AD.

Recently, the effects of MitoQ on mitochondria in several *in vitro* cell models were tested [114–117]. In cultured fibroblasts from Friedreich Ataxia patients, MitoQ prevented cell death known to be caused by endogenous oxidative stress [115]. In a study of PC12 cells, low concentrations of MitoQ (1μM) selectively inhibited serum deprivation-induced apoptosis in PC12 cells [116]. In a study of bovine aortic endothelial cells treated with glucose/glucose oxidase and lipid peroxide, MitoQ inhibited cytochrome *c* release, caspase 3 activation, and DNA fragmentation in greater percentages than did the corresponding untargeted counterparts such as vitamin E [117]. These studies suggest that MitoQ may reduce free radicals, decrease oxidative damage, and maintain mitochondrial function. Since oxidative damage is part of the known pathophysiology of AD, there is strong interest in determining whether mitochondrially targeted antioxidants decrease oxidative damage in the neurons of AD patients [6].

Smith et al [118] studied the effects of mitochondrially targeted antioxidants, including MitoQ, on laboratory mice. Some of the mice were fed with MitoQ for several weeks until MitoQ reached steady-state concentrations within all tissues assessed, including brain, heart, liver, and kidney tissues [118]. At that point, the concentration of MitoQ in the tissues was several hundred-fold higher than that in the bloodstream. Smith et al [118] found the uptake of MitoQ to be reversible, as observed by the rapid clearance of the simple

lipophilic cation methyltriphenylphosphonium from all organs when oral administration of MitoVitE was stopped. Using mass spectrometry, they found that orally administered MitoQ entered the bloodstream and distributed in an intact active form to tissues in the brain, heart, and liver. After the mice were fed with methyltriphenylphosphonium and MitoVitE, the levels of these accumulated antioxidants were in the range of 5 to 20 nmol/g (wet weight), or about 5 to 20 μmol/l in the tissues [118]. These high concentrations may be in a therapeutic range since MitoQ has been found to prevent oxidative damage in isolated mitochondria at lower concentrations (eg, 1 to 2.5 μmol/l [102, 103]). Therefore, the oral administration of well-tolerated doses of MitoQ may deliver potentially therapeutic concentrations to mitochondria in vivo [118].

Further, Adlam et al [119] recently examined the effect of MitoQ in a murine model of cardiac ischemia-reperfusion injury (a mitochondrial oxidative damage model). MitoQ fed to rats significantly decreased their heart dysfunction and mitochondrial damage after ischemia reperfusion. This protection was attributed to MitoQ in the mitochondria [119].

Since natural antioxidants given at high doses without side effects have been shown to decrease oxidative damage in AD mouse models and AD patients [33–43], there is a strong rationale for testing these mitochondrially targeted antioxidants in trials using AD cell and mouse models, and AD patients.

MitoVitE

MitoVitE is an antioxidant that consists of [2-3,4dihydro-6-hydroxy-2,5,7,8tetra-methyl-2H-1-benzopyran-2-yl] and triphenylphosphonium bromide [86]. MitoVitE is a derivative of vitamin E that is targeted to mitochondria, and it was developed to study mitochondrial oxidative damage. MitoVitE is rapidly taken up by mitochondria, and the uptake lasts for 15 min. Accumulation ratios of 5000–6000 unit have been achieved after incubating mitochondria with 1–20 μM MitoVitE [86]. MitoVitE is cytotoxic at 50 μM. The effects of MitoVitE have been tested in Jurkat cells. MitoVitE was found to reduce H_2O_2-induced caspase activity [120]; to prevent oxidative stress-induced cell death in cultured fibroblasts from Friederich Ataxia patients [115]; and at (1 μM) concentration, to inhibit cytochrome *c* release and caspase-3 activation, to inactivate complex 1, and to restore mitochondrial membrane potential and proteosomal activity in bovine aortic epithelial cells [117].

MitoPBN

MitoPBN is an antioxidant consisting of [4-[4 (1,1-dimethylethyl) oxidoimino]-methyl]phenoxy]butyl] and triphenylphosphonium bromide [104, 121]. A mitochondrially targeted analog of MitoPBN was prepared to determine the effect of ROS and mitochondrial function in mitochondria, based on a selective PBN (α-phenyl-N-*tert*-butyl nitrone) reaction with carbon-centered radicals [104]. Similar to MitoQ and MitoVitE, MitoPBN was rapidly taken up by mitochondria, with a resulting concentration ranging from 2.2 to 4.0 mM. It has been reported that MitoPBN blocks the O-induced activation of uncoupled proteins.

Recently, Poeggeler et al [122] developed an amphiphilic molecule, *N*-[4-(octa-*O*-acetyllactobionamidomethylene) benzylidene]-*N*-[1,1-dimethyl-2-(*N*-octanoyl) amido]-ethylamine *N*-oxide, (LPBNAH, a derivative of α-phenyl-N-*tert*-butyl nitrone) that exhibits profound antioxidant and neuroprotective activity and very efficiently antagonizes oxidotoxicity of primarily mitochondrial origin [122]. LPBNAH, when administered via food to rotifers, was found to decrease free radicals, to greatly enhance the survival of neurons, and to increase the life span of rotifers. The antiaging activity of LPBNAH exceeded that of other nitrone compounds, such as the parent compound PBN, by at least one order of magnitude. The development of such a neuroprotective antioxidant may lead to more safe and effective treatments of age-related diseases such as AD.

AMINO ACID- AND PEPTIDE-BASED MITOCHONDRIALLY TARGETED ANTIOXIDANTS

SS tetrapeptides are aromatic cationic peptides that contain the structural motif of alternating aromatic and basic amino acid residues, along with a 2′, 6′-dimethyltyrosine residue [86]. These tetrapeptides are mitochondrially targeted antioxidants. Their antioxidant properties are derived from the related compound 3, 5-dimethyphenolis, a known phenolic antioxidant [123]. SS tetrapeptides were originally We prepared to develop centrally acting opiopoid analgesics [124, 125]. The following SS tetreapeptide compounds have been developed for mitochondrial and cellular uptake studies: (1) Dmt-D-arg-Phe-Lys NH2 (SS-02); (2) Phe-D-Arg-Phe-Lys-NH2 (SS-20); (3) D-Arg-Dmt-Lys-Phe-NH2 (SS-31); and (4) Dmt-d-Arg-Phe-atnDAP-NH2 (SS-19) [86].

SS-02 neutralizes H_2O_2 and inhibits the oxidation of linoleic acid and low-density lipoproteins. SS-31 contains same amino acid residues, as does SS-02, but in an order that exhibits antioxidant properties. SS-02 is taken up by Caco-2 cells (derived from human colorectal adenocarcinoma), and its intracellular concentration is about 10 times greater than its extracellular concentration. SS-02 and SS-19 are rapidly taken up by isolated mitochondria from mouse liver, with accumulation in the mitochondria 105-fold. The incubation of mitochondria with FCCP reduced the uptake of SS-19 by 20%, suggesting partial potential-dependent uptake [86]. Treatment of SS-02 with digitonin showed that about 85% of SS-02 is present in the mitoplast (inner membrane plus matrix).

Recently, Zhao et al [106] developed peptide antioxidants that target the inner mitochondrial membrane. These antioxidants were used to investigate the role of ROS and the mitochondrial permeable transition of 3NP in cell death caused by the peptides [t-butylhydroperoxide (tBHP) and 3-nitropropionic acid (3NP)]. The structural motif of tBHP and 3NP centers on alternating aromatic and basic amino acid residues, with dimethyltyrosine providing scavenging properties. BHP and 3NP were found to be cell-permeable

and to concentrate at 1000-fold in the inner mitochondrial membrane. Peptide antioxidants potently reduced intracellular ROS and cell death caused by tBHP in neuronal N2a cells. These peptide antioxidants also decreased mitochondrial ROS production, inhibited mitochondrial permeability transition and swelling, and prevented cytochrome *c* release induced by Ca^{2+} in isolated mitochondria. In addition, peptide antioxidants inhibited 3NP-induced mitochondrial permeability transition in isolated mitochondria and prevented mitochondrial depolarization in cells treated with 3NP. ROS and mitochondrial permeability transition have been implicated in myocardial stunning associated with reperfusion in ischemic hearts. Peptide antioxidants were found to potently improve contractile force in an ex vivo heart model. It is noteworthy that peptide analogs without dimethyltyrosine did not inhibit mitochondrial ROS generation or swelling, and did not prevent myocardial stunning. These results clearly suggest that ROS underlies the cellular toxicity of tBHP and 3NP, and that ROS may mediate cytochrome *c* release via a mitochondrial permeability transition. Peptide antioxidants may be very beneficial in the treatment of aging and diseases associated with oxidative stress [106].

Further, Zhao et al examined the ability of a novel, cell-penetrating, mitochondrially targeted peptide antioxidant to protect against oxidant-induced mitochondrial dysfunction and apoptosis in two neuronal cell lines [105]. Treatment of neuronal cell lines with tBHP for 24 hours resulted in lipid peroxidation, significant cell death via apoptosis in both N2a and SH-SY5Y cells, phosphatidylserine translocation, nuclear condensation, and increased caspase activity. When treated with tBHP, the N2a and SH-SY5Y cells showed a significant increase in intracellular ROS, mitochondrial depolarization, and reduced mitochondrial viability. Concurrent treatment with < 1 nM SS-31 significantly decreased intracellular ROS, increased mitochondrial potential, and prevented tBHP-induced apoptosis.

The remarkable potency of SS-31 can be explained by its extensive cellular uptake and selective partitioning into mitochondria. Intracellular concentrations of [3H]SS-31 were 6-fold higher than extracellular concentrations. Studies using isolated mitochondria revealed that [3H]SS-31 was concentrated approximately 5000-fold in the mitochondrial pellet. By concentrating in the inner mitochondrial membrane, SS-31 became localized to the site of ROS production, and protected against mitochondrial oxidative damage and against further ROS production. SS-31 represents a novel platform for mitochondria-targeted antioxidants with broad therapeutic potential [105].

CONCLUSIONS

Recent advances in molecular, cellular, and animal model studies have revealed that mitochondria are the major source of free radical generation and of oxidative damage in aging and age-related neurodegenerative diseases. It is possible that age-related mitochondrial abnormalities and oxidative damage are major contributing factors for late-onset AD. To stop or delay the progression of late-onset AD, and also to reduce disease symptoms, several therapeutic strategies have been developed, including anti-inflammatory, antioxidant, and antiamyloid approaches. Among these, mitochondrial antioxidant therapy reduces AD pathology more than any other approach. However, until recently, a major limitation in developing antioxidant therapies for AD patients has been the inability to enhance antioxidant levels in mitochondria. There has been a breakthrough in the mitochondrial targeting of antioxidants. Mitochondrially targeted antioxidants have been developed, which preferentially enter the mitochondria—at several hundred-fold more than they enter natural antioxidants—where they rapidly neutralize free radicals and decrease mitochondrial toxicity. However, further research is needed to determine whether these mitochondrially targeted antioxidants can be used in mouse models of aging and in age-related neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's.

ABBREVIATIONS

3NP	3-nitropropionic acid
ABAD	alcohol dehydrogenase
Aβ	amyloid beta
ACh	acetylcholine
AD	Alzheimer's disease
ApoE	apolipoprotein E
APP	amyloid precursor protein
ATP	adenosine triphosphate
BChE	butylcholinesterase
ChAT	choline acetyltransferase
ETC	electron transport chain
H_2O_2	hydrogen peroxide
MCAT	mitochondrial catalase
MitoVitE	mitochondrially targeted vitamin E
MitoQ	mitochondrially targeted ubiquinone
MitoPBN	mitochondrially targeted *α*-phenyl-N-*tert*-butyl nitrone
N2a	neuroblastoma
NCAT	nuclear catalase
NFT	neurofibrillary tangle
$O_2^{\bullet -}$	superoxide anion
PBN	*α*-phenyl-N-*tert*-butyl nitrone
ROS	reactive oxygen species
SOD	superoxide dismutase
tBHP	t-butylhydroperoxide

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Review Article

Interplay Between Oxidative Damage, Protein Synthesis, and Protein Degradation in Alzheimer's Disease

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Protein synthesis and protein degradation are highly regulated cellular processes that are essential to maintaining cell viability. Numerous studies now indicate that protein synthesis and protein degradation are significantly altered in Alzheimer's disease (AD), with impairments in these two processes potentially contributing to AD pathogenesis. Alterations in steady state protein regulation may be a particularly important factor in regulating whether cells maintain homeostasis in response to oxidative damage, or conversely whether oxidative stress is induced by oxidative damage. The focus of this review is to discuss recent findings on each of these topics, and to discuss their importance to the onset and progression of AD.

INTRODUCTION

Alzheimer's disease and mild cognitive impairment

Alzheimer's disease (AD) is the leading cause of dementia in the elderly and is characterized by the presence of extensive senile plaque deposition and neurofibrillary pathology [10, 20]. Despite the numerous advances in our understanding of AD, the neurochemical alterations which are responsible for the onset and progression of AD have not been identified. A major obstacle to identifying such factors is the fact that studies involving AD brain tissue have been difficult to interpret. In particular, it has been difficult to distinguish which neurochemical events are playing a causal role in mediating neurodegeneration and neuropathology, and which events are occurring in response to the extensive neurodegeneration and neuropathology observed in AD brain. Because of this, there has been considerable interest in conducting studies in individuals who are in the earliest stages of AD, or individuals who are at the highest risk to develop AD, where the presence of little-to-no neuropathology allows for a clearer experimental interpretation of neurochemical studies.

A number of elderly individuals, who do not have dementia, develop cognitive deficits which are atypical of those observed in normal aging. Longitudinal analysis of these individuals has demonstrated that they convert to AD at a much higher rate than the at large elderly population [12, 13]. It is now thought that these individuals may represent a transitional state between dementia and normal aging, with such subjects being in the earliest stages of AD pathogenesis [12, 13]. Neuropathological studies in these individuals have revealed that these subjects exhibit an extensive overlap with the autopsy findings in older cognitively intact individuals. Because of this, it is believed that these individuals can be used to identify the neurochemical alterations which occur in the earliest stages of AD pathogenesis, and neurochemical alterations which precede the development of dementia and extensive AD neuropathology. Such neurochemical alterations may therefore represent the substrates for pathological and cognitive alterations observed in AD. Individuals in this cohort are now commonly referred to as having mild cognitive impairment (MCI) [12, 13].

Oxidative damage in AD and MCI

Studies from our laboratory and others have demonstrated that increased levels of protein oxidation [7, 16], lipid oxidation [14], and nucleic acid oxidation [2, 14] are all evident in AD. Recent studies have also found that increases in each of these forms of oxidative damage are present in MCI subjects [2, 7, 11, 19]. Interestingly, increases in each of these oxidative modifications preferentially occur in the brain regions involved in regulating cognition. Such an observation is consistent with oxidative damage contributing to the development of dementia. In support of this hypothesis, in studies involving MCI and early AD subjects the increases in protein oxidation were observed to be significantly and inversely correlated with word recall performance [7].

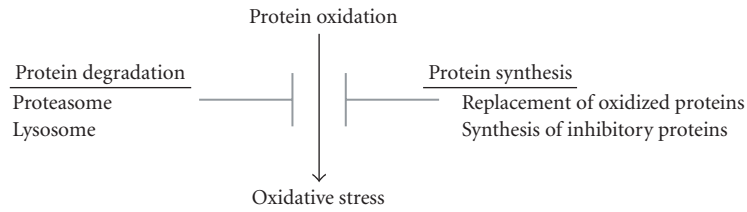

Figure 1: *Interplay between protein synthesis, protein degradation, protein oxidation, and oxidative stress.* Oxidized proteins are capable of inducing oxidative stress, with oxidative stress a potential mediator of AD pathogenesis. The ability of oxidized proteins to induce oxidative stress is inhibited by protein synthesis and protein degradation pathways. The generation of proteins to replace those which have been oxidized, and the synthesis of proteins which inhibits oxidized protein toxicity (ie, heat shock proteins), inhibits the induction of oxidative stress. Proteolytic pathways (lysosomal, proteasomal) degrade oxidized proteins and thereby ameliorate their ability to induce oxidative stress.

Elevations in oxidative damage in MCI and AD subjects are significantly higher than those of age-matched control subjects. This suggests that the elevations in oxidative damage within MCI and AD either represent an acceleration of the normal age-related generation of oxidative damage, or may indicate that alternative pathways for the generation of oxidative damage occur in MCI and AD subjects. Clarification of this issue is vital to our understanding of AD, and is likely important for the design of therapeutic interventions for AD.

It is well established that while elevations in oxidative damage occur in AD [14, 16], the amount of oxidative damage in an individual does not predict the presence or severity of AD. This same observation holds true for other age-related neurodegenerative disorders [3, 6], such as Parkinson's disease. This suggests that the ability of elevated levels of oxidative damage to induce oxidative stress is not solely dependent upon the gross levels of oxidative damage. It is likely that the development of oxidative stress is regulated in large part by the ability of cells to replace those macromolecules which have been damaged by oxidation, and the ability of cells to generate sufficient levels of inhibitory macromolecules, which inhibit the ability of oxidative damage to induce oxidative stress.

Protein oxidation, synthesis, and degradation in AD

While there are many different forms of oxidative damage in AD and MCI, protein oxidation is the one most likely to directly impact cellular homeostasis. This is based on the fact that proteins are directly responsible for the various enzymatic processes, and structural support, necessary for cellular homeostasis [17]. Oxidative modification of proteins is capable of inhibiting their normal function, inducing deleterious protein fragmentation, and promoting the ability of proteins to form promiscuous interactions which can lead to the development of protein aggregates [17]. The formation of highly oxidized or cross-linked proteins is likely to have negative effects on the proteolytic pathways (proteasomal and lysosomal), impairing the ability of these proteases to mediate bulk protein turnover [1, 4]. Maintaining low levels of protein oxidation is therefore likely to be a key and important part of maintaining the overall steady state protein kinetics in the cell.

As outlined above, the ability of protein oxidation to induce deleterious effects on a cell is ultimately regulated by the ability of cells to synthesize new proteins. Specifically, proteins are needed to replace those which have been oxidized, and to inhibit the initiation of oxidized protein toxicity. Failure to generate these two types of proteins would be expected to result in a progressive accumulation of aberrantly functional proteins, which would be expected to have direct and complex effects on the different cells of the brain (Figure 1).

Numerous studies indicate that in both AD and MCI the levels of protein synthesis are impaired [2, 9]. This inhibition appears to be due to deleterious oxidation of RNA molecules, as well as gross disturbances of the ribosome complex [2, 5, 9]. As outlined above, inhibition of protein synthesis would be expected to rapidly exacerbate the ability of oxidized proteins to induce oxidative stress. For example, the inability to replace oxidized proteins could increase the percentage of inactive or malfunctioning proteins in the cell to a level sufficient to induce cellular stress. Similarly, the threshold of oxidized proteins required to form protein inclusions and aggregates would be expected to be lower in cells that are impaired in their ability to synthesize inhibitory proteins (heat shock proteins, proteases). This potentially lethal combination of increased levels of oxidized proteins, and decreased levels of protein synthesis, almost certainly contributes to the oxidative stress believed to occur in AD.

It is possible that variability in the inhibition of protein synthesis helps to explain the inability of gross amounts of protein oxidation to predict the presence or severity of AD. For example, cells which have increasing levels of protein oxidation but are able to maintain sufficient levels of protein synthesis, there may be no induction of deleterious oxidative stress. Conversely, in cells where there is an inhibition of protein synthesis and an increasing amount of protein oxidation, there is likely to be the development of lethal oxidative stress. Experimental clarification of each of these issues, including the identification of which proteins are the most important to inhibiting the ability of oxidative damage to induce oxidative stress, is still very much needed.

Potential therapeutics

Based on the aforementioned studies we propose that interventions which increase protein synthesis, in particular the synthesis of beneficial proteins, may be useful in the treatment of AD. Two promising classes of pharmaceuticals are those which inhibit histone deacetylase (HDAC) activity, and compounds which regulate the mTOR pathway. Each of these types of compounds is known to potently stimulate protein synthesis [8, 18], and has been demonstrated to be neuroprotective. Interestingly, HDAC inhibitors have been demonstrated to not only suppress oxidative stress toxicity in vitro and in vivo, but have also been demonstrated to suppress the formation of neuropathology [8, 15, 18]. Identifying which proteins are increased in response to these experimental treatments, and which of these proteins are responsible for mediating neuroprotection, is important for our understanding of AD and the generation of potentially useful interventions for the treatment of AD and other age-related neurodegenerative disorders.

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Review Article

Roles of Cholesterol and Lipids in the Etiopathogenesis of Alzheimer's Disease

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Alzheimer's disease is the principal cause of dementia throughout the world and the fourth cause of death in developed economies.This brain disorder is characterized by the formation of brain protein aggregates, namely, the paired helical filaments and senile plaques. Oxidative stress during life, neuroinflamamtion, and alterations in neuron-glia interaction patterns have been also involved in the etiopathogenesis of this disease. In recent years, cumulative evidence has been gained on the involvement of alteration in neuronal lipoproteins activity, as well as on the role of cholesterol and other lipids in the pathogenesis of this neurodegenerative disorder. In this review, we analyze the links between changes in cholesterol homeostasis, and the changes of lipids of major importance for neuronal activity and Alheimer's disease. The investigation on the fine molecular mechanisms underlying the lipids influence in the etiopathogenesis of Alzheimer's disease may shed light into its treatment and medical management.

INTRODUCTION

Alzheimer's disease (AD), one of the major types of dementia in the elderly, is characterized by the formation of protein aggregates in the brain, namely paired helical filaments composed of hyperphosphorylated tau and senile plaques of the Aβ amyloid [1]. AD is a multifactorial disease, where four main factors appear to be involved in its pathogenesis, and influence tau pathology: (i) the action of Aβ_{1-42}, Aβ_{1-40}, and oligomers of these peptides [2]; (ii) oxidative stress molecules [3–5]; (iii) proinflammatory cytokines produced by activated glial cells [6]; and (iv) overproduction of NO by glial cells [7]. However, more recently a relevant link between changes in cholesterol homeostasis and AD has been evidenced [8–10].

The different factors triggering the degeneration of neurons modify various signalling pathways. Their actions appear to be mediated by the activation of the protein kinase systems cdk5/p35 and GSK3β, with the consequent hyperphosphorylation on tau [11]. The mechanisms involving the sequence of events after the neuronal insult by these molecules have been analyzed using as biological models either cell lines, primary cultured hippocampal cells, or transgenic mice models, such as Tg2576 [12] which expresses the Swedish mutation of the amyloid precursor protein APP, and other transgenic models of tau protein [13]. Aβ peptides and their oligomers induce alterations in the signalling cascades via activation of glial cells, or directly in neurons. Oxidative stress appears to be an early event in AD pathogenesis. The equilibrium between phosphatases and protein kinase activities is altered, and tau hyperphosphorylations occur as a consequence of deregulation in the cdk5 and MAP kinases signalling cascades [3]. These studies are consistent with some clinical findings in which cognitive decline in mild cognitive impairment (MCI) and AD patients, analyzed by neuropsychological tests, correlated with the levels of hyperphosphorylated tau markers in the cerebrospinal fluid [14, 15]. Moreover, these studies are relevant to the elucidation of the mechanisms involved in the etiopathogenesis of AD, and provide clues toward novel diagnostic approaches for this disease.

Lee et al [16] have provided evidence that points to alterations in rafts physiology in amyloid processing, a phenomenon, which appears to be modified by changes in cholesterol content in "lipid rafts," suggesting a direct link between cholesterol and AD. On the other hand, tau protein has also been found in rafts, and tau modifications by the Src kinase Fyn have been reported [16]. Fyn is present in rafts,

microdomains composed of cholesterol, and sphingolipids that participate in signal transduction systems. In addition, tau phosphorylation by Src kinases such as Fyn seems to be overactive in AD.

ETIOPATHOGENESIS OF ALZHEIMER'S DISEASE

Neuropathologically, AD has been characterized by extensive degeneration of cholinergic projection neurons of the basal forebrain nucleus basalis (NB), the presence of extracellular neuritic plaques mainly constituted by amyloid β-peptide (Aβ), intracellular deposits of neurofibrillary tangles formed by paired helical filaments (PHFs) containing the hyperphosphorylated tau protein, microglial-mediated inflammatory reaction, and neuronal death [1, 17, 18]. Even though, the pathogenic process leading to AD development has not been clearly defined; molecular and genetic factors are involved. In this review we emphasize the involvement of lipids components among the biochemical risk factors for AD.

Genetic factors and the familial AD

Among the genetic causes of AD, mutations and polymorphisms stand out in at least four genes. Mutations are associated with early-onset familial AD (EOAD) that account for 3% of all cases of AD and usually occur between the age of 30 and 60. These are different than the sporadic AD cases (more than 97%), even though genetic succeptibility and other molecular risk factors appear to be also involved. Alzheimer disease type 1 (AD1) is linked to mutations in the amyloid precursor gene (APP) [19]; while AD3 is caused by a mutation in the gene of presenilin-1 (PSEN1), located in chromosome 14 that encodes for a 7-transmembrane domain protein [20]; whereas AD4 accounts for mutations in the gene of presenilin-2 (PSEN2) on chromosome 1 [21] that encodes a similar 7-transmembrane domain protein. On the other hand, the late-onset of familial AD after age 65 is correlated with mutations AD2, related to the APOE4 allele on chromosome 19 [22], while AD7 and AD8 correspond to mutations that have been mapped to chromosomes 10p, 13p, and 20p, respectively [23, 24]. Mitochondrial DNA polymorphisms are also considered as a genetic risk factor [25, 26]. Furthermore, there is an association between a polymorphism in alpha-2 macroglobulin with low density lipoprotein-related protein-1 (LRP1), which is the receptor for A2M; and with APOE and APP [27, 28]. These studies suggest the possibility that all these proteins, A2M, LRP1, APOE, and APP, may participate in a common neuropathogenic pathway contributing to AD-related neurodegeneration.

Important links between the main biochemical events in AD

One of the hallmarks of AD is the observation of neurofibrillary tangles (NFT), intracellular filamentous aggregates of the microtubule-associated protein tau. Physiologic functions of tau stem from its ability to stabilize microtubules during axonal transport and its capability to help in the neurite growth [29]. Tau is regulated by phosphorylation. In a hyperphosphorylated status, tau detaches from the microtubules and, consequently, the microtubules fall apart and tau tends to aggregate in paired helical filaments (PHF), thus inducing breaks in the microtubular tracks and neuronal death [30]. Specific sites appear to be preferentially phosphorylated early in patients with AD, as, for example, the KXGS motifs targeted by the enzyme MARK, a serine-threonine kinase important for maintaining a polar network of microtubules, and, thus, cell polarity in neurons [31]. In addition to the intracellular NFT, the extracellular lesions are the amyloid plaques (or senile plaques) produced by the accumulation of amyloid (Aβ) beta peptides. Once cell-bound beta-amyloid precursor protein (APP) is cleaved by the β-secretase (BACE), it generates a soluble ectodomain sAPPβ and a C-terminal fragment CTFb, that is subsequently cleaved by γ-secretase originating neurotoxic soluble Aβ peptides that aggregate in oligomers to form these fibrillar structures [32]. APP interacts with multiple components of the nervous system mediating functions that include neuronal trophism, cell adhesion, neuronal migration, neurite outgrowth, cell-cell signalling, synapse formation, and plasticity. The active movement of APP within neurons contributes to transcription in the nucleus and apoptosis in the cytoplasm. Another cleavage of APP at the ε-site results in a fragment that can be stabilized by interaction with the factor Fe65. APP, Fe65, and Tip60 form a transcriptionally active complex that participates in gene transcription, thus making APP a gene regulator. However, a regulatory mechanism should exist to modulate APP levels since an excess of APP may lead to APP oligomerization, caspase activation, and neuronal apoptosis [33].

Oxidative damage and mitochondrial DNA alterations are involved in the neurodegeneration associated with AD. In AD, brain mitochondrial DNA point mutations have been found to appear specific to this condition. Some of these were associated with defects in oxidative phosphorylation. Additionally, the incidence of mitochondrial DNA mutations has been found to increase by 50% in AD patients [34, 35]. The mechanism that links mitochondrial alterations with neurodegeneration, as well as aging, is related to the oxidative and molecular damages that are being inflicted over time. Also, the oxygen-reactive species (ORS) scavenging mechanisms deteriorate with age and can be associated to functional deficits [36].

Even though, the early molecular events that occur in AD are not clear, the synapse loss is considered to be one of the morphological correlates related to the impairment of cognitive function observed in mid to late stages of AD [37]. It has been postulated that synaptic dysfunction precedes this synapse loss in AD [32]. In this regard, changes in the levels of proteins involved in synaptic vesicle biogenesis and/or recycling have been reported, like SNAP-25, syntaxin, and synaptotagmin in AD [38]. A second example is the critical reduction in the levels of dynamin 1 observed in AD brains, an essential protein in synaptic vesicle recycling [39]. This reduction is attributed to Aβ since it has been shown that

Aβ decrease the dynamin 1 levels involving calpain-mediated proteolysis and down-regulation of dynamin-1 gene expression [40].

General risk factors and the changes in lipids as a risk factor

Epidemiologic studies have evidenced several risk factors for AD. Age represents one of the stronger risk factors for AD [41]. The prevalence of AD doubles every 5 years after the age of 60, increasing from a prevalence of 2% among those 60- to 64-year-old to up to 50% of those aged 85 years and older [42]. Studies have shown that AD is more common among women than men by a ratio of 1.2 to 1.5 [43]. Another important risk factor is the presence of the apolipoprotein e-4 (APOE epsilon-4) allele [44]. Of its three forms, ε-2, -3, and 4, only the ε-4 allele increases the likelihood of developing AD. The lifetime risk of AD for an individual without the ε-4 allele is approximately 9%; the lifetime risk of AD for an individual carrying at least 1 e-4 allele is 29%. While representing a substantial risk of AD, the ε4/ε4 genotype is not sufficiently specific or sensitive to allow its use as a diagnostic test. Moreover, ε-4 allele appears to increase the risk of AD more in white and Asian populations than in black and Hispanic populations [45, 46]. Additionally, hypertension [47, 48], heart disease [49], obesity at midlife [50], smoking [51], elevated plasma homocysteine levels [52], diabetes [53, 54], as well as hypercholesterolemia [55, 56] are also considered as risk factors for AD.

In relation to hypercholesterolemia, several reports have shown that elevated serum cholesterol levels and elevated levels of Aß are linked with AD risk [57, 58]. Additionally a cluster of polymorphisms in cholesterol-related genes such as APOE, SOAT1, APOE 5′-untranslated region, OLR1, CYP46A1, LPL, LIPA, and APOA4 has been shown to correlate with levels of the brain cholesterol catabolite 24S-hydroxycholesterol in the cerebrospinal fluid conferring significant susceptibility to AD [59]. Studies using statins, lipid-lowering agents that inhibit HMG-CoA reductase: the key enzyme of the endogenous cholesterol synthesis, indicate that statins also can affect γ-secretase activity, thereby decreasing the breakdown of APP and reducing the risk of AD [60, 61]. Consequently, the proteolytic activity of γ-secretase is stimulated by neutral glycosphingolipids (cerebrosides), anionic glycerophospholipids, and sterols (cholesterol), showing the involvement of lipids and rafts in the modulation of BACE activity [62]. Furthermore statins exhibiting anti-inflammatory actions [63, 64] have been able to down-regulate the Aβ-mediated inflammatory response independent of cholesterol reduction [65]. These anti-inflammatory effects involve the functional inactivation of members of the Rho subfamily of small G-proteins, which regulate the actin-based cytoskeleton and participate in proinflammatory signalling pathways inducing cytokines and chemokines. This inactivation occurs through a mechanism that blocks the isoprenylation, a lipid modification of Rho-family members that facilitates specific interactions with cytoplasmic regulators, cellular membranes, and effectors [66, 67]. Recent findings demonstrate that the anti-inflammatory action of statins depends on the disruption of Rho family functions, as a consequence of reduction of isoprenoid cellular levels, preventing Rho family members from interacting with RhoGDI, resulting in increased levels of GTP-loaded G-proteins and reducing the Rac translocation to the plasma membrane [68]. In this context, Rac1 signalling as well as the enzyme BACE1 have been postulated as targets for developing novel therapies for AD [69]. These observations underlie a mechanism for the statin-mediated reduction in AD risk that involves down-regulation of neuronal APP processing and Aβ production and attenuation of microglia-mediated inflammation.

REGULATION OF TAU PROTEIN AND ITS POST-TRANSLATIONAL MODIFICATIONS

Since modified tau variants have been found in membrane domains, their interactions with lipids and the involvement of these molecules in AD are analyzed below. Tau is produced from a single gene in the chromosome 17 by alternative splicing, mainly during neuronal development [74]. The splicing products are 6 isoforms that differ in molecular weight according to the number of N-terminal repetitions and the number of microtubule binding sequences at their C-terminal domains [75]. The expression of tau isoforms also differs in central and peripheral nervous system and depends on the developmental stage. In rat brain tau starts its expression at the embryonic day 13, only the shortest isoform. From the postnatal day 8, this fetal tau isoform decreases, and the new isoforms, called "adult forms," start their expression. In the adult peripheral nervous system, there is another kind of tau, the high molecular weight isoform (110 kDa), which contains two additional expressed exons. In general, tau is a highly soluble protein, with a majority of hydrophilic residues; these characteristics mean that tau is a protein that has a natively unfolded structure, that confers resistance to heat and acid treatments.

Tau can be regulated post-translationally by phosphorylations, ubiquitinations, and O-glycosylations. This last consists of the addition of an O-linked N-acetylglucosamine (O-GlcNAc) on a Ser or Thr residues in the proximity of a Pro. Tau phosphorylation appears to be especially relevant considering that anomalous phosphorylations are involved in AD. The equilibrium between the protein kinases and phosphatases regulates the existence of these phosphorylations (Table 1).

Tau phosphorylations at Ser/Thr residues

Two different Serine-Threonine kinases have been described. (i) Proline-directed protein kinases that recognize consensus sequences that are followed by prolines. This group involves glycogen synthase kinase 3 (GSK3), cdk2 and cdk5; mitogen-activated protein kinases MAPK family, Erk1 and Erk2; and the stress-activated protein kinases (SAPK), JNK and p38 [11, 76]. These enzymes affect tau self-aggregation

Table 1: Summary of the different protein kinases implicated in Tau phosphorylation, the exact residues that are modified by them and the phosphatases that participate in the dephosphorylation process of each residue. Phosphatases are (1) PP1, (2) PP2A, and (3) PP2B. See [70–73].

Kinase	Phosphatase																		
	2, 3	2		2, 3	1, 2, 3	1, 2, 3	2, 3	3	3	1, 3		3				1, 2, 3	1, 2, 3	3	
PKA	—	—	—	—	—	—	—	—	S214	—	T234	S262	T293	S324	S356	—	S404	S409	S416
PKB	—	—	—	—	—	—	—	T212	S214	—	—	—	—	—	—	—	—	—	—
PKC	—	T123	—	—	—	—	—	—	—	—	—	—	—	—	—	S396	S404	—	S416
CaMKII	—	—	—	—	—	—	—	—	—	—	—	S262	—	—	S356	—	—	—	S416
$p110^{mapk}$	—	—	—	—	—	—	—	—	—	—	—	S262	—	—	—	—	—	—	—
JNK	—	—	T175	T181	—	S202	T205	T212	—	—	—	S262	—	—	—	S396	S404	—	—
p38	S46	—	T175	T181	—	S202	T205	T212	—	—	—	—	—	—	S356	S396	S404	—	—
SAPK3	—	—	—	T181	—	—	—	—	—	—	—	S262	—	—	S356	—	—	—	—
ERK2	S46	—	T175	T181	—	S202	T205	T212	—	—	—	—	—	—	—	S396	S404	—	—
GSK3β	—	—	T175	T181	S199	—	—	T212	—	T231	—	—	—	—	—	S396	S404	—	—
Cdk5	—	—	—	T181	—	S202	T205	T212		T231	—	—	—	—	—	S396	S404	—	—
Cdk2	S46	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

and phosphorylations occurring in the N-terminal and in the C-terminal tau moieties. (ii) Nonproline-directed protein kinases that recognize consensus sequences that are not followed by prolines. These include protein kinase C (PKC), protein kinase A (PKA), Ca^{+2}/calmodulin-dependent kinase II (CaM kinase II), Ser 262 kinase/$p100^{mapk}$, and microtubule affinity regulating kinase (MARK). These phosphorylations mainly occur at the tubulin-binding region of tau [77, 78].

Nonproline-directed serine-threonine phosphorylations of tau affect the microtubule affinity. Ser262/Ser356 sites, in the repeat domain of tau, are critical for tau binding to microtubules; therefore, when tau is phosphorylated in these residues, its affinity for microtubules diminishes. Tau phosphorylation status at these Ser residues plays a key role in the extension of cell processes [79]. Since tau stabilizes microtubules when it is bound to them, and neurites extension process needs the elongation of microtubules, then phosphorylations in Ser262 and Ser356 render microtubules less stable. Proline-directed serine-threonine phosphorylation of tau comprises almost 80% of total tau phosphorylation; it has a weak effect on microtubule binding and is regulated during neuronal development. This phosphorylation is enhanced in AD. It has been observed [79, 80] that the decrease in Ser/Thr-Pro phosphorylations favors the extension of processes, that is, when these sites are dephosphorylated. Phosphorylations are done by proline-directed protein kinases and mainly occur on the C-terminal region, thus modifying tau affinity for microtubules [78–80]. An imbalance in kinases triggers tau hyperphosphorylation, producing the paired helical filaments (PHFs), the basic components of neurofibrillary tangles (NFTs) [81].

Phosphorylations at Tyr residues

Tau can associate cell plasma membrane by its N-terminal region [82] without altering its microtubules binding. Tau can be phosphorylated by Fyn, a tyrosine kinase from the nonreceptor Src family kinases [83]. Fyn participates in the signal transduction system related to integrins having responses such as the Ras and Rho activations and the consequent actin reordering [84]. Bhaskar et al [85] have shown that Src also phosphorylate tau in Tyr 18 in vitro. In addition, Derkinderen et al [86] demonstrated that Abl phosphorylates tau in Tyr 394; both phosphorylations are present in PHFs. Tyr 18 is conserved in all tau isoforms and the sequence around this residue (GTYG) is close to the binding sequence of Fyn to its target (ETYG). Among the 7 PXXP motifs of tau, one has the major affinity to Fyn SH3 domain [84]. Moreover, Fyn has more affinity for 3R tau than for 4R tau, however, the low affinity for 4R tau is considerably increased by phosphorylations present in AD-like Ser 199/Ser202 and Ser 396/Ser 404, epitopes recognized by AT8 and PHF1 antibodies [85]. Since tau 3R plays an important role in neuronal development, its affinity with Fyn has a major function in this process. Meanwhile, tau 4R is mainly present in adult brain, and the increase in its affinity for Fyn may have an important effect in AD etiopthogenesis (Figure 1).

TAU PROTEIN IN PLASMA MEMBRANE

In the context of the involvement of lipids in AD, membrane tau appears to be relevant. The C-terminal tau domain is related to the binding of microtubules and actin filaments, participates in the stabilization of the cytoskeleton, and is a

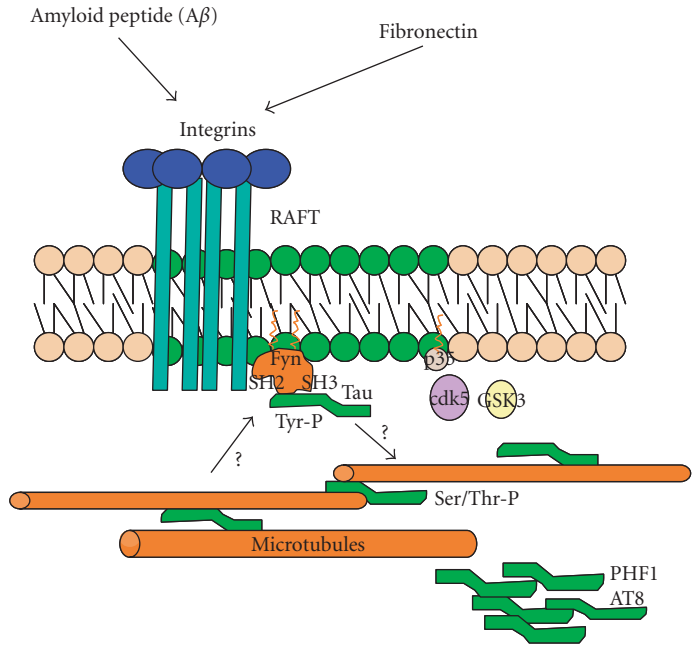

Figure 1

target for phosphorylations in Ser/Thr residues that regulate microtubule binding and the aggregation of tau in neurological diseases. The N-terminal moiety, is a projection of the protein and does not participate in the stabilization of microtubules and extension of neurites, as shown in deletion experiments [87, 88]. Brandt and Lee [87] showed that the projection domain is present in the plasma membrane of transfected PC12 cells with tau and its N-terminal domain. In nonneuronal models such as rat fibroblast (RAT-1), tau was not enriched in the periphery of the cell, indicating that its membrane localization requires the presence of neural-specific factors [87].

The association of tau to the plasma membrane is determined by its phosphorylation patterns [82]. Tau-1 antigens were detected in the membrane (tau dephosphorylated in Ser 199). When tau phosphorylation increases, it dissociates from membranes. Thus, tau phosphorylation status regulates its capability to bind to microtubules. Then, the pathological hyperphosphorylation of tau could lead migration of the subpopulation of tau associated to the plasma membrane to the cytosol, triggering its binding to microtubules or its aggregation. This is consistent with the lesser amount of tau phosphorylated at PHF1 and AT8 epitopes in the plasma membranes in AD [82].

It has been proposed that tau binds the plasma membrane in association with the membrane cortex, that is, the actin cytoskeleton present in the periphery of the cell [82]. Tau binds actin by the same amino acids used for the microtubule binding [88], and is regulated by the phosphorylation status. The accumulation of tau in the somatodendritic compartment produced in AD could be triggered by a loss of tau-cortical interaction as a result of the phosphorylation in PHF-like epitopes.

The phosphorylation pattern of tau is affected by phospholipids [89]: incubation of tau with phosphatidyl serine (PS), and MAP kinases showed that the phosphorylation of tau in PHF epitopes decreased, increasing the Tau1 reactivity. Phosphatidyl choline (PC) showed the same results, but phosphatidyl inositol (PI) did not. PS also reduced the cosedimentation of tau with microtubules in experiments of tau-tubulin association and enhanced the tau proteolysis by calpain. The lipid components of the plasma membrane are able by this way to alter tau function, making the tau subpopulation present in this compartment, different from the cytosolic tau.

In rafts, the exoplasmic leaflet is enriched with sphingomyelin and glycosphingolipids, the cytoplasmic leaflet is enriched with glycerolipids (phosphatidyl serine and phosphatidyl ethanolamine). Cholesterol is present in both leaflets. Some proteins such as those with GPI anchors are also present in rafts, leaving the GPI in the exoplasmic leaflet; also present in rafts are proteins with transmembrane domains, like the influenza virus proteins and haemaglutinin (HA) [90] and the palmitoylated such as Src family kinases. The hydrogen-bonding properties of glycosphingolipids with themselves and with the GPI anchor the GPI-linked proteins to stabilize the complexes formed in the microdomains. In addition, cholesterol has a planar shape that favors its organization in the membrane, also giving stabilization. The lipids can move in and out of rafts individually, making this system highly dynamic.

In oligodendrocytes, the myelination process is led in great part by Fyn. Fyn knockout mice lack correct myelination. Fyn interacts with tau, and tau interacts with microtubules. Both are present in the rafts in the oligodendrocytes (see [91]), forming a complex as evidenced by tau-Fyn

coimmunoprecipitation in raft and nonraft fractions. It was also observed that tubulin is capable of binding SH2 and SH3 domains of Fyn, whereas tau only binds the SH3 domain. When the cells were transfected with the N-terminal tau moiety containing the PXXP motif that interacts with Fyn, the number and length of processes diminished because tau N-terminal competes for the Fyn binding. Klein et al [91] proposed a model in which Fyn interacts with tau in rafts, and in turn tau interacts with microtubules allowing the extension process of oligodendrocytes when these cells contact neighbor neurons.

POTENTIAL ROLE OF LIPIDS AND CHOLESTEROL IN AD

Membrane lipids are essential for biological functions ranging from processes involving trafficking of molecules and signalling transduction. Lipids and cholesterol are transported through the blood stream by lipid-protein particles, lipoproteins that can be classified in different subsets according ot their density: high density (HDL), medium density (IDL), low density (LDL), and very low density (VLDL). LDL has an elevated fat proportion and participates in lipids transported from blood stream to peripheral tissues. HDL has a major protein fraction with respect to lipids, and takes part in reverse cholesterol transport from peripheral tissues to liver where conjugation and excretion occur. Cholesterol is transported in the plasma predominantly as cholesteryl-esters associated with lipoproteins. Cholesterol derived from diet is transported from the intestines to the liver within chylomicrons. Cholesterol can be synthesized de novo by the liver using acetyl-CoA as precursor molecule. In this biochemical pathway, the enzyme HMG-CoA reductase has a main role in the pharmacological intervention to diminish the endogenous cholesterol synthesis. Finally, cholesterol is excreted in the bile as free cholesterol or as bile salts following conversion to bile acids in the liver [92].

Brain cholesterol is mainly synthesized locally within the CNS. It is estimated that during CNS development, neurons synthesize most of the cholesterol needed for their growth and synaptogenesis. Later, when neurons are mature, they reduce their endogenous cholesterol's synthesis and become more dependent on cholesterol synthesized and secreted by the astrocytes [93]. Cholesterol in the CNS is turned over in a proportion of 0.7% of the total amount every day. Even though, it is not a great fraction, it is a relatively high amount of cholesterol considering that the CNS accounts for 2.1% of body weight and contains 23% of the total sterol in the whole body. The brain is therefore the most cholesterol-rich human organ. In addition, cholesterol accounts for 20–25% of the total lipids in neurons plasma membranes. Thus, neurons require a continuous supply of new cholesterol to maintain its constant concentration in the plasma membranes. Eukaryotic cells incorporate cholesterol through at least three mechanisms: (1) de novo synthesis within the cell from acetyl-CoA, which is the most important mechanism for neurons, (2) uptake of unesterified or esterified cholesterol from the external environment using the LDL receptors (LDLR), or (3) the Niemann-Pick C1-like protein (NPC1L1) (90).

LDLR bind particles that contain either apoE or ApoB-100 (remnants of chylomicrons, very low density lipoproteins (VLDL), and LDL) [94]. These particles are then processed through the clathrin-coated pit pathway to late endosomes and lysosomes. Then, the hydrolysis of the cholesteryl esters takes place and cholesterol becomes part of the metabolically active pool within the cell. At this final stage, cholesterol performs a variety of functions: it can be transferred to the plasma membrane, metabolized to other products, or act as a regulator of cell sterol metabolism [94]. Two other proteins; Niemann-Pick type C1 and C2 (NPC1 and NPC2), are also required to move the unesterified cholesterol to the metabolically active pool. However, their roles remain uncertain so far [92]. Current evidence indicates that cells with dysfunctional NPC1 or NPC2 accumulate unesterified cholesterol in late endosomes, which reflects a failure of cholesterol to exit efficiently this compartment and travel to the plasma membrane and endoplasmic reticulum [95]. This failure of cholesterol trafficking can be explained, in part, by its genetic basis, which involves mutations of either of two functionally related genes, NPC1 and NPC2, accounting, respectively, for 95% and 5% of the cases [95].

Cholesterol cannot pass directly through the blood-brain barrier (BBB). Firstly, it has to be oxidized into 27-hydroxycholesterol and/or 24S-hydroxycholesterol in the peripheral organs or in the CNS, respectively. As 24S-hydroxycholesterol is synthesized in the brain and spinal cord, concentrations of this late metabolite are higher in the CNS than in any other tissue. Some authors have even suggested that 24S-hydroxycholesterol peripheral levels can be used as a marker for AD [96]. In fact, during the early stages of AD, 24S-hydroxycholesterol levels are high in CSF and in peripheral circulation, even though the physiological explanation for this fact and its implications for AD are still unknown [96, 97]. The transport of excess cholesterol out of cells is mediated by ABCA1, a membrane protein that facilitates the formation of APOE-cholesterol-phospholipid (ApoE-Chol-PL) complex and requires the hydrolysis of ATP for its activity. Upon secretion of the APOE-Chol-PL complex into the extracellular environment, the complex may bind to LDL receptor-related protein (LDLRP) to be taken up by neurons or form HDL-like particles for transport to the systemic circulation [93].

Cholesterol influences the activity of the enzymes involved in the metabolism of the amyloid precursor protein and in the production of Aβ [58]. In some animal studies, dietary cholesterol accelerated Aβ deposition in the brain, whereas cholesterol-lowering drugs lowered it [58–60]. Also, in other in vivo studies a lower-cholesterol environment resulted in an increased production of soluble amyloid precursor protein [98]. The mechanism by which cholesterol affects Aβ production and metabolism is not fully understood. It has been suggested that possible mechanisms related to changes in plasma membrane stiffness and fluidity could explain the influence of cholesterol on enzymes like BACE 1. The high cholesterol content in lipid rafts, where these enzymes are located, could facilitate the clustering of α and β secretases with their substrates into an optimum configuration, thereby

promoting the undesirable pathogenetic cleavage of amyloid precursor protein [58, 69]. This suggests that the use of statins might decrease the levels of cholesterol in neurons thereby altering the organization of lipids rafts. However, Abad-Rodriguez et al have demonstrated that some of the most commonly used statins are poor penetrators of the blood-brain barrier [99], so their benefits in the prevention of AD development might derive from anti-inflammatory or antioxidant properties rather than a direct effect on cholesterol concentration in the plasma membrane of neurons. In neuronal and glial membranes, cholesterol is distributed asymmetrically among the two membrane leaflets. In normal physiological conditions, the plasma membrane cytofacial leaflet contains more than 85% of the membrane cholesterol in the synaptic plasma membrane, whereas the cholesterol content of the exofacial leaflet is low [93]. Some statins may raise this ratio by lowering the cholesterol content of the exofacial leaflet [100]. However, these effects of statins in the brain vary depending on the lipophilicity of each molecule. This suggests that, rather than reducing brain cholesterol, a feasible explanation for the effect of statins might be that they alter the cholesterol balance in the plasma membranes of brain neurons. However, the significance of these effects on the human brain is still unknown.

In vivo experiments have shown that there are differences between the effects of simvastatin, a lipophilic statin; and pravastatin, a hydrophilic statin, when given as a high-dose short-term treatment [70]. Simvastatin reduced the levels of lathosterol, a cholesterol precursor, but did not affect the formation of 24(S)-hydroxycholesterol. The HMG-CoA reductase and ABCA1 mRNA expression in the brain was significantly upregulated in animals treated with simvastatin compared to those treated with pravastatin or with placebo. These findings suggest that cholesterol synthesis is significantly affected by short-term treatment with high doses of lipophilic simvastatin, while whole brain cholesterol turnover is not disturbed [70].

Altogether, there is still no evidence for the net transfer of sterol from the systemic circulation into the brain. Details about the implications of cholesterol blood levels in the development of AD are therefore an open field for research. Niemann-Pick type-C disease (NPC) is a juvenile, fatal autosomal recessive neurovisceral lipid storage disorder. NPC is associated with a progressive neurodegeneration, thereby resulting in dementia caused by dysfunction of the neuronal network [101]. Hallmarks of NPC are ballooned neurons and massive neuronal loss with massive intracellular cholesterol accumulations both in human and in murine NPC brain [102].

Although NPC differs in major respects from AD, intriguing parallels exist in the cellular pathology of these two diseases, including neurofibrillary tangle formation, prominent lysosome system dysfunction, and influences of the apolipoprotein E4 genotype. In addition, accumulation of cleaved APP and Aβ peptides within endosomes has been observed in NPC [95]. In vivo experiments using murine models of NPC have demonstrated that tau protein is phosphorylated at epitopes considered to represent early stages of AD [102]. This strengthens the concept that an alteration in cholesterol metabolism could play a pivotal role in early stages of AD.

CHOLESTEROL IN LIPID MEMBRANE MICRODOMAINS (RAFTS) AS AN APPROACH TO STUDY ALZHEIMER'S DISEASE

Several protein systems use lipid rafts as a platform for signalling. "Lipid raft" is basically a definition given to those membrane domains rich in cholesterol and glycosphingolipids isolated from cells or cell membranes preparations through detergent and nondetergent methods [71]. Names like cholesterol enriched membranes (CEMs), glycosphingolipid enriched membranes (GEMs), detergent-insoluble, glycosphingolipid-enriched membranes (DIGs), and detergent-resistant membranes (DRMs) have been given to these rigid plasma membrane fragments based on their preferential lipid composition and/or nonionic detergent resistance properties [103]. Rafts can be invaginated or not depending on whether they are composed of a protein called caveolin. This protein interacts with the membrane making a hairpin loop and raising caveolae; these are considered as specialized forms of rafts implicated in cell transduction.

During the last ten years, numerous data obtained from a wide variety of biophysical and biological techniques have helped to explain the composition and function of lipid rafts. However, there are still many unanswered questions, especially in the area of cholesterol/lipids and cholesterol/proteins biochemical interactions and differential segregation. In the light of currently available scientific evidence, it seems clear that lipid rafts composition strongly depends on the "isolation" method used to obtain them [72, 104]. A plausible hypothesis to explain the differences occurring in cholesterol, glycosphingolipids, and proteins composition of lipid rafts, based on different isolation methods, has been recently described. In this hypothesis, scientific evidence is presented and discussed in order to support the existence of three different models of raft structure based on the utilization of different raft isolation protocols. The first two predict that all rafts isolated by the same method will have similar composition and the third predicts that rafts isolated by the same method could be heterogeneous in composition because the domains themselves are heterogeneous [105]. Conversely, Rajendran et al showed that, in lymphocytes, raft proteins like lck, lyn, and LAT were released from lipid rafts by treatment with methyl-β-cyclodextrin; whereas flotilins markers remained in detergent resistant membranes, suggesting that some rafts require less cholesterol than others to maintain their integrity, or that some rafts retain their cholesterol more effectively than the others by cholesterol sequestering agents [73].

It seems, therefore, of pivotal importance to focus on the raft isolation methods when discussing cholesterol rafts and their possible role in any pathophysiological event. Altogether, despite the heterogeneous composition found in

lipid rafts, it is widely accepted that they are membrane domains rich in cholesterol and glycosphingolipids [106, 107]. Lipid composition is approximately 1 : 1 : 1, phosphoglycerolipids: sphingolipids (including sphingomielin (SM) and glycosphingolipids (GSL)): cholesterol. In rafts isolated using detergent protocols, the phospholipid population is enriched in saturated acyl chains relative to the average for whole cell phospholipids [108]. Conversely, little consistent evidence has been provided regarding the mechanism underlying cholesterol selective migration, residence, and dynamics in these membrane lipid rafts.

Ipsen et al have proposed a lipid membrane spatial distribution theory that partially helped to explain the clustering of lipids into the cholesterol rich domains; they proposed that cholesterol and phospholipids form a liquid-ordered (Lo) phase characterized by a high level of molecular order in the lipid packaging [109]. According to this hypothesis, the Lo phase would coexist with the liquid-disordered phase (Ld) or liquid-crystalline phase, characterized by a high degree of disorder and very high lipid mobility, and with the gel phase, in which lipid molecules are virtually immobile. Lipids and proteins that "prefer" liquid-ordered phase (including both glycosphingolipids and glycosylphosphatidylinositol-anchored proteins) would segregate into the Lo-phase domains and thus into lipid rafts. However, this hypothesis appears to be controversial since, according to Brown [110], it does not have enough support. Importantly, a significantly lower fluidity was found in acyl chain of caveolae/raft domains when purified without the use of detergents [103].

In spite of its limitations, giant unilamellar vesicles (GUVs), an artificial membrane system, have provided a very useful tool to elucidate the biophysical mechanisms of raft assembly, especially allowing for the study of cholesterol and lipids dynamics in a noninvasive manner [111]. Application of FCS combined with confocal scanning microscopy allows for the determination of single molecule diffusion, chemical kinetics, and conformational equilibrium. Thus, it has become a valuable insight into lipid/cholesterol interactions and lipid dynamic organization during lipid rafts assembly [111, 112]. By applying these techniques, Kahaya et al [112–114] have established that both dioleoylphosphatidylcholine (DOPC) and DLPC, unsaturated phospholipids, gradually decrease their mobility as a function of cholesterol concentration [113]. When compared to DOPC, bigger changes in mobility were exhibited by DLPC, meaning that cholesterol would interact more intimately with the saturated phospholipids. They found that cholesterol interacts more strongly with SM than phosphatidylcholine (PC), implying that the latter interaction may have a role in the stiffness of SM membranes with respect to PC bilayers. In addition, in ternary mixtures, a weaker tendency to form extensive domains was observed for DOPC/dipalmitoylphosphatidylcholine (DPPC)/cholesterol as compared with DOPC/DSPC/cholesterol. In both cases, this tendency was much weaker than that for the DOPC/SM/cholesterol mixture. This reflects the weaker DPPC/cholesterol with respect to SM/cholesterol interactions. Altogether, it seems that cholesterol clearly interacts more strongly with sphingolipids than with phospholipids even for those that are unsaturated [114]. Interestingly, it has been found that ceramides, generated from SM by the action of SMase, displace membrane cholesterol due to a competition for associating with lipid rafts [115]. Other GUVs/FCS studies published by Kahya in 2003 [112] proved that the lipophilic fluorescent probe DiI-C18 was excluded from SM enriched regions, while the raft marker GM1 was present. Furthermore, cholesterol was shown to promote lipid segregation in DOPC-enriched liquid disordered and SM-enriched liquid ordered phases. Lipids mobility in SM-enriched regions significantly increased by increasing the cholesterol concentration.

Another feasible model to explain the generation of lipid rafts and the differential cholesterol distribution was proposed by Anderson and Jacobson and is based on protein/lipid interaction [116]. In this model, also named "the shell hypothesis," raft proteins like GPI-anchored proteins and selected transmembrane and peripheral proteins would be able to adopt a "shell" formation via interactions with cell membrane lipids. Caveolins, the proteolipid MAL, flotilins, and stomatin have been reported to interact with the bilayer and have an intimate contact with membrane lipids forming the so-called "lipid shells," which are thought to be thermodynamically stable and diffusible unit that coalesce based on protein-protein interactions. As a result, a larger functional unit called "lipid raft" would be created. However, it remains unknown which of the raft proteins are capable of interacting with lipids in a way to form these lipids shells.

Pankov et al [117] have hypothesized that integrins, important protein components of the cholesterol-enriched domains, may have a fundamental role in cholesterol levels and in the stability of lipid rafts. They demonstrated that integrin presence in fibroblast plasma membrane increased lipid rafts cholesterol and sphingomyelin content as well as the nonraft constituent phosphatidylethanolamine. Even though, they do not provide details about the mechanism underlying this fact, a likely explanation based on phosphatidylethanolamine thermodynamic incompatibility with cholesterol was proposed.

Rafts and caveolae contain several proteins implicated in signal transduction pathways [118, 119]. Thus, it is believed that rafts are implicated directly in transduction events; possibly allowing a group of proteins to participate in a certain pathway and isolating others, avoiding the crosstalk of different pathways. They could make the encounter of interacting proteins easier. Rafts are implicated in the integrin signalling and also in the processing of amyloid precursor protein (APP) to Aβ [120]. The secretases implicated in the cleavage of APP are present in rafts, and the processing in Aβ [121] is dependent on the cholesterol amount in the cell. It has been proposed that if the cell has more cholesterol, then it has more rafts, allowing the APP cleavage and the posterior accumulation of this peptide at the exterior of the cell [120].

AMYLOID PEPTIDE, APP, AND THEIR LINKS WITH RAFTS AND MEMBRANE LIPIDS

By using silver [122] and Congo-red staining [123] it was possible to see in the tissue affected with Alzheimer's disease the lesions produced in the cortical regions of the brain. The neurons are surrounded by a characteristic large, dark, and circular inclusion called Alzheimer's plaque. This plaque is made of amyloid deposits composed of a peptide of 4 kDa in a β-sheet conformation; this was called β-amyloid [124]. Later, it was discovered that the peptide was part of a 79 kDa protein with 695 amino acids recognized as an amyloid precursor protein (APP). Hydrophobic analysis of this protein revealed membrane-spanning segment, a C-terminal cytoplasmic domain, and an N-terminal extracellular domain; it is characterized as a ubiquitously expressed type-1 membrane glycoprotein [125]. APP undergoes alternative splicing originating three varieties depending on the amount of residues: APP_{695}, APP_{741}, APP_{751}, and APP_{770}. The two longer isoforms contain the exon 7, which encodes a serine protease inhibitor domain. This kind of inhibitors promotes the outgrowth of neurites providing a possible nonpathological function for this protein. The most common isoform present in the body is APP_{751}, but in the brain, the most abundant is APP_{695}. APP is widely expressed in cell surface, especially on neurons, but also in astrocytes, microglia, endothelial cells, smooth muscles, and all peripheral cells.

APP is processed by three enzymes called α, β, and γ secretases. The β-secretase is a member of the ADAM family of metalloproteases and the γ-secretase is a membrane-bound aspartyl protease, also called BACE. These secretases cleave the ectodomain of APP in different sites, resulting in two fragments named after the involved secretase: APPsβ and APPsα. After these cleavages, the α-secretase processes the transmembrane domain of the APP C-terminal fragments (α-CTF and β-CTF) producing two smaller fragments called p3 (for the α previous clip) and Aβ (for the β-clip). The first fragment dissolves easily within the brain, but the second does not dissolve and therefore it accumulates in the brain, forming the senile plaques. The Aβ fragments can have 40 or 42 residues ($A\beta_{1-40}$ or $A\beta_{1-42}$). The $A\beta_{1-42}$ is neurotoxic and more easily aggregates in plaques. Since the fragment produced by the previous cleavage of the γ-secretase does not produce Aβ or plaques, it is called "nonamyloidogenic pathway" and is neuroprotective. This is the major way of APP metabolism in most cells. Therefore, the stimulation of easier γ-secretase decreases the Aβ formation and protects from AD. There are several reagents that can stimulate this secretase, such as estrogen, testosterone, various neurotransmitters, growth factors, and the activation of protein kinase-C (PKC) by phorbol esters. β- and γ-secretases are considered as participants in the amyloidogenic pathway, especially γ-secretase because it is associated with presenilins. Two highly conserved residues in the transmembrane domains 6 and 7 of presenilins seem to be necessary for the normal γ-secretase activity. Since the presenilin genes PSEN1 and PSEN2 participate in familial AD, one possible treatment for the prevention of AD would be the control of γ-secretase activity. Both β- and $\tilde{\gamma}$-secretases are present in lipid rafts and these lipid microdomains containing cholesterol have been implicated in AD [126]. These membrane domains could function as a microenvironment, where APP is processed, but while the generation of Aβ seems to be dependent on rafts and what occurs there, the processing of the APP by the γ-secretase occurs outside the lipid rafts [120]. The levels of Aβ are not dependent on the cholesterol amount in the cell in vivo [121], due to cholesterol having no effect over γ-secretase activity, but the increment in the cholesterol at the membrane can increase the lipid rafts formation and thus allow the APP processing by BACE1 (γ-secretase) [126].

OXIDIZED LIPIDS AND CHOLESTEROL IN AD

Neurodegenerative disorders like Alzheimer's disease encompass oxidative modifications of lipids and cholesterol detected in serum and cerebrospinal fluid (CSF) of Alzheimer's disease patients (AD). The major protein modification by trans-4-hydroxy-2-nonenal (HNE) is the adduction to lysines, which was observed in postmortem brains of AD [127, 128]. The neurotoxic effect of HNE, a potent prooxidant, was determined in a cellular culture model. Acrolein is a more reactive product of the metal-catalyzed oxidation of polyunsaturated fatty acids [129] detected at increased level in the brain of AD and becomes neurotoxic to hippocampal neurons by a calcium-dependent mechanism [130].

Efforts have been made to find reliable biological markers for the initial phase in AD, even before the first clinical evidence for the disease. In this context, the detection of oxidative markers such as 4-HNE and 24S-hydroxycholesterol in plasma and urine samples can help in the early diagnosis of AD, thus facilitating the treatment approaches.

An elevated level of HNE, produced by the oxidation of arachidonic acid, was detected in the plasma of AD [131, 132] malondialdehyde, an end product of lipid peroxidation, and was increased in the plasma of AD patients versus age-matched and nutritionally evaluated control subjects [133]. Nevertheless, malondialdehyde failed as a predictive AD marker in several other studies [134, 135]. Elevated levels of 24S-hydroxycholesterol, produced via an enzymatic oxidation of brain cholesterol by CYP46, were detected in the plasma of AD compared with control age-matched volunteers [136, 137]. Concomitantly, treatment with statins lowered the levels of LDL-cholesterol and 24S-hydroxycholesterol in plasma [138], and it has been suggested that the oxysterol may be an important marker of AD risk instead of total cholesterol. In summary, 24S-hydroxycholesterol and HNE are good candidates to be a marker of AD.

A key role in AD pathology is now assumed to be vascular in origin due to hypoperfusion of microvasculature that induces hypoxia in brain tissue, events that are associated with oxidative stress [139]. Moreover, postmortem AD brains show atherosclerotic hypoperfused microvessels lesions that are closely related to oxidative stress markers and amyloid plaques. This scenario is completed by a recent work that has shown an induction of iNOS with a consequent

peroxynitrite production in astrocyte cell culture treated with LDL from AD patients [140], which is in agreement with an association among lipids/cholesterol, oxidative stress production and pathology of AD.

An interesting connection between cholesterol and amyloid-β-peptide ($A\beta_{1-42}$) has been demonstrated. $A\beta$: Cu^{2+} complexes oxidize cholesterol selectively at the C-3 hydroxyl group, catalytically producing 4-cholesten-3-one and therefore mimicking the activity of cholesterol oxidase [141]. Moreover, it was demonstrated that amyloid peptide precursor (APP) protein promotes cholesterol oxidation, yielding 7β-hydroxycholesterol, a proapoptotic oxysterol, but $A\beta_{1-42}$ was shown to be 200 times more potent as prooxidant [142, 143].

Sulfatides are a class of sulfated galactocerebrosides that mediate diverse biological processes including cell growth regulation, protein trafficking, signal transduction, adhesion, neuronal plasticity, and cell morphogenesis which has been characterized as potential AD marker. A decrease in sulfatide concentration in very mild dementia patients has been found [144] but a correlation with Alzheimer's disease should be done.

Interestingly, *knockout* mice for ApoE have between 61–114% more sulfatides and mice over-expressing ApoE4 exhibit 60% less sulfatides than the "wild type" counterparts. However, no modifications in the contents of phospholipids, sphingolipids, and cholesterol were detected, suggesting a novel role for ApoE in the brain in mediating sulfatides metabolism [145].

EXPERIMENTAL MODELS FOR AD AND THE STUDY OF THE ROLES OF LIPIDS

Studies revealed that an elevated serum cholesterol level is a risk factor for AD [146]. Based on this epidemiological data, a wide variety of in vitro and in vivo experimental models has been used to elucidate potential mechanisms underlying cholesterol/AD relationships. The involvement of cholesterol in modulating BACE activity was assessed, and it was observed that cholesterol stimulates the proteolytic activity of purified BACE in 100 nm unilamellar vesicles [147]. George et al reported that a diet which induced hypercholesterolemia increased APP intracellular domain and reduced soluble $A\beta$ in the transgenic mouse Tg2576, an AD mouse model that overexpresses human amyloid β-protein precursor [148].

Even though statins have pleiotropic effects, they have been widely used to study potential specific links between AD and cholesterol in a variety of AD experimental models [149]. Gender differences observed in the risk for AD development in humans led Park et al to assess the effect of lovastatin in male and female Tg2576 mice separately. Results showed a reduction of cholesterol levels in both sexes, but $A\beta_{1-40}$ peptide levels were increased in female mice only. As well, no changes were observed in the amounts of full-length, α-secretase processed amyloid precursor protein (APP) or presenilin 1 (PS1) in either sex [150].

Li et al (see [151]) reported that in the transgenic mice B6Tg2576, an AD mice model that also develops atherosclerosis, there is a positive relationship between the presence of aortic atherosclerotic lesions and cerebral β-amyloidosis. Atherogenic diets and spatial learning impairment were also positively correlated in this model. Cordle and Landreth demonstrated that statins reduce neuronal $A\beta$ production and inhibit the microgial mediated inflammation by means of a reduction of isoprenyl intermediates in the cholesterol biosynthetic pathway [152].

Recently, experiments performed with microglial cultures demonstrated that statins blocks $A\beta$ stimulated phagocytosis through inhibition of Rac1. In addition, these experiments paradoxically demonstrated that statins mediate the inactivation of G-proteins by increasing GTP loading of Rac and RhoA and by disrupting the interaction of Rac with its negative regulator RhoGDI [153].

MAIN CURRENT PHARMACOLOGICAL APPROACHES FOR AD

To understand the therapeutic approaches for AD based on the changes in lipids metabolism, it is worth to review the current pharmacological treatments for this disease. The cholinergic hypothesis of AD is based on the evidence that cholinergic brain deficits lead to alterations in attention and memory in animal models, healthy elderly, and cognitively impaired patients. Cholinesterase inhibitors have been widely accepted by clinical and basic researchers as a pivotal part of AD treatment. Furthermore, there is strong evidence supporting the improvement in cognitive and global function with the use of rivastigmine, donepezil, and galantamine [154]. However, a recent critical analysis of the scientific evidence has seriously questioned the benefits of these drugs in AD [153]. Several studies have established that in patients who are cognitively impaired, donepezil may delay the onset of AD [155]. Rivastigmine, which also inhibits butyrylcholinesterase, has shown benefits in mild to moderate AD in clinical trials, but side effects at high doses have led to the suspension of this therapy. A study designed to assess the effects of rivastigmine on the cognitive functioning of 92 patients suffering from dementia with Lewy bodies indicated that rivastigmine produces a significant benefit over placebo on tests of attention, working memory, and episodic secondary memory [156]. Galantamine, a competitive reversible inhibitor of acetylcholesterase and allosteric modulator of nicotinic receptors, has shown no great cognition improvements in mild to moderate AD, thus galantamine does not seem to be significantly better than donepezil [157].

In addition to its role as an excitatory neurotransmitter, glutamate can also be neurotoxic in excessive levels leading to disproportionate depolarization, influx of calcium into neurons, and cell death. Due to its role in memory impairment and dementia, glutamate has been considered a potential aggravating factor in AD [159]. On the other hand, three randomized clinical trials have shown that memantine, a glutamate antagonist causes just mild improvements in the cognitive performance of severe AD patients, though side effects were not very significant. Use of memantine has been shown to slow the decline of the treated patients, rather than cause

Table 2: Summary of the main clinical studies that have provided statins efficacy results in AD or other types of dementias. Dur: duration of the study (months), N: number of patients, SD: study design, PO: main primary outcomes or biological effects, AO: additional outcomes, SO: main secondary outcomes, Ref: references.

Drug dosage	SD	N	Dur	PO	SO	AO	Results	Ref
Different statins	Case control	284	72	Relative risk of AD (odd ratio)	—	—	Decrease relative AD risk (0.29)	[56]
Atorvastatin calcium 80 mg/day	Randomized, double-blind, placebo-controlled	63	12	ADAS cog and CGIC change score	ADAS cog, CGI, and NPI Scales	—	Improvements in PO, trends to improving in SO	[154]
Simvastatin 20 mg/day	Uncontrolled, open trial	19	3	CSF levels of βsAPP, αsAPP, Tau, phospho-Tau, Aβ_{1-42}, and plasma levels of Aβ_{1-42}	ADAS cog	—	βsAPP, αsAPP decreased, ADAS-cog slightly increased	[153]
Simvastatin 20 mg/day	Uncontrolled, open	19	12	CSF levels of Aβ_{1-42}, βsAPP, αsAPP, totAPP, and total Tau, plasma levels of Aβ_{1-42} ADAS cog	MMSE	—	No changes in CSF levels of Aβ_{1-42}, βsAPP, totAPP, total Tau, plasma levels of Aβ_{1-42}, ADAS cog, and MMSE, αsAPP increased	[158]
Pravastatin 40 mg/day	Randomized, placebo-controlled	5804	38.4	Coronary death, nonfatal myocardial infarction, fatal and nonfatal stroke	—	MMSE	No differences between treatment and controlled group	[155]
Simvastatin 40 mg/day	Randomized, placebo-controlled	20.536	60	Plasma levels of LDL, major coronary events, strokes, and revascularizations (separated into prior and not prior cerebrovascular disease)	Ischaemic and/or hemorrhagic stroke (separated into prior and not prior cerebrovascular disease)	TICS-m	No differences in the cognitive score between treatment and control groups	[156]

an actual improvement. Therefore, there seems to be no reason to expect promising results from memantine or better efficacy than cholinesterase inhibitors [160].

As we previously mentioned, the key event leading to cognitive impairment in a variety of AD is the formation of the peptide Aβ_{1-42}, which clusters into amyloid plaques in brains of patients with AD. β-secretase cleavage followed by γ-secretase proteolysis are both necessary to cleave APP into the toxic Aß$_{1-42}$ [1]. No sufficiently successful therapeutic strategies based on the inhibition of both β and γ secretases have been developed so far. γ-secretase inhibitors are already being tested in clinical trials, while β-secretase inhibitors are still in preclinical development. Other approaches include interfering with the deposition of the Aβ_{1-42} into plaques and enhancing its clearance, but none of these approaches has gained enough scientific and clinical evidence to support a feasible therapeutic intervention [161, 162].

It is widely accepted that hyperphosphorylated tau is the main component of neurofibrillary tangles (NFTs), a broadly known marker for AD neurodegeneration. Indeed, several reports point out that tau aggregation requires an anomalous previous hyperphosphorylation. Both the prolonged activity of glycogen synthase kinase-3 (GSK3β) as well as cdk5/p35 system deregulation are thought to be pivotal events in tau hyperphosphorylation [11, 12]. Therapeutic drugs like valproic acid and lithium, which are approved for other neurological and psychiatric disorders, have the ability to inhibit GSK-3β. However, according to clinical evidences, both the efficacy and tolerability are limited. Thus, current scientific evidence is still too weak to support a feasible pharmacological therapeutic avenue for AD [163].

CHOLESTEROL-LOWERING AGENTS (STATINS). ARE COGNITIVE IMPROVEMENTS THE BOTTLENECK?

Extensive critical revisions of AD treatments have been published by a number of authors in recent years [160–162] but none of them puts emphasis on the potential role of statins in AD based on the existing molecular and clinical evidence. Here, we have briefly summarized the main current pharmacological treatments. Clinical data regarding the use of statins and thier controversial conclusions are also presented in order to overview the problem and analyze its potential impact on AD treatment (Table 2).

The epidemiological data regarding the relationship between the use of statins and the risk of AD is controversial. Most of the preclinical results derived from cells and animal studies show a direct relationship between elevated cholesterol levels and molecular or cognitive markers of AD [15]. However, the current state of research in human studies is not solid enough to establish a certain and reliable causal relationship between the use of cholesterol lowering agents and the incidence of AD and other types of dementias [45, 160, 163, 164]. Concrete clinical evidence has been provided by Wolozin et al indicating that there is a 60 to 73% lower prevalence ($P < .001$) of probable diagnosed AD in patients taking lovastatin or pravastatin [165, 166]. Jick et al reported that patients of 50 years and older had lower risk of developing dementia, though not specifically Alzheimer's, when treated with statins. This lower relative risk was neither related to the presence or the absence of hyperlipidemia [56].

An open uncontrolled clinical trial with the simbastatin in 19 patients with AD settled that 20 mg/d of simbastatin for 12 weeks significantly reduced the cerebrospinal fluid (CSF) levels of β-secretase-cleaved amyloid precursor protein (βsAPP) and α-secretase-cleaved amyloid precursor protein (αsAPP). Conversely, CSF levels of tau, phosphotau, $A\beta_{1-42}$, and plasma levels of $A\beta_{1-42}$ remained unchanged. Cognitive capacity was measured using the disease assessment scale cognition (ADAS-cog). In this study, ADAS-cog score was just slightly increased in less than 50% of all patients [167]. Even though, this open observational study concluded that the simbastatin reduces $A\beta_{1-42}$ formation in AD patients, results were not consistent with a cognitive improvement due to the use of simbastatin.

A recently published pilot double-blind, placebo-controlled trial with a one year exposure to atorvastatin calcium 80 mg/d in 63 AD patients with an MMSE of 12–28 demonstrated that atorvastatin produces a significant beneficial effect at 6 months when evaluated using both geriatric depression scale and the ADAS cog. However, the secondary outcome measures ADAS cog, CGI, and neuropsychiatric inventory scales, evaluated at 12 months, were not consistent with certain protection against AD [168].

Another study demonstrated that simbastatin 20 mg/d for 12 months produces no changes in CSF levels of β-amyloid ($A\beta_{1-42}$), βsAPP, totAPP, and total tau as compared to the baseline, nor were there changes in $A\beta_{1-42}$ plasma levels. However, αsAPP was significantly increased suggesting that simbastatin could favor the nonamyloidogenic APP processing pathway [169]. Studies indicate that significantly more research is needed in order to clarify the roles of cholesterol therapeutic agents in the AD treatment. At the present stage of this research, there is cumulative evidence that the search of reliable biomarkers for AD, of importance for early AD diagnosis and monitoring the course of the disease, will allow the growth of therapeutical approaches, including both pharmacological treatment and cognitive rehabilitation of patients.

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Review Article

Targeting Gonadotropins: An Alternative Option for Alzheimer Disease Treatment

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Recent evidence indicates that, alongside oxidative stress, dysregulation of the cell cycle in neurons susceptible to degeneration in Alzheimer disease may play a crucial role in the initiation of the disease. As such, the role of reproductive hormones, which are closely associated with the cell cycle both during development and after birth, may be of key import. While estrogen has been the primary focus, the protective effects of hormone replacement therapy on cognition and dementia only during a "crucial period" led us to expand the study of hormonal influences to other members of the hypothalamic pituitary axis. Specifically, in this review, we focus on luteinizing hormone, which is not only increased in the sera of patients with Alzheimer disease but, like estrogen, is modulated by hormone replacement therapy and also influences cognitive behavior and pathogenic processing in animal models of the disease. Targeting gonadotropins may be a useful treatment strategy for disease targeting multiple pleiotropic downstream consequences.

BACKGROUND: ALZHEIMER'S DISEASE

Alzheimer's disease (AD), the primarily cause of senile dementia, is characterized by progressive memory loss, impairments in language and visual-spatial skills, episodes of psychosis, aggressiveness, and agitation, and ultimately death (reviewed in [1, 2]). AD is the most prevalent neurodegenerative disease, affecting approximately 4-5 million people in the United States and 15 million people worldwide. Given current population demographic predictions, it is estimated that by 2050, 50 million people will suffer from this devastating disease if no successful treatments are found [3]. The severity and the chronicity of this disease ultimately leads to institutionalization of patients, and thus results in a tremendous cost for the individual families and for society at large. Indeed, in the United States alone, the current cost of caring for patients with AD dementia is estimated at $100 billion per year and this will undoubtedly increase in coming years [4].

Unfortunately, to date, only palliative treatments of the symptoms are available and it is widely accepted that a better understanding of the etiology and disease pathogenesis is crucial for the development of new drugs capable of forestalling the progression of the disease. The leading hypothesis, the amyloid-β hypothesis, which is based on mutations in either the amyloid-β protein precursor (AβPP) or presenilins-1/2 (PSEN1/2) that affect the processing of amyloid-β and contribute to its accumulation in neurons and consequent formation of senile plaques [5], has come under increased scrutiny since manipulation of amyloid-β in cell or animal models does not yield the multitude of biochemical and cellular changes characteristic of the human disease. In fact, it is becoming increasingly evident that amyloid-β deposition may be a consequence rather than an initiator of the pathophysiological cascade [6–9]. Other mechanisms of disease, such as abnormally hyperphosphorylated bundles of tau protein found in neurofibrillary tangles [10], oxidativestress [11], metal ion deregulation [12],

and inflammation [13] also fail to completely explain all the abnormalities found in AD. Moreover, the lack of efficient therapeutic strategies based on such mechanisms only serves to emphasize the fundamental gap in our knowledge of disease.

CELL CYCLE DYSREGULATION: AN ALTERNATIVE HYPOTHESIS FOR ALZHEIMER'S DISEASE

There is increasing evidence for activated cell cycle in the vulnerable neuronal population in AD [14–16]. We suspect that the dysregulation of the cell cycle, in conjunction with oxidative stress, in hippocampal neurons leads to initiation of the pathophysiolological cascade of AD [17]. This hypothesis is supported by several neuronal changes seen in AD including the ectopic expression of markers of cell cycle [18], organelle kinesis [19], and cytoskeletal alterations such as tau phosphorylation [20]. Importantly, and suggestive of this pivotal effect, mitotic alterations are not only one of the earliest neuronal abnormalities found in AD but are also related to the majority of the pathological hallmarks, such as hyperphosphorylation of tau, amyloid-β accumulation, and oxidative stress (reviewed in [21]). To this end, a near identical phosphorylation of tau also occur when cells are mitotically active and phosphorylation is driven by cyclin-dependent kinases (CDKs) [22–25]. Therefore, one possibility is that cell cycle alterations could lead to tau phosphorylation and subsequent neuronal degeneration. In support of this hypothesis, several reports in the literature indicate that cell cycle markers are abnormally expressed in nerve cells with filamentous tau deposits. These markers include proteins cyclin D and Cdk4/Cdk6, involved in the G_0/G_1 transition, retinoblastoma protein, and the CDK inhibitors p15, p16, p18, and p19 [26–32]. Other markers such as cyclin E and Cdc25A, usually associated with G_1/S transition, have also been shown to be abnormally expressed in degenerating neurons [33–35]. Importantly, colocalization of different cell cycle markers with phosphorylated tau protein has also been demonstrated. In this regard, colocalization of cyclin B, Cdc2, Cdc25B, Polo-like kinase, Myt1/Wee1, and p27Kip1, all regulators of the G2/M transition, and some mitotic epitopes, such as phosphorylated histone H3, phosphorylated RNA polymerase II, PCNA, Ki67, and MPM2, has been demonstrated [27, 33, 34, 36–45]. Importantly some of these markers appear to precede the phosphorylation and aggregation of tau protein, suggesting a possible cause-and-effect relationship [37, 46, 47]. Moreover, these in vivo findings are supported by studies in cell models showing AD-like phosphorylation of tau protein in mitotically active cells [48–50] and also by the phosphorylation of recombinant tau by CDKs in vitro [51]. While cell cycle changes often precede tau phosphorylation, in a *Drosophila* model, cell cycle abnormalities appear to follow tau alterations [52]. Also, of relevance, experimental studies have established that inappropriate reentry into the cell cycle results in nerve cell death and reactivation of the cell-cycle machinery likely plays an important role in the apoptotic death of postmitotic neurons [53, 54]. Taken together, these findings indicate that cell cycle is intimately associated with AD and tau phosphorylation. However, as stated above, the chronology and mechanistic origin of tau phosphorylation remain to be clearly characterized.

There is also abundant evidence indicating that oxidative stress and free radical damage play key roles in the pathogenesis of AD [11, 55, 56]. Importantly free radicals, free-radical generators, and antioxidants act as crucial control parameters of the cell cycle [57] and have all been implicated in the development or halting of cell-cycle-related diseases such as cancer [58]. Therefore, one possibility is that oxidative stress and cell cycle dysregulation work synergistically in the development of AD [59]. In support of this notion, during the cell cycle, there is division and redistribution of cellular organelles such that mitochondrial proliferation is evident [60]. Mitochondrial proliferation is imperative for providing the energy needed for cell division, however, in cells where the cell cycle is interrupted or dysfunctional, cells incur a "phase stasis" to serve as a potent source of free radicals and cause redox imbalance [6], especially in those redox reactions involving calcium metabolism [61]. On the other hand, it is known that one pathway for oxidative stress mediated neuronal cell death is cell cycle reentry [62] and antioxidant treatments, most with potent cell-cycle inhibitory properties produce declines in tau phosphorylation [63].

Therapeutic interventions specifically designed to arrest cells at G_0 phase of the cell cycle, halt mitotic signalling cascades, or reduce the levels of endogenous or exogenous mitogens responsible for the aberrant mitosis in senescent neurons could have a tremendous success in AD treatment (reviewed in [21, 64]). Supporting this, nonsteroidal anti-inflammatory drugs (NSAIDs), which also possess antiproliferative properties, are useful to delay the progression of AD [65]. Likewise, antiapoptotic compounds such as resveratrol are well established in aging and AD. Resveratrol, a potent antioxidant of natural origin [66–69], may be of benefit in murine senescence and AD models and in some clinical studies in patients with AD [70]. Studies with animals also demonstrated protective effects of resveratrol against kainite-induced seizures [71] and its protective effects against brain injury due to ischemia/reperfusion in gerbil model [72]. Likewise, flavopiridol, a synthetic flavone closely related to a compound found in a plant native to India, *Dysoxylum binectariferum*, is a potent inhibitor of most CDKs, including CDK1, CDK2, CDK4, and CDK7 [73]. It induces growth arrest at either the G_1 and/or G_2 phases of the cell cycle in numerous cell lines in vitro by acting as a competitive binding agent for the ATP-binding pocket of CDK [73]. One consequence of this inhibition is a decrease in cyclin D1, the binding partner of CDK4 in G_1, by depletion of cyclin D1 mRNA resulting in a decrease in CDK4 kinase activity [74]. Importantly, the drug is in phase I and II clinical trials as an antineoplastic agent for breast, gastric, and renal cancers [75] and recent studies demonstrate its effectiveness on brain cancers such as gliomas [76]. These findings indicate that flavopiridol is a powerful CDK inhibitor as well as a potential therapeutic avenue for AD.

Notably, one powerful endogenous mitogen, luteinizing hormone (LH), a gonadotropin most often associated with reproduction, is particularly increased during aging and AD. Therefore another potential therapeutic option is to target age-related increases of this hormone in AD. The exploration of the link between gonadotropins such as LH and the etiology of AD and its potential value as a therapeutic avenue will be the focus of this review.

ARE SEX STEROIDS INVOLVED IN THE ETIOLOGY OF ALZHEIMER'S DISEASE?

Hormones of the hypothalamic-pituitary-gonadal (HPG) axis include gonadotropin releasing hormone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), estrogen, progesterone, testosterone, activin, inhibin, and follistatin. Each of these hormones is involved in regulating reproductive function by participating in a complex feedback loop. Briefly, this loop is initiated by the secretion of hypothalamic gonadotropin releasing hormone that stimulates the pituitary to secrete the gonadotropins LH and FSH. These gonadotropins are capable of stimulating oogenesis/spermatogenesis as well as the production of sex steroids which complete the feedback loop by reducing the gonadotropin secretion by the hypothalamus into the bloodstream [77].

Menopause and andropause are characterized by a dramatic decline in sex steroids resulting in an increase in the production of gonadotropins. To this end, in women, gonadotropins are considerably increased reaching a 3- to 4-fold increase in the concentration of serum LH and a 4- to 18-fold increase in FSH. Men also experience an increase of LH and FSH, but to a lower degree than those in women, resulting only in a 2-fold and 3-fold increase, respectively [78, 79]. Notably, the link between the HPG axis hormones and AD is not new as it has been hypothesized that the marked reduction in sex hormone levels during postmenopausal states results in various physiological and psychological changes associated with the development and progression of AD. In this regard, several epidemiological studies indicate that women have a higher predisposition to develop AD than do men [80–82] and treatment with hormone replacement therapy (HRT) reduced this risk in women [83, 84]. These gender differences, in addition to the capacity of HRT to reduce this risk in postmenopausal women, led researchers to investigate the role of female sex steroids, namely, estrogen, in the pathogenesis of AD.

To this end, estrogen can act as a neuroprotective agent by lowering the brain levels of amyloid-β [85], by ameliorating the nerve cell injury caused by amyloid-β [86], and/or promoting synaptic plasticity and growth of nerve processes [87]. Moreover, estrogen is also capable of reducing oxidative stress, increasing cerebral blood flow, and enhancing cholinergic function and glucose transport into the brain [88]. All of these effects have a well-known positive impact on the prevention and the amelioration of AD. However, recent prospective studies, including the Women's Health Initiative Memory Study (WHIMS), seem to contradict the previous promising observations regarding HRT. WHIMS, a randomized clinical trial designed to assess the incidence of dementia among relatively healthy postmenopausal women under HRT, showed a substantially increased overall incidence of dementia in postmenopausal women [89–91]. Since hormone therapy is relatively common for menopausal women, these latter findings have raised serious concerns about the long term efficacy and safety of HRT.

HORMONE REPLACEMENT: TIMING IS EVERYTHING

Many hypotheses have been postulated to justify the results of the WHIMS. To date, some aspects related to the form (estradiol versus conjugated equine estrogen (CEE)) and the route of administration (oral versus transdermal) of estrogen, the choice of progestin (natural versus synthetic progestins), the high doses administered, the type of treatment regimen (continuous versus cyclic) might be important points to be considered (reviewed in [92, 93]). For instance, the adverse effects on cognition are mainly attributed to the thromboembolic complications of oral CEE [94]. However, one aspect that has been overlooked and that is tightly linked to the timing of hormone therapy (ie, perimenopausal versus postmenopausal) is the release of, and capacity of, HRT to lower gonadotropins such as LH. In fact, it is only when one takes into account the role of these other hormones of the hypothalamic-pituitary-gonadal axis (reviewed in [77]), during a "critical period" around the onset of menopause and the years beyond that cognitive decline and susceptibility, onset, and progression of AD can be accurately characterized. To this end, chronic elevation of gonadotropins and decline in sex steroids leads to HPG axis shutdown. Therefore, HRT started in older women such as those of the WHIMS [89], while bringing estrogen to premenopausal levels, cannot decrease the levels of gonadotropins such as LH. On the other hand, HRT started during peri-menopause or early menopause, when the HPG axis feedback loop system is functional, does lead to a lowering of LH. Supporting this hypothesis, the levels of gonadotropins including LH are highest during peri-menopause and early menopause [95], when HRT has been observed to be most successful in preventing dementia [96, 97]. Likewise, studies also demonstrate that while cognitive decline can be rescued with estrogen therapy initiated immediately after menopause and ovariectomy (mimics menopause), however, unless subjects are previously primed with estrogen [98], estrogen replacement initiated after a long interval following menopause or ovariectomy is ineffective at rescuing cognition [99–101]. This later finding suggests that by priming, HPG-axis functionality is sustained and thus led to cognitive improvements after HRT. Likewise, estrogen becomes increasingly less effective at modulating LH expression and biosynthesis the longer that HRT is started after ovariectomy [102], a mechanism that is specifically mediated by the gonadotropin-releasing hormone (GnRH) receptor [103, 104]. Importantly, the ovariectomy findings parallel those observed during aging, such that estrogen feedback on LH secretion [105] and GnRH gene expression [106] is decreased. Whether

the beneficial AND detrimental effects of HRT are associated with menopause-driven gonadotropin changes is not yet fully known and is currently being examined in our laboratory. However, the above-cited evidence does indicate that timing, pituitary function, and estrogen-gonadotropin influences are more complex than previously thought. These findings may provide the reconciliating link between the contradicting data presented in the WHIMS and prior observational/epidemiological studies. Moreover, these data suggest a potential role for gonadotropins in the CNS, particularly on cognitive decline and AD pathogenesis and, more importantly, places gonadotropins as a potential therapeutic target for the treatment of AD.

EVIDENCE FOR A ROLE OF LH IN ALZHEIMER'S DISEASE

Epidemiological data supports a role of LH in AD. In this regard, and paralleling the female predominance for developing AD [81, 82, 107, 108], LH levels are significantly higher in females as compared to males [97] and LH levels are higher still in individuals who succumb to AD [109, 110]. Also important is the fact that, in Down syndrome, where the prevalence of AD-like etiology is higher in males than in females, that is, a reversal to what is observed in the normal population, males have higher serum LH levels compared to females [111, 112]. Therefore, LH allows an explanation for the reversing of the classical gender-predisposition in AD versus Down syndrome [113].

Like epidemiological data, direct experimental evidence also indicates that LH may be an important player in the development and progression of AD. In this regard, LH is capable of modulating cognitive behavior [114], is present in the brain, and has the highest levels of its receptor in the hippocampus [115], a key processor of cognition affected by aging and severely deteriorated in AD. Furthermore, we have recently examined cognitive performance in a well-characterized transgenic line that overexpresses LH [116–118] and have found that these animals show decreased cognitive performance when compared to controls [119]. Since other hormones in addition to LH are altered in the LH overexpressing mice, we also measured Y-maze performance in a well-characterized LH receptor knockout (LHRKO) strain of mice [120], which also have very high levels of LH, to begin to determine whether cognitive decline could be mediated by a specific LH mechanism (ie, the LH receptor). In this regard, LHRKO (−/−) mice performed indistinguishable from wild-type (+/+) mice. Therefore, the negative effects on cognition affected by high levels of LH were completely attenuated by knockout of the receptor. While comparing the TgLH-β and LHRKO animals should be done with caution (ie, different strain and background), changes in estrogen levels were unlikely to be responsible for the cognitive changes observed in this study since LH overexpressers show elevated rather than diminished levels of estrogen [116–118] and LHRKO mice show decreased levels of estrogen when compared to wild-type littermate controls [120]. On the other hand, both do show high LH but this is obviously a nonissue in the LHRKO animals. These findings support our hypothesis that modulation of cognition by estrogen is interrelated with the status of LH levels and function. Finally, recently we also found that experimentally abolishing LH in the AβPP transgenic mouse, an animal model of AD, using a selective GnRH agonist (leuprolide acetate) that has been shown to reduce LH to undetectable levels by downregulating the pituitary gonadotropin-releasing hormone receptors [121, 122], improved hippocampally related cognitive performance and decreased amyloid-β deposition in these mice when compared to aged-matched controls [123]. These findings, together with data indicating that LH modulates AβPP processing in vivo and in vitro [122], suggest that LH may be a key player in this disease.

Mechanistically, and as alluded to in the previous section, LH could be working via the modulation of cell cycle. LH is known to be a potent mitogen [124, 125] by acting through MAP kinases pathway [126]. In this regard, LH activates ERK [127] and other transcription factors [128] all involved in cell cycle [129], thus suggesting that high levels of this hormone could lead to the aberrant cell cycle reentry of neurons observed in AD.

CAN TARGETING LH BE THE NEW THERAPEUTIC AVENUE?

Findings discussed in this review indicate that targeting the release of LH may indeed be a successful strategy to prevent and forestall the progression of AD. As discussed above, preclinical data using leuprolide acetate leads to modulation of AβPP processing in normal mice [122] and cognitive improvement and decreased amyloid-β burden in AβPP transgenic mice [123]. More importantly, a recently completed phase II clinical trial shows stabilization in cognitive decline in a subgroup of AD patients treated with leuprolide acetate (http://clinicaltrials.gov/ct/show/nct00076440?orden=6). Specifically, female AD patients treated with high doses of leuprolide acetate (http://www.secinfo.com/d14D5a.z6483.htp, pages 56–64) showed stabilization in cognitive function and activities of daily living. These promising findings support the importance of LH in AD and give way for an alternative and much needed therapeutic avenue for this insidious disease.

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Review Article

The TGF-β1/Upstream Stimulatory Factor-Regulated PAI-1 Gene: Potential Involvement and a Therapeutic Target in Alzheimer's Disease

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Amyloid peptide (Aβ) aggregates, derived from initial β-site proteolytic processing of the amyloid precursor protein (APP), accumulate in the brains of Alzheimer's disease patients. The plasmin-generating cascade appears to serve a protective role in the central nervous system since plasmin-mediated proteolysis of APP utilizes the α site, eventually generating nontoxic peptides, and plasmin also degrades Aβ. The conversion of plasminogen to plasmin by tissue-type plasminogen activator in the brain is negatively regulated by plasminogen activator inhibitor type-1 (PAI-1) resulting in attenuation of plasmin-dependent substrate degradation with resultant accumulation of Aβ. PAI-1 and its major physiological inducer TGF-β1, moreover, are increased in models of Alzheimer's disease and have been implicated in the etiology and progression of human neurodegenerative disorders. This review highlights the potential role of PAI-1 and TGF-β1 in this process. Current molecular events associated with TGF-β1-induced PAI-1 transcription are presented with particular relevance to potential targeting of PAI-1 gene expression as a molecular approach to the therapy of neurodegenerative diseases associated with increased PAI-1 expression such as Alzheimer's disease.

INTRODUCTION

In patients with Alzheimer's disease (AD), plaques comprised of aggregated β-amyloid peptides (Aβ) accumulate in specific areas of the brain as a consequence of the proteolytic processing of the single-pass transmembrane amyloid precursor protein (APP) [1]. These Aβ deposits trigger prolonged inflammation, neuronal death, and progressive cognitive decline [2]. Aβ peptides are derived from APP by β-site cleavage by an aspartic protease (BACE) producing a membrane-bound COOH-terminal C99 fragment followed by a complex proteolytic event (involving presenilin and nicastrin) at the C99 transmembrane-localized γ position [3–5]. An alternative APP processing pathway also exists in which membrane-proximal (α-site) cleavage by matrix metalloproteinases (TACE, ADAM 10) replaces β position utilization producing a membrane-anchored C83 fragment. Subsequent γ-site processing of the C83 product results in generation of the nontoxic p3 peptide [3, 6].

The broad-spectrum protease plasmin also degrades Aβ [7–9] and activation of plasmin decreases Aβ peptide levels [10]. Plasmin-mediated proteolysis of APP, moreover, appears to involve the α site (either as a direct or indirect target) resulting in decreased Aβ production, thus suggesting a protective role for the plasmin cascade in the central nervous system. Indeed, plasmin levels in the brains of AD patients are considerably reduced [10] further supporting a causal relationship between deficient activity of the plasmin-generating proteolytic system and accumulation of Aβ in the progression of AD.

PLASMIN-ACTIVATING SYSTEM IN ALZHEIMER'S DISEASE

Several members of the serine protease inhibitor (SERPIN) superfamily exhibit neurotrophic, neuroprotective, or neuropathophysiologic activities depending on the specific cell type and pathways involved [11]. These include SERPINF1, SERPINI1 (neuroserpin), SERPINE1 (plasminogen activator inhibitor type-1; PAI-1), SERPINE2 (nexin-1), and SERPINA3 [11]. PAI-1, in particular, has multifunctional roles in the central nervous system as it both maintains neuronal cellular structure and initiates signaling through the ERK pathway [12]. PAI-1 directly influences the plasmin-dependent pericellular proteolytic cascade by regulating the conversion of plasminogen to plasmin by urokinase- and tissue-type plasminogen activators (uPA/tPA) (Figure 1).

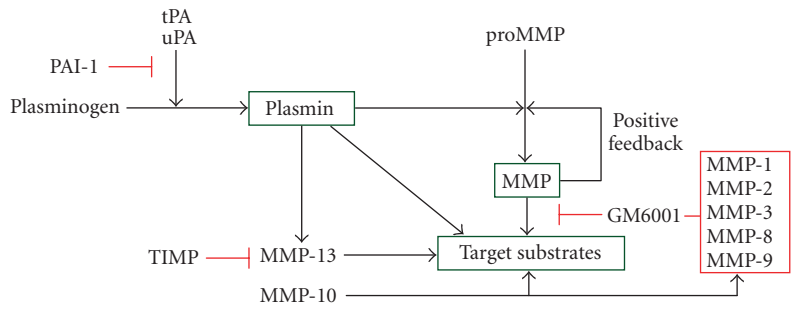

Figure 1: tPA and uPA convert plasminogen to the active, broad-spectrum, protease plasmin both at the cell surface and in the immediate pericellular space. Plasmin, in turn, degrades target substrates (eg, APP, *Aβ*) directly as well as indirectly through downstream activation of matrix metalloproteinases (MMPs). Inhibition of MMP activity (GM6001, TIMP) has confirmed their participation in plasmin-initiated proteolysis. Most importantly, this cascade is effectively attenuated by overexpression (or exogenous addition) or PAI-1 which blocks tPA and uPA catalysis inhibiting, thereby, plasmin generation.

PAI-1 immunoreactivity in the central nervous system of AD patients was associated with senile plaques and ghost tangle structures [13] consistent with the earlier colocalization of PA and PAI-1 in plaque structures [14] which are sites of sustained inflammation [15]. Recent findings in Tg2576 and TgCRN8 transgenic mice, engineered to express brain-targeted Swedish mutant Aβ and the double Swedish/V717F mutant Aβ, respectively, under control of the hamster prion promoter and exhibit age-dependent Aβ plaque development (at 12 and 3 months, resp) as well as cognitive deficiencies [16], established that tPA activity was significantly decreased compared to controls [17]. This decline correlated with corresponding increases in PAI-1 expression specifically in areas of the brain where tPA activity was reduced (hippocampus, amygdala). Since direct Aβ peptide injection increased PAI-1 expression and whereas Aβ removal from the hippocampal region required both tPA and plasminogen, it appears that a functional tPA-plasmin axis is required for Aβ clearance [17]. While PAI-1 may be neuroprotective in specific acute injury settings (eg, tPA-triggered neuronal apoptosis) [18], chronically elevated PAI-1 levels likely promote Aβ accumulation by inhibiting plasmin-dependent degradation (Figure 1).

CONTROLS ON TGF-β1 TARGET GENES: THE PAI-1 MODEL

Several reports described elevated TGF-β1 levels in brain biopsies from patients with Parkinson's disease, AD, and stroke [20–22]. This growth factor is likely to influence the onset and progression of AD at several levels. Increased brain expression of TGF-β1 correlates with Aβ angiopathy, and transgenic mice that overexpress TGF-β1 in astrocytes exhibit early onset Aβ deposition [23]. TGF-β1, moreover, induces astrocyte APP expression through a TGF-β1-responsive AGAC Smad-binding element in the APP promoter; subsequent Aβ production, moreover, was enhanced by TGF-β1 signaling [24]. Since the PAI-1 gene is also transcriptionally upregulated by TGF-β1 [19, 25], the coordinate overexpression of PAI-1 and increased Aβ generation in response to elevated TGF-β1 in the brains of AD patients may dispose to disease progression [26]. Collectively, these findings raise the possibility that targeting TGF-β1-inducible genes (eg, PAI-1, APP) may have therapeutic benefit in the setting of AD.

The regulation of TGF-β1-activated genes (ie, PAI-1) is largely transcriptional [19, 25, 27, 28] with the PAI-1 gene subject to complex combinatorial expression controls involving the major transcription effectors p53, Sp1, and members of the MYC family [19, 29, 30]. Prominent TGF-β-response elements in the human PAI-1 promoter include the hexanucleotide E box motif (5′-CACGTG-3′; as in the PE1, PE2, HRE-2 sites) and closely related sequences recognized by the basic helix-loop-helix/leucine zipper (bHLH-LZ) transcription factors of the MYC family (eg, MYC, MAX, TFE3, USF-1, and USF-2) [31–36]. This E box element likely functions, therefore, as a "platform" for recruitment of both positive and negative regulators of PAI-1 expression [37–39]. Recent UV crosslinking and tethered DNA affinity chromatographic analyses identified the bHLH-LZ protein upstream stimulatory factor-1 (USF-1) as a major PAI-1 E box-recognition factor [40]. Specific E box mutations that ablate USF-1 binding to a PAI-1 target deoxyoligonucleotide probe (CA → TC) effectively attenuated TGF-β1-stimulated PAI-1 promoter-driven CAT reporter activity [36]. The human PAI-1 promoter, however, harbors several additional TGF-β-responsive elements, including three E box-adjoining Smad sequences located just 5′ of the PE2 site [32, 33, 35, 41]. Since an engineered two-base-pair mutation CA**CG**TG → CA**AT**TG in a serum-responsive PAI-1 E box attenuated growth state as well as TGF-β1-dependent transcription [36], this same dinucleotide substitution was incorporated into a luciferase reporter construct bearing the immediate 806 base pairs of the human PAI-1 5′ upstream region. Initial truncation approaches did, in fact, confirm that a major (albeit not the only) TGF-β1-responsive element resided within the most proximal 606 base pairs of the human PAI-1 promoter [19]. Specific disruption of the PE2 region E box element by site-directed mutagenesis significantly attenuated TGF-β1-mediated PAI-1 transcription (Figure 2)

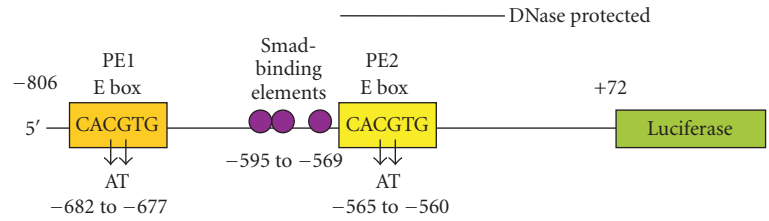

Figure 2: Topography of the PAI-1p806-Luc reporter construct illustrating the two (PE1 and PE2) E box sequences. Site-directed mutagenesis and luciferase assays clearly indicated that an intact E box at the PE2 site is required for maximal TGF-β1-induced PAI-1 transcription in human epithelial cells [19].

[19]. The consequences of USF binding to the PAI-1 PE2 E box site may be more complex, however, than simple motif occupancy. Indeed, certain E box-recognition factors including USF-1 and USF-2 effectively induce DNA bending. Cooperative interactions between Sp1 and/or p53 with USF proteins, for example, may be dependent on USF-initiated modifications to DNA conformation allowing distally spaced factors important in expression control to interact with resulting effects on PAI-1 transcription [42].

SEQUENCE REQUIREMENTS FOR USF OCCUPANCY OF THE PE2 REGION E BOX MOTIF

Chromatin immunoprecipitation recently confirmed that the PAI-1 PE2 E box site is an *in vivo* USF target motif [38]. Since an intact consensus PE2 region E box sequence is necessary for a maximal transcriptional response to growth factors [19], it was important to identify any additional requirements for PE2 E box-occupancy that might influence site residence including the Smad-binding AGAC elements implicated in TGF-β1-dependent APP expression [24]. PE1 and PE2 probes recognition appeared dependent solely on an intact 5′-CACGTG-3′ motif since nuclear factor binding to individual PE1 and PE2 target constructs was successfully blocked by short double-stranded deoxyoligonucleotides containing a consensus E box flanked by non-PAI-1 sequences whereas a mutant E box (5′-CAATTG-3′) "bait" failed to compete [19]. It was important, however, to confirm these results using site-specific mutants within the context of native PAI-1 promoter sequences (eg, the PE2 region backbone) in order to assess the potential contributions of the Smad-binding elements, E box flanking nucleotides (such as the AAT trinucleotide "spacer" between the PE2 E box and the first upstream Smad site), and the CACGTG motif to nuclear protein binding (Figure 2). A recent study established that the major protein/DNA interactions in the PE2 segment were, in fact, E box-dependent and did not require accessory sites since mutation of all three Smad-binding sites (AGAC → CTTG) or removal of the ATT spacer did not affect USF occupancy of the PE2 region E box [19]. While the CACGTG "core" is a target for occupancy by at least seven members of the bHLH-LZ transcription factor family (USF-1, USF-2, c-MYC, MAX, TFE3, TFEB, TFII-I), USF proteins have a preference for C or T at the −4 position in the presence of $MgCl_2$ [43]. Indeed, the human PAI-1 gene has a T at the −4 site of the PE2 region E box as well as a purine at +4 and −5 and a pyrimidine at +5 ($A_{-5}T_{-4}C_{-3}A_{-2}C_{-1}G_{+1}T_{+2}G_{+3}G_{+4}C_{+5}$), all of which facilitate USF binding [43]. Chromatin immunoprecipitation of the PE2 region E box site in the human PAI-1 gene, moreover, indicated a dynamic occupancy by USF subtypes (USF-1 versus USF-2) as a function of growth state [44]. This motif was clearly a platform for USF-1 binding in quiescent cells. An exchange of PE2 E box USF-1 homodimers with USF-2 homo- or USF-1/USF-2 heterodimers, furthermore, closely correlated with PAI-1 gene activation. This switch may well determine the transcriptional status of the PAI-1 gene in quiescent versus growth factor-stimulated culture conditions [38, 45]. Site occupancy and transcriptional activity additionally require conservation of the PE2 core E box structure as the CACGTG → CACGGA and TCCGTG dinucleotide substitutions (in the rat gene) [36] and a CACGTG → CAATTG or TCCGTG replacement (in the human gene), with retention of PAI-1 flanking sequences, resulted in loss of both competitive binding and growth factor-dependent reporter activity [19, 44]. The CACGTG → TCCGTG mutation is particularly relevant since bHLH proteins with E box-recognition activity have a conserved glutamate important for interaction with the first two nucleotides (CA) in the E box motif [46]. These data are also consistent with the known hexanucleotide preference (CACGTG or CACATG) of USF proteins [39, 47, 48]. To further dissect the role of USF in TGFβ1-mediated PAI-1 transcription *in vivo*, a dominant-negative USF construct (A-USF) was implemented for molecular genetic targeting [19]. A-USF effectively titers away functional USF proteins by forming highly stable interactions with native USF proteins; such USF/A-USF heterodimers, however, are unable to bind DNA due to replacement (in the A-USF construct) of the basic DNA-binding residues with an acidic domain [49]. A-USF transfection effectively attenuated TGF-β1-induced PAI-1 expression establishing the importance of USF family transcription factors in PAI-1 gene control [19, 50, 51].

MAPPING THE TGF-β1-INDUCED PAI-1 SIGNALING AND TRANSCRIPTIONAL CONTROL NETWORKS: OPPORTUNITIES FOR THERAPEUTIC INTERVENTION

The molecular mechanisms associated with the TGF-β1-initiated E box-dependent PAI-1 gene control and the collateral Smad-mediated APP induction/TGF-β-directed Aβ

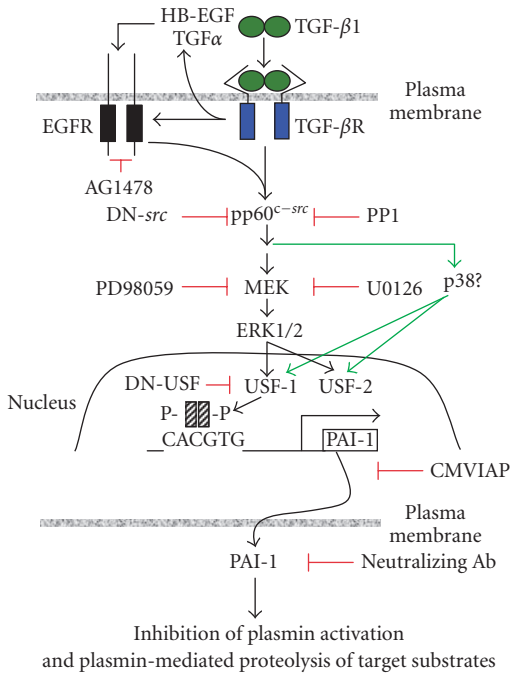

FIGURE 3: Pathways involved in the regulation of PAI-1 expression and function in response to TGF-β1 stimulation. Positve influences are depicted in black arrows; effective inhibitors defined pharmacologically or by use of dominant-negative constructs (DN) are highlighted in red (detailed in [50]). CMVIAP = PAI-1 antisense expression vector.

processing in specific central nervous system cell types remain to be clarified. The available data, however, clearly suggest that these two responses to TGF-β1 are linked in the pathophysiology of human neurodegenerative disease. It has become apparent, moreover, that PAI-1 overexpression is a likely major contributory if not a causative event in AD progression. Our current understanding of the pathways utilized by the TGF-β1 to stimulate the PAI-1 transcription (*summarized in Figure 3*) indicate that this growth factor activates a kinase cascade, at least partially as a function of epidermal growth factor receptor mobilization (either through the release of the appropriate ligands or the direct receptor transactivation), involving MEK, ERK1/2, and perhaps p38 [49–51]. Pharmacological approaches, use of dominant-negative constructs, and kinase assays suggest that *src* family kinases and *ras* GTPase are upstream of MEK-ERK-p38 in this model of induced PAI-1 expression [50, 51]. MAP kinases, in turn, interact with nuclear transcription factors including members of the USF family that, once phosphorylated, bind as dimers to E box motifs in the PAI-1 promoter to modulate gene expression [19, 26, 38, 40, 50, 51]. Genetic perturbation of PAI-1 synthesis in specific areas of the brain (with dominant-negative USF or PAI-1 antisense vectors) or delivery of PAI-1 neutralizing antibodies may effectively stimulate uPA- and/or plasmin-dependent target substrate degradation (eg, Aβ) or at least attenuate the rate of Aβ accumulation (Figures 1 and 3). The continued identification of regulatory points in the PAI-1 expression control network (Figure 3) and recent identification of TGF-β1-response sites (as well as the involved nuclear factors) in the APP and PAI-1 promoters [19, 38, 40, 52] may provide new molecular targets for the therapy of neurodegenerative syndromes associated with PAI-1 upregulation. Indeed, specific SERPINS (including PAI-1) have already been suggested as potential novel therapeutic targets for stroke and cerebral ischemia [12]. The TGF-β1 gene, furthermore, is also USF-regulated [53] suggesting that interference with USF-dependent transcriptional events may have widespread therapeutic implications.

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Review Article

The Creatine Kinase/Creatine Connection to Alzheimer's Disease: CK Inactivation, APP-CK Complexes, and Focal Creatine Deposits

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Cytosolic brain-type creatine kinase (BB-CK), which is coexpressed with ubiquitous mitochondrial uMtCK, is significantly inactivated by oxidation in Alzheimer's disease (AD) patients. Since CK has been shown to play a fundamental role in cellular energetics of the brain, any disturbance of this enzyme may exasperate the AD disease process. Mutations in amyloid precursor protein (APP) are associated with early onset AD and result in abnormal processing of APP, and accumulation of Aβ peptide, the main constituent of amyloid plaques in AD brain. Recent data on a direct interaction between APP and the precursor of uMtCK support an emerging relationship between AD, cellular energy levels, and mitochondrial function. In addition, recently discovered creatine (Cr) deposits in the brain of transgenic AD mice, as well as in the hippocampus from AD patients, indicate a direct link between perturbed energy state, Cr metabolism, and AD. Here, we review the roles of Cr and Cr-related enzymes and consider the potential value of supplementation with Cr, a potent neuroprotective substance. As a hypothesis, we consider whether Cr, if given at an early time point of the disease, may prevent or delay the course of AD-related neurodegeneration.

FUNCTION AND SUBCELLULAR LOCALIZATION OF THE CREATINE KINASE ISOENZYME FAMILY MEMBERS IN BRAIN

Large amounts of energy are required to maintain the signalling activities of the cells in the central nervous system (CNS). The dominant share of energy consumption in the brain can be assigned to brain function-related processes, for example, for maintenance of membrane potential by the Na^+/K^+-ATPase, Ca^{2+} homeostasis by the Ca^{2+}-ATPase, neurotransmitter processing, intracellular signalling, and axonal as well as dendritic transport [1]. Mechanisms to facilitate energy transfer within cells that require fluctuating high energy levels, such as those in skeletal muscle, heart, and brain, include the juxtaposition of intracellular sites of ATP generation with sites of ATP consumption, as well as the transfer of high-energy phosphates between these sites by the creatine kinase (CK)/phosphocreatine (PCr) system [1, 2].

CK is categorized into four isoforms based on its tissue expression (muscle or brain) and subcellular distribution (cytosolic or mitochondrial). In sarcomeric muscle, dimeric cytosolic muscle-type CK (MM-CK) is localized to the M-band [3], the sarcoplasmic reticulum (SR) [4, 5], and the plasma membrane. At these sites, MM-CK is functionally coupled to the myofibrillar acto-myosin ATPase [6–8], the SR Ca^{2+}-ATPase [4, 5], and the plasma membrane Na^+/K^+ ATPase [9], respectively, and utilizes PCr for local in situ regeneration of ATP. In the brain, the dimeric cytosolic form of CK is called brain-type CK (BB-CK). The octameric mitochondrial CK (MtCK) is classified into two forms: sarcomeric muscle form (sMtCK) and brain form called ubiquitous MtCK (uMtCK) [10, 11]. Both MtCKs are located in the mitochondrial intermembrane space [12], along the entire inner membrane and also at peripheral contact sites [13], where inner and outer membranes are in close proximity [14, 15]. There, MtCK can directly transphosphorylate

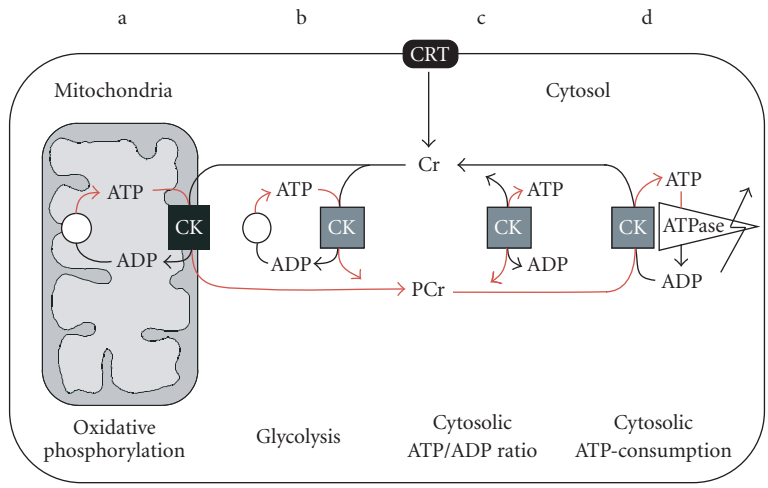

Figure 1: The CK/PCr system.

intramitochondrially produced ATP into PCr [16], which is then exported into the cytosol.

uMtCK is always coexpressed with dimeric cytosolic BB-CK [17] at various levels throughout the entire brain. In cerebellum, both of these CK isoforms are found highly concentrated in the glomeruli structures of the cerebellar granular layer. However, the level of BB-CK is much higher than uMtCK in cerebellar Bergmann glial cells. In addition, both isoforms are highly expressed in the choroid plexus and in hippocampal granule and pyramidal cells. The hippocampus is important for learning and memory and is most severely affected in AD [18].

Generation of ATP, hence CK activity, is critical for CNS function. Neurons require a great amount of ATP to maintain membrane polarization, Ca^{2+} influx from organelles, processing of neurotransmitters, intracellular signalling systems, and axonal and dendritic transport [1]. Interestingly, CK is specifically associated with these important processes. On a subcellular level, BB-CK has been found in association with synaptic vesicles [19] and synaptic plasma membranes [20]. On the other hand, supporting glial cells also require ATP for neurotransmitter uptake. In the rat hypothalamus, BB-CK plays an essential role in regenerating ATP for glutamate clearance during excitatory synaptic transmission [21]. Therefore, the number of synapses and synaptic plasticity can be profoundly regulated by ATP levels in neuronal and non-neuronal cells [22].

During brain development, there is a coincidence in the timing of maximal expression of BB-CK and myelin basic protein in the cerebellum which is an indication for a role of BB-CK in myelination [23]. Both BB-CK and uMtCK levels are increased in a coordinated fashion during postnatal brain development [24]. In brain, CK has been shown to be associated with synaptic membranes [25] and to facilitate glutamate uptake into vesicles [26], thus being directly involved in the energetics of neurotransmitter uptake. CK has also been shown to be associated with acetylcholine receptor-rich membranes [27] and to be involved in quantal release of acetylcholine in synaptosomes [28]. Further, CK together with enolase are part of a complex which is involved in axonal transport [29] and thus support the energetics of these transport events. CK has also been shown to be functionally coupled to the Na^+-K^+ ATPase [9, 30], as well as to the ATP-gated K^+-channel [31, 32]. This seems to be important due to the fact that about 50% of total brain energy is used by the Na^+ ion pump [1]. In addition, CK knock-out mice display a significant neurological phenotype [33, 34]. Based on these findings, a functional CK/PCr energy shuttle system has been proposed [35], where BB-CK and uMtCK would constitute an efficient energy buffering and shuttle system in brain [36], similar to that observed in muscle.

THE CREATINE KINASE/PHOSPHOCREATINE SHUTTLE SYSTEM IN THE BRAIN

As mentioned above, the major energy-consuming process in neural cells is the transport of ions by the Na^+/K^+-ATPase [37]. Even though the cellular pools of ATP are rather small and the movement of ATP within cells by diffusion is slow [1], no significant change in overall ATP levels can be detected during activation of excitable tissues [38]. This is because ATP is continuously and efficiently replenished from the large pools of PCr through the CK reaction, as has been shown in detail in muscle cells [39–42]. The CK isoenzymes catalyze the reversible transfer of the high-energy N-phosphoryl group of phosphocreatine (PCr) to ADP to yield ATP. The concept of the creatine kinase/phosphocreatine (CK/PCr) shuttle system (Figure 1) describes the functional association of CK isoenzymes with discrete intracellular compartments at sites of ATP production and utilization. Thus, PCr and Cr serve as cytosolic energy transducers to connect these intracellular sites and together with precisely localized CK isoenzymes constitute an organizational feature that increases the efficiency of energy metabolism [1, 39, 41].

The CK/PCr system functions as a temporal and spatial energy buffer, as well as a regulator of cellular energetics [39, 42, 43]. It maintains high global ATP/ADP ratios by preventing a rise in intracellular free ADP and thus preserves the thermodynamic efficiency of ATPases even at high cellular ATP turnover [36]. By this mechanism, an inactivation of cellular ATPases by rising [ADP] is avoided and a net loss of adenine nucleotides is prevented [39, 44, 45]. Thus, the CK/PCr system is a rapidly available source for ATP transport and resynthesis not only in muscle but also in the brain. The high activity of CK in the brain, together with high concentrations of its substrates, PCr and Cr, as well as the phenotype of mice deficient in brain-type CK isoforms [34, 35] and the effects of Cr supplementation on brain function (see below) strongly indicate that CK is a key enzyme in brain energy metabolism [46] and that PCr is an important energy reservoir and energy transport molecule [47]. A schematic drawing of the subcellular micro-compartmentation of CK enzymes and their colocalization with ATP-producing and -consuming sites within the cell is depicted in Figure 1 (from Schlattner U and Wallimann T. Metabolite channeling: creatine kinase micro compartments, to (Lennarz WJ, and Lane MD, eds.) *Encyclopedia of Biological Chemistry.* Vol 2. New York, USA: Academic Press; (2004):646–651; with permission by ELSEVIER Publishing Company).

Isoenzymes of CK are found in different compartments such as mitochondria (a) and the cytosol (b)–(d) in a soluble form (c) or associated to a different degree to ATP-delivering processes, for example, mitochondrial oxidative phosphorylation (a) or glycolysis (b) or to ATP-consuming processes, like ATPases or other ATP-requiring or ATP-regulated processes (d). A large cytosolic PCr pool up to 30 mM is built up by CK using ATP generated by oxidative phosphorylation (a) or glycolysis (b). PCr is then used to buffer global (c) and local (d) ATP/ADP ratios. In cells that are polarized and/or have a very high or localized ATP consumption, these CK isoenzymes, together with easily diffusible PCr, also maintain an energy shuttle between ATP-providing and ATP-consuming processes (a), (d). Metabolite channeling occurs where CK is associated with ATP-providing or ATP-consuming transporters, ion pumps, or enzymes that are operating also in brain (a), (d). Cr is synthesized mostly in kidney and liver. Cells can take up Cr from the blood stream by a specific creatine transporter CRT. In brain, CRT is prominently localized at the blood-brain barrier, but is also seen on the plasma membrane of neurons [48–51].

Details on the importance of CK and its substrates for brain function are revealed by recent studies on the neurological and behavior phenotype of CK knockout mice [33, 34]. Mice lacking the expression of one CK isoform, cytosolic BB-CK or uMtCK, display abnormalities in formation and maintenance of hippocampal mossy fibre connections and in behaviors such as habituation, spatial learning, and seizure susceptibility [34]. On the other hand, adult mice lacking both BB-CK and uMtCK, the so-called CK double knockout mice, display reduced body weight and are severely impaired in spatial learning in both dry and wet maze, and display lower nest building activity and acoustic startle reflex responses [34]. Morphological analysis of CK double knockout brains revealed a reduction of brain weight and hippocampal size, a smaller regio-inferior area, and relatively larger supra-pyramidal and intra-infra-pyramidal mossy fiber area [34]. These results suggest that the lack of both brain-specific CK isoforms renders the synaptic circuitry less efficient in coping with sensory or cognitive activity related challenges in the adult brain and fully support the physiological importance of CK for normal brain function.

CREATINE SYNTHESIS AND UPTAKE IN BRAIN

In vertebrates, Cr is synthesized mostly in the liver and kidney and is then transported through the blood and taken up by target tissues with high energy demands. Cr biosynthesis involves two sequential steps catalyzed by L-arginine: glycine amidinotransferase (AGAT) and S-adenosylmethionine:guanidinoacetate N-methyltransferase (GAMT) [51]. It has been shown that a certain amount of Cr is synthesized endogenously in the developing brain [51–53], and recently both AGAT and GAMT, as well as creatine transporter (CRT) have been identified and localized in distinct cell populations of the developing brain [51, 52]. GAMT immunoreactivity is very strong in oligodendrocytes, moderate in astrocytes, and not detected in embryonic neurons. These observations led to the conclusion that Cr in neurons is derived in part from local glial populations surrounding the neurons, indicating a novel neuron-glial relationship involving Cr trafficking [49]. However, the majority of Cr seems to get taken up continuously through the blood-brain barrier by CRT [48], which works against a huge Cr gradient [54]. Nevertheless, certain brain cells seem to have the capacity for endogenous Cr biosynthesis, especially in the developing brain [51]. Patients with genetic CRT-deficiency lack any detectable Cr in the brain [55] and have severe neurological phenotypes including hypotonia, developmental speech delay, autism, and brain atrophy [55, 56]. These cases emphasize the importance of the substrates of CK, Cr, and PCr, for normal brain function in man.

DISTURBED ENERGY METABOLISM IN NEURODEGENERATIVE DISEASES

A common feature of severe neurodegenerative disorders, such as Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and AD are mutations in nuclear or mitochondrial DNA. This leads to secondary mitochondrial dysfunction accompanied by a more or less severely disturbed energy metabolism as well as a disturbed energetic status of the brain [57]. Cellular energy reserves are important for normal brain function, however, the cellular energy state also appears to play a key role in regulating and initiating apoptosis or necrosis of brain cells, since mitochondria are known to be essential in controlling specific apoptotic pathways [58]. For example, in patients with ALS, a chronically deficient intake of energy [59], increased mitochondrial volume (swelling), oxidative damage, and decreased complex I activity have been observed [60]. Further,

a loss of mitochondrial membrane potential and chronically elevated cytosolic Ca^{2+}-levels accompany these observations [61].

One of the most important hallmarks in the pathogenesis of senile dementia of the Alzheimer type (AD) is the marked decrease of cerebral glucose metabolism [62] caused by disturbed acetyl-CoA synthesis and critically lowered oxidative phosphorylation [63]. Measurement of local cerebral glucose metabolism by positron emission tomography (PET) has become a standard technique to study dementia [64]. By this method, a regional impairment of cerebral glucose metabolism in neocortical association areas in the brains of AD patients could be shown [64]. In addition, cortical acetylcholine esterase activity is significantly lower in patients with AD compared to age-matched normal controls [64]. A decrease of the oxidative energy metabolism in senile dementia and the resulting ATP deficit may thus change protein degradation, synaptic transmission and ion homeostasis [63]. Furthermore, disturbed function and abnormal morphology of mitochondria are also associated with AD and PD [65].

A global decrease in cerebral metabolic rate also occurs in AD and in other dementias, and the AD brain is characterized by a variable, but often marked, loss of neurons, a deposition of extracellular plaques, and intracellular neurofibrillary tangles [66–68]. Impaired energy metabolism [68] and altered cytochrome c oxidase activity are among the earliest detectable defects in AD [69–72]. Most recently, focal deposits of Cr have been discovered in AD [73], suggestive of a perturbed energetic status and deregulated Cr synthesis and/or uptake (see further below).

MITOCHONDRIAL ASPECTS OF NEURODEGENERATION

The involvement of mitochondria in neurological disorders is frequently discussed. It is known that pathological states and mitochondrial dysfunction often lead to the excessive generation of free radicals and subsequent oxidative damage [74]. Studies of AD patients have identified decreased complex IV activity and mitochondrial DNA mutations [75, 76]. Recently, a role for mitochondria has been indicated in Aβ-induced apoptosis. The Aβ-binding alcohol dehydrogenase (ABAD) has been reported to interact with Aβ in the mitochondria of AD patients and transgenic mice [77] and to potentiate Aβ-induced apoptosis and free-radical generation in neurons. Furthermore, in brains from patients with autopsy-confirmed AD and clinical dementia ratings before death, the activity of tricarboxylic acid cycle (TCA) enzymes (pyruvate dehydrogenase complex, isocitrate dehydrogenase, and the alpha-ketoglutarate dehydrogenase) of mitochondria were significantly decreased. Changes in TCA cycle activities correlated with the clinical state of the disease, suggesting a coordinated mitochondrial alteration [77]. Recently, a structural and functional interplay between dendritic mitochondria and spines/synapses was discovered in in vitro cultured neurons [78]. A small fraction of mitochondria is present within dendritic protrusions of cultured neurons [78]. Interestingly, in these cultured neurons, Cr supplementation enhances mitochondrial activity and causes a higher density of spines and synapses. Remarkably, the ability of neurons to form new excitatory synapses in response to stimulation is also correlated with increased activity of dendritic mitochondria [78]. Neuronal activity itself affects the motility, fusion/fission balance, and subcellular distribution of mitochondria in dendrites, depending on calcium influx. This seems to be physiologically relevant, because repetitive depolarization that stimulates synapse formation causes the redistribution of mitochondria into dendritic protrusions [78]. These results suggest a local involvement of mitochondria in synapse formation and development. Taken together, these findings are in agreement with the concept that the characteristic loss of synapses in disorders like AD arises in part from mitochondrial dysfunction [79].

PERTURBED CK FUNCTIONS AND LOWER PCR/CR RATIOS ARE LINKED TO NEURODEGENERATIVE DISEASES INCLUDING AD

Oxidative alterations of proteins and lipids have been implicated in the progression of neurodegenerative disorders [80, 81]. Protein carbonyls, considered a marker of protein oxidation, are increased in AD [82]. Using a proteomic approach, BB-CK, glutamine synthase (GS), and ubiquitin carboxy-terminal hydrolase l-1 (UCH L-1) were identified as the three major specifically oxidized proteins in AD brains [83]. Oxidative modification of CK rapidly inactivates the enzyme and results in abnormal partitioning of CK between the soluble and pellet fractions [84]. As a consequence, CK activity in AD brain homogenates is decreased by 86%, as shown by [alpha 32P]8N3ATP incorporation into the enzyme, but the expression level of CK is decreased by less than 14% [84]. These findings can be explained by the fact that all CK isoforms possess a highly reactive cysteine residue that is specifically modified by sulfhydryl reagents or oxygen or peroxynitrite radicals [85]. Loss of BB-CK activity [82, 84, 86, 87], resulting from its oxidation [87], implies that the maintenance of a healthy cellular energy state is perturbed in the AD brain and that energy supply in glia cells, neurons, and synaptic elements is altered. This is corroborated by one study, where Alzheimer's patients were found to have reduced levels of brain PCr in early stages of the disease and decreased oxidative metabolism in later stages compared to healthy persons, indicating that the AD brain is under energetic stress [88].

FORMATION AND PRESENCE OF FOCAL CREATINE DEPOSITS IN AD BRAIN

The recent discovery of Cr deposits in the brains of transgenic APP mice by Fourier transform infrared microspectroscopy (FTIR) in frozen and desiccated brain slices [73] raises many questions concerning its role in AD. At present, it is not known whether the creatine exists as precipitated microcrystals or as localized, sequestered pools in vivo. In this section, we speculate on some possible origins for these deposits.

Cr is a very prominent compound in both muscle and brain, where total Cr (PCr plus Cr) may reach 50 mM or 20 mM, respectively, the latter being strongly dependent on the region of the brain [36]. Intracellularly, under normal energetic conditions, 2/3 of total Cr is in the form of energy-rich PCr and 1/3 in the form of Cr. During cellular energy stress, the PCr/Cr ratio decreases. Cr is less insoluble than PCr, having a solubility limit of roughly 100 mM in aqueous solution, depending on temperature and pH. Thus, in glycolytic skeletal muscle that is highest in total Cr, this solubility limit is nearly reached if all PCr was converted to Cr. In brain this is less likely, given the lower total Cr concentration. However, it is conceivable that upon massive destruction of neurons in AD accompanied by lysis and cell death, significant amounts of Cr are set free within the region of brain cell destruction. Thus, if concentrated, Cr might precipitate in the extracellular space of the brain, giving rise to focal deposits in vivo. However, no such neuronal loss is observed in the transgenic APP mice, nor are there obvious changes in cell morphology that would support such a hypothesis. In fact, the focal Cr deposits are seen in frozen brain sections that have to be desiccated before FTIR microspectroscopy measurements [73]. It is therefore not unreasonable to suppose that under in vivo (hydrated) conditions, the elevated Cr exists in solution, for example, inside intact cells or in vacuoles or other subcellular compartments, and that this Cr only solidifies into focal microcrystals when the tissue is dried.

Another explanation is based on a breakdown in the synthesis and/or neuronal uptake pathway of Cr. Some glial cells, especially oligodendrocytes, synthesize Cr, which is then released to be taken up by neurons [49, 51]. AD is accompanied by inflammation and an increase in the number of glial cells, providing an additional source of Cr. Neurons normally take up Cr from the extracellular space by CRT. If, however, neurons were energetically stressed, Cr uptake could be limited, because the uptake of Cr via CRT is accompanied by concomitant Na^+ and Cl^- cotransport into the cell [54]. This Na^+ has to eventually be pumped out of the cell by the ATP-driven Na^+/K^+-ATPase that also uses a major part of cellular energy [30]. Thus, if Cr uptake into neurons was hampered, the net result would be a slow accumulation of Cr in the extracellular space.

A third possibility is that the oxidation of BB-CK and uMtCK [85] limits the formation of PCr, which is in turn depleted to support ADP to ATP conversion, thus favoring the generation of excess Cr.

Our recent data (see below) raise yet another possibility that uMtCK targeting to the mitochondria may be disrupted by a loss of APP function [89], which in turn would result in a decrease in synthesis of PCr and a concomitant accumulation of Cr within cells.

Since the microdeposits of Cr detected by FTIR microspectroscopy are found distributed focally across large regions of the hippocampus in the transgenic AD model mice [73], occasionally colocalized on the edges of AD plaques, it is likely that at least some of the Cr deposits are not intracellular, but rather in the extracellular space. They could be generated either by Cr leakage from dying cells or by impaired Cr trafficking between glia and neurons, as outlined above. However, irrespective of primary course, the factor triggering Cr deposits would be a disturbed energy charge of neurons under stress.

INTERACTION AND COMPLEX FORMATION OF APP WITH MITOCHONDRIAL CREATINE KINASE

Recent biochemical data from our laboratories provide a direct link between APP and uMtCK and shed new light on putative molecular mechanisms that lead to energetic abnormalities in AD brain (discussed above) [89]. Using a functional proteomic screen, in which APP interacting proteins were isolated based on their biochemical affinity and identified by peptide mass fingerprinting, we found that the short cytoplasmic tail of APP family proteins interacts with several different mitochondrial targeted proteins (see [89] and Li and Homayouni, unpublished observations). This interaction was of high affinity toward the preprotein forms, containing the N-terminal signal sequence, of the mitochondrial proteins. Importantly, coexpression of APP C-terminal regions dramatically stabilized uMtCK in cultured cells. APP family proteins are type-I transmembrane glycoproteins that undergo sequential N- and O-linked glycosylation in the ER/Golgi pathway. Using co-immunoprecipitation assays, we found that uMtCK associated with the full-length but lower molecular weight APP proteins, suggesting that the interaction occurs prior to maturation of APP proteins. Immunohistochemical analysis indicated that APP and uMtCK colocalize in ER/Golgi and not in mitochondria of cultured primary neurons as well as in transiently transfected nonneuronal cells. These results raise the possibility that APP family proteins may function as cytoplasmic chaperone-like proteins to stabilize mitochondrial proteins such as uMtCK. Indeed, APP is induced and accumulates in the ER/Golgi of cultured cells after treatments that induce oxidative metabolic stress or ER stress via disruption of the ER folding machinery, thus affecting protein maturation and causing accumulation of unfolded proteins within the ER lumen [90, 91]. In turn, some evidence indicates that APP induction can play a protective role against cell stress and axonal injury [91, 92].

Why then only mutations in APP, and not its other family members, have been linked to AD? We propose that mutations in APP result in a dual attack on the mitochondria. First, these mutations enhance the generation of Aβ peptide [93], which were shown to be directly toxic to mitochondria through an interaction with ABAD [77]. Second, based on our recent data, we speculate that the loss of the normal function of APP in targeting of uMtCK, and perhaps other mitochondrial proteins to the mitochondria, would result in a further compromise of mitochondrial function in the affected neurons. This hypothesis is consistent with a stochastic model proposed by Clarke and colleagues [94], in which the mutated cell exists in an altered steady state and upon a random insult initiates a cascade of events resulting in cell death. It has been shown that mutations in APP

increase the vulnerability of cells to oxidative stress [91, 95]. Although, these findings pertain to APP mutations, which are linked only to early-onset AD, they suggest a more general mechanism for the pathogenesis of AD involving dysregulation of mitochondrial function [65, 96, 97].

NEUROPROTECTIVE EFFECTS OF CR SUPPLEMENTATION FOR NEURODEGENERATIVE DISEASES

Over the past decade, the ergogenic benefits of synthetic Cr monohydrate have made it a popular dietary supplement, particularly among athletes [98]. The anabolic properties of Cr also offer hope for the treatment of diseases characterized by muscle weakness and atrophy, as well as for rehabilitation [99]. In serum-free cultured cells, Cr supplementation has been shown to protect rat hippocampal neurons against glutamate and Aβ toxicity [100]. In an animal model of traumatic brain injury (TBI), it has been shown that Cr supplementation protects against neuropathology of TBI through mechanisms involving maintenance of mitochondrial bioenergetics and preservation of ATP levels [101]. This is due to the fact that newly entered Cr is phosphorylated inside the cell by the catalytic activity of CK, leading to an increased PCr/ATP ratio and, thus, a higher energy charge in the cell. Brustovetsky et al demonstrated neuroprotective effects of PCr and Cr pretreatments against energetic deprivation caused by 3NP and glutamate excitotoxicity in cultured neurons [102]. There, surprisingly, extracellular PCr was more efficacious than Cr. This could be explained by the fact that PCr is able to bind to and stabilize cell membranes [103].

Recent data using human fetal striatal and mesencephalic tissue identified Cr as a potent natural survival and neuroprotective factor for GABA-ergic neurons in a model for HD [104] and of dopaminergic neurons in a model for PD [105–107]. Cr is also beneficial in animal models of cerebral ischemia [108–110] and spinal cord injury [111, 112]. In the G93A transgenic mouse model for ALS, long-term Cr supplementation extends life span, significantly improves motor coordination [113], and reverts the cholinergic deficit in some forebrain areas at an intermediate stage of ALS [114]. In rat and mouse models of cerebral ischemia, oral Cr administration resulted in neuroprotection and remarkable reduction in ischemic brain infarction [109, 115]. Postischemic caspase-3 activation and cytochrome *c* release were significantly reduced in creatine-treated mice. Cr administration buffered ischemia-mediated cerebral ATP depletion [115]. These authors suggest that a prophylactic Cr supplementation, similar to what is recommended for an agent such as aspirin, may be considered for patients in high stroke-risk categories.

Supplementation with Cr has been used as an adjuvant to a therapeutic scheme in numerous diseases associated with muscle and neuromuscular degeneration. To date, two clinical pilot trials to test the efficacy of Cr monohydrate in ALS have been completed without any measurable improvements in overall survival or in a composite measure of muscle strength [116, 117]. However, these pilot studies were powered only to detect a 30–50% or greater change in rate of decline of muscle strength. These trials raised new questions about the optimal dosage of Cr and its beneficial effects on muscle fatigue, a measure distinct from muscle strength. A large multicenter clinical trial is currently underway to further investigate the efficacy of Cr monohydrate in ALS and to address these unresolved issues. To date, evidence shows that Cr supplementation at 5–10 grams over a time period of 12 months has a good safety profile and is well tolerated by patients with ALS.

In a trial with HD patients, Cr supplementation lowered brain glutamate levels [118]. Very recent data from a randomized, double-blind, placebo-controlled study with 64 subjects with Huntington's disease (HD), 8 g/day of Cr administered for 16 weeks, show that Cr was well tolerated and safe. Serum and brain Cr concentrations increased in the Cr-treated group and returned to baseline after washout. Intriguingly, serum 8-hydroxy-2'-deoxyguanosine (8OH2'dG) levels, an indicator of oxidative injury to DNA, were markedly elevated in HD, but were reduced significantly by Cr treatment [119]. In patients with a novel cytochrome b mutation, Cr supplementation attenuated the production of free radicals and the paracrystalline intramitochondrial inclusions [120] brought about by crystallization of overexpressed MtCK inside mitochondria [121]. The rationale for the use of Cr along with available evidence from animal models and clinical trials for ALS and related neurodegenerative or neuromuscular diseases have been described in [122]. Thus, it is obvious that Cr as a simple nutritional supplement shows a great potential for neuroprotective effects in various neuromuscular and neurodegenerative diseases.

RATIONALE FOR CREATINE SUPPLEMENTATION IN ALZHEIMER'S DISEASE

Very recent data by Snyder et al show that Aβ addition to cortical neurons in cell cultures leads to internalization of NMDA-receptors with concomitant dephosphorylation of the NMDA receptor subunit NR2B at Tyr 1472 [123]. Since it has been shown earlier that Cr supplementation significantly protects neurons against Aβ neurotoxicity [100], it can be inferred that Cr may indirectly benefit AD patients by reducing the effects of Aβ toxicity and NMDA-receptor internalization. It may also alleviate the deterioration of glutamatergic neurotransmission and synaptic plasticity that are vital for learning and memory. For example, treatment of hippocampal neurons with 20 mM Cr significantly increased both basal and activity dependent synaptogenesis [78]. In two studies, Cr supplementation has been shown to improve mental concentration [124], as well as memory and learning [125] in healthy human subjects. It is possible that this will also be true for early stage AD patients.

Given the evidence for metabolic dysfunction in AD, we hypothesize that Cr supplementation at an early time point of the disease might be useful in compensating for the disturbed energy metabolism in subjects with AD by replenishing the energy pools, activating mitochondrial respiration

[126, 127] and protecting cells from apoptosis [127, 128]. Although Cr cannot increase energy charge if CK is damaged, for example, by oxidative damage (see below), very early in the course of AD, CK is still functioning to some extent, so it is reasonable to assume that Cr may be of benefit in those early phases. Further, CK isoenzymes are known to be prime targets of oxidative damage by free radicals [85–87] that are a hallmark of many neuromuscular and neurodegenerative diseases. The substrate Cr, together with MgADP or MgATP, upon forming a transition state complex in the active site of CK, has a protective effect against inactivation of CK isoenzymes by free radicals, such as oxygen radicals and peroxynitrite. In the case of MtCK, Cr in the presence of nucleotide, additionally prevents the dissociation of native octameric MtCK into dimers [85]. Thus, an elevation of the intracellular concentration of Cr by Cr supplementation may confer additional protection to CK and concomitantly delay the free-radical induced inactivation of the CK system in brain that is seen in AD [87].

Cr might be expected to improve energetic conditions for all cells, as well as for "at risk neurons," in animal models of neurodegenerative diseases, providing temporary protection. Such protection would occur in a vital time period when cell fate is still in balance, or perhaps precritical, before secondary excitotoxicity might threaten weakened neurons. Such protection by Cr, however, could only be expected if the CK system were not compromised in a significant way such that PCr would still be synthesized by CK. An additional mechanism by which Cr may exert neuroprotection, could be through activation of AMPK in the brain, in a manner similar to that recently shown in muscle cells [129]. Since AMPK is a cellular energy sensor and fuel gauge, this would lead to short-term and long-term compensatory reactions to help the cells recruit more energy sources, for example, by up-regulation of glucose transport and elevation of fatty acid oxidation [130].

Lastly, Cr may exert neuroprotection by reducing protein aggregation. For example, Cr was found to reduce transglutaminase-catalyzed protein aggregation, in vitro, [131] a process thought to be relevant for the formation of protein aggregate formation in several neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's disease.

Thus, one may postulate that Cr supplementation, in combination with other established clinical interventions, may be a very valuable adjunct therapy for patients at an early stage of the disease progression. However, additional studies are needed first to address the questions of where exactly the microcrystalline Cr deposits are located, for example, intra- or extracellularly, and whether they are associated with specific structures of the brain. In addition, it would be important to be able to quantify Cr in these deposits. Since these focal Cr microdeposits are observed in the brain of transgenic APP mice, as well as AD patients [73], there is a valid concern that supplementation with extra Cr might exacerbate rather than ameliorate this situation. However, as reasoned above, Cr, if given at an early time point of disease, may prevent or delay the formation of Cr deposits that are a consequence of cellular pathology. In any case, Cr supplementation should be tested first on the transgenic APP mice in which the Cr deposits have been found. In the long term, if warranted by the outcome of such tests, further trials with AD patients could be performed. If successful, this cheap and safe intervention, involving as a nutritional supplement, may extend a huge socioeconomic benefit by improving the quality of life of AD patients and lowering exploding health care costs.

ABBREVIATIONS

3NP	3-nitropropionic acid
8OH2'dG	8-hydroxy-2'-deoxyguanosine
Aβ	amyloid beta peptide
ABAD	Aβ-binding alcoholdehydrogenase
AD	Alzheimer's disease
AGAT	L-arginine:glycine amidinotransferase
ALS	amyotrophic lateral sclerosis
AMPK	AMP-stimulated protein kinase
APP	amyloid precursor protein
BB-CK	cytosolic brain-type creatine kinase
CK	creatine kinase
CNS	central nervous system
Cr	creatine
CRT	Na^+ and Cl^- dependent creatine transporter
FTIR	Fourier transform infrared
GAMT	S-adenosylmethionine:guanidinoacetate N-methyltransferase
HD	Huntington's disease
MM-CK	cytosolic muscle-type creatinekinase
PCr	phosphocreatine
PD	Parkinson's disease
PET	positron emission tomography
sMtCK	sarcomeric mitochondrial creatine kinase
SR	sarcoplasmic reticulum
TBI	traumatic brain injury
TCA	tricarboxylic acid
UCH L-1	ubiquitin carboxy-terminal hydrolase l-1
uMtCK	ubiquitous mitochondrial creatine kinase

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Research Article

Heme Deficiency in Alzheimer's Disease: A Possible Connection to Porphyria

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Mechanisms that cause Alzheimer's disease (AD), an invariably fatal neurodegenerative disease, are unknown. Important recent data indicate that neuronal heme deficiency may contribute to AD pathogenesis. If true, factors that contribute to the intracellular heme deficiency could potentially alter the course of AD. The porphyrias are metabolic disorders characterized by enzyme deficiencies in the heme biosynthetic pathway. We hypothesize that AD may differ significantly in individuals possessing the genetic trait for an acute hepatic porphyria. We elaborate on this hypothesis and briefly review the characteristics of the acute hepatic porphyrias that may be relevant to AD. We note the proximity of genes encoding enzymes of the heme biosynthesis pathway to genetic loci linked to sporadic, late-onset AD. In addition, we suggest that identification of individuals carrying the genetic trait for acute porphyria may provide a unique resource for investigating AD pathogenesis and inform treatment and management decisions.

INTRODUCTION

AD is a progressive and invariably fatal neurodegenerative disease and the leading cause of senile dementia [1]. Synaptic and neuronal loss best correlates with cognitive decline [2]. Metabolic imbalance in diseased neurons may contribute to neuropsychiatric symptoms that include delusions and hallucinations, anxiety, mood disorder, and sleep disturbance that are common in AD [3]. Mechanisms that cause AD are unknown. Recently we proposed a hypothesis that explains why elevated plasma homocysteine is a risk factor for AD [4, 5]. Implicit in that hypothesis is development of neuronal heme deficiency, and evidence of heme deficiency in AD brains has been reported [6]. Here, we extend this theme by considering the possible impact of porphyria on AD. The porphyrias are metabolic disorders characterized by enzyme deficiencies in the heme biosynthesis pathway. We propose that an understanding of porphyria may provide novel insights into AD pathogenesis.

GENERAL CONSIDERATIONS

Molecular and biochemical aspects of the porphyrias and their diagnosis and treatment are the subject of several excellent reviews [7–13]. Eight enzymes are required for *de novo* heme biosynthesis. With the exception of 5-aminolevulinic acid synthase [ALAS, EC 2.3.1.37], the initial and rate-limiting enzyme of the heme biosynthesis pathway, deficiency in one of the other seven enzymes is associated with a specific form of inherited porphyria [10]. Four of the hepatic porphyrias, so-called because liver is the major site of expression of the enzymatic defect in heme biosynthesis, are designated "acute" porphyrias because clinical expression of the disease is associated with an acute neurologic syndrome (the acute attack or porphyric crisis) [8, 11]. These are the extremely rare Doss porphyria (deficiency of ALA dehydratase, EC 4.2.1.24), acute intermittent porphyria (deficiency of porphobilinogen deaminase, EC 4.3.1.8), hereditary coproporphyria (deficiency of coproporphyrinogen oxidase, EC 1.3.3.3), and variegate porphyria (deficiency of protoporphyrinogen oxidase, EC 1.3.3.4). Acute intermittent porphyria is generally the most common form of acute hepatic porphyria encountered. Significantly, enzyme deficiencies are present in other organs, including the brain, and the enzyme deficiency is life-long.

Acute neurologic syndrome associated with clinical attacks of acute hepatic porphyria can include both neuropsychiatric symptoms and neurodegenerative change [8, 11, 12]. Neuropsychiatric symptoms that include anxiety, insomnia, confusion, hallucinations, agitation, and paranoia (so-called

porphyric encephalopathy-8) underscore CNS involvement. Autonomic neuropathy may underlie severe abdominal pain and cardiovascular symptoms. In severe cases, a peripheral neuropathy resembling Guillain-Barre syndrome can develop [8, 11]. Clinical attacks of acute porphyria can be induced in latent individuals by a variety of environmental factors including many common medications, nutritional factors, restricted carbohydrate and calorie intake, smoking, and hormones such as progesterone; lists of safe and unsafe drugs are available [9, 11, 12]. A common mechanism of inducing agents is believed to be greatly increased hepatic heme demand. Thus, biosynthesis of cytochrome P450 enzymes that utilize heme as a prosthetic group can be induced as much as 40–50-fold in liver by drugs such as barbiturates [11]. Increased heme demand results in the induction of ALAS and increased synthesis of the heme precursor, 5-aminolevulinic acid [ALA]. In individuals who have inherited a partial deficiency in one of the enzymes of the heme biosynthesis pathway, that enzyme and not ALAS is the rate-limiting step in heme biosynthesis. Then, ALA and other heme precursors can accumulate. Moreover, heme biosynthesis is insufficient to meet demand and heme deficiency is unresolved. Acute attacks are treated with infusions of glucose and hemin [9, 12]. Hemin restores the regulatory heme pool. This suppresses hepatic ALAS induction and the overproduction of ALA and other heme precursors. Glucose infusion may also suppress ALAS but by a different mechanism. Fasting, which can induce the acute attack, appears to activate transcriptional coactivator PGC-1α (via a cAMP/CREB pathway) and PGC-1α greatly increases hepatic ALAS expression by activating transcription factors NRF-1 and FOXO1 [14, 15]. In addition, ALAS may respond directly to cAMP [14, 15]. Glucose appears to antagonize both pathways. Abdominal pain and psychotic symptoms resolve quickly upon timely treatment of the acute attack but peripheral neuropathy can require months to resolve and recovery is often incomplete [11].

The pathogenesis of nervous system dysfunction in the acute attack remains unclear. There are two predominant hypotheses [8, 11]. One suggests functional heme deficiency develops during the acute attack, in liver and possibly in neural tissues, and impairs critical cell processes dependent on hemoproteins such as energy production by the mitochondrial electron transport chain. Studies utilizing mice deficient in porphobilinogen deaminase, an experimental model of acute intermittent porphyria, underscore the importance of functional heme deficiency in nervous tissue in the development of motor neuropathy [16–18]. The second hypothesis suggests that heme precursors and their metabolites accumulate to toxic levels during the acute attack. ALA, in particular, is implicated because it is produced excessively in all the acute hepatic porphyrias and may have neurotoxic properties [8, 11]. Excessive ALA production occurs in lead poisoning due to lead-mediated inhibition of ALA dehydratase, and also in hereditary infantile tyrosinemia (type I) in which the enzyme defect leads to endogenous production of the ALA dehydratase inhibitor, succinylacetone [19]. In both diseases, neuropsychiatric symptoms that resemble those of the acute attack occur [8, 11]. While recent clinical studies underscore the potential importance of excessive hepatic production of heme precursors as the primary cause of the neurologic complications in the acute porphyric attack [20, 21], induced elevation of plasma ALA in a human volunteer, by itself, did not produce symptoms of porphyria [22]. Clearly, many details are unresolved [8, 11].

DOES PORPHYRIA OFFER INSIGHT ON AD?

We hypothesize that heme deficiency is important in AD pathogenesis and that AD may differ significantly in individuals possessing the genetic trait for an acute hepatic porphyria because there is the potential to develop more severe heme deficiency. Figure 1 schematically depicts this hypothesis.

AD-related factors may create an imbalance in neuronal heme supply and demand. In AD, heme supply may be reduced. Aging is the greatest risk factor for development of AD, and at least in rat brain, heme biosynthesis declines in normal aging [25]. Nutritional factors could be important. Pyridoxine deficiency in the aged could contribute to age-related decrease in heme biosynthesis because pyridoxal phosphate is a cofactor for ALAS [10]. Glycoxidation reactions are prominent in AD brain [26], and glycoxidation reactions might inactivate enzymes required for heme biosynthesis [27]. Moreover, heme biosynthesis requires mitochondrial integrity and mitochondrial damage is prominent in AD [28]. In AD, heme demand may be increased. Mitochondrial damage would necessitate increased mitochondrial turnover and *de novo* synthesis of heme-containing proteins such as cytochromes. Moreover, mitochondrial damage may itself be caused by heme deficiency [29, 30] thus creating a vicious cycle further impairing heme biosynthesis. Glycoxidation reactions could promote degradation of heme proteins [31]. Heme degradation may be favored over heme biosynthesis in AD neurons because of chronically elevated HO-1 [32, 33]. Factors unique to AD could also increase heme demand. Thus, amyloid-β [Aβ] binds heme, which may contribute to development of a functional heme deficiency [6] and affect Aβ toxicity by inhibiting Aβ aggregation [34, 35]. We speculate that the reduced capacity to synthesize heme in individuals with porphyria could exacerbate such an imbalance in heme supply and demand.

In addition, the two proposed mechanisms of nervous system dysfunction in the acute porphyric attack, functional heme deficiency and toxic accumulation of ALA, have cellular effects that could be important in AD pathogenesis. Inhibition of heme biosynthesis produced senescence-associated changes in gene expression in cultured mouse cortical neurons [36] and was proapoptotic in NGF-induced PC12 cells [37]. Increased oxidative stress, which is one of the earliest observed events in AD pathogenesis [38], and heme deficiency may help explain several pathophysiological features of AD including mitochondrial abnormalities and impaired energy metabolism, cell cycling and cell signaling abnormalities, neuritic pathology, and abnormal expression of iron regulatory protein 2 (IRP2) [5]. ALA is a source of

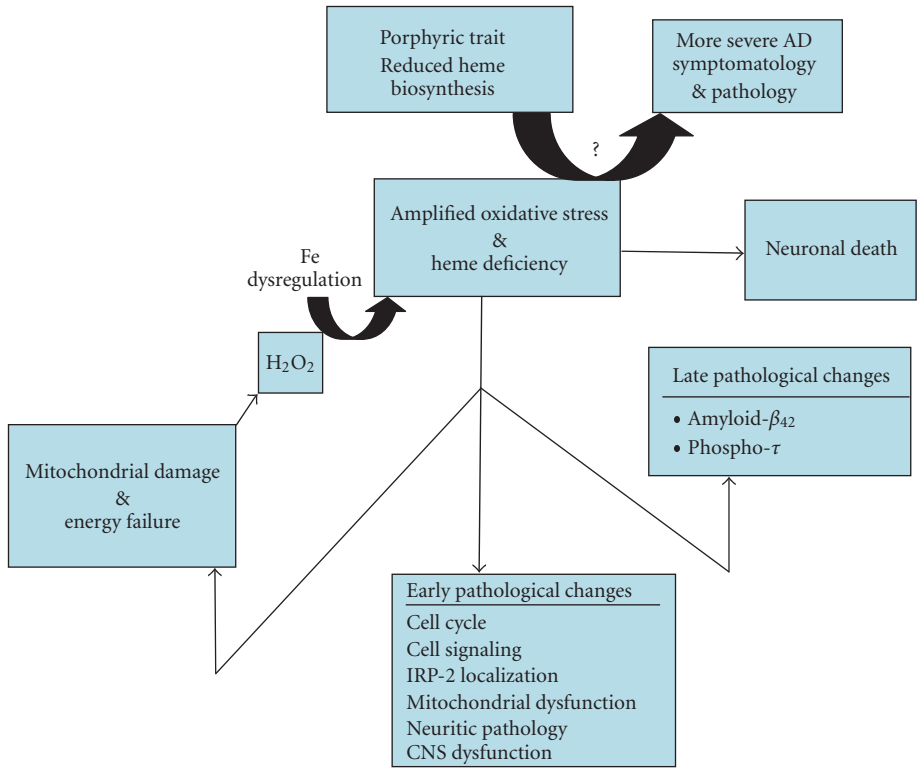

Figure 1: AD and porphyria. Oxidative stress and free radical damage occur early in AD [23]. Disruption in iron homeostatic mechanisms contributes to oxidative damage in AD [24]. A consequence of oxidative stress predicted by the ferric cycle hypothesis is heme deficiency [4, 5]. Moreover, AD-related factors such as accumulation of amyloid-β may limit heme bioavailability [6]. We hypothesize that reduced capacity for cells to synthesize heme, in individuals with the genetic trait for acute hepatic porphyria, contributes to development of heme deficiency (and possibly oxidative stress). AD-related pathological change and neuropsychiatric and behavioral symptoms associated with AD may be more severe in these individuals.

oxygen free radicals in the presence of heavy metals such as iron [39]. The product of iron-catalyzed oxidation of ALA, 4, 5-dioxovaleric acid, is an effective alkylating agent of guanine moieties in DNA *in vitro* [40, 41], and ALA-induced mitochondrial and nuclear DNA damage has been shown in several cell lines including PC12 cells [42]. Moreover, ALA may disrupt normal iron sequestration by ferritin. It released iron from ferritin *in vitro* [43, 44] and caused oxidative damage to the ferritin molecule [45]. As in the toxic mechanism proposed for homocysteine in AD pathogenesis [4, 5], ALA may make available a catalytic metal that can promote oxidative stress.

DOES PORPHYRIA INCREASE THE RISK OF AD?

Clinically overt acute hepatic porphyria (predominantly acute intermittent porphyria) is relatively rare with a prevalence of perhaps 5 per 100,000 [12]. However, the prevalence of the genetic trait for acute porphyria is far greater because perhaps 90% of affected individuals are clinically latent [7, 12]. In a Finnish population the estimated prevalence of porphobilinogen deaminase deficiency, the biochemical defect in acute intermittent porphyria, was 1 per 500 [46]. Using gene analysis to supplement enzymatic analysis, the estimated prevalence of porphobilinogen deaminase deficiency in a French population was 1 per 1675 [47]. Consistent with the possibility that deficiency in heme biosynthesis could increase susceptibility for AD is the intriguing observation that the chromosomal location of genes encoding enzymes in the heme biosynthesis pathway correlate with genetic loci linked to sporadic, late-onset Alzheimer's disease (maximum lod score $\geqslant 1$) [48] (Table 1). However, the significance of this observation is unclear. In cases such as deficiency in porphobilinogen deaminase, heme deficiency alone may be insufficient to cause AD but could contribute to disease progression when superimposed on other disease processes. However, effects may be indirect and not related to heme levels. For example, the proximity of the ALA dehydratase gene to an AD-related locus is noted in Table 1. ALA dehydratase-porphyria is an extremely rare form of acute hepatic porphyria. Moreover, ALA dehydratase activity is far in excess of the activities of other enzymes in the heme biosynthetic pathway and for that reason > 95% loss of activity is needed before clinical symptoms of porphyria develop [12]. However, ALA dehydratase is also a high K_m enzyme. Under AD-associated conditions, toxic levels of ALA may possibly accumulate and contribute to AD pathogenesis. While interesting, any relationship between heme deficiency

Table 1: Chromosomal locations of genes encoding enzymes of the heme biosynthesis pathway and genes linked to development of late-onset Alzheimer's disease.

Heme biosynthetic enzymes	Location[1]	Closest AD-related loci[2]
ALAS-1 (EC 2.3.1.37)	3p21	3p14, 3p26
ALA-dehydratase (EC 4.2.1.24)[3]	9q34	9q34
Porphobilinogen deaminase (EC 4.3.1.8)[3]	11q23.3	11q25
Uroporphyrinogen III synthase (EC 4.2.1.75)	10q25.3	10q21–10q25
Uroporphyrinogen decarboxylase (EC 4.1.1.37)	1p34	1p31–1p36
Coproporphyrinogen III oxidase (EC 1.3.3.3)[3]	3q12	3q28
Protoporphyrinogen oxidase (EC 1.3.3.4)[3]	1q22	1q23, 1q24
Ferrochelatase (EC 4.99.1.1)	18q21.3	18q22

[1] from Meissner et al [10].
[2] From Table 1 by Kamboh in [48].
[3] A deficiency is associated with a specific form of acute hepatic porphyria.

and AD is speculative. If the genetic trait for one of the acute hepatic porphyrias is a risk factor for AD, why has this relationship gone unnoticed? The answer simply may be that the majority of individuals with the biochemical defects of acute porphyria are clinically latent, and that many genetic and environmental factors likely contribute to the development of sporadic, late-onset AD.

CONCLUSIONS

AD may differ significantly in individuals who have the genetic trait for acute hepatic porphyria because there is the potential to develop more severe neuronal heme deficiency and possibly accumulate ALA and other heme precursors. Epidemiological data confirming a link between AD and porphyria would be an important test of the hypothesis. AD progression (from disease-free state, to mild cognitive impairment, to AD) in individuals with a genetic trait for acute hepatic porphyria could be compared with AD progression in an unaffected cohort. Testing for the presence of a genetic trait for acute porphyria in individuals diagnosed with mild cognitive impairment or early AD might identify a unique subset of AD patients. Management decisions may need to be adjusted in such individuals to avoid potential sensitivity to common medications and novel therapeutic agents which, if porphyrinogenic, could exacerbate porphyria and possibly AD symptoms. Approaches such as these could yield significant new information on AD pathogenesis and treatment.

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Review Article

Signaling, Polyubiquitination, Trafficking, and Inclusions: Sequestosome 1/p62's Role in Neurodegenerative Disease

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Aggregated misfolded proteins are hallmarks of most neurodegenerative diseases. In a chronic disease state, including pathologic situations of oxidative stress, these proteins are sequestered into inclusions. Accumulation of aggregated proteins can be prevented by chaperones, or by targeting their degradation to the UPS. If the accumulation of these proteins exceeds their degradation, they may impair the function of the proteasome. Alternatively, the function of the proteasome may be preserved by directing aggregated proteins to the autophagy-lysosome pathway for degradation. Sequestosome 1/p62 has recently been shown to interact with polyubiquitinated proteins through its UBA domain and may direct proteins to either the UPS or autophagosome. P62 is present in neuronal inclusions of individuals with Alzheimer's disease and other neurodegenerative diseases. Herein, we review p62's role in signaling, aggregation, and inclusion formation, and specifically as a possible contributor to Alzheimer's disease. The use of p62 as a potential target for the development of therapeutics and as a disease biomarker is also discussed.

INTRODUCTION

Many neurodegenerative diseases such as polyglutamine repeat diseases, Lewy bodies in Parkinson disease, Alzheimer's disease, tauopathies, and others share abnormal accumulation of ubiquitinated proteins into aggregates and inclusions as a hallmark feature of the disease pathology. The molecular basis for the accumulation of these inclusions remains poorly defined; however the aberrant accumulation of aggregated proteins and disturbances in protein degradation suggest a common underlying mechanism. These inclusions share amyloid-like structure and several biochemical features such as: ubiquitin, aggregated proteins, proteasome subunits, chaperones, and other proteins that become trapped through their association with aggregated proteins. Here we review the role of aggregates, protein turnover, and the ubiquitin proteasome system (UPS), and focus on the role of a recently discovered proteasomal shuttling protein, sequestosome 1/p62, and its role in neurodegeneration. We also discuss the potential of employing p62 as a biomarker for neurodegenerative disease and as a potential target for therapeutic development.

MISFOLDING AND THE UPS PATHWAY

In normal cells, large amounts of newly synthesized proteins are defective "off-pathway" products. Even with abundant molecular chaperones, nearly 30% of nascent proteins are misfolded due to mutations or inefficient assembly [1]. To correct these mistakes, misfolded proteins can either be degraded via the ubiquitin proteasome pathway (UPS) shortly after their synthesis or they may form aggregates of high molecular weight oligomers [2]. The ultimate fate of misfolded proteins depends on kinetic partitioning between these two competitive pathways [3]. Because aggregates are more stable than the improperly folded protein, to degrade misfolded substrates effectively the proteasome must win the competition for the misfolded substrates before they have an opportunity to aggregate. Under normal conditions, accumulated proteins are removed promptly before any damage can be caused to the cell. However under certain situations in nerve cells, accumulated proteins are prone to form inclusion bodies which are the hallmarks of several neurodegenerative diseases [4, 5]. Increasingly it is becoming apparent that these

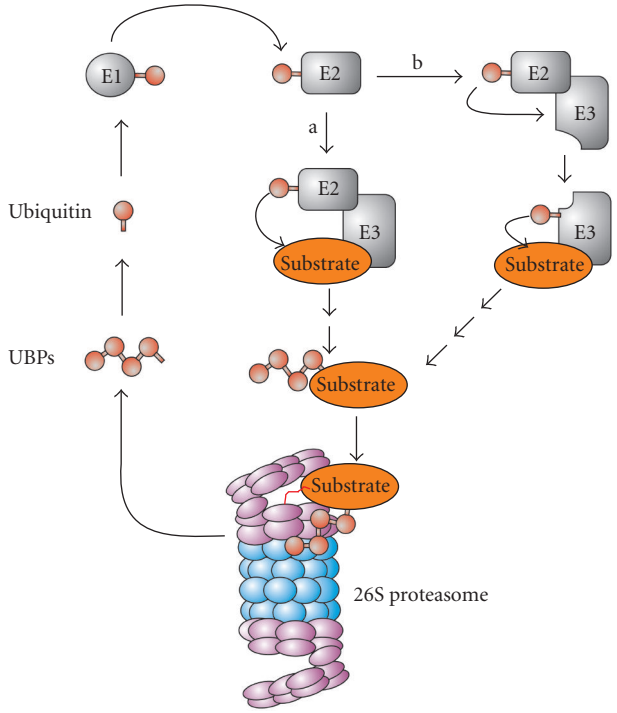

FIGURE 1: The pathway of ubiquitin-linked degradation of proteins and the 26S proteasome (see [84]).

inclusions/aggresomes may be the sites for sequestration of aggregated proteins. Herein, we will attempt to clarify the function and toxicity of protein aggregates and inclusions.

The UPS is responsible for the degradation of proteins and it serves as a cellular quality control system that tags misfolded proteins with ubiquitin for degradation by the 26S proteasome. Protein degradation via UPS involves two steps: (1) covalent attachment of polyubiquitin chains to target proteins, and (2) degradation of the tagged proteins by 26S proteasome complex with release of free and reusable ubiquitin (Figure 1). Ubiquitin is a protein that forms different chains with itself [6, 7] and serves as a signal through covalent attachment to other proteins. Three enzymes are involved in ubiquitination of substrates, eventually resulting in the formation of a bond between the C-terminus of ubiquitin (Gly76) and the ε-amino group of a substrate lysine residue. Ubiquitin-activating enzyme (E1) forms a thiol ester with the carboxyl group of Gly76, activating the C-terminus of ubiquitin. The activated ubiquitin molecule is carried by ubiquitin-conjugating enzyme (E2) and transferred to the substrate lysine residue by ubiquitin-ligases (E3) (Figure 2) [8]. Additional ubiquitin molecules can be added to form polyubiquitin chains. The terminal carboxyl of each ubiquitin is linked to the ε-amino group of a lysine residue of an adjacent ubiquitin in the chain. Ubiquitin can form chains in vivo at all seven lysine residues (K6, K11, K27, K33, K29, K48, K63) (Figure 3) [9]. Polyubiquitin chains linked through K48 are a primary signal for protein degradation [8]. By comparison, K63-linked chains are involved in DNA repair, ribosome function, mitochondrial DNA inheritance, the stress response and targeting of proteins for endocytosis [8]. However, it should be noted that a model substrate tagged with K63-linked tetra-ubiquitin could effectively signal substrate degradation [10]. A chain of at least four ubiquitin moieties attached to a target protein are required for substrate recognition and subsequent degradation by the 26S proteasome [11].

The ubiquitin conjugation cascade contains a large family of E2s and an even larger set of E3s. For example, in budding yeast there is one E1, eleven E2s, and more than twenty E3s [7]. The large number of E3 enzymes may reflect the extraordinary diversity of the ubiquitinated substrates in eukaryotes. All E3 enzymes belong to three protein families: homologous to E6AP carboxy terminus (HECT), really interesting new gene (RING), and UFD2 homology (U-box) proteins. Those E3s share a common E2-binding domain and a substrate-interacting domain. One remarkable feature of the ubiquitin conjugation pathway is the modulation of target protein selection. The substrate specificity depends mainly on the identity of E3 [7]. On the other hand, biochemical studies have shown that the identity of E2 can influence the recognition of specific structures of a polymeric ubiquitin modification [12], indicating that the specificity of the E2/E3 interaction may determine the final selection of the target substrate.

The 26S proteasome is a multimeric protease complex that plays a central role in protein degradation through both ubiquitin-dependent and ubiquitin-independent mechanisms. The 26S proteasome complex consists of a 20S core particle which is proteolytically active, and one or two 19S regulatory caps which are responsible for recognition, unfolding, deubiquitination, and translocation of substrate proteins into the lumen of the core particle (Figure 4) [13].

The 20S proteolytic core consists of four stacked rings with two outer α-rings embracing two central β-rings. The outer rings are each composed of seven different alpha subunits and the inner rings of seven different beta subunits (Figure 5). The overall structure of the 20S core resembles a barrel with dimensions of 15 nm in length and 11 nm in diameter. A central proteolytic chamber is formed by two face-to-face β-rings and is separated by 3 nm wide β-annuli. Three subunits $\beta1$, $\beta2$, and $\beta5$ form the catalytic site. β-subunits gain proteolytic activity by autolytic processing of the N-terminal propeptides and the exposure of a critical threonine residue. Access to the chamber requires reorganization of the N-terminal H0 helices of the α-subunits which normally form a seal by interacting with side chains. The N-terminus of the $\alpha3$-subunit plays a critical role in the seal formation. Addition of the 19S cap can induce channel opening, and the 19S ATPase, Rpt2, plays a key role in this process.

Another component of the 26S proteasome, known as the 19S regulator, which is composed of 17 or 18 subunits, is responsible for recognition, unfolding, deubiquitination, and translocation of substrate proteins into the lumen of the core particle, where the substrate is degraded [13]. In high salt concentrations, the 19S regulator breaks down into two subcomplexes; the lid and the base. The base consists of 6 ATPases (Rpt1 to Rpt6) that share a high level of similarity

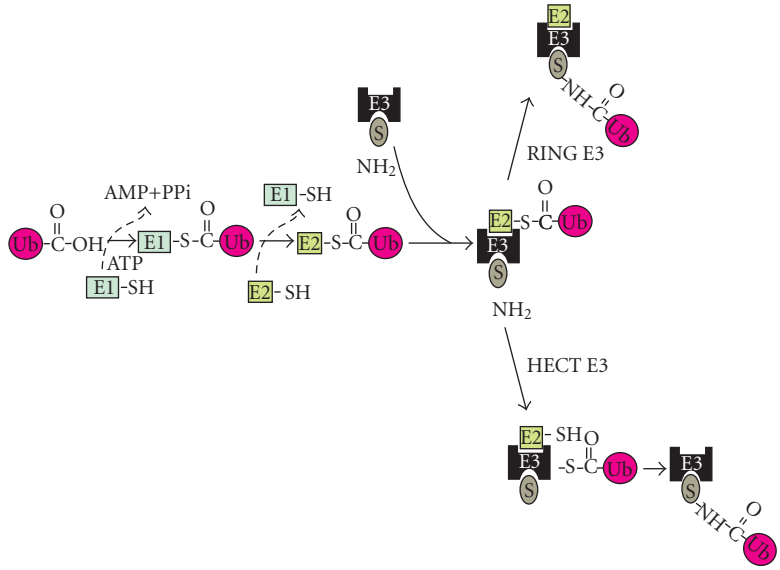

Figure 2: A schematic representation of substrate ubiquitination (see [85]).

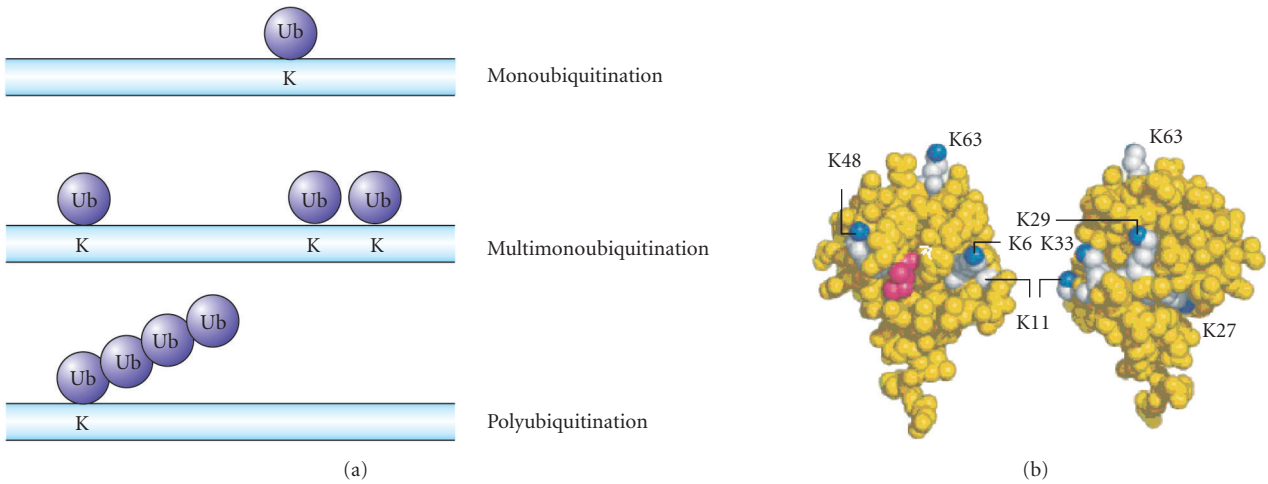

Figure 3: (a) Ubiquitin can be added as a single molecule at one or more sites. Alternatively, the branched chains of polyubiquitin may be formed. (b) Space-filled model of ubiquitin indicating the seven lysine residues (see [86]).

to one another. The ATPases form a six-membered ring that interact directly with the α-ring of the 20S proteasome. Protein substrates need to pass through the center of this ring in order to enter the catalytic chamber of 20S proteasome. In addition, the ATPase ring is involved in the antichaperon activity required to unfold the protein substrates. The base also includes non-ATPase subunits, such as Rpn10 (S5a), which contains an ubiquitin interacting motif (UIM). The lid subcomplex consists of eight non-ATPase subunits, where Rpn11 plays a key role in the recycling of ubiquitin by cleaving the ubiquitin chain from the protein substrate.

In addition to the standard proteasomes, cells are able to produce immunoproteasomes as a transient response to cytokines IFN-γ or TNF-α [14]. IFN-γ induces biosynthesis of proteasome maturation protein (POMP) and proteasomal β5i subunit low molecular weight protein 7 (LMP7), accelerating the assembly of the immunoproteasome in which three catalytic subunits are replaced by homologous subunits (β1i, β2i, β5i) [15]. The immunoproteasome may participate in generating antigenic peptides displayed on MHC-class I molecules [16], but are not limited to this function. Recent observations reveal that in familial amyotrophic lateral sclerosis (fALS) patients, impaired degradation of mutant SOD1 is associated with a decrease in the constitutive proteasomes and an increase in the immunoproteasome level, resulting in selective motor neuron degeneration [17]. In Huntington's disease (HD), high levels of immunoproteasome subunits (LMP2 and LMP7) have also been observed, and are associated with neurodegeneration, indicating that immunoproteasomes may play a yet to be

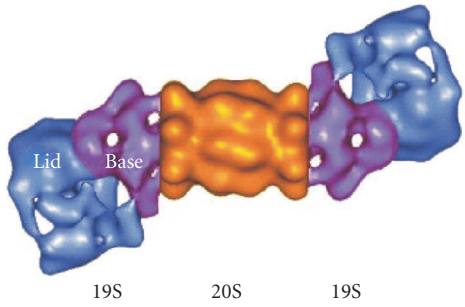

Figure 4: The structure of the 26S proteasome complex. (http://www.biw.kuleuven.be/dtp/cmpg/pgprb.htm.)

defined role in the pathogenesis of neurodegenerative diseases [18].

TARGETING POLYUBIQUITINATED PROTEINS FOR TURNOVER

Polyubiquitin chains are a signal that targets proteins for degradation by the proteasome complex. Recognition of polyubiquitinated proteins by the 26S proteasome plays a critical role in protein degradation. Presentation of a polyubiquitinated substrate to the 26S proteasome takes place through ubiquitin-interacting proteins, such as S5a, Dsk2, Rad23, and p62 [19, 20] (Figure 6). Ubiquitin-interacting proteins involved in ubiquitination/deubiquitination generally have ubiquitin-association (UBA) domains that can directly bind to ubiquitin (Figure 7). Previous studies have shown that most UBA domains bind the polyubiquitin chains rather than the monoubiquitin ones. Some UBA domains even discriminate further binding K63-linked polyubiquitin chains rather than K48-linked chains [21]. However, the interaction between ubiquitin and the UBA domain is a low-affinity interaction. NMR chemical shift mapping shows that ubiquitin specifically, but weakly, binds to a conserved hydrophobic epitope on the UBA domain, while the UBA domains can bind to the hydrophobic patch on the surface of the five-stranded β-sheet of ubiquitin with different orientations [22]. The weak interaction may enable rapid assembly and disassembly between polyubiquitin and the shuttling protein. UBA-containing proteins might contribute to the regulated capture and transient stabilization of proteins that are otherwise constitutively degraded [23]. Recent findings have shown that a functional UBA domain is required for the localization of these shuttling proteins into aggregates [24], suggesting a common mechanism of ubiquitin-mediated sequestration of essential ubiquitin-binding proteins into aggregates.

In addition to the UBA domains that bind the polyubiquitin chains, shuttling proteins commonly contain a ubiquitin-like domain (UbL) that binds the proteasome [20, 25, 26]. These proteins are able to shuttle the polyubiquitinated substrates to the 26S proteasome for degradation [27]. Ataxin-3, a proteasome-associated factor, has been shown to interact with the shuttling protein Rad23 to mediate the degradation of ubiquitinated substrates, suggesting an important role for shuttling proteins in the UPS [28]. Since each type of polyubiquitin chain forms a different conformation [29], and each UBA domain may recognize specific types of polyubiquitin chains, shuttling the protein may present chain-specific polyubiquitinated substrates to the proteasome for degradation. A recent study of the polyubiquitin interaction properties of thirty UBA domains reveals that these domains can be classified into four groups [30]: those with linkage specific characteristics, those which bind different chains, those which are nondiscriminatory, and those which do not bind ubiquitin. Moreover, it is possible that non-UBA sequences may modulate interaction properties in the UBA domain [30].

PROTEIN AGGREGATES AND NEURODEGENERATION

Protein turnover is dependant on a functional UPS. Failure to remove the polyubiquitinated proteins may lead to the accumulation of aggregated proteins [31]. The capacity of the ubiquitin proteasome pathway can be exceeded either by overexpression of substrates or by a decrease in proteasome activity. In cultured cells, proteasomal inhibitors can cause the aggregation of an overexpressing disease-associated protein [32], indicating that dysfunction of the proteasome might be a factor that initiates the formation of inclusions. A recent study has shown that a wide-range of nondisease-associated proteins is found in inclusions when cells were treated with proteasomal inhibitors. These proteins include ubiquitinated or nitrated α-tubulin, SOD-1, α-synuclein, and 68K neurofilaments [33]. Transient expression of two unrelated aggregation-prone proteins caused nearly complete inhibition of the UPS, indicating that protein aggregation can directly impair the UPS function [34]. A positive-feedback mechanism has been proposed to explain the turnover point of protein aggregation. Impaired proteasome function may result in an increase in protein aggregates, which leads to a further decline of proteasome activity. It is important to note that UPS impairment is not the result of steady-state sequestration of the UPS components, or simple substrate competition [35]. Direct physical interaction between proteasome and aggregates is not required for UPS impairment, indicating that protein aggregates may influence the activity of the proteasome in a currently unknown manner.

Aggregated proteins may sequester to form inclusions also referred to as an aggresome, where molecular chaperones, proteasome subunits, ubiquitin, and intermediate filament (IF) proteins colocalize [3]. The formation of aggresomes occurs at the microtubule organizing center (MTOC) and is considered to be a process distinct from the protein aggregation [36, 37]. The formation of cytoplasmic inclusion bodies requires active transport of misfolded proteins along microtubules, with redistribution of the IF protein to form a cage surrounding the core of aggregated, ubiquitinated protein [31]. Protein misfolding can be prevented or even reversed by chaperones. If chaperone activity fails, then the proteins must be degraded before aggregation takes place.

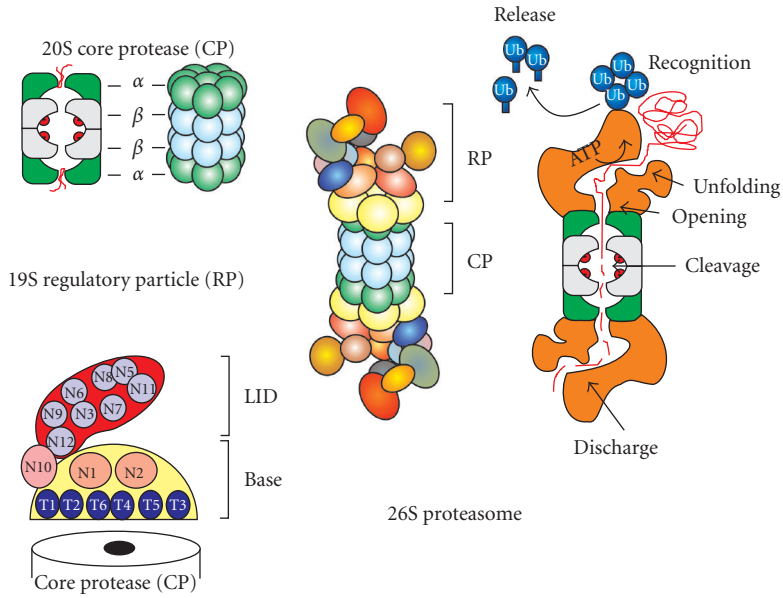

Figure 5: The structure of the 20S core and 19S regulatory particle. (http://plantsubq.genomics.purdue.edu/plantsubq/html/guide.html.)

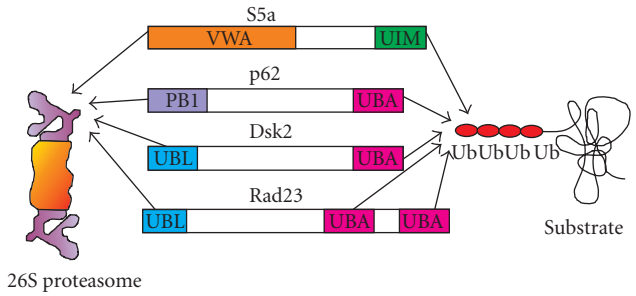

Figure 6: Presentation of ubiquitinated substrates to the 26S proteasome (see [87]).

Thus, aggresomes may serve as sites to sequester polyubiquitinated/misfolded proteins. In this regard, the UPS functions as a sensor to control degradation of misfolded proteins that tend to aggregate through exposure of hydrophobic sequences [31]. Should excessive amounts of protein aggregates accumulate, they may negatively impair the function of the UPS [34, 35]. Thus, it is critical to sequester aggregated proteins so as to preserve the UPS function.

Recent evidence has shown that early protein aggregates may be toxic to neuronal cells. Soluble dimers and trimers, and protofibrils or fibrils of amyloid beta (Aβ) peptide are cytotoxic [38]. On the other hand, although the early prefibrillar disease-associated protein aggregates are harmful to cells, the mature fibrils are relatively harmless [39]. In addition, a previous study reveals that UPS impairment is independent of inclusion body formation [35]. Altogether, these observations suggest that the inclusions/aggresome pathway are likely to be cytoprotective by recruiting misfolded proteins effectively isolating them within the cell. In further support of this mechanism, it has been reported that inclusion body formation predicts improved survival and leads to decreased levels for the highly aggregating protein huntingtin [40].

The accumulation of protein aggregates and formation of inclusion bodies are associated with many age-related neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, and others, suggesting that there are direct links between protein aggregates and the resulting pathology [4] (Figure 8). The accumulation of ubiquitin conjugates may reflect the failed attempt of the UPS to remove damaged proteins [41]. An important component linked to aberrations in UPS and to the pathogenesis of Parkinson's disease is Parkin, an E3 ubiquitin ligase [42]. A previous report reveals that Parkin can associate with Rpn10 (S5a), a subunit of the 26S proteasome, indicating that Parkin may transfer ubiquitin conjugates for proteasomal degradation [43]. Defects in Parkin may result in the accumulation of its substrates, such as α-synuclein, and contribute to the pathogenesis of PD.

There is a strong evidence demonstrating that proteasome inhibition by pharmacological treatment enhances inclusion formation in cellular models. However, there is less support for the notion that aggregates directly inhibit the proteasome in any disease state. More studies are needed in animal models to critically evaluate the role of protein aggregates and inclusions on proteasome function.

SEQUESTOSOME 1/P62, TRAFFICKING, AND INCLUSION FORMATION

Other aggregate-prone proteins participate in neurodegenerative diseases such as Alzheimer's disease (AD) (Figure 8). There are two types of protein deposits in AD: extracellular amyloid plaques rich in Aβ peptides, and intracellular

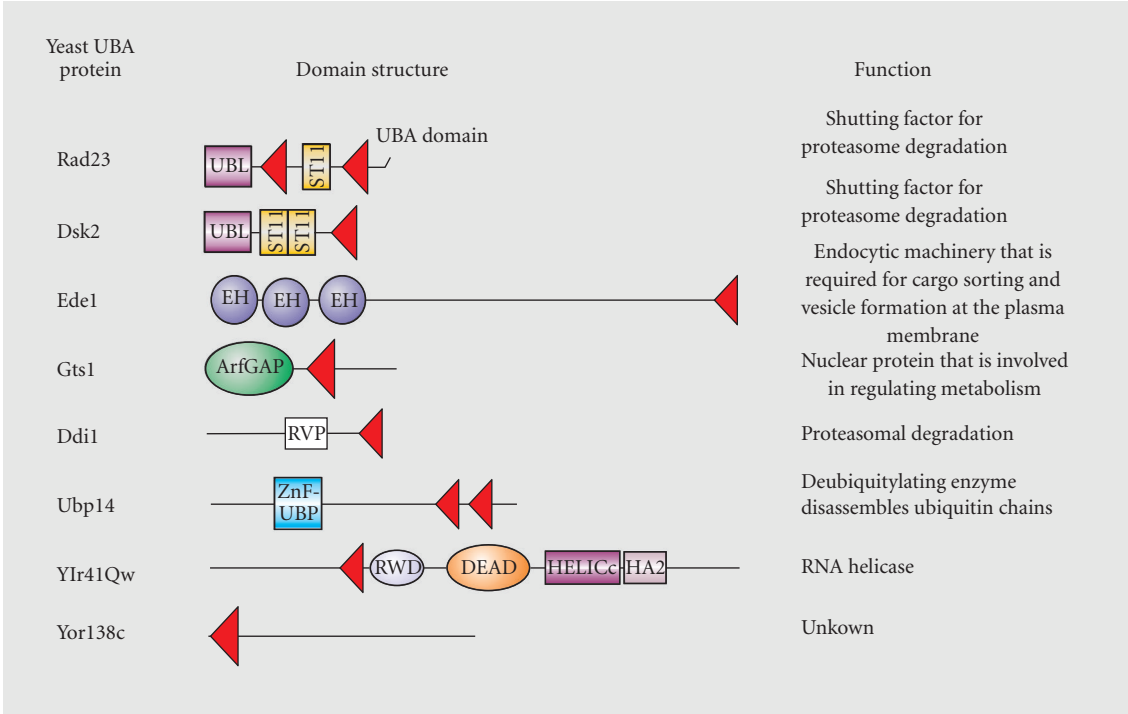

Figure 7: Proteins containing UBA domains have diverse structure and function (see [86]).

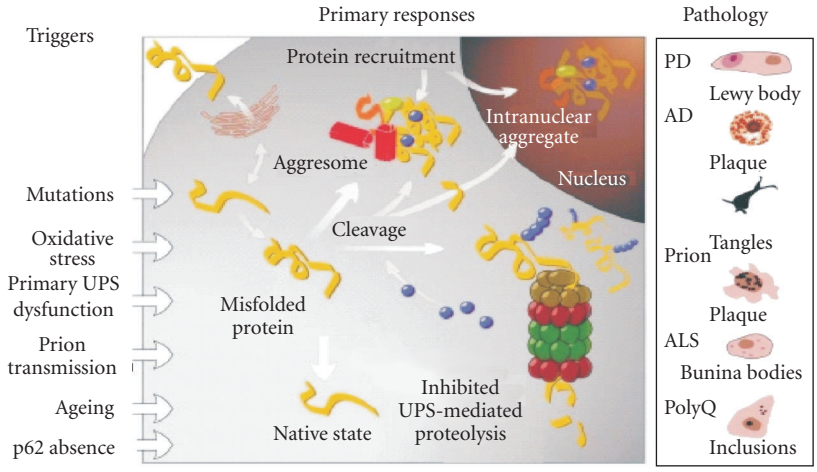

Figure 8: The UPS and pathogenesis of neurodegeneration (see [41]).

neurofibrillary tangles containing hyperphosphorylated polyubiquitinated tau [44]. Previous study in our lab has shown that p62 can shuttle K63-polyubiquitinated tau for proteasomal degradation. Disturbing tau trafficking may result in the accumulation of insoluble/aggregated tau in the brain, contributing to AD [45]. Aβ peptides are produced by proteolytic cleavage of the amyloid precursor peptide (APP). In solution, Aβ peptides may undergo self-assembly leading to the transient appearance of soluble protofibrils and eventually to insoluble fibrils [46]. Recent proteomic study of amyloid plaques recovered from AD brain revealed that a total of 488 proteins coisolated with plaques. Moreover, 26 proteins were enriched in plaques by comparison with surrounding non-plaque tissues, including proteins involved in cell adhesion, cytoskeleton and membrane trafficking, chaperones, kinase/phosphatase, and regulators [47].

Sequestosome 1/p62 is a highly conserved protein that was initially identified as a phosphotyrosine-independent ligand of the src homology 2 (SH2) domain of $p56^{lck}$ [12]. Sequestosome 1/p62 contains a ubiquitin-associated (UBA) domain at its C-terminus (Figure 9), which can selectively bind K63-polyubiquitinated proteins [20]. The ability of p62

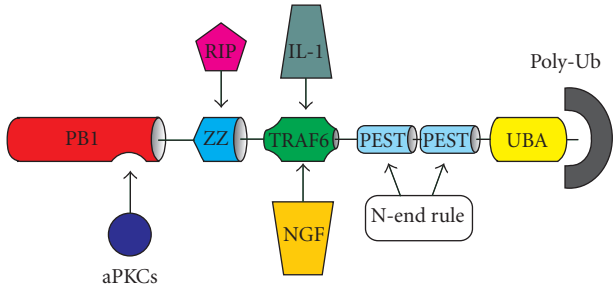

Figure 9: A schematic diagram showing the domain organization of p62 protein.

to interact selectively with K63-polyubiquitinated proteins [20] supports the idea that sequences in the holoprotein may modulate the UBA interaction properties [30]. The UBA domain of the human p62 protein forms a compact three-helix bundle. A $Pro^{392} \rightarrow$ Leu substitution mutation can modify the UBA domain by extending the N terminus of helix 1. This modification affects interaction of p62's UBA domain with polyubiquitin chain binding, and may contribute to Paget's disease of bone [49]. In addition, recent studies reveal that p62 protein lacking a UBA domain fails to form aggregates in HEK cells with impaired survival responses. This indicates that the UBA domain is critical for sequestering polyubiquitinated proteins [20], which is in keeping with a role for sequestration of polyubiquitinated proteins as an underlying contributor to inclusion formation [24].

Sequestosome 1/p62 also contains a PB1 domain that binds aPKC, a ZZ finger, a binding site for the RING finger protein TRAF6, and two PEST sequences (Figure 9) [48]. With multiple protein-protein interaction motifs, p62 is also considered a scaffold [48], and has been extensively studied in the context of neurotrophin signaling. The N-terminus of p62 protein can directly interact with the proteasome subunit component [20], and the localization of protein substrates to the proteasome is sufficient for degradation [50]. Thus, p62 is also viewed as a shuttling protein, playing an important role in sequestering polyubiquitinated substrates, interacting with ubiquitinated substrates through its UBA domain and the proteasome through its N-terminus [51]. In support of a shuttling role for p62, we have observed that p62 is necessary for both tau and TrkA interaction with the proteasome [45, 51]. In addition, we find that p62 $-\backslash-$ mice possess AD-like neurodegeneration [Babu and Wooten, *unpublished*]. Likewise depletion of nerve growth factor (NGF) results in AD-like neurodegeneration in anti-NGF transgenic mice [52]. Interestingly, decreased membrane TrkA expression has been correlated with decline in performance on the mini mental state exam and may serve as a marker for late stage AD [53]. The correlation between the phenotype of the p62 $-/-$ mice and the anti-NGF mice is suggestive of an overlap in pathways wherein p62 and NGF function. Further studies will be needed to sort out the exact mechanism whereby p62 regulates the trafficking of TrkA.

The N-terminal PB1 domain of p62 is involved in p62 self-interaction, and in interaction with other proteins that possess a PB1 domain [54]. However, the PB1 domain can assume a ubiquitin fold and this may be the basis of the N-terminus of p62 interacting with the proteasomal subunit S5a [20]. Overexpression of p62 results in large inclusions, while depletion of p62 retards protein degradation and leads to accumulation of nondegraded aggregated polyubiquitinated proteins [20]. We have shown that cells which possess p62 inclusions possess enhanced survival characteristics [55]. This finding supports the growing idea that inclusions are sites for sequestration of misfolded proteins that are being triaged for degradation. In this regard, p62 has been localized to ubiquitin containing inclusions in Alzheimer's disease containing tau [56]. Since p62 has been shown to be necessary for tau interaction with the proteasome [45], it is possible that polyubiquitinated tau may accumulate in the absence of p62. Studies are underway to examine p62's role in tau trafficking. Culture of mouse embryo fibroblasts from wild type or p62 $-/-$ mice challenged with either chloroquine, a lysosomal inhibitor, or MG132, a proteasomal inhibitor, reveals that p62 is necessary for inclusion formation under proteasome impaired conditions (Figure 10). Altogether, these findings suggest that p62 plays a key role in trafficking, regulation of aggregation and inclusion body formation. It is likely that p62 containing inclusions observed in AD and other neurodegenerative diseases contain proteins destined for degradation. In the absence of p62 these proteins would be expected to accumulate in their misfolded polyubiquitinated state and contribute to neurodegeneration.

The genomic structure of p62 reveals the presence of a CpG island and multiple binding sites for SP-1, AP-1 NF-κB, and Ets-1 family transcription factors in the promoter region, suggesting that p62 transcription may be regulated by these factors [57]. In this regard either inhibition of the proteasome or increases in free radicals have been shown to induce p62 expression [58]. Moreover, inhibition of p62 transcription blocked proteasomal-induced sequestration of ubiquitinated proteins, and the enlargement of inclusions [59]. These studies support the idea that p62 is intimately involved in the formation of inclusions and in the protection of cells from the toxicity of misfolded proteins by enhancing inclusion formation [20, 55]. Thus, inclusions may arise as a protective mechanism against stress conditions. Indeed, we find that cells overexpressing p62 possess large inclusions and enhanced survival [20, 55].

Sequestosome 1/p62 may also act as a scaffold of TRAF6 [48, 60]. In this regard, p62 could serve as a site for TRAF6 dependent K63-polyubiquitination of target substrates and in the activation of transcription factor NF-κB. Interestingly, TRAF6 colocalizes into inclusions along with p62 [20]. A recent study in our lab has shown that the p62-UBA domain is required for TRAF6 polyubiquitination, suggesting that p62 may carry ubiquitin chains needed for the activation/autoubiquitination of TRAF6 [60]. When the interaction between p62 and TRAF6 was disrupted by competitive inhibitory peptide, the formation of p62/TRAF6 inclusions in cultured cells was suppressed and survival diminished [20, 45]. Therefore, p62 regulates activation of NF-κB through

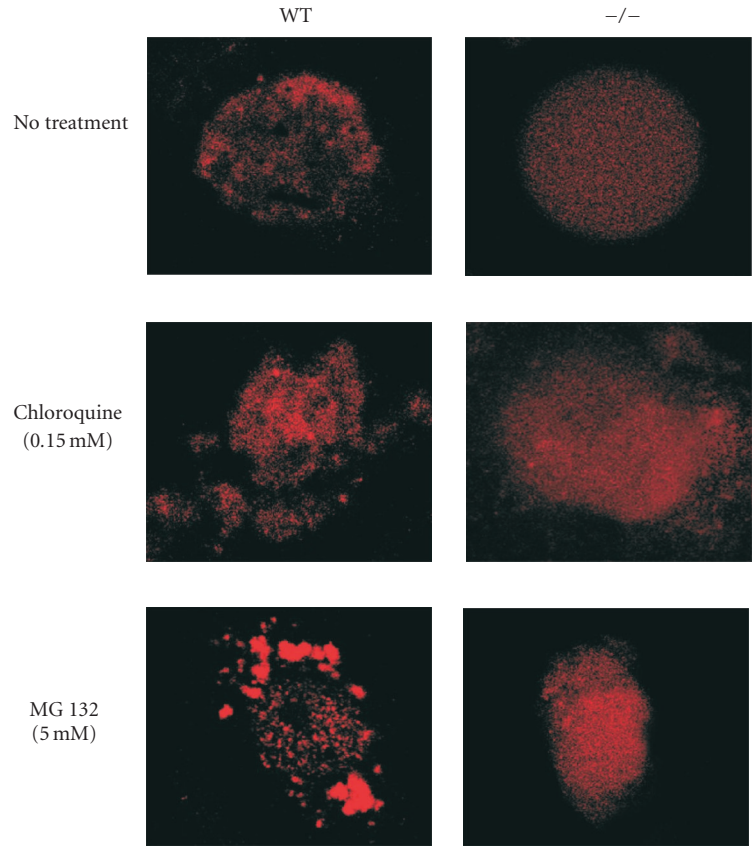

Figure 10: Mouse embryo fibroblasts (MEF) from wild type (WT) or p62 −/− mice were treated with chloroquine or MG132 as shown. After 24 hours, the cells were fixed and stained with primary antibody to ubiquitin and secondary antibody to Texas Red.

recognition of TRAF6-catalyzed polyubiquitin chains and/or recruitment of TRAF6 to a microenvironment to enhance protein ubiquitination.

In addition, p62 can form a ternary complex with aPKC and PAR-4 (a stress induced transcript) [61]. Interaction of aPKC with PAR-4 blocks its activity; however, p62 can antagonize PAR-4-induced aPKC inhibition [61]. Moreover, recent studies have shown that p62 can modulate association of Akt with aPKC [62]. Thus, p62 through interaction with aPKC likely regulates cell survival and death signaling through aPKC/PAR-4 [61] and aPKC/Akt [62]. Also, PAR-4 has recently been shown to interact with BACE and regulates the production of Aβ peptide [63].

Therefore, it would not be surprising to observe that in situations where PAR-4 is induced during oxidative stress or injury that low expression of p62 might compromise the neuron and contribute to development of neurogeneration. Excessive accumulation of misfolded proteins is known to provoke oxidative stress and induce PAR-4 expression [61]. Moreover, oxidatively modified proteins are resistant to proteolysis and may further enhance accumulation of aggregated proteins [64]. We hypothesize that decline in expression of p62 could serve as a biomarker for those individuals at risk for developing neurodegenerative disease. In this model, aging and oxidative stress along with diminished expression of p62 would define a threshold where proteins fail to properly fold or triage for degradation, survival signaling is impaired, and the neurodegenerative disease phenotype is manifested. Interestingly mutation in valsolin-containing protein (VCP), a ubiquitin binding protein involved in UPS trafficking, has been linked to frontotemporal dementia [65]. We speculate that p62, as well as other ubiquitin binding proteins, may be candidate-genes for detailed genetic analysis. The goal here would be to examine potential polymorphisms that may serve as risk determinants for neurodegenerative disease. In keeping with this hypothesis, recent genetic analysis of the ubiquilin 1 gene (UBQLN1) revealed that certain genetic variants increase the risk of AD [66]. Similar to VCP and p62, UBQLN1 encodes a protein that serves as a shuttling protein to deliver polyubiquitinated proteins to the proteasome for degradation.

RELATIONSHIP OF MALLORY BODIES TO INCLUSION BODIES

Mallory bodies (MB) are a disease-associated type of aggresomes/inclusion consisting of excessive accumulation of keratin and are characteristic of alcoholic steatohepatitis (ASH) and nonalcoholic steatophepatitis (NASH). These inclusion share abnormal liver morphology observed in Wilson's

disease (WD), Indian childhood cirrhosis (ICC), and idiopathic copper toxicosis (ICT) [67]. ICC and ICT individuals develop copper induced cirrhosis of the liver by consumption of contaminated water or milk [67]. WD mutations in ATP7B lead to abnormal copper accumulation in various tissues, particularly the liver [67]. Copper-mediated oxidative stress may also play a pathogenic role in chronic neurodegenerative diseases such as Alzheimer's disease. Sequestosome 1/p62 is an integral component of MB [68] and of the Mallory like-inclusions found in WD, ICC, and ICT, suggesting that p62 may play a role in their formation. The formation of MBs can be induced by prolonged alcohol intoxication and chronic metabolic disturbances [68]. Impaired protein degradation has been implicated as an underlying factor in alcoholic liver disease similar to its role in chronic neurodegenerative disorders [67–69]. Moreover, removal of p62 blocked the formation of MBs, whereas over expression enhanced their formation [69]. MBs contain high molecular weight polyubiquitin conjugates as well [69], which suggest that these are sites for sequestration of polyubiquitinated/misfolded proteins. As previously mentioned, p62 expression is transcriptionally regulated, in particular to agents of oxidative stress [58, 59]. Oxidative stresses causing alterations in mitochondria are well recognized as contributors to Alzheimer's disease [70, 71]. Oxidatively damaged mitochondria are removed by a process of autophagocytosis [72], a process that declines with age. Altogether, these findings strongly suggest that p62 plays a role in the formation of MBs which may have similarity to inclusion bodies observed in neurodegenerative disease.

AUTOPHAGY AS A ROUTE TO PROTEIN DEGRADATION

The cellular trafficking network that takes place involves movement of proteins from one intracellular compartment to another. In some instances receptor proteins in late endosome are deubiquitinated by chain-specific deubiquitinating enzymes at that site [73], while in others it appears that proteins traffic to the proteasome for chain removal and are then transported to the lysosome for degradation [74]. Sequestosome 1/p62 has been shown to be a component of the late endosomes [75, 76], and is able to sort proteins, such as TrkA to the endosome [77]. Under stress conditions late endosomes may fuse with autophagosomes [78], a process that involves bulk phase sequestration of cytoplasmic proteins. Aggregated proteins can be removed by the process of autophagy [79], a process that is impaired in AD [80]. Since the UPS may be impaired by protein aggregates [34, 35], it is reasonable to propose that autophagy could serve as a mechanism that cells hold in reserve for the removal of protein aggregates. In this regard α-synuclein can be degraded by both UPS and autophagy [81]. Therefore as aggregates arise, cells could degrade these proteins by autophagy while preserving the function of the UPS. Numerous studies now indicate that cells may attempt to compensate for impairments in one form of proteolysis (UPS) by dramatically elevating an alternate form of protein degradation (autophagy) [82]. Recently, p62 has been shown to bind light chain 3 (LC3), a protein that is tightly associated with the autophagosomal membrane [83]. Under stress conditions p62 would thereby link polyubiquitinated aggregated proteins to the autophagic machinery, facilitating their clearance. Indeed this has been found to be the case for clearance of mutant huntingtin [83]. Since p62 is localized to late endosomes [75, 76], p62 through interaction with LC3 may be needed in the recruitment of proteins for autophagy. Therefore decline in p62 expression would not only lead to the accumulation of polyubiquitinated proteins, but also to an absence of autophagosomes which in the appropriate genetic environment may further contribute to an absence of inclusions and accumulation of misfolded proteins. Currently we are studying whether neurons isolated from p62 $-/-$ mice fail to form autophagosomes and the effects this may have.

FUTURE DIRECTIONS FOR THERAPEUTIC TARGETS

Recent targets for therapy include reducing protein misfolding and blocking aggregation. Strategies that promote degradation of misfolded proteins, such as: (1) enhanced expression of chaperones; (2) overexpression of E3 ligases; (3) enhanced expression of shuttling proteins, such as p62; and last, (4) up regulation of proteasome activity and/or autophagy Since protein oxidation and aggregation are intimately linked [64, 70], it is likely that more than one approach will be needed to effectively remove aggregated proteins and treat neurodegeneration. Clearly, early diagnostic markers are needed to effectively time intervention and treatment.

CONCLUSIONS

Great strides have been made in the past ten years toward understanding the pathological, cellular, biochemical, biophysical, and molecular bases of targeting proteins for degradation. Two mechanisms have been studied which promote the removal of aggregated/misfolded/polyubiquitinated proteins: the UPS and autophagy. Both mechanisms are regulated by p62. The observation that p62 plays an intimate role in the regulation of protein signaling, polyubiquitination, and trafficking suggests that further study of its role as a regulator of oxidative stress, neuropathology, and neurotoxicity in the brain is warranted. There is clearly a need to learn more about p62 in the context of aging, genetic background, and environmental factors. The convergence of these elements will determine the onset and severity of neurodegeneration. For AD, the greatest risk factor for the disease is age. The known AD genes (PS1, PS2, APOE, APP) account for less than half the genetic variance in the disease, suggesting there are many other risk determinant genes yet to be identified. Altogether, these findings will contribute to the development of more effective means for treating AD and for assessing those who might benefit from therapeutic intervention.

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Research Article

Parkinson's Disease in Relation to Pesticide Exposure and Nuclear Encoded Mitochondrial Complex I Gene Variants

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Parkinson's disease (PD) is a common age-related neurodegenerative disorder thought to result from the integrated effects of genetic background and exposure to neuronal toxins. Certain individual nuclear-encoded mitochondrial complex I gene polymorphisms were found to be associated with ∼ 2-fold risk variation in an Australian case-control sample. We further characterized this sample of 306 cases and 321 controls to determine the mutual information contained in the 22 SNPs and, additionally, level of pesticide exposure: five distinct risk sets were identified using grade-of-membership analysis. Of these, one was robust to pesticide exposure (I), three were vulnerable (II, III, IV), and another (V) denoted low risk for unexposed persons. Risk for individual subjects varied > 16-fold according to level of membership in the vulnerable groups. We conclude that inherited variation in mitochondrial complex I genes and pesticide exposure together modulate risk for PD.

INTRODUCTION

Parkinson's disease (PD) is a common age-related neurodegenerative disorder thought to result from the integrated effects of genetic background and exposure to neuronal toxins (eg, MPTP, rotenone and other insecticides). Rarely, it is caused by mendelian mutations (*α-synuclein*, *DJ-1*, *PINK1*, and *LRRK2*). Tremor, rigidity, slowness of movement and postural instability are the predominant symptoms due to the selective loss of pigmented dopamine-producing neurons in the substantia nigra pars compacta region of the brain. Whether familial, age-related, or consequent to neuronal toxin exposure, impaired mitochondrial complex I (NADH:ubiquinoneoxidoreductase; EC 1.6.5.3) is found [1–17]. Mitochondrial complex I consists of at least 46 subunits, seven encoded in the mitochondrial genome and the remainder, at least 39, encoded in the nucleus.

One testable hypothesis is that PD risk is modulated by inherited sequence variation in complex I genes. Mellick et al [1] addressed this possibility: they screened for single nucleotide polymorphisms (SNPs) in nuclear encoded complex I genes. A total of 22 SNPs (16 genes) polymorphic among Australians were investigated (306 PD patients; 321 control subjects). Statistically significant associations, and ∼ 2-fold variation in risk, were observed for *NDUF* genes *A*1, *A*10, *A*6, and *S*4, when taken individually. None of these associations would have survived correction for multiple comparisons. Although information on pesticide exposure, sex, and age at onset was available, it was not used in the association analysis due to the limited sample size and the larger number of multiple comparisons that would have been generated.

We extend this work by jointly investigating the 22 SNP genotypes found for these 306 PD patients and 321 control subjects, avoiding multiple comparisons, and considering level of pesticide exposure and age at onset. The goals were to identify combinations of alleles robust to pesticide exposure, others that are especially vulnerable, and to quantify risk for individuals. This was accomplished using grade-of-membership analysis or GoM.

Using GoM, two sets of parameters are simultaneously estimated by maximum likelihood (see "Methods"). One set represents a specified number of extreme pure type groups. Here, each of the five groups has distinct frequencies for the SNP genotypes, level of toxin exposure, and PD status according to age. The other set of parameters represents the degree of similarity of each subject to the groups. These

graded membership scores range from zero, that is, denoting no resemblance to the group, to one, that is, the individual matches the group exactly, and sum to one for each person. The scores can be input into logistic models to quantify disease risk and produce 95% CI. This approach has identified highly predictive sufficient genetic risk sets for Alzheimer's disease [18] and multilocus genotypes specific to breast cancer and fibroadenoma [19].

Five model-based groups relevant to PD status were identified. Of these, one set of complex I polymorphisms was robust to pesticide exposure (I), three sets were vulnerable (II, III, IV), and another (V) denoted low risk for unexposed persons. Risk for individuals varied > 16-fold according to level of membership in the vulnerable groups. We conclude that inherited variation in mitochondrial complex I genes and pesticide exposure together modulate risk for PD.

METHODS

The specific details of the case-control sample and the genotyping methods used have been reported previously [1].

Study subjects

Onset ranged over six decades of age among the 306 case subjects (decade of age: %): 30–39: 3%, 40–49: 12%, 50–59: 31%, 60–69: 36%, 70–79: 16%, 80+ : 2%. The cases did not have symptoms of other neurological conditions, for example, change in cognition suggestive of Alzheimer's disease onset. The 321 control subjects did not have any symptom of parkinsonism (decade of age: %): 30–39: 1%, 40–49: 6%, 50–59: 22%, 60–69: 36%; 70–79: 31%, 80+ : 4%.

Environmental exposures

A structured questionnaire was used to probe for exposures to environmental toxins including insecticides, herbicides, fungicides, solvents, heavy metals [20]. However, this study limited exposure assessment to self-reported exposures to pesticides (ie, insecticides, herbicides, and fungicides). Responses were coded as 0 = no exposure, 1 = limited exposure, and 2 = regular exposure at least weekly for six months. Pesticide exposure was more common among men and cases (men: 63% vs 55%; women: 46% vs 39%), especially regular exposure (men: 20% vs 9%; women: 5% vs 3%).

Genetic determinations

SNPs in nuclear genes that encode mitochondrial complex I proteins were identified from the HGVbase as of July 2001 [1]. The 22 of 70 identified SNPs polymorphic among 16 randomly selected healthy Australian subjects are investigated (Table 1). SNP determinations were made using the DASH method [21, 22]. The major allele at each locus was coded as "*a*" and the minor allele "*b*" yielding genotypes *aa*, *ab*, and *bb*. Multilocus genotypes were coded, for example, *aa:ab*, for genes having more than one SNP. Infrequent (0.018) missing values were ignored in the data analysis.

Table 1: The genes and SNPs investigated.

Number	Gene	SNP (HGV base ID)
1	DLST	SNP000002340 (A/G)
2	NDUFA1	SNP000005157 (G/C)
3	NDUFA1	SNP000008196 (T/C)
4	NDUFA1	SNP000008197 (T/G)
5	NDUFA10	SNP000015174 (G/A)
6	NDUFA10	SNP000020002 (A/G)
7	NDUFA6	SNP000005146 (C/T)
8	NDUFA7	SNP000005158 (C/T)
9	NDUFA8	SNP000005147 (A/G)
10	NDUFA8	SNP000008968 (G/A)
11	NDUFB4	SNP000019034 (C/T)
12	NDUFB7	SNP000005144 (C/G)
13	NDUFB8	SNP000005127 (C/A)
14	NDUFB9	SNP000005142 (C/T)
15	NDUFS1	SNP000005158 (G/T)
16	NDUFS1	SNP000005159 (A/G)
17	NDUFS2	SNP000018866 (T/A)
18	NDUFS4	SNP000005133 (A/G)
19	NDUFS4	SNP000005178 (G/A)
20	NDUFS7	SNP000005156 (T/C)
21	NDUFS8	SNP000005155 (C/T)
22	NDUFV2	SNP000000182 (C/T)

The data analytic approach

Detailed clinical genetic profiles were identified using grade-of-membership analysis or GoM [23–25]. Case subjects were considered according to age (< 60, 60–69, 70+) and environmental exposure $(0, 1, 2)$; control subjects were coded according to exposure regardless of age, that is, 12 categories total.

GoM can be described after first identifying four indices. One is the number of subjects I $(i = 1, 2, \ldots, I)$. Here, $I = 627$ subjects were identified. The second index is the number of variables J $(j = 1, 2, \ldots, J)$. There are $J = 17$ variables. Our third index is Lj: the set of response levels for the Jth variable. This leads to the definition of the basic GoM model where the probability that the ith subject has the Ljth level of the Jth variable is defined by a binary variable (ie, $y_{ijl} = 0, 1$). The model with these definitions is

$$\text{Prob}\left(y_{ijl} = 1.0\right) = \sum_{k} g_{ik}\lambda_{kjl}, \tag{1}$$

where the g_{ik} are convexly constrained scores (ie, $0.0 \le g_{ik} \le 1.0; \sum_k g_{ik} = 1.0$) for subjects and the λ_{kjl} are probabilities that, for the Kth latent group, the Ljth level is found for the Jth variable. The procedure thus uses this expression to identify K profiles representing the pattern of $J \times Lj$ responses found for I subjects.

The parameters g_{ik} and λ_{kjl} are estimated simultaneously using the likelihood function (in its most basic form).

$$L = \prod_i \prod_j \prod_l \left(\sum_k g_{ik} \cdot \lambda_{kjl} \right)^{y_{ijl}}. \quad (2)$$

In the likelihood y_{ijl} is 1.0 if the Ljlth level is present and 0.0 if it is not present. Decade of age provided starting values.

Information on sex was available to further characterize the groups. One option in the likelihood is to separate calculations for "internal" (here, clinical and genetic) and "external" (here, sex) variables. For internal variables, maximum likelihood estimations (MLE) of g_{ik} and λ_{kjl} are generated and the information in internal variables is used to define the K groups. For external variables the likelihood is evaluated (and MLE of λ_{kjl}; generated) but the information is not used to redefine the K groups, that is, the likelihood calculations for likelihood equations involving the g_{ik} are disabled for external variables so that the g_{ik}, and the definition of the K groups, is not changed.

Next, three age-specific logistic models (< age 60, 60–69, 70+) were constructed to estimate the risk for PD according to membership in the vulnerable groups II, III, and IV. For this use, the graded membership scores were categorized from 1 (< 0.20 membership) to 5 (> 0.80 membership) representing 0.20 increments.

RESULTS

Five GoM groups represent the data, displayed in Table 2. Group I was robust to toxin exposure. Groups II, III, and IV were vulnerable. Group V had limited toxin exposure and was at low risk. Each group had a distinctive set of SNP genotypes for nuclear genes that encode mitochondrial complex I subunits.

Robust to pesticide exposure (I)

Low risk for PD despite limited toxin exposure carried a distinctive genetic signature of infrequent genotypes: X-linked *A1 aa:ab:ab* or *bb:aa:aa*, *A6 bb, A8 bb:a-*, *A10 ab:aa*, *B4 ab*, *B8 ab* or *bb B9 ab*, *S1 ab:aa*, and *S4 bb:ab*. The group consisted predominantly of females (84% chance). There was some chance of being affected at ages 70 or older after regular exposure to toxins despite this protective signature.

Early onset, regular exposure (II)

Group II was affected before age 60 and vulnerable to regular pesticide exposure. Its genetic signature consisted of *A6 aa*, *A8 ab:ab, A10 aa:bb*, *S1 bb:a*, the common *S2 ab* genotype found also for group I, and *DLST bb.*

Early onset PD, limited exposure (III)

Group III also had high risk for PD with onset before age 60(86%), at limited pesticide exposure (43%). This vulnerability was associated with *A6 ab*, *B7 bb*, and *DLST aa*. Homozygous *V2 bb* was found (22%) for this group only.

Late onset PD, limited exposure (IV)

Group IV was at risk for PD (63%) at ages 60 to 69, at limited pesticide exposure (70%). It had genetic signature *A8 aa:aa, B7 ab, S1 bb:bb*, *S2 bb*, *S4 bb:aa*, and *S8 bb*.

Low risk, no exposure (V)

Group V represents low risk for PD when not exposed to pesticides. Two SNP genotypes stand out as determinants: *V2 ab* (QRF = 1.47—the highest genetic influence score) and *S4 aa:aa* (QRF = 1.25). QRF stands for "question relevance score" denoting the relative importance of the variable in determining the group.

Informative variables

No one genetic variable dominated. An information statistic H, related to Shannon's information statistic (Bell Laboratories) was estimated for each variable: values close to zero indicate that the variable was not useful. Three of four SNPs deemed statistically significant in chi-square analyses ($A6 : \mathbf{H} = 0.92$, $A10 : \mathbf{H} = 0.68$, $S4 : \mathbf{H} = 1.13$) [1] were identified as being highly informative. The fourth, $A1$, had limited heterozygosity and low H score (0.19). Nonetheless, $8196b + 8197b$ distinguished robust group I from the other groups. Additional loci were highly informative: *B7 bb* was associated with risk (III), *aa* with protection (I, V) (H = 1.07); *A8* 5147 *bb* was protective (I); risk was associated with 8968 *ab* for persons exposed to pesticides (II) (H = 0.84), among others as shown in Table 1.

RISK FOR PARKINSON'S DISEASE

Figure 1 shows the membership distributions of case and control subjects in each age group (< age 60, 60 to 69, 70+). Few subjects exactly matched the respective groups (N = 0, 1, 1, 3, 1). Most divided membership, for example, had SNP genotypes found for several of the extreme pure type groups shown in Table 1. Relatively few subjects had membership scores of 0.60 or higher (0.60–0.79: green; 0.80–1.00: gray). Nonetheless, several trends were apparent: cases occurring before age 60 tended to resemble groups II and III more than the control subjects. Cases at ages 60 to 69 resembled group IV. Cases at age 70 and older did not over-represent II, III, and IV. Instead, both cases and controls most strongly resembled group V, that is, there may be a survival advantage for this set of polymorphisms. Note the consonance of membership distributions for control subjects in each age group.

The odds of PD, for subjects in each age group, were predicted by membership in groups II, III, and IV in logistic models (Figure 2). Early onset PD was significantly predicted by groups II "early onset, regular exposure" and III "early onset, limited exposure" (OR (95% CI): 2.7 (1.8 to 4.0) and 4.0 (2.5 to 6.5)), respectively. Note that the confidence limits do not include the neutral reference value of one, which would denote no risk. Hence, even limited resemblance of subjects to either group, that is, membership score 0.20–0.39 versus < 0.20, carries statistically significantly increased risk.

Table 2: Probabilities for each variable outcome found for GoM groups I to V. Group I: *"robust to pesticide exposure,"* group II *"early onset PD, regular exposure,"* group III: *"early onset PD, limited exposure,"* group IV *"late onset PD, limited exposure,"* group V *"low risk, no exposure."*

Variable & outcome	Sample	freq.	I	II	III	IV	V	H-value
Disease status & pesticide exposure								1.41
PD, < age 60	None	8.77	—	—	57	—	—	
	Limited	10.21	—	62	29	—	—	
	Regular	3.51	—	38	—	—	—	
PD, ages 60–69	None	8.61	—	—	—	31	—	
	Limited	7.02	—	—	—	25	—	
	Regular	2.07	—	—	—	7	—	
PD, ages 70+	None	4.63	—	—	—	—	**13**	
	Limited	3.03	—	—	—	—	**8**	
	Regular	0.96	8	—	—	—	—	
Control	None	28.07	—	—	—	—	**79**	
	Limited	20.26	85	—	—	37	—	
	Regular	2.87	7	—	14	—	—	
A1:5157-8196-8197	*aa:aa:aa*	90.16	57	100	100	90	94	0.19
	aa:ab:ab	5.41	3.7	—	—	—	—	
	aa:bb:bb	2.46	—	—	—	10	—	
	ab:aa:aa	1.15	—	—	—	—	6	
	bb:aa:aa	0.82	6	—	—	—	—	
A6:5146	*aa*	46.62	—	**100**	—	—	82	0.92
	ab	42.93	—	—	**100**	84	—	
	bb	10.45	100	—	—	16	18	
A7:5148	*aa*	68.76	100	100	32	100	—	0.57
	ab	27.21	—	—	49	—	100	
	bb	4.03	—	—	20	—	—	
A8:5147-8968	*aa:aa*	36.16	—	—	—	**88**	55	0.84
	ab:aa	34.36	42	57	100	—	—	
	ab:ab	14.50	—	43	—	—	32	
	bb:aa	7.17	46	—	—	—	—	
	bb:ab	6.35	12	—	—	**6**	13	
	bb:bb	1.47	—	—	—	**5**	—	
A10:15174-20002	*aa:aa*	21.64	—	**33**	31	15	29	0.68
	aa:ab	30.66	**23**	—	52	56	34	
	aa:bb	17.21	—	**67**	—	—	—	
	ab:aa	16.18	**77**	—	—	—	—	
	ab:ab	14.31	—	—	17	29	37	
B4:19034	*aa*	79.39	—	100	100	97	100	0.43
	ab	19.97	**100**	—	—	—	—	
	bb	0.64	—	—	—	3	—	
B7:5144	*aa*	25.69	100	—	—	—	100	1.07
	ab	50.57	—	100	—	100	—	
	bb	23.74	—	—	100	—	—	
B8:5127	*aa*	62.86	—	100	100	45	65	0.39
	ab	32.15	**71**	—	—	55	35	
	bb	4.98	**29**	—	—	—	—	
B9:5142	*aa*	92.08	50	100	100	100	100	0.16
	ab	7.92	50	—	—	—	—	

Table 2: Continued.

S1:5158-5159	*aa:aa*	30.54	13	35	—	58	31	0.65
	ab:aa	12.48	48	—	32	—	—	
	ab:ab	37.60	40	25	68	—	69	
	bb:aa	1.64	—	7	—	—	—	
	bb:ab	7.72	—	33	—	—	—	
	bb:bb	10.02	—	—	—	42	—	
S2:18866	*aa*	44.64	—	—	100	53	100	0.81
	ab	45.62	100	**100**	—	—	—	
	bb	9.74	—	—	—	47	—	
S4:5133-5178	*aa:aa*	25.28	—	—	—	—	**97**	1.13
	ab:aa	26.09	—	50	68	—	—	
	ab:ab	25.77	—	38	32	57	—	
	bb:aa	7.62	—	—	—	43	—	
	bb:ab	11.67	100	—	—	—	—	
	bb:bb	3.57	—	12	—	—	**3**	
S7:5156	*aa*	28.62	—	48	60	29	—	0.40
	ab	55.79	100	52	9	28	100	
	bb	15.59	—	—	31	42	—	
S8:5155	*aa*	69.03	100	83	66	25	85	0.21
	ab	25.84	—	17	34	52	15	
	bb	5.13	—	—	—	22	—	
V2:182	*aa*	66.07	100	100	78	100	—	0.60
	ab	30.68	—	—	—	—	**100**	
	bb	3.25	—	—	22	—	—	
DLST:2340	*aa*	24.23	—	—	**86**	—	—	0.72
	ab	57.84	100	18	**14**	100	100	
	bb	17.93	—	82	—	—	—	

Note: influential genotypes in determining the group are indicated in bold. The question relevance factor (QRF) score for that variable and group was > 1.20.

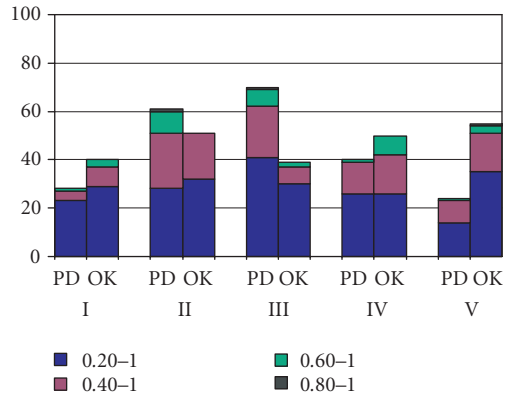
Each 0.20 increment multiplies risk: successive increments in group II membership carry risks of 2.67, 7.13, and 29.0. Successive increments in group III membership carry risks of 4, 16, and 64. Higher levels of risk are predicted by, for example, 0.5 membership in each of groups II and III.

Onset at ages 60 to 69 was significantly predicted by groups II "early onset, regular exposure" and IV "late onset, limited exposure" (OR (95% CI): 1.6 (1.1 to 2.3) and 2.63 (1.8 to 3.8)). Successive increments in group II membership carry risks of 1.6, 2.6, and 4.0. Successive increments in group IV membership carry risks of 2.63, 6.9, and 18.2. Higher levels of risk are predicted by, for example, 0.5 membership in each of groups II and IV.

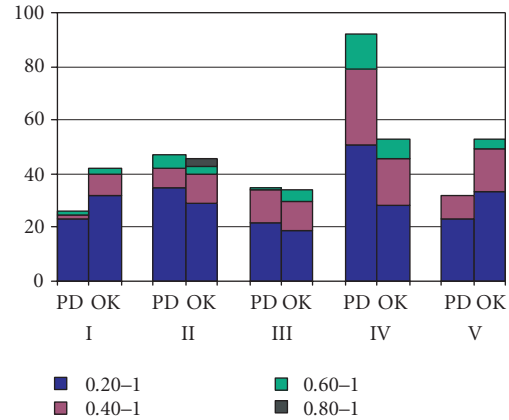
The model was not predictive at ages 70 and older, that is, the global hypothesis that the parameter values were zero could not be rejected ($P = .15$).

DISCUSSION

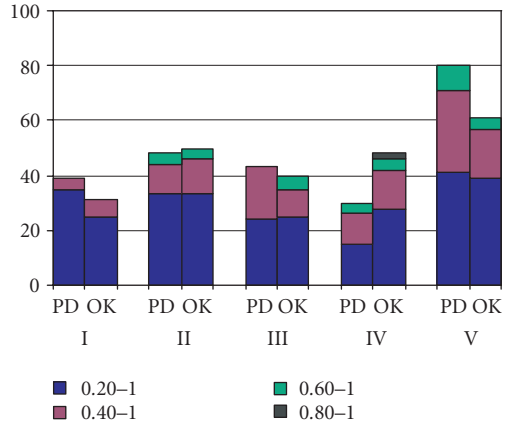
The object of this study is the mutual information contained in multiple SNPs located in nuclear genes that encode mitochondrial complex I subunits, level of toxin exposure, and Parkinson's disease status according to age. A prior investigation of individual SNPs in the study sample [1] found relative risks of about two for PD associated with certain SNPs located in *NDUF* genes *A*1, *A*10, *A*6, and *S*4. This more inclusive analysis replicated these findings and yielded better estimates of risk in relation to the available information. Specifically, five model-based groups were identified that represented robustness to pesticide exposure (I), vulnerability to regular (II) and limited exposure (III, IV) and low risk in the absence of exposure (V). The robust group consisted predominantly of females and carried a set of less frequent alleles including one on the X chromosome. The vulnerable groups differed according to age at onset (< age 60 for II and III; age 60 to 69 for group IV) and level of toxin exposure (limited or regular for II, none or limited for groups III and IV). Even the low risk groups I and V had some level of risk for PD at ages 70 and older. Thus the mutual information investigated using GoM was more informative in terms of age, sex, and toxin exposure compared to straightforward association analysis.



(a) Subjects under age 60: 141 PD cases, 91 controls



(b) Subjects aged 60 to 69 years: 111 PD cases and 116 controls



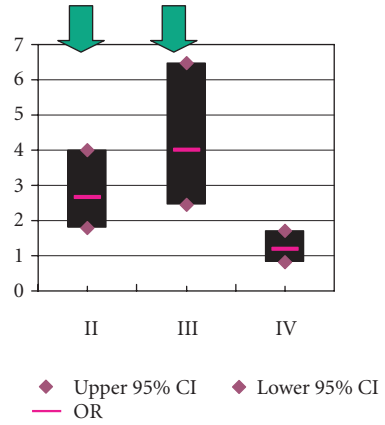
(c) Subjects age 70 or older: 54 PD cases, 114 controls

Figure 1: Individuals have assigned membership scores in the groups. These continuous scores ranging from 0 to 1 have been categorized as < 0.20, 0.20–0.39, 0.40–0.59, 0.60–0.79, and 0.80 to 1.00. Control subjects in each age group have similar frequency distributions of membership. Case subjects < age 60 over-represent membership in groups II and III, group IV at ages 60 to 69. Cases at age 70 and older over-represent the groups I and V.

This approach, avoiding multiple comparisons which would have decimated each of the individual associations [1], was able to estimate risk for individuals according to the level of membership in the vulnerable groups. At ages < 60, statistically significant 3-fold and 4-fold elevation in risk was found for persons who had limited (0.20–0.39) resemblance to groups II and III, respectively, compared to those having very little (< 0.20) resemblance. Successive increments in group II membership carry risks of 2.67, 7.13, and 29.0. Successive increments in group III membership carry risks of 4, 16, and 64. Higher levels of risk are predicted by, for example, 0.5 membership in each of groups II and III. Hence, clinically relevant and statistically significant results were obtained.

Taking the Rotterdam cohort as a guide, incidence increases from 0.3 per 1000 person-years at ages 55 to 65 years to 4.4 per 1000 person-years at ages 85 years and older [27]. The incidence of symptoms of parkinsonism was similar for men and women, but men more often met diagnostic criteria (male-to-female ratio, 1.54; 95% CI, 0.95 to 2.51), hence, the great care taken in this study to consider age at onset. Because men were more often exposed to pesticides compared to women, sex, per se, was not used to determine the risk groups.

Biological interpretation is not straightforward, yet the results lend further credence to the believe that faulty combinations of mitochondrial complex I subunits pose significant risk for age-related PD, presumably, by reduced ATP production and increased production of reactive oxygen species. The results imply that certain persons are robust and others vulnerable to PD when exposed to pesticides. Measurement of pesticide exposure information was structured, but imperfect: memory fades; duration beyond six months was not investigated. One feature of GoM, the identification of extreme types, minimizes this problem by filtering a lack of fidelity in the data. To the extent that groups are misidentified, risk estimates would be expected to be biased toward the null,



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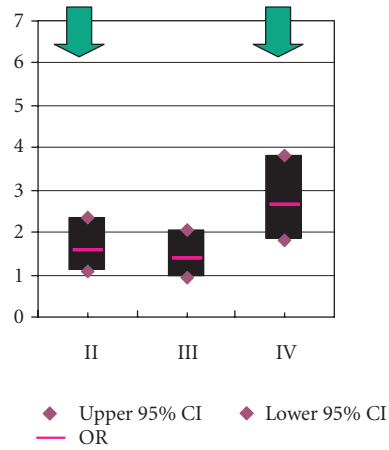

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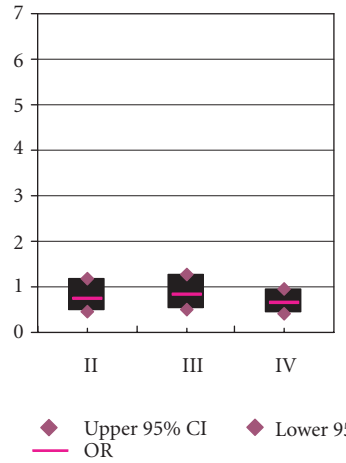

(c) Subjects age 70 or older: 54 PD cases, 114 controls

Denotes statistical significance: the 95% CI for the odds ratio does not include the neutral value of one.

(d)

FIGURE 2: The odds of PD were estimated for each age group (< age 60, 60 to 69, 70+) in logistic models. The predictors were membership scores in the vulnerable groups II, III, and IV. The scores were coded categorically from 1 to 5 representing 0.20 increments. Risk multiplies for each increment of 0.20. For example, at ages < 60, successive increments in group III membership carry risks of 4 (0.20–0.39), 16 (0.40 to 0.59), and 64 (0.60 to 0.79).

that is, underestimate risk. The ability of GoM to interpret mixtures of genetic, clinical, and pathologic data is further demonstrated in these referenced papers [28–34].

In summary, fuzzy latent class analysis was employed to identify sets of polymorphisms located in nuclear genes encoding mitochondrial complex I proteins associated with PD, and effect modification by toxin exposure. Even partial resemblance to the identified risk sets carried appreciable risk for PD. This form of analysis may prove a particularly useful way for hypothesis generation and subsequent investigation of specific gene x gene and gene x environment interactions in relation to common sporadic PD.

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Mini-Review Article

Screening Pesticides for Neuropathogenicity

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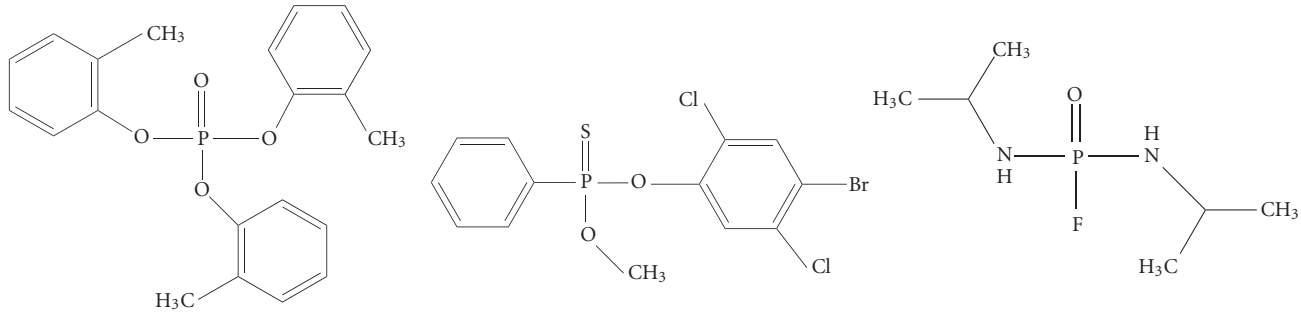
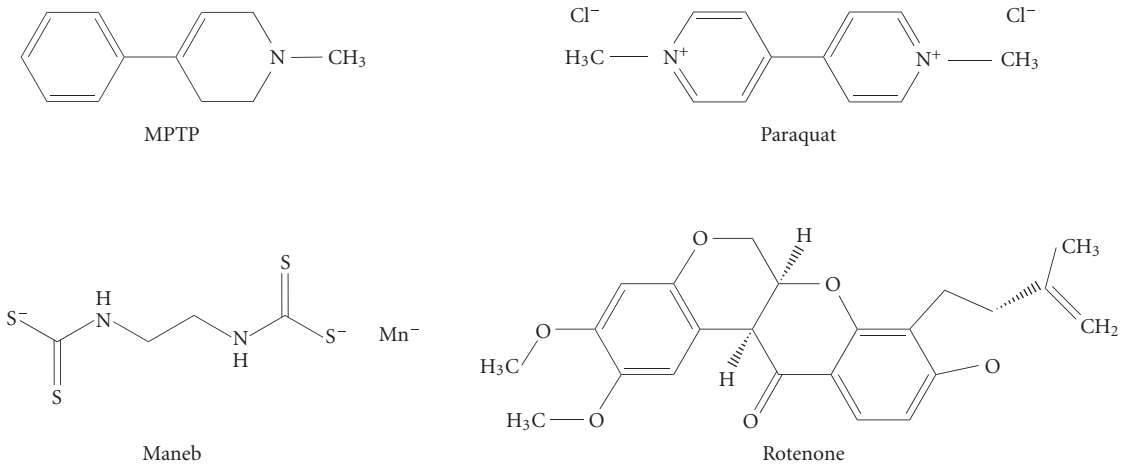
Pesticides are routinely screened in studies that follow specific guidelines for possible neuropathogenicity in laboratory animals. These tests will detect chemicals that are by themselves strong inducers of neuropathogenesis if the tested strain is susceptible relative to the time of administration and methodology of assessment. Organophosphate induced delayed neuropathy (OPIDN) is the only known human neurodegenerative disease associated with pesticides and the existing study guidelines with hens are a standard for predicting the potential for organophosphates to cause OPIDN. Although recent data have led to the suggestion that pesticides may be risk factors for Parkinsonism syndrome, there are no specific protocols to evaluate this syndrome in the existing study guidelines. Ideally additional animal models for human neurodegenerative diseases need to be developed and incorporated into the guidelines to further assure the public that limited exposure to pesticides is not a risk factor for neurodegenerative diseases.

INTRODUCTION

In the early 1980s, there was an unfortunate human situation in which drug abusers developed Parkinsonism syndrome [1] following exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, see Figure 1) that is a by-product in an attempt to chemically synthesize heroin. Although earlier researchers sought links between manganese exposure and Parkinsonism [2], the MPTP incident greatly increased the interest in correlating environmental exposure to contaminants and human neurodegenerative diseases. Human exposure to pesticides is essentially unavoidable in modern life both in the developed nations and more increasingly in the developing ones. Worldwide pesticide sales and usage in both 2000 and 2001 were in excess of five billion pounds. In the United States alone there were about 1.2 billion pounds of pesticides used including insecticides, herbicides, fungicides, rodenticides, but not including wood preservatives, special biocides, and chlorine/hydrochlorides [3]. Human exposure to pesticides depends upon many factors and often agricultural workers have the highest rates of exposure as they apply pesticides to crops. Spray drift and migration of the pesticides to potable water as well as residues in food stuffs and residues resulting from home and garden applications are also very significant sources of exposure. Many insecticides are neurotoxic by design with targets being acetylcholinesterase (organophosphates and carbamates), the Na^+ conductance channel (DDT, pyrethrins, and pyrethroids), the acetylcholine receptor (nicotinics), the GABA receptor (emamectin), Ca^{++} channels (ryanodine), and some agents such as rotenone that affects mitochondrial function and also may affect the nervous system. If a poisoned individual recovers from the initial toxicity following a single dose of anticholinesterase inhibitors (with the exception of some organophosphates) or agents that act on transmitter receptors, and when the chemical is rapidly metabolized and excreted, there is usually no established pathological or neurodegenerative change although there are many anecdotal reports of persistent subtle effects (see reference [4]). The trauma of the acute poisoning incident may have some psychological effects that may not actually be related to the neuropharmacology of the agent. The consequences of chronic exposure to pesticides, whether they are designed to act on the nervous system as are insecticides or are herbicides designed to be specific for plants, may be causing effects in humans through their known or yet to be discovered effects in the nervous system. Over the past decade there has been a growing body of literature that suggests pesticides as being risk factors either for possibly initiating or facilitating the progression of neurodegenerative diseases (eg, see Table 1). Theoretically humans may have the initiation of the diseases triggered by exposure to a pesticide or a pesticide in combination with other environmental contaminants. In some cases, it is possible that individuals with a genetic predisposition for a neurodegenerative disease may be at an increased risk to exposure to



(a) Chemicals associated with OPIDN



(b) Chemicals proposed to be associated with Parkinson's syndrome

Figure 1: Chemical structures.

pesticides that might initiate the disease. In other cases where the initiating event in either normal or genetically susceptible persons is caused by a spontaneous event or another chemical exposure, the progression following its initiation may be facilitated to various degrees by exposure to pesticides.

The potential toxicity of pesticides is evaluated in laboratory animals prior to registration and updated in the reregistration process in a series of required or conditionally required studies that follow specific guidelines [5]. Partly as a consequence of the discovery that MPTP caused a neurodegenerative disease as well as the interest in the possibility that there is increased susceptibility associated with prenatal and neonatal exposures, there has been increased testing as a part of the registration/reregistration process to attempt to determine the potential effects of pesticides on the nervous system. As a result, a series of special neurotoxicity study guidelines were developed in the early 1990s. These guidelines for special neurotoxicity testing together with other more general study guidelines that also assess for effects on the nervous system are listed in Table 2.

OVERALL GOAL OF THE STUDY GUIDELINES AND RISK ASSESSMENT

In classical terms, the goal of the study guidelines is to characterize the toxicity of the pesticide and to identify the most sensitive endpoint in the most sensitive species. Once this endpoint is selected from the pesticide's toxicity database including the required studies following the guidelines in Table 2, nonguideline studies that are either conducted at the registrant's own initiative or as recommended by the USEPA as well as studies from the open literature, a risk assessment is performed. Traditionally the risk assessment is based on the no observable adverse effect level (NOAEL) for this endpoint coupled with available or estimated exposure data. The NOAEL is adjusted by uncertainty factors to further assure the safety of the chemical to humans. First, a factor of 10 X for intraspecies variation based on the assumption that within species some individuals may be 10 times more sensitive than the tested group is employed. Another 10 X factor for interspecies variation based on the assumption that humans may be 10 times more sensitive than the most sensitive laboratory animal species is also employed. Another 10 X

Table 1: Selected examples of human neurodegenerative and other neurological diseases both demonstrated and possibly attributed to pesticides.

Disease	Pesticide (reference)	Association with humans	Guidelines for assessment
Organophosphate-induced delayed neuropathy (OPIDN)	Organophosphates cholinesterase inhibitors. (8-review)	Strong. Actual association demonstrated	Yes—hen studies
Parkinson's disease	Paraquat [20–25], maneb [26, 27], rotenone [28–30], organochlorines [31–34], also [35]	Not firmly established but circumstantial evidence	No
Alzheimer's disease	No specific pesticide—agricultural workers [36, 37]	One case study—association not proven. Epidemiological study with 68 cases—no association concluded	No learning and memory not assessed in older animals
Amyotrophic lateral sclerosis	2-4-dichlorophenoxy-acetic acid [38, 39]	Report of increased relative risk among employees in manufacturing. Agricultural workers have higher rates	No specific test but several tests would detect neurological and muscular degeneration
Autism	No specific pesticide [22]	Suggestion that impaired metabolism of pesticides may be associated with increased incidence of autism	No, but certain patterns in the DNT study may be an indicator
Psychiatric disorders	Organophosphates [40–42]	Authors claim of positive association in epidemiological studies and EEG changes in humans and monkeys	No

factor may be applied if it is determined that the database is incomplete or there is no NOAEL for the most sensitive endpoint. When there is an evidence of developmental toxicity in fetuses or neonatal animals at lower doses than parental or adult toxicity, an additional FQPA (Food Quality Protection Act) 10 X (reducible to 3 X or 1 X depending upon the circumstances) safety factor is applied to assure the protection of fetuses, newborns, and children. It should be noted that the application of the uncertainty factors is to the NOAEL and the lowest observable adverse effect level (LOAEL) is always higher than the NOAEL. Thus, a total uncertainty factor of 100 applied to an NOAEL is in reality a factor of 300 from a dose where there is an effect when the LOAEL is a dose three times higher than the NOAEL. In order to eliminate or compensate for some of the limitations of the NOAEL and LOAEL approaches, statistical methods have been developed to determine a benchmark dose (BMD) that accounts for gaps in dose spacing or account for a study not showing an NOAEL [6]. The uncertainty factors as described above (except for not having an NOAEL) are applied to the BMD.

A risk assessment for a chemical with an NOAEL based on liver toxicity at the LOAEL that has evidence of neuropathogenicity in laboratory animals at higher doses than at the LOAEL will be protective against the neuropathogenesis although neuropathogenicity was not the basis for the selection of the NOAEL. The protective nature of both the NOAEL and BMD approaches to risk assessment assumes that most other potential target organs in humans will only be affected at higher doses than the most sensitive endpoint in laboratory animals. If humans are especially susceptible to neuropathogenesis resulting from exposure to a certain pesticide, the endpoint based on animal studies may underestimate the risk to humans. However, the minimum 100 X uncertainty factor plus any additional factors would only in rare cases not be protective against such neuropathogenesis in humans having extreme sensitivity to the chemical. To date, although this may be debatable by some, there is no *known* neuropathological condition caused or facilitated by pesticides that should not be protected against by the current approach to risk assessment as outlined above provided that the pesticide does not interact with other environmental contaminants, drugs, or naturally occurring chemicals to render neuropathogenicity. The principle of selecting the most sensitive endpoint in the most sensitive test animal species and using either the NOAEL or BMD and applying uncertainty factors to drive down exposure is still the basis for risk

Table 2: List of guideline studies both general and especially designed to assess for neurotoxicity.

Guideline	Study title	When required	Species [1]	Protocol timing	Nonpathology [2]	Histopathology
General toxicity assessment						
870.1100 870.1200 870.1300	Acute oral, dermal, and inhalation toxicity	For all chemicals (inhalation not required for some)	Rat or rabbit	Acute dose to young adults and observations for 14 days	Clinical signs, body weight and mortality	Gross necropsy only
870.3100 870.3150	Subchronic oral, dermal, and inhalation dosing	For most chemicals	Rat or dog	90 days dosing starting with young adult	Clinical signs, body weight and hematology, clinical chemistry and urinalysis and mortality	Generally hematoxylin and eosin
870.3700	Prenatal developmental	For most chemicals	Rat or rabbit	Gestation days 6–16 for rats and 6–18 for rabbits	Clinical signs, body weight, uterine data and pup data and mortality	Gross dissection for visceral and skeletal assessment of the pups
870.3800	Reproductive	For chemicals with food uses and chronic exposure	Rat	Continuous from premating through gestation, lactation and adults for two generations	Reproductive performance, clinical signs, body weight and mortality, pup growth and development	Necropsy and histopathology of the reproductive organs
870.4100 870.4200 870.4300	Chronic dosing and carcinogenicity	For chemicals with food uses or where chronic exposure is expected	Rat, dog, and mouse	6 months for dogs, 18 months of more for mice and 24 months for rats	Clinical signs and mortality	Generally the same as for subchronic
Special studies for organophosphate-induced delayed neuropathy						
870.6100	Acute delayed neurotoxicity for OPIDN	All organophosphate AChE inhibitors	Hen (8–14 months of age)	Single limit dose of 2 gm/kg or near lethal dose (ie, LD_{50}). Atropine can be used to prevent death by AChE inhibition. Must have 6 survivors. Sacrifice at day 21	Gait assessment neuropathy toxic esterase acetylcholinesterase	Whole body perfusion. Sections of medulla oblongata, spinal cord (rostral cervical, midthoracic, and lumbosacral), and peripheral nerves. "Appropriate" myelin and axon specific stains
870.6100	Subchronic delayed neurotoxicity for OPIDN	For organophosphate AChE inhibitors when the acute study is inconclusive		Limit dose of 1 gm/kg. Three dose levels required. Establish NOAEL and LOAEL		

Table 2: Continued.

Guideline	Study title	When required	Species [1]	Protocol timing	Nonpathology [2]	Histopathology
Special neurotoxicity assessments in rats*						
870.6200a	Acute neurotoxicity screen	Conditionally required	Rat	Single dose to young adults with assessments at predosing, optimum time of effect and at days 7 and 14	FOB and Motor Activity at pretest, time to peak effect and days 7 and 14	In situ perfusion with fixative. Paraffin embedding acceptable for CNS but plastic embedding required for peripheral. GFAP immunohistochemistry, Bodian's stain and Bielschowsky's silver methods recommended in addition to standard stains
870.6200b	Subchronic neurotoxicity screen		Rat	Daily dosing with live assessments at predosing, weeks 4, 8 and 13 but histopathology at week 13 only	FOB and Motor activity at pretest, 4th, 8th and 13th weeks	
870.6300	Developmental neurotoxicity		Rat	Gestation and lactational exposure. Optional direct pup gavage exposure. Maternal assessments. Pup assessments before weaning and at day ~21 and day ~60	FOB, motor activity. Learning and memory. Acoustic startle response. Brain weight and morphometric	
870-6500	Schedule-controlled operant behavior	Rarely conducted or required. Recommended for chemicals showing neurotoxicity in other studies that would be further characterized by this special test. Test can be combined with other guideline studies	Rat	Open	Special operant behavior	Not specified
870.6850	Peripheral nerve function		Rat		Peripheral nerve conduction velocity and amplitude	Not specified
870.6855	Neurophysiology: sensory evoked potentials		Pigmented rat strain		Implantation of electrodes in brain followed by visual, auditory or somatosensory stimuli evaluated	Not specified

Most commonly used species.
For organophosphates and carbamates, plasma cholinesterase and RBC and brain acetylcholinesterase are periodically assessed.
*The guidelines recommend rats to be tested but other species can be tested under special circumstances.
Copies available at http://www.epa.gov/pesticides (see under Science and Policy-Test Guidelines).

assessment although some may consider this principle outdated. The toxicity database as generated by the required studies is intended to be a thorough screening process and is not intended to be an in-depth assessment of any organ including the nervous system unless special inclusions are made. However, when there are justifications to believe that the toxicity for a given chemical is being underestimated by the standard set of required toxicity studies, and validated methods for additional testing are available, these additional studies can be recommended to further characterize the toxicity.

An inherent problem with the guidelines for neurotoxicity studies is that the rat, dog, mouse, or rabbit may not provide a model for certain types of neurotoxicity that humans may be especially sensitive to. No matter how much testing is done in animals, such toxicity will not be detected prior to exposure to humans. The case of aplastic anemia is one example of there not being an animal model for prediction of a particular type of toxicity. It is estimated that one person in 30–40,000 is susceptible to the aplastic anemia caused by the antibiotic chloramphenicol [7]. There might also be cases of unusual human susceptibility to a neuropathogen of similar low frequency and there would be no way to detect them using the current battery of studies. Unlike the chloramphenicol model where the dosage was intentional and monitored, exposure to pesticides is much smaller and the actual amounts, times, and frequencies of pesticide exposure are not known.

The guidelines (Table 2) for the more general acute (870.1100 for oral, 870.1200 for dermal, and 870.1300 for inhalation), subchronic (870.3100 for rodents and 870.3150 for nonrodents—usually dogs), prenatal development (870.6300) and reproductive (870.3800) and chronic toxicity in rats and dogs (870.4100) and carcinogenicity in rats and mice (870.4200 or 870.4300) are nonspecific in their description of methods recommended for evaluation of the histopathology of the nervous system. The more obvious neurotoxicity would be detected by observation of the behavior of the animals based on daily cage-side evaluations if the technical staff is appropriately trained to look for and detect changes in behavior. The only instructions in the nonacute studies for histopathology preparation apply to all tissues and are not specific for nerve tissue: "tissues and organs designated for microscopic examination should be fixed in 10 percent buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hours prior to trimming." No commentary on the special stains to be used is provided. Hematoxylin and eosin are routinely used.

SPECIAL STUDIES FOR ASSESSMENT OF NEUROTOXICITY IN RATS

The studies designed for specific assessment of potential neurotoxicity in rats include the series 870.6200 for acute and subchronic screening in adults and 870.6300 for developmental neurotoxicity (DNT). The latter study includes exposure to pups in utero and during lactation either via lactation or by direct gavage exposure to the pups. These studies include cage-side observations for the more obvious clinical signs and for functional observational battery (FOB)[1] which assess the animal for motor and sensory effects. The technical staff making these observations is supposed to be especially trained to detect subtle changes in clinical signs indicative of neurotoxicity and typically is unaware whether the animal was dosed with the test material or otherwise. For these studies, the instructions for histopathological evaluation of the nervous system are more specific than for the general screening studies. "Tissues should be prepared for histological analysis using in situ perfusion and paraffin and/or plastic embedding procedures. Paraffin embedding is acceptable for tissues from the central nervous system. Plastic embedding of tissue samples from the central nervous system is encouraged, when feasible. Plastic embedding is required for tissue samples from the peripheral nervous system. Subject to professional judgment and the type of neuropathological alterations observed, it is recommended that additional methods such as Bodian's and Bielchowsly's(*sic*) silver methods, and/or glial fibrillary acidic protein (GFAP) immunohistochemistry be used in conjunction with more standard stains to determine the lowest dose level in which neuropathological alterations are observed."

In the developmental neurotoxicity study (870.6300), pups (11 or 21 day old depending on the length of lactational exposure) and adults (about 62 days old) derived from dams exposed to the pesticide from day 6 of gestation through lactation (at least to day 10 but many laboratories continue dosing up to the time of parturition) via lactation or by direct gavage dosing during the lactation period are examined histologically. In addition to histopathology, an abbreviated FOB assessment, learning and memory and motor activity and acoustic startle responses are all evaluated in the pups at about weaning time and again as young (about 60 days) adults. Histology of pups is different from the 870.6200 studies in that the brain is fixed by immersion rather than in situ. The guidelines for the neurotoxicity screening studies and the developmental neurotoxicity study provide references for more detailed instructions for histopathology and behavior assessments. The laboratory conducting the study is responsible for selecting the techniques and stains to be used.

Positive control studies such as with trimethyl tin or acrylamide for neurohistopathology as well as positive controls such as amphetamine and haloperidol for motor activity and scopolamine for learning and memory are currently recommended to assure the susceptibility of the strain and the competence of the laboratory personnel conducting the study. The argument for also considering nonchemical agents to evaluate the proficiency of a laboratory in the use of a test (eg, memory) has been made [8].

These screening studies should detect alterations of the nervous system that occur within the limited time frames of testing with respect to the age of the rat when tested provided that the relatively pure strains of rat used are susceptible to

[1] The FOB assessment in the series 870.6300 is less detailed than for the series 870.6200. In particular, there is no requirement to include grip strength or landing foot splay.

any neurotoxicity that could be induced by the chemical. Consequently there are limitations with regard to their predictive value for the major neurodegenerative diseases which are associated with older humans such as Parkinson's and Alzheimer's. In particular, only young adults are assessed in the 870.6200 screening studies and exposure to the rats is in utero and up to the first three weeks of life in the series 870.6300 developmental neurotoxicity study. The rats are not kept on the study to determine if the in utero exposure predisposed them to development of neuropathological conditions in the later stages of life or if a challenge by the test chemical would be worse if the rats were not exposed in utero.

Three other studies (870.6500, scheduled operant behavior, 870.6850, peripheral nerve function, and 870.6855, neurophysiology: sensory evoked potentials) are rarely conducted but can be used to further characterize indications of neurotoxicity suggested in either the general or the special neurotoxicity guideline studies or based on the pesticide's structure and predicted activity relationships.

There are no guidelines for studies with monkeys which may have a similar level of susceptibility to neurotoxicants that may produce or facilitate human neuropathogenesis. Reasons for this are that studies with monkeys are expensive and only limited numbers of animals can be used.

NONGUIDELINE STUDIES

Studies from the open literature or studies conducted by the pesticide industry that either do not have protocols consistent with the guidelines or that are conducted to address a specific question are grouped together as nonguideline studies. Such nonguideline studies can provide endpoints for risk assessment when peer review determines that they are of acceptable scientific merit. It is, however, difficult to request that companies conduct special nonguideline studies without sufficient justification that the study is validated to render data useful for risk assessment purposes.

A recurring problem with nonguideline studies is that they often use routes of test chemical administration not related to human exposure scenarios. Intraperitoneal, intravenous, and intramuscular modes of administration may be very useful in attempting to determine the mode of action of a chemical. Such data are important in understanding the possible molecular basis for the neuropathogenesis. However, extrapolating data from these routes of administration to human exposure by the dietary, dermal, or inhalation routes is problematic.

The use of nonguideline studies with purposeful dosing of human volunteers to assist in the risk assessment for pesticides is done on a case by case basis following both scientific and ethical review. Such studies with human volunteers are occasionally conducted with pesticides that may cause transitory effects such as cholinesterase inhibition but certainly not to see if a neurodegenerative condition results. Epidemiological studies that attempt to correlate the incidence of certain types of diseases with pesticide exposure to humans derived from surveys of the subjects' personal history provide insight into the possibility that exposure to the pesticide may be related to the onset and progression of neuropathogenesis. These studies, however, can only suggest a possible relationship because the subjects are also simultaneously exposed to many other chemicals and there is no real way to determine the actual extent to which the subjects were exposed to the suspect pesticide chemical or if exposure occurred during the critical times to affect the onset or progression of the neuropathological condition.

STUDY GUIDELINES FOR ASSESSING ORGANOPHOSPHATE-INDUCED DELAYED NEUROPATHY

The only established neuropathy in humans associated with pesticides is organophosphate-induced delayed neuropathy (OPIDN) caused by certain but not all organophosphate insecticides and some other organophosphates not used as insecticides. A recent review on OPIDN provides more detailed information on the history and development of this model [9]. Documentation that OPIDN affects humans dates back to the early part of the last century when a major incident occurred during the prohibition years in the USA as a result of consumption of a Jamaican ginger alcoholic drink that was later demonstrated to be contaminated with tolyl phosphate esters. It is estimated that some 20,000 persons were affected to various degrees with a syndrome that was called *Ginger jake paralysis* or *jake leg*. The classical work of M. Smith and R.D. Lillie [10] of the US Public Health Service in the 1920s and 30s demonstrated that the phosphate contaminants were responsible for the condition and could reproduce the syndrome in rabbits, dogs, monkeys, and calves. In human exposure to ginger jake, the condition was described as "the initial flaccidity, characterized by muscle weakness in the arms and legs giving rise to a clumsy, shuffling gait, was replaced by spasticity, hypertonicity, hyperreflexia, clonus, and abnormal reflexes, indicative of damage to the pyramidal tracts and a permanent upper-motor neuron syndrome. In many patients, recovery was limited to the arms and hands and damage to the lower extremities (foot drop spasticity and hyperactive reflexes) was permanent, suggesting damage to the spinal cord" [4]. Validation that organophosphate insecticides cause OPIDN in humans comes partly from an incident concerning workers manufacturing the insecticide mipafox following an accident [11]. Domestic animals are also susceptible to OPIDN as indicated by the poisoning of water buffalo in Egypt [12] by the insecticide leptophos. A review of the possible association between leptophos with OPIDN in humans [13] describes problems in distinguishing between leptophos and other contaminants as the cause of OPIDN.

Considerable research on the structure of organophosphate insecticides that can cause this neuropathy has been done [9]. Of the tolyl phosphate contaminants in the ginger product, it was later determined that only one, the ortho-isomer, was responsible for the toxicity, indicating the highly specific chemical structural nature of the induction of this syndrome. Figure 1 presents some of the chemical structures of organophosphates that are known to cause OPIDN.

Research on biochemical approaches has led to the discovery that inhibition of "neuropathy target esterase" (NTE) by the organophosphates that cause the delayed-type neuropathy has provided a basis for screening of new organophosphate candidates for development as insecticides [14]. Chemicals showing higher levels of inhibition of NTE are reported to have a good correlation with development of OPIDN.

The hen was determined to be a relatively very susceptible species but the laboratory rat and mouse were not appreciably susceptible to OPIDN. The hen provides a model for assessing the potential for an organophosphate to cause neurotoxicity and is used in the acute and repeat dose study guidelines (870.6100). It is necessary to use adult domestic hens 8–14 months of age since the chick has a lower sensitivity [15]. In the acute study, a near lethal dose is administered usually by gavage and the hen may be protected by atropine from the inhibitory effects of the organophosphate on acetylcholinesterase. Following dosing, the hens are observed for their gait characteristics including the ability to walk up an incline and after 21 days are sacrificed and examined histologically. The repeat dose study is conducted when there is an evidence of OPIDN in the acute study or when there is an evidence of inhibition of NTE. The focus of the repeat dose study is to determine the NOAEL and LOAEL for OPIDN and it includes control, low, mid, and high (maximum 1 gm/kg) doses. The guidelines provide the following details for histopathological examination of the nervous system. "Tissues should be fixed by whole body perfusion, with a fixative appropriate for the embedding media. Sections should include medulla oblongata, spinal cord, and peripheral nerves. The spinal cord sections should be taken from the rostral cervical, the midthoracic, and the lumbosacral regions. Sections of the proximal regions of both of the tibial nerves and their branches should be taken. Sections should be stained with appropriate myelin- and axon-specific stains." The guidelines recommend that TOCP (tri-ortho-cresyl phosphate, Figure 1) be used as a positive control to assure the susceptibility of the hens. Not all hens are equally susceptible to OPIDN [16].

No new organophosphate insecticides have been introduced in recent years and either organophosphate insecticides that were demonstrated to cause OPIDN have been phased out or their uses have been greatly restricted. New organophosphates, however, may in the future be needed for control of certain pests that become resistant to currently registered pesticides. OPIDN is not considered to be related to another known human neurodegenerative disease. However, the OPIDN model may be very useful in studying the progressive degeneration of the nervous system following initiation of the nerve degeneration that may be applied to human neurodegenerative diseases if the underlying mechanisms of OPIDN can be elucidated and compared with human diseases.

PESTICIDES AND PARKINSONISM SYNDROME

Parkinson's disease (PD) is regarded as the second most common neurodegenerative disorder in humans and affects about 2% of the population over the age of 60 years. Clinically, PD is a disorder of motor function characterized by tremor, slow and decreased movement (bradykinesia), muscular rigidity, poor balance, and problems in gait [17]. Pathologically, PD patients show loss of dopaminergic neurons in the substantia nigra pars compacta and frequently have Lewy bodies, eosinophilic intracellular inclusions composed of amyloid-like fibers and α-synuclein [18]. PD may have a genetic basis for susceptibility for an early onset form but the occurrence of the more prevalent late onset form does not have an established genetic basis [19]. The latter form may result from a multitude of different factors including insults from xenobiotics and an individual's inherent sensitivity or differences in the metabolism and pharmacokinetics of the xenobiotics. Since the discovery that MPTP [1] could cause PD like syndrome, interest in the herbicide paraquat, which has some structural similarity to MPTP (Figure 1) led to the possibility that this herbicide could be a risk factor in the PD syndrome [20–25]. Factors such as exposure from living in rural areas, farming, drinking water from wells and exposure to agricultural chemicals have been investigated and claimed as support for an association between paraquat and increased PD. Interest in the herbicide maneb as a possible risk factor for PD developed because of its reported effects on dopamine whereas it was demonstrated to enhance the effects of the active metabolite of MPTP or MPP+ [26, 27]. Rotenone, a pesticide that is an inhibitor of mitochondrial Complex I function, has also been implicated for being associated with PD based partly on work that associates the mode of action of MPTP or its principal metabolite MPP+ with an effect on mitochondrial Complex I function [28–30].

Organochlorine insecticides as well as tricyclohexyl and triphenyl tin inhibit various ATPases in nerve membranes including one enzyme species that also shows a bell-shaped curve for activation and then inhibition of activity by Mn^{++} and it was earlier suggested [31] that inhibition of ATPases might be related to an environmental factor in Parkinson's disease etiology. DDT and dieldrin persist in the body and once ingested can remain there indefinitely. Mobilization of DDT or dieldrin from fat stores as the body ages to critical areas associated with PD might be a factor in its development or progression. An association between dieldrin presence and PD syndrome [32] was reported based on a small number of patients examined. Heptachlor has also been demonstrated to affect dopamine function [33] in laboratory animals.

The association between PD and pesticides is a controversial issue and the USEPA does not currently consider that pesticides are risk factors in this disease. A recent comprehensive review of this issue, supported in part by industry but published in a peer reviewed journal, led the authors to conclude "that animal and epidemiological data reviewed do not provide sufficient evidence to support a causal association between pesticide exposure and PD" [43].

If a pesticide was causing or affecting a PD like syndrome in susceptible laboratory animals, the signs of tremor, slow and decreased movement, muscular rigidity, problems in gait would be expected to be detected in the screening process if all of the appropriate studies were requested and conducted.

The available studies for paraquat, maneb, or rotenone do not show obvious indications of these signs at least not at their LOAELs in the strains tested. The histopathological effects would probably not be so obvious within the limited assessment for histopathology in the current study guidelines since the substantia nigra is a relatively small section of the brain and would require special assessment to determine if there were test chemical induced changes in the dopamine dependent cells within it. Chronic exposure for paraquat is currently based on "chronic pneumonitis" in dogs with a conventional 100 fold uncertainty factor. Maneb is currently regulated for chronic exposure based on its effects on the thyroid in rats at the LOAEL plus a 1000 fold uncertainty factor including an extra 10 X because of an incomplete database. Endpoints for rotenone are currently being reevaluated and the reports of its association with PD syndrome being considered for future testing but the current LOAEL is not based on indications of neuropathogenesis.

Historically, the rat has limitations as an in vivo model for PD and attempts to study the effects of either Mn^{++} or MPTP in this species resulted with some but limited data. A detailed review of the development of animal models for PD and other neurodegenerative diseases is beyond the scope of this review and there are no suitable models for incorporation into the guidelines. Reviews of neurotoxicant induced models of PD in the rat have been published recently in 2004 [44] and 2005 [45] and provide comprehensive discussions of the many problems associated with trying to develop an animal model. A review of the development of animal models in mice has also been presented [46] and limitations of this species including genetically engineered strains are discussed [47]. Factors such as the low susceptibility of rodents to PD like syndrome or a narrow or limited vulnerable age or the differences in metabolism and access to the critical sites by the critical form of the toxic agents as well as the cumulative effects and the influence of combinations of chemicals all contribute to problems in developing animal models for predicting a chemical's potential to be a risk factor for PD.

One important consideration in the development of animal models for PD concerns the question: what is the goal of the model? For example, some models are developed to further understand the neurochemical events associated with the initiation and progression of the disease in order to develop therapy. Other models may have the goal of establishing a basis for risk assessment. One of the criticisms of some of the developing models that they do not mimic the disease in humans closely enough is not necessarily detrimental to the goal of providing data for risk assessment. This is because if the model shows an effect suggestive at all of neuropathogenesis it would be important in the hazard characterization of the chemical. Thus, genetically manipulated mice that spontaneously develop PD like syndrome whether it mimics the human condition exactly or not would be an important addition to the guidelines. The suspect chemicals could be tested in these strains to see if the spontaneous rates of the syndrome are increased, occur at an earlier onset time, or are worse in the presence of the pesticides.

In vitro data using rat or other animal tissue preparations can be very useful for providing data on mechanisms but not always generalize to the in vivo situation. One such example is the effect of paraquat which was suspected as causing PD like syndrome based on its structural similarity to MPTP/MPP+ that does not have the same affinity for the dopamine transporter or cause inhibition of mitochondrial complex I in in vitro studies indicating that paraquat has as effect on dopamine neurons that is unique from rotenone and MPTP [48]. It is still possible that an NTE like model such as for predicting OPIDN could be developed based on in vitro studies. Limitations associated with in vitro models based on animal tissue include that in real life, exposure is not just to the single chemical but to complex mixtures, in vitro studies do not reflect the cumulative effects of the pesticide or the temporal aspects of the initiation or progression of the disease.

Another animal model for induction of PD involves mice and their early exposure and later challenge based on work with paraquat and maneb [49]. The mice exposed as fetuses during pregnancy were reported to be more susceptible to indications of PD when challenged later in life by these pesticides. This implied that an initial injury predisposed the animals to susceptibility in later stages of life. This model is based on the "Barker hypothesis" or its expanded form for Parkinsonism where it is postulated [50] that early exposure to chemicals destroys certain critical cells in the substantia nigra to levels below those needed to sustain function associated with advancing age. In these studies, combinations of paraquat and maneb were used assessing the mutual influence of each. The role of early life environmental risk factors in PD has been independently reviewed [51].

As indicated above, a problem with attempting to assess for the effects of pesticides as risk factors of neuropathogenesis by the study guidelines is that some of the literature reports associating pesticides with PD imply that combinations of pesticides or other agents rather than the individual pesticides are the risk factors. Extensive justification would be needed before studies with combinations of xenobiotics could be requested to provide data for risk assessment. Establishing what combinations of chemicals should be tested, how long the tests should run for and what relative doses of each chemical to be tested would be a task in itself and interpreting the data with regard to which chemical is really the contributing factor would be problematic.

OTHER NEUROLOGICAL DISORDERS

Table 1 lists Alzheimer's disease (the most common neurodegenerative disease), amyotrophic lateral sclerosis, autism and psychiatric disorders as possibly being related to pesticide exposure. Tests for learning and memory through the life cycle including the later months near study termination in chronic or cancer studies might be considered for incorporation into the guidelines to attempt to assess for at least some aspects of Alzheimer's disease. Alzheimer's disease may have both genetic and environmental factors [52] and animal models of Alzheimer's disease are being developed [53]

but their usefulness for evaluating risk associated with pesticide exposure has not been established. Many factors may influence the progression of Alzheimer's disease and a very recent report indicates that persons with higher levels of education have faster rates of cognitive decline [54]. Animal models of autism are being developed [55] and the endpoints of (i) lower sensitivity to pain and higher sensitivity to nonpainful stimuli, (ii) diminished acoustic prepulse inhibition, (iii) locomotor and repetitive/stereotypic-like hyperactivity combined with lower exploratory activity, and (iv) decreased number of social behaviors and increased latency to social behaviors are considered possible indicators of a drugs association with autism based on studies with valproic acid. The developmental neurotoxicity study (DNT, 870.6300) can assess for some of these parameters but there are no inclusions in the current guidelines for DNT studies for assessing social behaviors. If a pesticide caused neurological or muscular degeneration, it could possibly aggravate amyotrophic lateral sclerosis but would be regulated based on its NOAEL to be protective. Although neuropsychiatric disorders may not be strictly within the description of neurodegenerative disease, there has been a continuous debate over the possibility that organophosphate poisoning causes neuropsychiatric sequella [40]. A review of this topic is beyond the scope of this manuscript. Nonguideline studies with monkeys [41] with sarin have been reported to produce long-lasting EEG changes that are claimed to confirm an earlier observation of changes in the human EEG patterns [42] following organophosphate exposure. The persons exposed to sarin (a potent cholinesterase inhibitor) gas in the Tokyo subway incident in the mid 1990s have been assessed periodically and reports indicate possible neurological effects either related to the gas itself of post traumatic stress disorder [56–58].

OVERALL ASSESSMENT OF NEUROTOXICITY STUDY GUIDELINES FOR CHARACTERIZING RISK FOR NEUROPATHOGENICITY

Strength of the neurotoxicity study guidelines. The neurotoxicity study guidelines provide a screening procedure that should detect pesticides that are strong inducers of neuropathogenesis in the animal strains and species tested relative to the time of administration and ages of the tested animals. The guidelines are adaptable and as more and better techniques and models (such as genetically manipulated strains) are developed these models can be incorporated into the guidelines. Humans would have to be inherently especially more sensitive to a neuropathological response to a pesticide or there would have to be other contributing factors from the real world if they were to develop neuropathological conditions as a result of the low level of exposure that is set by the selection of the most sensitive endpoint in the most sensitive species as determined by the battery of required studies and other available data and the application of uncertainty factors to drive down exposure.

Weaknesses or limitations of the study guidelines. Several inherent weaknesses in the study guidelines can be identified. Most of these reflect a disparity between the stringent conditions of laboratory testing and real world exposure. These include the following.

(i) Pesticides are tested individually. Thus, the interaction and cumulative effects of the individual pesticide with the many other pesticides, xenobiotics, drugs, and natural foodstuffs are not assessed.

(ii) Relatively pure strains of standardized laboratory animals are tested meaning that a neuropathological condition will be detected only if that particular strain is sensitive to the chemical. The human population is very diverse with varying degrees of sensitivity to a given chemical. The standardized strains do not have a predisposition to develop neuropathogenesis such that it cannot be assessed if there is a potential for the pesticide to accelerate the progression of a neuropathological condition once started.

(iii) Healthy young animals on diets optimized for their health would be the least susceptible to a toxic insult are used for testing. There is a wide variation in diet and disparity in ages and in the level of health that makes humans possibly more susceptible.

(iv) Temporal conditions are not fully evaluated such as early in utero and fetal exposure affecting the animal to be more sensitive to an insult by the chemical in the later phases of its life.

(v) Neuropathogenesis may result from the destruction of only a very small structure of the brain (ie, the pars compacta of the substantia nigra) and such changes in structure may be missed in routine histopathological assessment of the brain. This is an important concept since the laboratory animal may have a higher tolerance to destruction of the brain area than the human and the animal may not show clinical signs until there is a major destruction but the human may show clinical signs after only minimal or moderate destruction.

(vi) Laboratory animals are not the same as humans. Detection of neuropathogenesis in animals does not mean that the human will develop the same lesion. Conversely, failure of animals to develop a neuropathological condition does not mean that the human will.

All of the factors above for weaknesses or limitations apply in to the study guidelines in general such as for assessing for cancer and developmental toxicity and these limitations are well recognized. In essence, studies conducted following the guidelines, as imperfect as they may be, plus other available data are what risk assessments are based on. Epidemiological data come later.

SUMMARY

Pesticides are individually tested in a series of studies with established guidelines with laboratory animals to determine if they have the potential for neuropathogenicity. Thus, the neuropathological effects of chemicals that are strong inducers of neuropathogenesis in the species of animals tested and if tested at the critical susceptible times will be detected in the battery of studies required for registration and reregistration. Additional testing in animals can be conducted based

on suggestions of neuropathogenesis from existing studies or based on structure activity relationships to further characterize a neuropathological condition possibly associated with the pesticide. Currently, the endpoints determined by the completed battery of required and other studies and the use of uncertainty factors in risk assessment are designed to provide a reasonable protection against possible neuropathogenesis of pesticides to humans. If humans are uniquely susceptible or the timing for test chemical administration in the animal studies is not appropriate, or if the pesticide must interact with other chemicals, potential effects in humans could be missed but the inclusion of the uncertainty factors is designed to protect against such possibilities by driving down exposure. The discovery that humans are susceptible to OPIDN resulted from accidental exposure to an organophosphate led to the development of the hen model for OPIDN testing which is the only model for neuropathy that is purposefully assessed for in routine screening studies. Had this accident not happened, there might be an occasional incident of persons developing the OPIDN syndrome today without knowing its cause. The OPIDN model is unlike the major human neurodegenerative diseases since OPIDN starts soon after exposure while Parkinson's and Alzheimer's may require long intervals between exposures and onset or they may require a natural onset before pesticides can facilitate their progression. Therefore, the possibility that pesticide exposures can be risk factors for neurodegenerative diseases needs to be considered in epidemiological models, whether alone or in combination with other factors. Development of animal models to more completely assess for possible relationships between pesticide exposure and neurodegeneration in humans need to be developed and validated to render data useful for risk assessment. Animal models with strains genetically engineered to be susceptible to known human neurodegenerative diseases may eventually be developed and validated and be important additions to the guidelines for neurotoxicity assessment.

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Mini-Review Article

Mechanisms of Neuronal Death in Synucleinopathy

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α-synuclein is a key molecule in the pathogenesis of synucleinopathy including Parkinson's disease and multiple system atrophy. In this mini-review, we mainly focus on recent data obtained from cellular models of synucleinopathy and discuss the possible mechanisms of neurodegeneration. Recent progress suggests that the aggregate formation of α-synuclein is cytoprotective and that its precursor oligomer (protofibril) may be cytotoxic. The catechol-derived quinones are the candidate molecules that facilitate the oligomer formation of α-synuclein. Furthermore, the cellular membranes are shown to be the primary targets injured by mutant α-synucleins, and the mitochondrial dysfunction seems to be an initial step in the neuronal death.

INTRODUCTION

α-synuclein is a 140 amino acid brain protein, mainly localized in presynaptic terminals [1, 2]. Although the detailed physiological functions of α-synuclein are still elusive, recent studies suggest that it plays a key role in synaptic functions cooperated with cysteine-string protein-α (CSP α), which contains a typical domain for HSP40-type molecular cochaperones [3]. In the subgroup of neurodegenerative disorders termed "synucleinopathies," α-synuclein is known to polymerize into fibrils and to accumulate in pathologic hallmark inclusions, such as lewy body (LB), lewy neuritis (LN), and glial cytoplasmic inclusions (GCIs). The LB and LN are characteristic of Parkinson's disease (PD), and point mutations or gene multiplications of α-synuclein are responsible for familial PD [4–6]. Moreover, transgenic flies overexpressing mutated human α-synuclein showed progressive locomotor disability with dopaminergic neuronal cell death with intracytoplasmic inclusions [7]. These findings suggest that abnormal α-synuclein metabolism plays a key role in neurodegenerative processes in PD and other synucleinopathies, but the precise underlying mechanisms still remain unknown [8]. To elucidate the possible roles of α-synuclein in neurodegeneration, we have developed cells that overexpress wild-type or mutant α-synucleins in dopaminergic or inducible catechol-quinone producing cell lines [9, 10].

Aggregate formation of α-synuclein and cell death

The inclusions in synucleinopathies were proved to be composed of β-sheet rich fibrils formed by nitrated species of α-synuclein [11]. Several lines of evidence suggested that reactive oxygen species (ROS) play a key role in the conformational change of α-synuclein and the following aggregate formation [12–15]. We developed human dopaminergic SH-SY5Y cells overexpressing wild-type or mutant α-synucleins, and established experimental models of intracellular aggregate formation following the exposure to various ROS [9]. The aggregates thus formed were immunopositive for ubiquitin, nitrotyrosine, and dityrosine, and positive for thioflavin S staining, which was in good agreement with the pathological features of inclusion bodies in synucleinopathies [9]. The γ-tubulin and molecular chaperones coexisted as well, suggesting that the aggregate formation was associated with the intracellular transport system for protein turnover responses against the toxic effects of misfolded proteins. Such mechanisms are called "aggresome" and are suggested to represent one of the cytoprotective responses [16–18]. Interestingly, the recent study on huntingtin showed that inclusion body formation reduced the risk of neuronal death [18]. However, it is still controversial whether the aggregate formation of α-synuclein has cytotoxicity in the neuronal cell or sequesters toxic species.

We established a cellular model in which intracellular α-synuclein aggregations were efficiently formed in response to various types of ROS exposure [9, 19]. Under these conditions, a significant number of cells showed caspase 3 activation [19]. To explore possible relationships between the aggregate formation and apoptosis, first we investigated whether α-synuclein aggregates colocalized with activated caspase-3 using a double immunostaining method. Following the combined exposure of the cells to a no donor and rotenone, α-synuclein aggregates were efficiently formed in the cytoplasm as previously reported [9]. Surprisingly, immunocytochemical analyses revealed that the aggregate positive cells did not show any caspase 3 activations and, conversely, that caspase 3 activated cells did not contain any α-synuclein aggregates [19]. Iron was able to induce α-synuclein aggregates more effectively than any other ROS inducers and no donors, suggesting the iron plays a key role in the aggregate formation [9]. When using both ROS and no inducers, the addition of ferric iron triggered further aggregate formation, but cells positive for activated caspase 3 were not coincident with aggregate positive cells. In quantification experiments, it was revealed that caspase 3-positive cells were decreased by the addition of ferric iron. On the other hand, by chelating ferric iron, the aggregate formation was decreased with concomitant increases of caspase 3 activation. These data suggest that the ferric iron plays a key role in the α-synuclein aggregation [19]. Furthermore, these data also imply that the aggregate formation may be cytoprotective against various cellular insults including oxidative stress [19, 20].

Possible interaction between α-synuclein and dopamine-quinone derivatives

Since α-synuclein is ubiquitously expressed at high levels in all brain regions [21], the mechanisms responsible for the preferential and selective neurodegeneration of dopaminergic neurons in the substantia nigra remain to be determined. Previous studies suggested that the specific vulnerability of dopaminergic neurons may be linked to the cytotoxic oxidative potential of dopamine [22]. Highly reactive oxygen species (ROS) are generated not only in dopamine oxidation but also during the decay of catechol-derived orthoquinones which covalently incorporate into a variety of molecules including proteins and nucleic acids [23]. On the other hand, previous reports demonstrated that α-synuclein might regulate dopamine metabolism by direct interaction with the tyrosine hydroxylase [24], the dopamine transporter [25] and vesicular monoamine transporter (VMAT2), key proteins in the regulation of the dopamine content within nerve terminals [26, 27]. Therefore, the pathological metabolism of α-synuclein may be closely linked to the misregulation of dopamine, consequently leading to neuronal death. In support of this notion, catechol-derived orthoquinones (eg, dopamine-quinone or DOPA-quinone) accelerate and stabilize the formation of α-synuclein protofibrils by inhibiting the conversion of toxic protofibrils into fibrils [28, 29].

To shed light on the pathophysiological mechanisms underlying α-synuclein-mediated neurodegeneration in dopamine neurons, we developed novel neuronal cell lines coexpressing α-synuclein (wild-type or A53T) and tyrosinase that produces catecholamines and their oxidized metabolites [30, 31]. Investigating the effects of wild-type or mutant α-synuclein expression, we found that the coexpression of wild-type and A53T mutant α-synuclein in tyrosinase-overexpressing cells exacerbated DNA damage and successive apoptotic cell death compared to the cells overexpressing CAT or antisense α-synuclein. Both wild-type and A53T mutant α-synucleins coexpressed with tyrosinase resulted in the gradual accumulation of high-molecular weight complexes immunopositive for α-synuclein. This band, possibly representing oligomerized forms, corresponded to the size of α-synuclein tetramer and was also detected by the NBT/glycinate redox-cycling staining, suggesting that it was modified by quinones [32].

Moreover, during these processes, the mitochondrial membrane potential was specifically decreased without the activation of MAP kinases [32]. Although the underlying mechanism(s) of neuronal cell death following the coexpression of tyrosinase and α-synuclein are still elusive, it is likely that α-synuclein modified by the oxidized catechol metabolites forms cytotoxic intermediates, that is, "protofibrils". Recent reports suggested that protofibrillar α-synuclein tightly binds to lipid bilayers and increases the membrane permeability by forming pore-like structures [33–35]. While the membranous structures damaged by protofibrils in dopaminergic nerve terminals remain unknown, intracellular organelles, such as synaptic vesicles and mitochondria, are possible candidates. In this regard, disruption of synaptic vesicle membranes would result in an increase of the cytoplasmic dopamine levels that would trigger the further accumulation of dopamine-quinone and dopamine-derived oxyradicals and thus lead to a vicious cycle. Likewise, mitochondrial enzymes in the electron transport chain and the functional permeability transition pores are impaired by dopamine oxidation products [36] making it plausible that the early damage of mitochondria observed in this cellular model reflects the actions of α-synuclein protofibrils and the subsequent increase of the membrane permeability in the presence of oxidized catecholamine metabolites [27].

Membrane injuries may trigger neurodegeneration

We further analyzed the resting membrane potential and whole-cell membrane conductance using the ramp voltage in the cell lines expressing wild-type or mutant α-synuclein [37]. Interestingly, the cells expressing A53T α-synuclein have the most depolarized membrane potential. By the application of the ramp voltage under the whole cell voltage-clamp condition, we obtained an almost linear current-voltage (I-V) relationship in each cell line. The slope of the I-V relationship in the cells expressing mutant α-synuclein was significantly steeper than that in the cells expressing the vector alone or wild-type α-synuclein, indicating that the expression of mutant α-synuclein results in higher ion

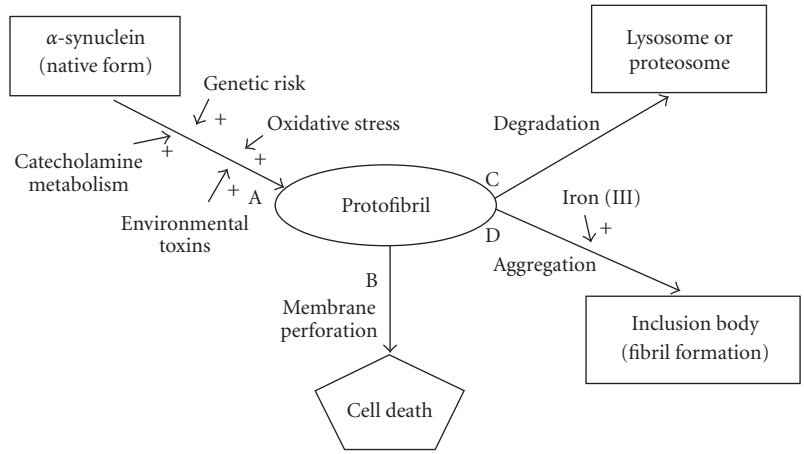

Figure 1: Possible mechanisms of neurodegeneration in synucleinopathy.

permeability of the plasma membrane [37]. Because it has been suggested that abnormal intracellular calcium homeostasis plays a crucial role in the pathogenesis of neurodegenerative disorders [38], the intracellular free calcium concentrations in α-synuclein-transfected cells were quantified using a calcium indicator dye, fura-2 [39]. Notably, both the intracellular calcium concentrations under basal conditions and after depolarization induced by potassium chloride application were significantly higher in the mutant α-synuclein expressing cells than in cells expressing the empty vector or wild-type α-synuclein [37]. These results suggest that mutant α-synuclein is involved in the perturbation of the intracellular calcium homeostasis.

Taken together, our data from cellular models of synucleinopathy suggest that oligomer or protofibril, but not aggregate or fibril, formation of α-synuclein plays a key role in the pathomechanisms of synucleinopathy (Figure 1). The iron specifically triggers the aggregate formation of α-synuclein, but this seems to be a cytoprotective process [19]. The cytotoxic protofibril formation may be facilitated by not only gene mutations, but also the modification of α-synuclein by catechol-derived quinones [28, 32]. The cellular membranes are the primary targets injured by protofibrils [37], and the mitochondrial dysfunction seems to be an initial step in the neurodegeneration of synucleinopathy [32]. Obviously, protofibrils or oligomers of α-synuclein are heterogeneous in size and stability and exist as mixtures in the cytosol. Therefore, at present it is difficult to specify the detailed molecular structures that may be responsible for the cellular injuries. However, from these data, it is plausible that the reduction of the protofibril pool may rescue neurons from death (Figure 1). If this is the case, not only the acceleration of degradation (C in Figure 1) but also the facilitation of aggregate formation (D in Figure 1), may be a novel strategy for the treatment of synucleinopathy. Of course, the most important way would be to decrease the input (A in Figure 1) into the protofibril pool.

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Review Article

Gaucher Disease and the Synucleinopathies

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Several recent observations suggest a connection between Gaucher disease, the inherited deficiency of glucocerebrosidase, and the synucleinopathies. Rare patients have been observed who develop both Gaucher disease and parkinsonism. Autopsy studies on these subjects reveal synuclein-positive Lewy bodies and inclusions. An increased incidence of synucleinopathies also has been noted in relatives of Gaucher probands. In complementary studies, screening of patients with parkinsonism has identified a greater than expected frequency of glucocerebrosidase mutations. These glucocerebrosidase mutation carriers have a wide spectrum of associated parkinsonian phenotypes, ranging from classic L-dopa-responsive Parkinson disease to a phenotype more characteristic of Lewy body dementia. Despite this association, the vast majority of Gaucher carriers and patients with Gaucher disease never develop parkinsonism. However, mutations in this gene are likely to be a contributing risk factor in subjects otherwise prone to developing synucleinopathies.

INTRODUCTION

The synucleinopathies, including Parkinson disease (PD), diffuse Lewy body dementia (DLBD), Lewy body variant of Alzheimer disease (LBVAD), and multiple system atrophy (MSA), are devastating adult-onset neurodegenerative diseases that affect millions of people worldwide. New insights into the genetics and pathophysiology of certain synucleinopathies have arisen from an unexpected source: a rare Mendelian disorder. Gaucher disease (GD) (MIM 230800, 230900, and 231000), the most common of the lipidoses, is the recessively inherited deficiency of the lysosomal enzyme glucocerebrosidase (EC.3.2.1.45). Affected individuals store the lipid glucocerebroside within lysosomes of macrophages, resulting in characteristic-appearing Gaucher cells. Associated clinical manifestations include hepatosplenomegaly, anemia, thrombocytopenia, easy bleeding, and bruisability, bony involvement and, in some cases, pulmonary involvement. Gaucher disease is classified into three major clinical types depending upon the degree of nervous system involvement. Patients with type 3, or chronic neuronopathic GD, have a varying degree of systemic involvement with at least one neurological manifestation; patients with type 2, or acute neuronopathic disease, have severe neurological involvement leading to death perinatally or in the first years of life. Type 1, the most common form, has no associated neurological symptoms by definition. In recent years, a small subgroup of patients has been identified that develop parkinsonian manifestations in adulthood. Several different and complimentary strategies have been used to investigate this association (Figure 1).

CLINICAL DESCRIPTIONS OF SUBJECTS WITH GAUCHER DISEASE AND PARKINSONISM

The first indications of a relationship between Gaucher disease and parkinsonism appeared in the literature as scattered case reports describing patients with Gaucher disease who developed early-onset, treatment-refractory parkinsonism [1–3]. Then, in 2003, a cohort of 17 such individuals was assembled, that included Ashkenazi Jewish probands as well as patients with diverse ethnicities [4]. The patients in this series had relatively mild Gaucher manifestations with a mean age at diagnosis of 35 years. In contrast, their parkinsonian symptoms had a rather early onset, with a mean age at diagnosis of 48 years. These individuals exhibited classic features, including asymmetric tremor, rigidity, akinesia and, at times, dementia. Four subjects in this series were treated with enzyme replacement therapy (ERT) with recombinant human glucocerebrosidase without any improvement or slowing of parkinsonian symptoms. It was also noted that some of these probands had a positive family history of parkinsonism in hetrozygous relatives.

Several other papers have described Gaucher probands with differing degrees of parkinsonian manifestations [5, 6]. These ranged from mildly affected subjects diagnosed in their 70's and 80's, to early onset subjects who developed dementia in their 40's. The spectrum appears to include both L-dopa-responsive and -resistant patients. Initial presentations have included the more classic unilateral tremor and others with progressive rigidity. The rate of progression also has been quite variable.

PATHOLOGICAL FINDINGS

The most consistent pathology observed in the brains from patients with neuronopathic type 2 and type 3 GD has been the periadventitial accumulation of Gaucher cells [7]. Significant neuronal loss with atropic neurons has been described in the basal ganglia, nuclei of the midbrain, pons and medulla, cerebellum, dentate nucleus, and hypothalamus [8, 9]. A recent neuropathological survey identified unique patterns of disease in neuronopathic patients, consisting of neuronal loss and gliosis specific to the hippocampal layers CA2-4 and layer 4b of the calcarine cortex [10]. Even in subjects with type 1 GD, which, by conventional definition, spares the CNS, astrogliosis of CA2 was noted.

In four individuals with Gaucher disease and parkinsonism, Lewy bodies were observed (Figure 2), as well as the involvement of hippocampal layers CA2-4 [10, 11]. Two of the patients had numerous intraneuronal, synuclein-positive inclusions in CA2-4, reminiscent of the brainstem-type Lewy bodies seen in the substantia nigra (SN) of idiopathic Parkinson disease. The other two patients lacked these hippocampal inclusions, but exhibited a Lewy body distribution consistent with diffuse Lewy body dementia. All four subjects exhibited hippocampal CA2-4 gliosis, depletion of SN neurons, SN gliosis, and brainstem-type Lewy bodies in the SN.

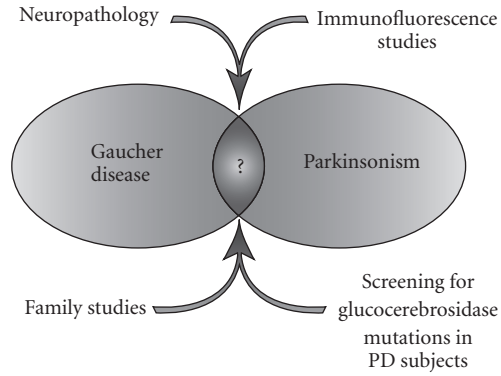
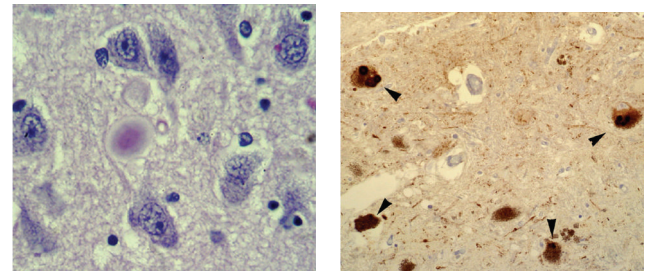


Figure 1: Several different strategies are employed to elucidate the relationship between Gaucher disease and parkinsonism. From Sidransky [38].



(a) (b)

Figure 2: Lewy bodies seen in a patient with Gaucher disease and parkinsonism. (a) CA2 hippocampal region demonstrating pyramidal cell neurons with a brainstem-type Lewy body. H&E stain, ×400 magnification. (b) Synuclein-immunoreactive Lewy bodies in hippocampal CA4 neurons, indicated by arrowheads. Synuclein antibody, Ventana, ×200 magnification. From Tayebi et al [4].

GLUCOCEREBROSIDASE MUTATIONS IDENTIFIED IN SUBJECTS WITH PARKINSON DISEASE

A molecular study was initiated to screen DNA extracted from brain tissue of individuals with pathologically confirmed, idiopathic Parkinson disease for alterations in the glucocerebroside gene (GBA). Remarkably, glucocerebrosidase mutations were detected in such subjects more often than expected, given the carrier frequency of Gaucher disease [11]. Direct sequencing of the entire glucocerebrosidase gene in 57 DNA samples collected from five different American brain banks revealed mutant alleles in eight (14%), including two homozygotes and six heterozygotes. Five of these had one or more alleles with the common N370S mutation, which is specifically associated with nonneuronopathic Gaucher disease. Four additional subjects carried E326K and T369M, which are considered polymorphic alterations in GBA. Brain samples from 44 age-matched controls without pathological evidence of PD were also sequenced and two were found to have the E326K allele, but no mutations were identified. In this series, the individuals with glucocerebrosidase mutations tended to be among the younger subjects screened, and most had documented Lewy bodies. Subsequently, DNA from 26 additional brain samples collected in Britain were sequenced and two (8%) carried glucocerebrosidase mutations [12]. These findings suggested that mutations in GBA, even in heterozygous individuals, might be an inherited risk factor for the development of parkinsonism.

These findings have since been substantiated by studies in other patient populations around the world. In many of the subsequent studies, however, the results were obtained by screening cohorts of patients diagnosed with Parkinson disease for one or more specific GBA mutations. Aharon-Peretz et al [13], in a clinic-based series of 99 Ashkenazi patients from Northern Israel with classic PD, screened blood samples for six common mutations in glucocerebrosidase. They identified 31 patients (31.3%) carrying glucocerebrosidase mutations N370S or c.84insG, including three N370S homozygotes. This frequency was over five-fold higher than the frequency of GBA mutations detected in their two control groups, which were composed of 74 patients with Alzheimer disease and 1543 Ashkenazi controls, respectively.

Both control groups were far from ideal, as significant clinical overlap exists between subjects carrying the diagnoses of PD and AD, and individuals included in the Ashkenazi control group were of mixed ages and had not been screened clinically to determine their neurological status. While the number of PD probands with glucocerebrosidase mutations was quite remarkable, it would be premature to attempt to estimate the relative risk of developing parkinsonism in an Ashkenazi individual with one or two glucocerebrosidase mutations.

A second report [14] focused on 160 Ashkenazi Jewish probands with Parkinson disease and 92 clinically evaluated, age-matched controls of Jewish ancestry from a New York City clinic. Each subject was screened for the N370S mutation. Seventeen probands (10.7%) with N370S were identified, including two homozygotes, as compared to 4.3% of controls, but these results did not reach statistical significance. While this study was limited, in that only one glucocerebrosidase mutation was considered, the frequency of mutations was considerably lower than that described in the Israeli cohort. Clearly, larger-scale studies with appropriate controls are warranted in the Ashkenazi population.

Investigators from Toronto [15] screened for seven glucocerebrosidase mutations, including two very rare alleles, among 88 unrelated Caucasian subjects of Canadian origin with clinically diagnosed parkinsonism. This cohort was selected for an early age of onset or a positive family history, and was compared to 122 clinically screened controls. Mutations were identified in 5.6% of the cohort with Parkinson's disease, as compared to 0.8% of the controls.

In a fourth study conducted in probands with early-onset Parkinson disease from Venezuela, the entire glucocerebrosidase gene was sequenced in 33 subjects and in 29 screened controls [16]. Four unrelated probands (12%) carried three different glucocerebrosidase mutations. Each of the four were L-dopa-responsive, and the ages at onset ranged from 29 to 47 years of age.

Thus, independent studies, despite differences in design and ascertainment, have detected mutations in glucocerebrosidase in subjects with parkinsonism at a frequency higher than expected in some populations. The approximate carrier frequency for GBA mutations is estimated at 0.0343 in the high-risk Ashkenazi Jewish population, and at 0.006 in the general population [7].

PHENOTYPIC FEATURES OF GAUCHER HETEROZYGOTES WITH PARKINSONISM

The phenotypic features encountered among subjects with parkinsonism carrying GBA mutations are quite varied. The group from Israel [17] compared the clinical characteristics of 40 subjects with Pd and at least one mutant GBA allele with those of 108 subjects with PD without an identified GBA mutation. They concluded that the overall clinical manifestations, including initial presentation and the extent of rigidity, tremor, bradykinesia, hallucinations, and dementia, were not significantly different in the two groups. All of the reported subjects had a favorable response to L-dopa. These authors also did not find a significant difference in age at onset, sex, or family history of PD. In contrast, subjects with GBA mutations identified by the brain bank screenings tended to have an earlier age at onset [11]. The series from Venezuela identified GBA carriers with very early-onset parkinsonism [16]. Moreover, recent analyses have suggested a high incidence of GBA mutations in subjects who died with the diagnosis of diffuse Lewy body dementia [18]. Thus, at present, the phenotype appears to include a wide spectrum of parkinsonian features ranging from classic L-dopa-responsive PD to those with early-onset symptoms or prominent dementia.

PARKINSONIAN MANIFESTATIONS AMONG RELATIVES OF PATIENTS WITH GAUCHER DISEASE

Another indication that heterozygotes for Gaucher mutations may be at risk for the development of parkinsonism has arisen from family studies. In a small pilot project, all patients with Gaucher disease seen in the Gaucher clinics at the National Institutes of Health over an 18-month period were questioned specifically regarding a possible family history of Parkinson disease or dementia. These interviews resulted in the identification of ten families in which obligate or confirmed carriers of GBA mutations developed parkinsonian manifestations [19]. Often, these individuals were the parent or the grandparent of the Gaucher proband.

One illustrative example was the extended family of a 7-year-old proband with type 3 Gaucher disease, where, in the paternal lineage, multiple family members spanning several generations developed parkinsonism. Both affected and unaffected relatives were examined and DNA samples were collected. It was found that in this family, heterozygosity for mutation L444P correlated with Parkinson disease. In the nine other smaller pedigrees, an obligate or confirmed carrier was shown to have parkinsonism. This study, therefore, lends additional support to the conclusion that mutations in the glucocerebrosidase gene, even in carriers, may contribute to the development of parkinsonism.

POSSIBLE MECHANISMS FOR THE ASSOCIATION OF GAUCHER DISEASE AND THE SYNUCLEINOPATHIES

Alpha-synuclein pathology in the brain is a feature of several prevalent neurodegenerative disorders, including Parkinson disease, dementia with Lewy bodies, the Lewy body variant of Alzheimer disease, and rare conditions such as multiple system atrophy and neurodegeneration with brain iron accumulation (NBAI-1) [20]. These disorders all demonstrate abnormal fibrillization and accumulation of proteinaceous, insoluble alpha-synuclein inclusions in neurons and glia, indicating a shared cellular pathology in the handling and clearance of alpha-synuclein. Alpha-synuclein is one of several proteins with a high propensity to aggregate. While this protein has little or no detectable secondary structure in solution and is considered to be natively unfolded, binding of alpha-synuclein to a number of ligands and proteins alters this native state and leads to partially folded conformations [21]. The end products of alpha-synuclein aggregation

are insoluble polymers or fibrils, considered necessary for the formation of Lewy bodies. Lewy bodies, however, contain other proteins, including cytoskeletal-associated proteins such as tau. A common pathological finding in the synucleinopathies is abnormal accumulation of hyperphosphorylated alpha-synuclein, either with or without tau [22], suggesting that tau may contribute to the aggregation process. One hypothesis is that mutated glucocerebrosidase also could contribute to aberrant fibrillization of proteins responsible for neurodegeneration.

It has been demonstrated that mutations in alpha-synuclein result in aberrant aggregation [23]. In addition, increased expression of wild-type alpha-synuclein through gene triplication can cause rare genetic forms of parkinsonism through a toxic gain-of-function that leads to neuronal death [24]. The pathology associated with synuclein mutations is much more widespread and may resemble DLBD. Similarly, the pathology observed in both Gaucher homozygotes and heterozygotes encompasses the spectrum of synucleinopathies, including DLBD, which might provide further support for the hypothesis that glucocerebrosidase contributes to aggregation of alpha-synuclein through a gain-of-function mechanism.

All synuclein mutations promote formation of oligomers, referred to as protofibrils. Protofibrils are still soluble, but can form annular structures which are toxic and might cause membrane damage [25, 26]. Soluble forms of alpha-synuclein, such as the native wild-type form and, possibly, protofibrils, are degraded via a lysosomal degradation pathway, called chaperone-mediated autophagy (CMA) [27]. Cuervo and colleagues noted that wild-type alpha-synuclein has a pentapeptide motif shared by other proteins that use this pathway for degradation. Experiments in PC12 cells demonstrated that alpha-synuclein binds to the chaperone molecule hsc70 in the cytosol, and is then internalized via a receptor, Lamp2a, on the lysosomal membrane. Lysosomal inhibitors such as ammonium chloride blocked this process. In contrast, mutant alpha-synuclein could still form complexes with the chaperone, but failed to internalize and remained bound to the receptor. This occupation of Lamp2a subsequently could inhibit the degradation of other CMA substrate proteins, resulting in a cellular log-jam. Mutations in glucocerebrosidase, therefore, might cause lysosomal dysfunction or interfere with receptor binding of alpha-synuclein at the lysosomal membrane, resulting in cell toxicity.

Other evidence indicates that the ubiquitin-proteasome system (UPS) may be compromised in PD [28, 29]. The accumulation and aggregation of potentially cytotoxic proteins in Lewy bodies suggest generalized protein mishandling and subsequent proteolytic stress. Thus, another mechanistic possibility is that GBA mutations that result in misfolded protein might overwhelm the UPS ability to degrade other abnormally accumulated proteins, including alpha-synuclein.

Another proposed mechanism for the association of GD and the synucleinopathies involves the potential role of lipids. Alpha-synuclein adopts a helical conformation when bound to lipid membranes, which would inhibit the conversion into fibrillar forms [30]. Other studies, however, indicate that lipids can also promote alpha-synuclein aggregation and toxicity through formation of protofibrils [31]. Alpha-synuclein has been shown to bind brain-derived glycosphingolipids that contain glucocerebroside as their core structure [32]. Therefore, potential changes in membrane lipid structure due to accumulation of the substrates glucocerebroside and/or the more toxic glucosylsphingosine might enhance both aggregation and cytotoxicity of synuclein, leading to the pathology associated with glucocerebrosidase mutations. As Gaucher carriers generally have no demonstrable lipid accumulation, however, this mechanism appears to be less likely.

THERAPIES

At present, available treatment options for the synucleinopathies provide no more than a temporary slowing of neurodegeneration and the concurrent functional deficits. The ability to prevent or delay onset is the best medical strategy, but progress in the early diagnosis and treatment of these disorders has been limited by an incomplete understanding of etiology and pathogenesis.

While enzyme replacement therapy is available for patients with Gaucher disease, there is no evidence that this treatment has any benefit for subjects with parkinsonism carrying GBA mutations. First, the recombinant enzyme does not cross the blood-brain barrier and has limited utility in the treatment of the neurological symptoms encountered in type 2 and 3 patients. Furthermore, several subjects with Gaucher and parkinsonism have received ERT with no improvement or slowing of their neurological manifestations [4, 5]. As heterozygotes have sufficient glucocerebrosidase activity to prevent glucocerebroside storage, additional enzyme would not be expected to have significant impact. Likewise, therapies designed to reduce substrate accumulation are not likely to be of benefit. If misfolding or impaired trafficking of mutant glucocerebrosidase, however, contributes to the protein aggregation encountered in the synucleinopathies, it is possible that specific chemical chaperone therapy could be of use in these patients.

IMPLICATIONS FOR GENETIC COUNSELING

The broader implications of these preliminary findings have the potential to generate considerable alarm. Clearly, caution is recommended in translating these findings to the patient community. From the general experience of treating thousands of patients with Gaucher disease, it is evident that the vast majority never develop Parkinson disease [33]. Furthermore, it is evident that the majority of Gaucher carriers do not have parkinsonism. Even in families where Gaucher disease and parkinsonism is found, not all carriers develop a neurodegenerative disorder [19]. Currently, especially in light of the different frequencies reported in these studies, [11, 13–16] it would be prudent to counsel families that mutations in this gene are just one of a multitude of potential risk factors that contribute to the development

of parkinsonism. There also are no current therapies for Gaucher disease that would likely to be of benefit to at-risk individuals.

For clinicians, however, awareness of this association may enable a better ascertainment of its frequency. Questions regarding a family history of tremor or dementia should be included in the evaluation of all patients with Gaucher disease. In addition, patients evaluated in Parkinson disease clinics should be asked whether any relatives have Gaucher disease.

The concept of heterozygous individuals being at risk for other disorders is not unique to Gaucher disease. There is growing evidence that heterozygosity for a Mendelian disorder may be a risk factor for the development of other common complex diseases [34–37]. These preliminary data suggests that heterozygosity for Gaucher mutations could be an additional inherited risk factor in an individual otherwise prone to parkinsonism. Explorations into the molecular and pathophysiological mechanisms underlying this association are being pursued aggressively, and will lead to a better understanding of both disorders.

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Review Article

HIV Neurotoxicity: Potential Therapeutic Interventions

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Individuals suffering from human immunodeficiency virus type 1 (HIV-1) infection suffer from a wide range of neurological deficits. The most pronounced are the motor and cognitive deficits observed in many patients in the latter stages of HIV infection. Gross postmortem inspection shows cortical atrophy and widespread neuronal loss. One of the more debilitating of the HIV-related syndromes is AIDS-related dementia, or HAD. Complete understanding of HIV neurotoxicity has been elusive. Both direct and indirect toxic mechanisms have been implicated in the neurotoxicity of the HIV proteins, Tat and gp120. The glutamatergic system, nitric oxide, calcium, oxidative stress, apoptosis, and microglia have all been implicated in the pathogenesis of HIV-related neuronal degeneration. The aim of this review is to summarize the most recent work and provide an overview to the current theories of HIV-related neurotoxicity and potential avenues of therapeutic interventions to prevent the neuronal loss and motor/cognitive deficits previously described.

GENERAL MECHANISMS OF HIV NEUROTOXICITY

Just over a decade ago, the first reports of HIV-related neurotoxicity were published. Two proteins associated with the AIDS virus, gp120 (a coat glycoprotein) and Tat (transactivation) have been shown to be neurotoxic. The HIV-associated protein gp120 was shown to be neurotoxic to cultured dopamine neurons [1]. Exposure to gp120 for 3 days reduced the ability of neurons to transport dopamine and decreased the size of the dendritic tree. The neurotoxicity of Tat was first identified by Nath et al [2] when they described the reactive epitope of Tat as being $Tat_{31–61}$. Full-length Tat is 86–104 amino acids in length and the analysis of peptides of differing, overlapping, lengths did not yield toxic responses in primary neuronal culture. Shortly after this report, Cheng et al [3] reported that Tat was neurotoxic to fetal neurons through a calcium-dependent mechanism. One postulated mechanism for Tat toxicity is via increased oxidative stress. Direct intrastriatal injections of Tat results in a significant increase in carbonyl formation [4]. Increased gliosis has been observed, indicating neuronal death and infiltration by glia [4–6]. Cellular damage and death following Tat administration have also been linked to an increase in apoptosis [7, 8]. Other mechanisms for Tat neurotoxicity include altered calcium homeostasis [7, 9], stimulation of TNF-α and NF-κB [10], stimulation of glutamate receptors [11], and activation of nitric oxide synthase and stimulation of nitric oxide production [12]. Similar to Tat, gp120 has been shown to be neurotoxic via multiple pathways. Both in vivo and in vitro, gp120 administration has been shown to induce apoptosis [13, 14]. Antagonism of glutamate receptors, primarily the NMDA subtype, attenuates gp120-induced toxicity [11, 15]. Activation and stimulation of the nitric oxide synthesis pathways has also been reported following exposure to gp120 [15].

Biomarkers of oxidative stress have consistently been detected in brain tissues and cerebrospinal fluid of patients with HIV-associated dementia [16]. The role for HIV-1 proteins in the development of oxidative stress associated with HIV-1 infection was proposed [17]. It is still debated whether the oxidative stress in HIV is attributable to direct interactions of HIV-1 proteins with neural cells or whether it results from chronic inflammatory reaction induced by the exposure of the CNS tissue to virotoxins. However, it is evident that neurotoxic HIV-1 proteins released from cells harboring HIV-1 may directly trigger oxidative stress, both in cell culture [7, 18] and in animal models [4, 6]. Even a transient exposure to HIV-1 proteins may be sufficient to trigger a cascade of events that leads to neuronal degeneration [19]. Thus, Tat is an important mediator of neurotoxicity in the HIV-infected brain and investigation of its role in HIV-associated neurodegeneration is important for understanding of the pathogenesis of HIV cognitive and motor dysfunction.

MICROGLIA/ASTROCYTES AND OPIOIDS

Involvement of microglia and astrocytes in HIV-related neurotoxicity has been established. Yet, whether the effects observed due to microglia involvement are a direct result of HIV-1 stimulation or a byproduct of infection remains to be elucidated. Parallels can be drawn between microglia involvement in neurological disorders such as HIV-related dementia, multiple sclerosis, and Alzheimer's disease [20]. In each disorder, microglia involvement includes the inflammatory process and the release of cytokines, chemokines, and nitric oxide. In addition to the release of damaging chemokines and cytokines, the tumor suppressor transcription factor, p53, has been shown to be necessary to induce apoptosis [21]. This could provide a novel pathway for HIV induction of neuronal apoptosis and cell death. A quite different profile is observed with astrocytes. When astrocytes express Tat, survival is promoted via increased antioxidant mechanisms, but Tat is released into the extracellular space where the adjacent neurons can take up Tat where axonal transport can take it to distal sites where it will elicit toxic effects [22, 23]. As neurons die, reactive gliosis takes place. This is characterized by an increase in glial fibrillary acidic protein (GFAP) staining. An increase in GFAP staining has been reported in cells exposed to Tat [24]. Astrocytosis could be particularly relevant in individuals abusing intravenous drugs. Astrocytes express opioid receptors and stimulation of the mu-subtype of the opioid receptor family potentiates Tat toxicity in neurons and astrocytes [25]. In addition to intravenous drug use, stimulation of mu-opioid receptors acting synergistically with HIV proteins to elicit neurodegeneration has greater impact regarding the use of opioid analgesics for the relief of pain. The use of morphine, codeine, fentanyl, and so forth, would be prohibitive in patients afflicted with HIV due to the increased risk for synergistic toxicity. Administration of morphine has been shown to upregulate the expression of the CCR3 coreceptor and CCR5 receptor in astrocytoma cells, which would increase viral binding and trafficking of the virus and promote viral infection [26]. Collectively, mu-stimulating opioid ligands would increase the receptors necessary for the transmission of the virus (CCR3 and CCR5), and potentiate the neurotoxicity observed in the presence of gp120. Recently, investigations into other opioid subtype-selective (kappa and delta) and their possible functionality as therapeutic interventions have begun (refer to "potential therapeutic interventions"). The role of astrocytes and microglia in the pathogenesis of HIV-related neurotoxicity is becoming increasingly apparent. From harboring the virus, to secreting proinflammatory chemokines and cytokines, to potentiating the spread of the virus and the viral particles, astrocytes and microglia play an integral role in HIV-related neurotoxicity.

CHEMOKINES

Proinflammatory chemokines function to exacerbate HIV neurotoxicity as an extension of astrocytes and microglia function. Chemokine involvement has long been suspected due to the actions of microglia following exposure to HIV-related proteins. The two prominent chemokine receptors that are involved in HIV neurotoxicity are the CXCR4 and CCR5 receptors, both of which are expressed on microglia [27]. The CXCR4 receptor belongs to the family of G-protein-coupled receptors and is believed to signal through a Gi/Go mechanism. Stimulation of these receptors results in an elevation in intracellular calcium, an effect which is reduced when pretreated with pertussis toxin to inactivate the receptor [28]. Initial hypotheses included gp120 direct interactions with neurons resulting in apoptosis, stimulation of astrocytes/microglia resulting in indirect effects on neurons, or possibly both mechanisms. Apoptosis induced by gp120 can be completely attenuated in the presence of the tripeptide, TKP, which prevents activation of microglia via CXCR4 receptors [29]. Antibodies to CXCR4 also prevented neuronal apoptosis due to caspase-3 activation [30]. Interestingly, the density of the CXCR4 receptor is inversely related to the concentration of fibroblast growth factor (FGF). As concentrations of FGF are increased, the density of CXCR4 receptors is reduced [31]. These effects are not due to direct actions of FGF on CXCR4 receptors, but through the FGF receptor kinase signaling pathway. Inhibitors of FGF receptor kinase attenuate the effects of FGF on CXCR4 receptor density [31]. Collectively, the effects of FGF in HIV encephalitis may be mediated through the inverse regulation of CXCR4 receptor expression. Similarly, stimulation of CHP100 cells, which constitutively express CXCR4 and CCR5 receptors, with phorbol 12-myristate-13-acetate downregulates CXCR4 and CCR5 and decreases the extent of gp120-induced cell death [32]. Alpha and beta chemokine ligands which are naturally released from microglia and astrocytes such as SDF1alpha, MIP1alpha, MIP1beta, and "regulated on activation, normal T-cell expressed and secreted" (RANTES) all interfere with the action of gp120 at CXCR4 and CCR5 receptors, and thus reduce cellular apoptosis and death [29, 30, 32]. SDF-1 results in a rapid release of tumor necrosis factor-alpha (TNF-alpha), which in turn exerts autocrine and paracrine effects on nearby cells. The release of TNF-alpha increases the synthesis of prostaglandins by activating the arachidonic acid cascade resulting in derangement of astrocytes-glial-neuronal communications [33]. In addition to increased prostaglandin formation, TNF-alpha increases the expression of proteinase-activated receptor-2 (PAR-2) which is responsible for cell survival [34]. Mice lacking PAR-2 are more susceptible to Tat-induced toxicity than wild-type [34]. SDF-1 promotes cell survival, which is not what would be expected considering that SDF-1 and gp120 are both agonists at the CXCR4 receptor. This discrepancy could be explained by differential effects on the p53 system. Stimulation of CXCR4 by gp120 stimulates p53 and increases the phosphorylation of Apaf-1 (proapoptotic) in neurons [35]. In addition, gp120 regulates the phosphorylation of p53 and regulates the expression of MDM-2 and p21 [35]. These effects are reversed by the p53-inhibitor pifithrin-alpha. Contrary to these findings, SDF-1 stimulates the acetylation of p53 and promotes the production p21.

Therefore, stimulation of the CXCR4 receptor could result in activation of different p53 targets dependent on the stimulating ligand, which will determine whether activation of CXCR4 exerts a positive or negative influence on neurons. Collectively, these data demonstrate the importance of astrocytes/microglia in the pathogenesis of HIV-related neurotoxicity. Early hypotheses focused on the involvement of glutamate as an integral component of HIV neurotoxicity. Although glutamate is a major toxin involved in neuronal death, it is now apparent that the CXCR4 receptor mediates cell death via non-glutamate mechanisms [36]. Investigation into the development of CXCR4 antagonists, in conjunction with NMDA antagonists, would offer better protection against gp120-induced cellular death.

GLUTAMATE AND EXCITATORY AMINO ACIDS

As discussed in the previous section, CXCR4 receptors appear to have a major role in the development of HIV-related neurotoxicity. Another component of this toxicity is the role of glutamate in cellular death. The toxic component secreted from HIV-1infected mononuclear phagocytes has been characterized as being a glutamate [37]. Some of the earliest studies demonstrated the addition of the N-methyl-D-aspartate (NMDA-) receptor antagonist memantine [38]. The addition of memantine not only increased the viability of neurons, but improved the dendritic arborization and synaptic density. The toxic effect is due to both the stimulation of NMDA receptors and the subsequent elevation in intracellular calcium. Coadministration of D-2-amino-5-phosphonopentanoic acid (APV) or MK-801 (both NMDA receptor antagonists) or removal of calcium from the culture media all decrease cell death associated with gp120 exposure [39]. One hypothesis for gp120 effects on glutamate homeostasis involves the attenuation of glutamate uptake, and concomitant increase in glutamate release [40]. Both gp120 and Tat have been postulated to work through a glutamate-mediated mechanism. Nath et al [11] demonstrated that coadministration of both gp120 and Tat exerted a synergistic potentiation of toxicity in monkeys. The concentrations of both gp120 and Tat were at subtoxic concentrations, yet the addition of the two proteins together resulted in a significant amount of cell death. To determine if these effects could be mediated by glutamate, the addition of memantine completely blocked the toxicity exerted by the gp120/Tat combination [11]. The toxic effects of Tat alone have also been linked to glutamatergic effects. Exposure of rat cortical neuronal cultures to low (nanomolar) concentrations of Tat or gp120 (picomolar) increases the uptake of calcium into neurons and eventually leads to cell death [41, 42]. Coadministration with the glutamate antagonists completely blocked the observed increase in ^{45}Ca influx and cell death [41, 42]. Administration of non-glutamate drugs had no effect on calcium influx or cellular death. In addition to the reports of direct alterations in glutamate homeostasis leading to cellular dysfunction, other endogenous glutamate agonists have been shown to be elevated in HIV infected individuals. One compound that has been shown to be elevated in HIV-patients is quinolinic acid. Activated macrophages produce quinolinic acid and this toxin has been implicated in a variety of neurological disorders. Macrophages exposed to Tat significantly increased their production of quinolinic acid, which correlated with an increase in indolamine 2,3-dioxygenase induction [43]. Interestingly, production of quinolinic acid occurs as part of the kynurenic acid pathway. Kynurenic acid is neuroprotective and functions as a glutamate antagonist [44]. A shift of the synthesis pathway favoring quinolinic acid results in neurotoxicity. Recent studies have added new insight into the mechanisms of HIV neurotoxicity involving the glutamate system [45, 46]. Not only does glutamate function through direct excitotoxic mechanisms by stimulating NMDA receptors, but also glutamate can induce the production of free radicals by inhibiting the uptake of cystine uptake and thus reducing the formation of glutathione [45]. Activated microglia express the glutamate transporter EAAT-1 and the density of this transporter increases with progression through the disease process. It would be suspected that this would provide protection against toxicity by increasing glutamate uptake and may in fact provide protection for a period of time. Later in the disease, when cognitive/motor deficits are more apparent and dementia begins, dysfunction of activated microglia could play a role in the neuronal apoptosis and death normally observed later in the disease [45]. Although it has been accepted that Tat toxicity involves a glutamate component, the exact mechanism by which this occurs has not been elucidated. Chandra et al [46] demonstrated that Tat releases Zn^{2+} from its binding site on the NMDA receptor. When Zn^{2+} is bound to the NMDA receptor, the receptor has reduced capacity to conduct Ca^{2+} through its channel. When Zn^{2+} is removed, even at neuroprotective concentrations, the NMDA receptor is activated; there is an increase in Ca^{2+} influx resulting in increased activity of an intracellular cascade leading to cellular death.

APOPTOSIS, CALCIUM, AND NITRIC OXIDE

As previously discussed, microglia involvement in HIV-related neurotoxicity involves oxidative stress, alterations in calcium homeostasis, activation of NMDA receptors, and apoptosis. Stimulation of NMDA receptors result in an influx of extracellular calcium. Elevations in intracellular calcium would activate nitric oxide synthase in neurons (nNOS, or NOS-1). Increases in nitric oxide can react with cellular superoxide forming the damaging peroxynitrite. This is further compounded by the contribution of nitric oxide from microglia which is inducible (iNOS or NOS-2). The involvement of nitric oxide in a variety of neurodegenerative diseases can be robust [47]. Polazzi et al [12] reported that Tat acts at the level of the iNOS gene to increase production of nitric oxide from activated microglia. Therefore, Tat does not act simply to increase the production of nitric oxide, but at the genomic level to induce the production of iNOS and interacts with NF-κB and interferon-gamma to potentiate the damage resulting from the nitric oxide that is produced [12]. This cycle could potentially form a "feed-forward" cycle where overstimulation of NMDA receptors

leads to alterations in calcium homeostasis, increases in nitric oxide formation, neuronal death, microglia activation, and additional nitric oxide being induced from the activated microglia [48]. Inhibitors of the NMDA receptor (MK-801) or iNOS (1400 W) all reduced gp120-induced neurotoxicity [48]. These findings suggest an important role of iNOS in the development of HIV-related neurotoxicity. Changes in intracellular calcium regulation may involve Na^+/H^+ exchangers and L-type calcium channels [41]. Addition of amiloride attenuated the intracellular rise in intracellular calcium in both neurons and astrocytes. Blockade of L-type calcium channels with nimodipine, diltiazem, and $CdCl_2 + NiCl_2$ also significantly decreased the rise in intracellular calcium in neurons, with no effect in astrocytes. This differential effect on neurons and astrocytes could afford protection against calcium-related toxicity if appropriate drugs are used to distinguish between neuron and astrocytes actions. One possible mechanism for this increase in intracellular calcium involves Tat modulation of phosphoinositide (PI) turnover [9]. Exposure of cultured fetal neurons to Tat resulted in elevated intracellular calcium that is attenuated by antagonizing the effects of inositol 1,4,5-triphosphate on releasing calcium from intracellular stores. Specifically, Tat functions through a pertussis-toxin sensitive phospholipase C mechanism to increase intracellular calcium leading to glutamate receptor-mediated calcium influx and subsequent dysregulation of calcium homeostasis [9].

Multiple mechanisms can lead to cellular apoptosis. It is accepted that both Tat and gp120 exposures will elicit significant cellular damage and death, but the apoptotic mechanisms differ between the two proteins. Both gp120 and Tat have been shown to induce apoptosis in neuronal culture. TGF-beta1 reverses the alterations observed in calcium homeostasis and reduces the number of neuronal cells dying from apoptotic cell death following exposure to gp120 [49]. TNF-alpha augments the effects of Tat on apoptosis which seem to be mediated in part by oxidative stress [50]. Administration of antioxidants will partially reverse the effects of Tat, yet a component exists which is not due to oxidative stress. Using HIV-1 isolates, cell lines which overexpress Bcl-2 or Bcl-xL are protected from damage whereas the wild-type cells are significantly compromised [51]. Addition of a Bcl-2 antagonist to the overexpressing cells completely reversed the neuroprotection. Collectively, the Bcl-2 pathway is an important pathway for the pathogenesis of HIV-related neurodegeneration, and modulation of this pathway could afford protections against neuronal damage. Another pathway with importance for HIV neurodegeneration involves TNF-alpha and the TNF-related apoptosis-inducing ligand (TRAIL). In HIV infection, human monocyte-derived macrophages exhibit an increase in TRAIL levels and these cells are associated with neurons which are caspase-3 positive [52]. Analysis of specific protein actions has demonstrated that gp120 works in part through enhancement of COX-2 expression and activation of interleukin-1 converting enzyme (caspase-1). Antagonists of caspase-1 activity, acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK) and t-butooxycarbonyl-L-aspartic acid benzyl ester-chloromethylketone (Boc-Asp-(OBzl)-CMK) block the release of interleukin-1beta and attenuate apoptosis [53]. Blockade of the inducible form of COX-2 with NS-398 also prevents the rise in cellular apoptosis [53]. Intraventricular administration of gp120 for up to 7 days resulted in an elevation in the expression of caspase-3 in rat brain [54]. Singh et al [55] expanded these findings to confirm those of Acquas et al [54] that demonstrated gp120 activation of caspase-3, but extended them to include the actions of Tat which appeared to promote apoptosis via an endonuclease G mechanism. The caspase inhibitor Z-DEVD-FMK significantly reduces gp120-induced elevations in caspase-3, but had no significant effect on Tat toxicity. These findings are the first to suggest that although both gp120 and Tat elicit robust neurotoxicity and cellular apoptosis, they can work through different apoptotic mechanisms.

STEROIDS

Sex steroids

Postmenopausal women who are infected with HIV are at risk for experiencing dementia and Parkinson's-like symptoms associated with low levels of estrogen [56] leading to speculation that estrogen may attenuate the development of these debilitating symptoms [57]. 17β-estradiol (E_2)has been generally observed to be neuroprotective in cell culture [58, 59]. A potential mechanism for E_2 protection may be the phenolic A ring, which would act as a direct free radical scavenger [60]. The concept of neuroprotection by E_2 has expanded beyond the realm of simple steroid-receptor interactions to include interactions with unidentified cell-surface receptors and direct modulation of neurotransmitter function [58, 59]. Potential exists for direct effects of E_2 as a free radical scavenger, interactions with cell-surface receptors, or with its nuclear binding site. From these studies, the latter appears to be unlikely since nuclear effect would commonly occur over hours or days. It is clear that E_2 affords some level of neuroprotection in all culture models, and this protection is dependent on a complex system of interactions from the extracellular to the nuclear space.

Collectively, previous reports have helped to partially elucidate the mechanisms of Tat and gp120 toxicity. Recent work has indicated that E_2 may slow the transmission of HIV [61], protect against the synergistic toxicity of Tat and gp120 [62], reduce Tat-induced inflammation in endothelial cells [63], and may prevent neuronal death in the presence of HIV-1 protease [64]. The synergist toxicity following gp120 and Tat exposure can be extended to synergistic toxic effects of cocaine. Subtoxic concentrations of gp120 and Tat, plus physiologically relevant concentrations of cocaine, resulted in a significant increase in cell death [65]. This effect is blocked in the presence of E_2, but not 17α-E_2. Progesterone afforded minimal protection, yet surprisingly, testosterone exhibited protection on the level of E_2 [65]. The effects of both E_2 and testosterone were reversed by ICI-182,780, a selective estrogen antagonist, suggesting interactions with the cell-surface E_2 receptors. Not only does E_2 protect against oxidative cell

death, but also evidence suggests that it prevents apoptosis, through both a Bcl/Bax mechanism and interleukin-1beta [24, 66]. This inhibition is reversed by both ICI-182,780 and tamoxifen, E_2 antagonists. Further work is needed on this topic to determine the efficacy of E_2 neuroprotection with regard to HIV neurotoxicity [67].

Glucocorticoids

The ability of glucocorticoids to potentiate HIV neurotoxicity has long been known, but the mechanisms of this action are poorly understood. Synthetic glucocorticoids such as prednisone and dexamethasone potentiate the effects of gp120 on intracellular calcium concentration [68, 69]. This is particularly deleterious considering that these synthetic glucocorticoids are routinely used to treat *Pneumocystis carinii* pneumonia, which is commonly observed in AIDS patients. A possible mechanism for this action is via reduction in energy (ATP) levels which reduces mitochondrial membrane potential and promotion of extracellular calcium increases. Energy supplementation has been shown to ameliorate these effects of glucocorticoids [68]. These studies have been replicated in vivo using physiological concentrations of cortisol, resembling concentrations observed during stress with similar outcomes, increased mobilization of intracellular calcium, and increased depletion of ATP [70]. Concentrations of glucocorticoids and gp120, which are nontoxic independently, cause significant toxicity when coadministered in striatal cultures [69]. Administration of gp120 alone results in significant increases in reactive oxygen species, whereas coadministration with cortisol increases the amount of lipid peroxidation [71]. It is clear that glucocorticoids exacerbate HIV, and gp120, toxicity by potentiating increases in intracellular calcium and reducing cellular ATP levels. When combined, these effects predispose the cell to oxidative damage and death.

STRIATAL TOXICITY, DOPAMINE, AND DEMENTIA

HIV dementia is a subcortical dementia associated with dysfunction in the basal ganglia [72]. Currently, there are multiple HIV proteins that have been reported to exert neurotoxic effects. Several groups have demonstrated the presence of Tat protein in brains of patients with HIV encephalitis by immunostaining [73, 74]. Additionally, mRNA levels for Tat are also elevated in brain tissue of patients with HIV dementia [73]. Once released, Tat and gp120 have been shown to exert toxicity through an oxidative stress pathway [4–6, 75]. One neurological system that is involved in gp120- and Tat-induced neurotoxicity is the dopaminergic system. Patients develop symptoms of dopamine deficiency and exhibit heightened sensitivity to dopaminergic selective drugs as well as psychostimulants which act on dopaminergic neurons in the basal ganglia [76]. Other neurotransmitter systems that interact with the dopaminergic system, such as glutamatergic and opioid [77], also increase the sensitivity of the basal ganglia to the neurotoxic properties of gp120 and Tat. Therefore, the basal ganglia and the dopaminergic system would be a likely target for the development of therapeutic agents to attenuate or prevent the development of HIV-associated dementia.

Administration of gp120 to striatal cell cultures elicits a toxicity that is reversed by coadministration of a glutamate antagonist [1]. Toxicity of Tat to striatal neurons is due, in part, to direct actions on the neuron which is not subject to desensitization [3]. The dementia that has been observed in HIV patients only occurs in about 20% of all infected people. Comparison of brain extracts from demented versus non-demented patients revealed that supernatants from demented patients showed significantly greater toxicity compared to supernatants obtained from nondemented patients [78]. One underlying mechanism for HIV-related dementia and Parkinson-like symptoms could be loss of dopamine transporters/function following gp120 and Tat exposure. Recently, Wang et al [79] reported that patients suffering from HIV-associated dementia exhibited a significant 13%–20% reduction in dopamine transporter density compared to seronegative controls. This study was the first to demonstrate HIV-induced damage to dopaminergic terminals in humans, which was inversely related to viral load (load increases, transporter density decreases). Additional studies are needed to further elucidate these mechanisms and offer insight into possible therapeutic interventions to prevent/attenuation HIV-associated neurotoxicity to dopamine neurons.

POTENTIAL THERAPEUTIC INTERVENTIONS

Currently, there are no therapeutic interventions which can be used to attenuate the development of HIV neurotoxicity and/or dementia. Based on the data that has been discussed, logical choices would be ligands which block some of the mechanisms associated with gp120 and Tat toxicity. This could include antagonizing NMDA glutamate receptors, blocking the intracellular rise in calcium, inhibition of NOS, or blocking the effects of microglia/chemokines. One of the first avenues pursued was antagonism of the NMDA receptor. A low concentration of NMDA, plus arachidonic acid enhances toxicity in neuronal culture. It is possible that gp120 activates phospholipase A2 to increase arachidonic acid release, which then sensitizes the NMDA receptor to the actions of glutamate. This effect is reversed by memantine, an NMDA receptor antagonist [80, 81]. Memantine is currently clinically used to treat spasticity and Parkinson's disease and may provide therapeutic relief of some of the symptoms of HIV neurotoxicity. Further work is warranted to more completely elucidate the function of memantine in the CNS and determine its usefulness in treating HIV neurotoxicity. A drawback to memantine use is that it will only be effective if used in a prophylactic manner. After neuronal death occurs, and the onset of dementia is apparent, memantine would have little therapeutic usefulness other than to slow the progression of dementia.

Opioid compounds have yielded the mixed results when attempting to determine their interaction with the gp120 or the Tat. Compounds which would normally be considered

mu-preferring have been shown to potentiate the toxicity of gp120 and Tat [25]. Conversely, other kappa- and delta-preferring opioid and nonopioid analgesic agents have been shown to be protective. The nonopioid compound, flupirtine, produces analgesia, but also possesses anticonvulsant and muscle-relaxant properties and has been shown to be cytoprotective against gp120-induced toxicity [80]. The kappa-preferring agonist, U50,488, suppresses microglia release of quinolinic acid [82]. Suppression of quinolinic acid release will decrease stimulation of NMDA receptors and consequently reduce excitotoxicity in neurons. We have shown that the peptidergic delta agonist, DPDPE, reduces oxidative stress in SK-N-SH cells exposed to Tat [83]. Our results also extend to the nonpeptidergic agonist, SNC-80, and the effects of both agonists are reversed by delta-preferring antagonists, suggesting a receptor-mediated mechanism for inhibition of oxidative stress [83]. Interestingly, opioids which are abused (morphine, heroin, and their derivatives) act primarily at the mu-subtype, which is the subtype primarily responsible for synergistic toxicity with gp120 and Tat. Agents which act at kappa- and delta-opioid receptors may provide viable analgesic options for individuals that are HIV infected.

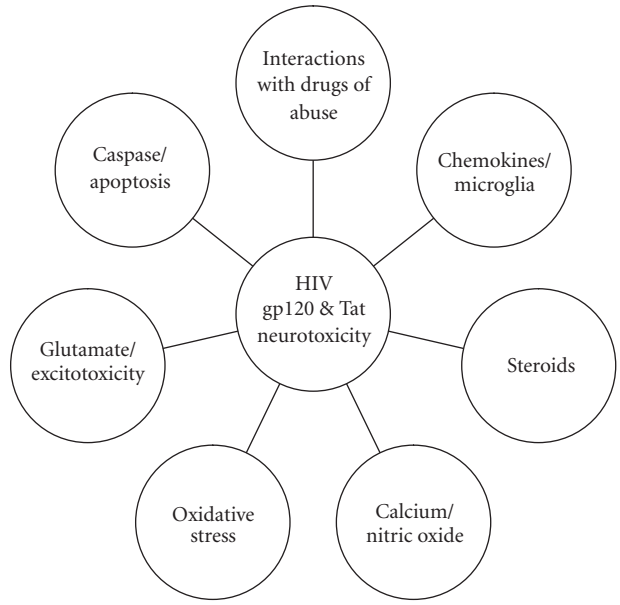
Another area that has garnered a great deal of interest in the last decade has been the effects of steroids on the neurotoxicity of HIV proteins. It has become clear that hormones released in response to stress, namely cortisol, potentiate the toxicity of gp120 and Tat [69, 70]. Intermediate (prednisone) and long-acting (dexamethasone) glucocorticoids are often prescribed for respiratory-symptoms-associated opportunistic diseases associated with HIV which could compound HIV-related neurodegeneration. More interest has been given to the protective effects of estrogen. We have shown that estradiol reduces the oxidative stress elicited by gp120 and Tat in SK-N-SH cells and this effect is reversed by the estrogen antagonist ICI-182,780 [84]. This work supported previous reports that estradiol attenuates the toxicity following HIV-1 protease administration [64], gp120 administration [85, 86], or coadministration of gp120 and Tat [62]. There have been mixed results concerning the effects of other sexsteroids such as progesterone, testosterone, and their derivatives. It appears that whether these hormones are protective or potentiating depends on the assay system that is being utilized and the manipulations that are used to optimize the assay. Further work on the effects is necessary to fully understand its protective potential in HIV model systems. Questions that must be asked are (1) are the effects of estradiol selective for estradiol, or can other "estrogens" elicit the same effect, and (2) are estradiol effects mediated by cell-surface receptors, intracellular receptors, or possible due to the structural nature of estradiol. Areas of current research that appear to be yielding exciting results are the use of selective-estrogen receptor modulators (SERMs) and the use of plant-derived estrogens. Early studies have shown that administration of some of these compounds can prevent, or attenuate, HIV protein-induced toxicity without the side effects associated with estrogen, such as promotion of estrogen-dependent tumors in females or feminization of males (unpublished observations).

A wide array of other approaches has been used to try and prevent the pathogenesis of HIV-related neurodegeneration and dementia. One compound that has yielded interesting results is CPI-1189 in the treatment of HIV-related dementia. CPI-1189 works through an unknown mechanism, but appears to ameliorate TNF-alpha toxicity by increasing activation of ERK-MAP kinase [87]. CPI-1189 also attenuates culture toxicity in the presence of quinolinic acid and gp120 [87]. Excitement about CPI-1189 has waned after a more recent clinical trial has indicated that CPI-1189 is well tolerated, but did not demonstrate a significant improvement in neuropsychological measures [88]. Inhibition of matrix metalloproteinase with prinomastat reduced neuronal toxicity following exposure to supernatants from brain-derived Tat sequences obtained from demented patients [89]. The hypothesis put forth by Johnston et al [89] was that the particular Tat sequence from demented patients resulting in a higher level of toxicity due to induction of matrix metalloproteinases. Prophylactic treatment with lithium has also been postulated to be neuroprotective [90]. Pretreatment or coadministration of lithium to neuronal cultures exposed to gp120 was protected via a phosphatidylinositol 3-kinase/Akt pathway, but treatment following gp120 exposure was ineffective [90]. Targeting oxidative damage is another therapeutic avenue that has been explored. Compounds such as L-deprenyl, didox, imidate, diosgenin, and ebselen all prevented oxidative damage following exposure to CSF from HIV demented patients [91]. Human lipidated apoE3 has also been shown to protect neurons from Tat-induced toxicity via prevents of Tat-induced oxidative stress [92, 93]. Also Tat and apoE compete for the same binding site, resulting in increased extracellular Tat; the ability for apoE to prevent Tat-induced oxidative stress may outweigh the increase in extracellular Tat. More investigation into this effect would be warranted prior to making any definitive conclusions regarding the effectiveness of apoE. Involvement of chemokines, and in particular the CXCR4 and CCR5 receptors, in HIV-neurotoxicity and dementia has been demonstrated by many investigators. One hypothesis put forth involves the blockade of these receptors, leading to subsequent reductions in toxicity. Use of a novel CXCR4 antagonist, neomycin B hexa-arginine, has been shown to effectively cross the blood-brain barrier and reduce gp120-induced toxicity through a CXCR4-mediated mechanism [27].

Collectively, there appears to be no definitive therapeutic treatment for HIV-neurodegeneration and dementia. Some of the above compounds, such as the estrogenic compounds and the CXCR4 antagonist, neomycin B hexa-arginine, have shown some promise. Use of brain-derived neurotrophic factor (BDNF) has yielded favorable results, although delivery of a peptide into the brain offers some challenges [94]. A great deal of additional work is still necessary to determine the true effectiveness of any of these therapeutic approaches.

SUMMARY AND CONCLUSIONS

It is clear from the literature and ongoing studies that considerable work needs to be done to further elucidate the



Scheme 1: *Schematic of various into HIV neurotoxicity and the major factor involved.*Although not an inclusive list, these are the major factors in the pathogenesis of HIV neurotoxicity and the development of dementia. The surrounding circles are not meant to imply that each factor is exclusive of other factors since considerable overlap exists between each of the components.

mechanisms of HIV neurotoxicity and the development of HIV-related dementia. It is also apparent that the development of therapeutically useful drugs will be delayed due to the complexity of the systems involved with HIV, and gp120/Tat in particular, toxicity. In Scheme 1, factors implicated in HIV neurotoxicity are depicted demonstrating the complexity of mechanisms underlying HIV neurotoxicity. Each of the surrounding circles also represent current avenues being pursued in the development of clinically relevant compounds to attenuate or prevent the progression of HIV neurotoxicity and dementia.

In sum, interest in elucidating the toxicity cascades for gp120 and Tat toxicity is great. Understanding these cascades is tantamount to developing therapeutic agents which could attenuate or prevent the neuronal degeneration associated with late-stage HIV infection. Agents with effects on multiple cascades are the most likely agents to provide relief from the progression of neuronal degeneration and may very likely come from sources not yet examined. An example could be the effects of lithium on gp120 toxicity as previously reported. Fully understanding the differences between individuals who are demented and those who are not will add significant amounts of information to our knowledge base. If the next decade has even greater productivity than the last, significant and robust advancements can be achieved.

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