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Employing New Cellular Therapeutic Targets for Alzheimer's Disease: A Change for the Better?

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Abstract

Alzheimer's disease is a progressive disorder that results in the loss of cognitive function and memory. Although traditionally defined by the presence of extracellular plaques of amyloid-β peptide aggregates and intracellular neurofibrillary tangles in the brain, more recent work has begun to focus on elucidating the complexities of Alzheimer's disease that involve the generation of reactive oxygen species and oxidative stress. Apoptotic processes that are incurred as a function of oxidative stress affect neuronal, vascular, and monocyte derived cell populations. In particular, it is the early apoptotic induction of cellular membrane asymmetry loss that drives inflammatory microglial activation and subsequent neuronal and vascular injury. In this article, we discuss the role of novel cellular pathways that are invoked during oxidative stress and may potentially mediate apoptotic injury in Alzheimer's disease. Ultimately, targeting new avenues for the development of therapeutic strategies linked to mechanisms that involve inflammatory microglial activation, cellular metabolism, cell-cycle regulation, G-protein regulated receptors, and cytokine modulation may provide fruitful gains for both the prevention and treatment of Alzheimer's disease.

Keywords

β-Amyloid; Akt; caspases; erythropoietin; Forkhead transcription factors; metabotropic; microglia; nicotinamide; Wnt

PATHOPHYSIOLOGY AND MEDICAL IMPLICATIONS OF ALZHEIMER'S DISEASE

Neuronal injury during Alzheimer's disease predominantly occurs in the hippocampus and the cortex. The disorder leads to a progressive deterioration of cognitive function with loss of memory and is characterized by two pathologic hallmarks that consist of extracellular plaques of amyloid-β peptide aggregates and intracellular neurofibrillary tangles composed of hyperphosphorylated microtubular protein tau. The β-amyloid deposition that constitutes the plaques is composed of a 39−42 amino acid peptide (Aβ), which is the proteolytic product of the amyloid precursor protein (APP) (Maiese, K and Chong, ZZ, 2004).

With an aging population, medical costs for neurodegenerative disease parallel a progressive loss of economic productivity with increasing morbidity and mortality. The cost of physician

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services, hospital and nursing home care, and medications also proceeds at an exponential rate ultimately resulting in an annual deficit to the economy that is greater than $380 billion. The most significant portion of this economic loss is composed of only a few neurodegenerative disease entities with Alzheimer's disease representing a significant portion. For example, the annual cost per patient with Alzheimer's disease is estimated at $174, 000 with an annual aggregate cost of $100 billion (McCormick, WC *et al*., 2001, Mendiondo, MS *et al*., 2001).

EXPLORING NOVEL MECHANISMS OF CELL INJURY

A variety of cellular mechanisms can lead to the generation of Alzheimer's disease. At present, no definitive therapy for the prevention or resolution of this disease exists. New investigations have begun to focus on pathways of oxidative stress that involve cellular pathways that may mediate cell injury at levels below the development of β-amyloid aggregates and neurofibrillary tangles. In this review, we consider alternative cellular pathways that involve oxidative stress and apoptotic injury for the development of therapeutic strategies against Alzhemier's disease. These pathways can originate with the proto-oncogene Wnt and the serine-threonine kinase Akt and involve mechanisms related to cellular metabolism, cell cycle regulation, metabotropic glutamate modulation, and cytokine modulation.

Oxidative Stress and Alzheimer's Disease

Oxidative stress represents a significant pathway that leads to the destruction of both neuronal and vascular cells. Oxidative stress occurs as a result of the production of reactive oxygen species (ROS). ROS consist of oxygen free radicals and associated entities that include superoxide free radicals, hydrogen peroxide, singlet oxygen, nitric oxide (NO), and peroxynitrite. The production of ROS, such as peroxynitrite and NO, can lead to cell injury through cell membrane lipid destruction and cleavage of DNA (Vincent, AM and Maiese, K, 1999, Wang, JY *et al*., 2003). ROS result in the peroxidation of cellular membrane lipids (Siu, AW and To, CH, 2002), peroxidation of docosahexaenoic acid, a precursor of neuroprotective docosanoids (Mukherjee, PK *et al*., 2004), the cleavage of DNA during the hydroxylation of guanine and methylation of cytosine (Lee, DH *et al*., 2002), and the oxidation of proteins that yield protein carbonyl derivatives and nitrotyrosine (Adams, S *et al*., 2001). In addition to destroying cellular integrity, ROS can inhibit complex enzymes in the electron transport chain of the mitochondria resulting in the blockade of mitochondrial respiration (Yamamoto, T *et al*., 2002).

Oxidative stress precipitates apoptotic cellular injury that consists of both nuclear DNA degradation and membrane phosphatidylserine (PS) exposure (Chong, ZZ *et al*., 2003, Vincent, AM *et al*., 1999, Witting, A *et al*., 2000). Membrane PS exposure and DNA fragmentation are independent processes that lead to apoptosis. The biological role of membrane PS externalization can vary in different cell populations. In many cell systems, membrane PS externalization can become a signal for the phagocytosis of cells (Chong, ZZ *et al*., 2003, Chong, ZZ *et al*., 2004, Hoffmann, PR *et al*., 2001, Kang, JQ *et al*., 2003). In neurons, cells expressing externalized PS may be removed by microglia. In contrast, membrane PS exposure also can function in vascular cells to activate coagulation cascades. The externalization of membrane PS residues in endothelial cells (ECs) can promote the formation of a procoagulant surface (Dombroski, D *et al*., 2000, Fadok, VA *et al*., 2001, Maiese, K and Vincent, AM, 2000). Furthermore, oxidative stress can significantly increase chromosomal aberrations and micronuclei (Bresgen, N *et al*., 2003, Chong, ZZ *et al*., 2002) and lead to the activation of apoptotic pathways (Chong, ZZ *et al*., 2002, Lee, DH *et al*., 2002) in ECs. ECs that are exposed to oxidative stress incur both DNA fragmentation and membrane PS externalization during exposure to insults, such as hypoxia, oxidants, and free radicals (Aoki, M *et al*., 2001, Burlacu, A *et al*., 2001, Chong, ZZ *et al*., 2002, Chong, ZZ *et al*., 2002, Lin, SH and Maiese, K, 2001).

Exposure to ROS can precipitate apoptosis in neurons and ECs through multiple cellular pathways. Oxidative stress, such as NO or hydrogen peroxide, results in nuclei condensation and DNA fragmentation (Chong, ZZ *et al*., 2003, Goldshmit, Y *et al*., 2001, Pugazhenthi, S *et al*., 2003, Vincent, AM *et al*., 1999). In neurons, NO exposure produces apoptotic death in hippocampal and dopaminergic neurons (Chong, ZZ *et al*., 2003, Sharma, SK and Ebadi, M, 2003, Vincent, AM and Maiese, K, 1999, Witting, A *et al*., 2000). Injury during NO exposure also can become synergistic with hydrogen peroxide to render neurons more sensitive to oxidative injury (de la Monte, SM *et al*., 2003, Wang, JY *et al*., 2003). Hydrogen peroxide also results in neuronal injury through impaired mitochondrial function and increased levels of pro-apoptotic gene products, such as CD95/Fas (de la Monte, SM *et al*., 2000, Pugazhenthi, S *et al*., 2003, Vaudry, D *et al*., 2002). Externalization of membrane PS residues also occurs in neurons during anoxia (Chong, ZZ *et al*., 2002), NO exposure (Chong, ZZ *et al*., 2003), or during the administration of agents that induce the production of ROS, such as 6-hydroxydopamine (Salinas, M *et al*., 2003).

Oxidative stress is believed to lead to both the onset and progression of Alzheimer's disease. Transient hypoxia in sporadic Alzheimer's disease can lead to mitochondrial dysfunction, impaired membrane integrity, and APP cleavage (Chen, GJ *et al*., 2003). During the progression of Alzheimer's disease, lipid peroxidation (Butterfield, DA *et al*., 2002), protein oxidation (Choi, J *et al*., 2003), and DNA oxidation (Lovell, MA *et al*., 1999) have been reported. Furthermore, in mice overexpressing APP, the brain Aβ deposits that are characteristically found in Alzheimer's disease co-localize with an array of oxidative stress markers (Smith, MA *et al*., 1998), suggesting that there exists a close correlation between oxidative stress and Aβ deposition.

Aβ has been found to result in the generation of ROS, such as hydrogen peroxide, through metal ion reduction and to lead to oxidative toxicity in neurons (Huang, X *et al*., 1999). Free radical generation by Aβ is strongly influenced by the aggregational state of the peptides (Monji, A *et al*., 2001). Given the link between Aβ deposition and oxidative stress, agents that modulate ROS may be potentially useful in the therapy of Alzheimer's disease. For example, application of the free radical antioxidant vitamin E has been demonstrated to prevent neurotoxicity from Aβ (Subramaniam, R *et al*., 1998).

Other evidence exists that suggests cellular injury during Alzheimer's disease may result from not only increased insults from oxidative stress, but also from impaired cellular repair mechanisms following oxidative injury. Significant elevations of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, are observed in intact DNA in the cerebrospinal fluid of Alzheimer's patients. Yet, levels of free 8-OHdG, which are generated during normal cellular repair mechanisms, are depleted significantly in the cerebrospinal fluid of Alzheimer's patients (Lovell, MA *et al*., 1999).

Cellular oxidative pathways that proceed through apoptosis appear to be a predominant factor in the cell loss observed during Alzheimer's disease. Accumulating evidence has been obtained from human and *in vitro* models of Alzheimer's disease suggesting that apoptosis contributes to the neuronal loss during the disease. Data from *in situ* TUNEL (terminal deoxynucleotidyl transferase nick-end labeling) assays of brain tissues from Alzheimer's patients demonstrate neuronal demise consistent with apoptotic cell death. A correlation between the incidence of TUNEL-positive cells and plaque density was also observed (Colurso, GJ *et al*., 2003). Levels of the apoptotic marker prostate apoptosis response-4 (Par-4) also has been shown to be significantly increased in the brains of patients with Alzheimer's disease (Guo, Q *et al*., 1998). Other lines of evidence link apoptotic cellular injury with APP and its proteolytic product Aβ. In *in vitro* studies, expression of familial Alzheimer's disease mutants of APP result in apoptotic neuronal injury (McPhie, DL *et al*., 2003). It is the cytoplasmic domain of

APP that can lead to sustained apoptosis through c-Jun N-terminal kinase pathways (Hashimoto, Y *et al*., 2003). Additional studies have illustrated that direct application of Aβ to neuronal cells can lead to chromatin condensation, DNA fragmentation, and membrane PS exposure characteristic of apoptosis in cultured neurons (Fig. **3**).

Inflammatory Mechanisms During Alzheimer's Disease

In patients with Alzheimer's disease, microglial cells co-localize with the perivascular deposits of β-amyloid. In addition, microglial activation has been observed to occur in concert with the evolution of amyloid plaques (Maiese, K *et al*., 2004). The generation of oxidative stress by microglia during Aβ deposition further suggests that microglia may play an important role in the pathogenesis of Alzheimer's disease. Studies with microglia stimulated by phorbol myristate acetate have demonstrated the release of superoxide radicals. Application of scavenger agents for ROS, such as superoxide dismutase or deferoxamine mesylate, in the presence of activated microglia can prevent cellular injury. These studies suggest that oxidative stress generated by microglia can be responsible for cellular injury (Tanaka, M *et al*., 1994).

Microglia may lead to cellular damage in Alzheimer's disease not only through the generation of ROS products (Sankarapandi, S *et al*., 1998), but also through the production of cytokines and the demise of neighboring neurons and ECs (Benzing, WC *et al*., 1999, Mehlhorn, G *et al*., 2000). Microglia promote the production of pro-inflammatory and neurotoxic factors, such as TNF and interleukin-1ß, free radicals such as NO and superoxide (Sankarapandi, S *et al*., 1998), and fatty acid metabolites such as eicosanoids that can precipitate cell death (Liu, B and Hong, JS, 2003). Cytokines are produced by microglia in response to toxic stimulation, such as interleukins and tumor necrosis factor-α (TNF-α). TNF-α production by microglia may be linked to neurodegeneration by increasing the sensitivity of neurons to free radical exposure (Combs, CK *et al*., 2001).

Microglial activation in Alzheimer's disease patients has been identified through glial cultures in autopsy specimens (Lue, LF *et al*., 1996). Expression of markers that are indicative of microglial activation was found to be significantly increased in patients with Alzheimer's disease (Rogers, J and Lue, LF, 2001). Application of a position emission tomography marker [^{11}C](R)-PK11195 for microglial activation in patients with mild and early Alzheimer's disease also has demonstrated microglial activation in regions of the entorhinal, parietal, and cingulate cortex, suggesting that microglial activation is an early event in the pathogenesis of the disease (Cagnin, A *et al*., 2001).

One of the major pathogens of Alzheimer's disease, namely Aβ, can lead to inflammatory cell injury through a variety of routes. Aβ can not only precipitate a significant inflammatory response with microglial activation and the secretion of TNF-α (Bornemann, KD *et al*., 2001), but also Aβ can elicit the neuronal expression of inducible nitric oxide synthase, peroxinitrite production, and neuronal apoptosis during an acute inflammatory response (Combs, CK *et al*., 2001). Interestingly, in patients with Alzheimer's disease, microglial cells co-localize with the perivascular deposits of Aβ. Microglial activation has been observed to occur in concert with the evolution of amyloid plaques (Sheng, JG *et al*., 1997). Ultrastructural three-dimensional reconstruction of human classical plaques in different stages of development illustrates that the number of microglia in the amyloid plague parallels a progressive increase in fibrillar deposition and the size of fibrillar plague (Wegiel, J *et al*., 2000). The generation of oxidative stress by microglia during Aβ deposition suggests that microglia may play an important role in the pathogenesis of Alzheimer's disease.

Microglia are monocyte-derived immunocompetent cells that enter the central nervous system during embryonic development and function similar to peripheral macrophages for the phagocytic removal of apoptotic cells. A variety of possible mechanisms may regulate the

phagocytosis of cells that have entered the apoptotic pathway. Some studies identify the generation of annexin I and membrane PS exposure that appears to be necessary to connect an apoptotic cell with a phagocyte (Arur, S *et al*., 2003). Secreted factors by either apoptotic or phagocytic cells, such as milk fat globule-EGF-factor 8 (Hanayama, R *et al*., 2004), fractalkine (Hatori, K *et al*., 2002), and lipid lysophosphosphatidylcholine (Lauber, K *et al*., 2003) also have been shown to assist with the phagocytic removal of injured cells.

The translocation of membrane PS residues from the inner cellular membrane to the outer surface appears to be critical for the removal of apoptotic cells (Fadok, VA *et al*., 2001, Kang, JQ *et al*., 2003, Maiese, K *et al*., 2000). The phospholipids of the plasma membrane are normally in an asymmetric pattern with the outer leaflet of the plasma membrane consisting primarily of choline-containing lipids, such as phosphatidylcholine and sphingomyelin, and the inner leaflets consisting of aminophospholipids that include phosphatidylethanolamine and PS. The loss of membrane phospholipid asymmetry leads to the externalization of membrane PS residues and serves to identify cells for phagocytosis (Chong, ZZ *et al*., 2003, Hoffmann, PR *et al*., 2001, Kang, JQ *et al*., 2003, Maiese, K and Chong, ZZ, 2003).

Expression of the phosphatidylserine receptor (PSR) on microglia works in concert with cellular membrane PS externalization to activate microglia. Cells, such as neurons or ECs, exposed to ROS can lead to the induction of both microglial activation and microglial PSR expression. Treatment with an anti-PSR neutralizing antibody in microglia prevents this microglial activation (Chong, ZZ *et al*., 2003, Kang, JQ *et al*., 2003) and application of PS directly results in microglial activation that can be blocked by a PSR neutralizing antibody (Chong, ZZ *et al*., 2003, Kang, JQ *et al*., 2003), suggesting that both PS exposure in target cells and PSR expression in microglia are necessary for microglial recognition of apoptotic cells in the nervous system. Recognition of cellular membrane PS by the PS-specific receptors on microglia may require cofactors, such as Gas6 (Nakano, T *et al*., 1997) or other agents, such as integrin and lectin (Witting, A *et al*., 2000).

The Wnt Pathway may be Essential During Alzheimer's Disease

Wnt proteins, named after the Drosophilia protein “wingless” and the mouse protein “Int-1”, represent a large family of secreted cysteine-rich glycosylated proteins. This family of proteins are intimately involved in cellular signaling pathways involved in embryonic cell patterning, proliferation, differentiation, orientation, adhesion, survival, and apoptosis (Maiese, K *et al*., 2004, Nelson, WJ and Nusse, R, 2004). Wnt binds to Frizzled transmembrane receptors on the cell surface to activate downstream signaling events. These involve at least two intracellular signaling pathways that are considered of particular importance. One pathway controls target gene transcription through β-catenin, generally referred to as the canonical pathway that involves Wnt-1, Wnt-3a, and Wnt-8 and functions through β-catenin-dependent pathways. Another pathway pertains to intracellular calcium (Ca^{2+}) release which is termed the non-canonical or Wnt/ Ca^{2+} pathway consisting primarily of Wnt-4, Wnt-5a, and Wnt-11 that functions through non-β-catenin-dependent pathways, such as the planar cell polarity (PCP) pathway and the Wnt-Ca^{2+}-dependent pathways (Katoh, M, 2002).

As one of the best characterized members of the Wnt family, Wnt-1 was first identified as a proto-oncogene in mammary carcinomas, but recently has been illustrated to play a critical role in neuronal development (Tang, K *et al*., 2002). Wnt functions by binding to the transmembrane receptor Frizzled and the co-receptor lipoprotein related protein 5 and 6 (LRP-5/6) (Wehrli, M *et al*., 2000) followed by recruitment of disheveled, the cytoplasmic bridging molecule, to inhibit glycogen synthase kinase (GSK-3β) (Ikeda, S *et al*., 1998, Papkoff, J and Aikawa, M, 1998). The inhibition of GSK-3β prevents phosphorylation of β-catenin and its degradation. The free β-catenin translocates to the nucleus where it activates

lymphocyte enhancer factor (Lef) and T cell factor (Tcf) (Ishitani, T *et al*., 2003) leading to stimulation of Wnt – response genes.

In some cell systems, Wnt-1 signaling has been associated with the control of apoptosis (Fig. **1**). Wnt-1 prevents apoptosis through β-catenin / Tcf transcription mediated pathways (Chen, S *et al*., 2001,Rhee, CS *et al*., 2002). Over-expression of exogenous Wnt-1 results in the protection of cells against c-myc induced apoptosis through induction of β-catenin, cyclooxygenase-2, and Wnt-1 induced secreted protein (WISP-1) (You, Z *et al*., 2002). Wnt-1 signaling also can inhibit apoptosis through prevention of cytochrome c release from mitochondria and the subsequent inhibition of caspase 9 activation (Chen, S *et al*., 2001). The adenomatous polyposis coli (APC) gene, a member of the Wnt pathway, appears to represent another mechanism that regulates apoptosis. The APC gene functions to cleave β-catenin leading to the down-regulation of transactivation of Tcf/Lef (Tetsu, O and McCormick, F, 1999). Without Tcf/Lef activity, APC is then permitted to increase the activities of caspase 3, caspase 7, and caspase 9 and lead to the cleavage of poly (ADP-ribose) polymerase (PARP) to enhance the vulnerability of cells to apoptosis (Chen, T *et al*., 2003).

Loss of Wnt signaling can play a role in Alzheimer's disease (Fig. **1**). Neurotoxicity of β-amyloid deposition of the 39−42 amino acid peptide (Aβ) in hippocampal neurons during Alzheimer's disease has been linked to increased levels of GSK-3β and loss of β-catenin. Decreased production of Aβ can occur during the enhancement of protein kinase C (PKC) activity (Savage, MJ *et al*., 1998) which may be controlled by the Wnt pathway (Garrido, JL *et al*., 2002). The proteolytic processing of APP during Alzheimer's disease has been closely linked to the Wnt pathway through presenilin 1 (PS1) and disheveled. PS1 is required for the processing of APP and has been shown to down-regulate Wnt signaling and interact with β-catenin to promote its turnover (Soriano, S *et al*., 2001). Dishevelled, a known downstream transducer of Wnt signaling pathway, also can regulate the α-secretase cleavage of APP through PKC/mitogen-activated protein kinase dependent pathways, increasing soluble production of APP (sAPP) (Mudher, A *et al*., 2001). Overexpression of mouse dishevelled-1 and −2 inhibits GSK-3β mediated phosphorylation of tau protein and may thus prevent formation of neurofibrillary tangles during Alzheimer's disease (Wagner, U *et al*., 1997). Thus, disheveled may increase neuronal protection during neurodegenerative disorders through sAPP production and reduction in tau phosphorylation. As a result, modulation of the Wnt pathway as well as its downstream constituents may offer novel therapeutic approaches to tackle neurodegeneration in Alzheimer's disease.

Protein Kinase B (Akt) Controls a Series of Pathways Necessary for the Prevention of Cellular Injury During Neurodegenerative Disease

Three family members of the serine/threonine kinase protein kinase B (PKB), or Akt named after the oncogene *v-Akt*, have been identified (Chong, ZZ *et al*., 2004). They are PKBα or Akt1, PKBβ or Akt2, and PKBγ or Akt3. Akt belongs to the AGC (cAMP-dependent kinase/protein kinase G/protein kinase C) superfamily of protein kinases and consists of three functional domains. The N-terminal pleckstrin homology (PH) domain provides binding sites for membrane phospholipids, which is involved in the recruitment of Akt to the plasma membrane. The catalytic domain of Akt has specificity for serine or threonine residues of proteins that are substrates for Akt. The C-terminal hydrophobic motif (HM) functions to provide a docking site for the activation of kinases.

Akt is expressed in mammals, but can vary in the level of expression in a variety of tissues and cells. Akt1 is the most highly expressed isoform. Although Akt2 is expressed at a lower level than Akt1, significant expression of Akt2 occurs in insulin-responsive tissues, such as skeletal muscle, liver, heart, kidney, and adipose tissue (Altomare, DA *et al*., 1995). This distribution for Akt2 expression may suggest a critical role for this protein in insulin signaling pathways.

In the central nervous system, the expression of Akt1 and Akt2 can be observed at increased levels during development, but is gradually decreased during postnatal development (Owada, Y *et al*., 1997). Yet, in the adult brain, expression of Akt1 and Akt2 is initially weak with a dramatic increase in the expression of Akt1 mRNA and Akt1 protein in cells that are subjected to injury (Chong, ZZ *et al*., 2004, Kang, JQ *et al*., 2003, Owada, Y *et al*., 1997), suggesting that Akt may play an important role during paradigms that involve cell injury. Different from Akt1 and Akt2, Akt3 is only expressed in a limited number of tissues, such as in the brain and testes, with lower expression evident in skeletal muscle, pancreas, heart, and kidney (Nakatani, K *et al*., 1999).

Once activated, Akt can provide protection against cellular injury (Fig. **1**). The phosphorylation of two major residues, Thr^{308} and Ser^{473}, are considered necessary for the activation of Akt. Activity of Akt is achieved through phosphorylation by phosphoinositide-dependent kinase 1 to confer protection against genomic DNA degradation (Chong, ZZ *et al*., 2002,Wick, A *et al*., 2002,Yamaguchi, A *et al*., 2001) and membrane PS exposure (Chong, ZZ *et al*., 2002,Chong, ZZ *et al*., 2003,Kang, JQ *et al*., 2003). Early studies have demonstrated that over-expression of Akt in cerebellar granule neurons prevents apoptosis during growth factor withdrawal (Dudek, H *et al*., 1997). In contrast, the expression of a dominant-negative Akt or inhibition of PI 3-K attenuates cell survival normally supported by growth factors (Chong, ZZ *et al*., 2002,Maiese, K *et al*., 2004). Subsequent work has illustrated an important role for Akt for the survival of various cell types during injury paradigms. Akt promotes cell survival during free radical exposure in primary hippocampal neurons (Chong, ZZ *et al*., 2003,Matsuzaki, H *et al*., 1999), neuronal cell lines (Kang, JQ *et al*., 2003,Kang, JQ *et al*., 2003), and cerebral vascular endothelial cells (ECs) (Chong, ZZ *et al*., 2004,Chong, ZZ and Maiese, K, 2004). Enhanced Akt activity can foster cell survival against several other toxic insults that include matrix detachment (Rytomaa, M *et al*., 2000), DNA damage (Chong, ZZ *et al*., 2002,Chong, ZZ *et al*., 2004,Henry, MK *et al*., 2001,Kang, JQ *et al*., 2003), anti-Fas antibody administration (Suhara, T *et al*., 2001), oxidative stress (Chong, ZZ *et al*., 2004,Kang, JQ *et al*., 2003), and transforming growth factor-β (TGF-β) application (Conery, AR *et al*., 2004).

Akt also prevents inflammatory cell demise through extrinsic cellular mechanisms that involve membrane PS exposure and the subsequent activation of microglia. Enhanced Akt activity can prevent cellular membrane PS externalization in both neurons and ECs during a variety of insults that involve anoxia, free radical exposure, and oxygen-glucose deprivation (Chong, ZZ *et al*., 2004, Li, F *et al*., 2004, Maiese, K *et al*., 2004). In addition, Akt appears to employ the modulation of membrane PS externalization to prevent microglial activation (Kang, JQ *et al*., 2003). Activation of Akt can prevent membrane PS exposure on injured cells and block the activation of microglia that are exposed to media taken from cells that over-express active, phosphorylated Akt during cellular injury (Kang, JQ *et al*., 2003, Kang, JQ *et al*., 2003). Cytoprotective agents, such as nicotinamide and erythropoietin (EPO), also employ mechanisms that involve Akt to regulate microglial activation and proliferation (Li, F *et al*., 2004, Maiese, K *et al*., 2003, Maiese, K *et al*., 2004). These protective agents block membrane PS exposure on cells and possibly prevent the shedding of membrane PS residues that is known to occur during apoptosis (Simak, J *et al*., 2002). In addition to targeting the activity of membrane PS exposure and microglial activation, Akt also may directly address cellular inflammation by inhibiting several pro-inflammatory cytokines, such as TNF-α (Fontaine, V *et al*., 2002).

Downstream Substrates of Akt Include the The Forkhead Transcription Factors, Glycogen Synthase Kinase-3β, and Bad

The forkhead transcription factor (FOXO3a, FKHRL1) represents one cellular pathway that is centrally controlled by Akt. Activation of FOXO3a can result in apoptotic cellular degeneration

in a transcription-dependent manner following its translocation to the nucleus (Brunet, A *et al*., 1999, Dijkers, PF *et al*., 2002, Gilley, J *et al*., 2003). FOXO3a activation has been demonstrated to decrease mitochondrial membrane permeability ($\Delta\psi_m$) and can result in cytochrome c release (Yu, C *et al*., 2003). Akt inhibition of FOXO3a requires its phosphorylation that results in the association of FOXO3a with 14−3−3 protein and retention of FOXO3a in the cytoplasm, rendering it ineffective to target genes in the nucleus and thus blocking apoptosis. During periods of oxidative stress in the nervous system, an initial inhibitory phosphorylation of FOXO3a at the regulatory phosphorylation sites (Thr^{32} and Ser^{253}) (Brunet, A *et al*., 1999, Rena, G *et al*., 1999) can occur (Chong, ZZ *et al*., 2004). However, loss of phosphorylated FOXO3a expression appears to subsequently result over a 12 hour period, possibly by caspase degradation, which potentially can enhance the vulnerability of neurons to apoptotic injury during neurodegenerative disorders, such as Alzheimer's disease (Chong, ZZ *et al*., 2004).

In addition, Akt controls the activity of glycogen synthase kinase-3β (GSK-3β), a serine/threonine kinase that is specifically expressed in the central nervous system. Although phosphorylation of GSK-3β at Ser^9 by Akt results in its inactivation, it is important to note that phosphorylation of GSK-3β at Thr^{216} results in an enhanced activity of the enzyme, which can occur during neuronal degeneration (Bhat, RV *et al*., 2000). Interestingly, GSK-3β has been shown to be involved with the neurotoxicity of Aβ during Alzheimer's disease. In brains of Alzheimer's patients, GSK-3β expression is present in the cytoplasm of pretangle neurons and its expression coincides with the development of neurofibrillary changes (Pei, JJ *et al*., 1999). On a cellular level, Aβ exposure in cultured hippocampal neurons can activate GSK-3β (Takashima, A *et al*., 1998). In addition, application of antisense oligonucleotides against GSK-3β and the GSK-3β inhibitor, lithium, can prevent cellular injury mediated by Aβ (Alvarez, G *et al*., 1999, Takashima, A *et al*., 1993).

GSK-3β also can regulate APP processing and the phosphorylation of tau. GSK-3β facilitates Aβ release by increasing the cellular maturation of APP (Aplin, AE *et al*., 1997), a process believed to occur during the early onset of Alzheimer's disease (Citron, M *et al*., 1994). GSK-3β is also one of the protein kinase candidates that can phosphorylate the tau protein. Hyperphosphorylated tau is the major component of neurofibrillary tangles that consist of paired helical filaments. GSK-3β appears to be necessary for sequential phosphorylation of tau at sites that are required for the formation of a paired-helical-filaments (Zheng-Fischhofer, Q *et al*., 1998). Overexpression of GSK-3β in transgenic mice results in the hyperphosphorylation of tau in hippocampal neurons and pre-tangle-like somatodendritic localization of tau (Lucas, JJ *et al*., 2001). If one removes GSK-3β activity through the inhibitor lithium, hyperphosphorylation of tau is blocked and tau binding to microtubules is promoted to yield microtubule assembly (Hong, M *et al*., 1997, Munoz-Montano, JR *et al*., 1997). Tau hyperphosphorylation by GSK-3β may require caspase 3 cleavage of APP (Fig. **2**). The generation of the C-terminal fragment C31 results from the cleavage of APP at the caspase site D720 of the C-terminus by caspase 3 (Nishimura, I *et al*., 2002). Once generated, C31 enhances glycogen synthase kinase-3β expression and tau protein phosphorylation (Kim, HS *et al*., 2003).

Akt inactivates Bad, a pro-apoptotic Bcl-2 family member, through phosphorylation of its serine residues. Bad is a Bcl-2 homology 3 (BH3)-only subfamily member of Bcl-2 proteins that are associated with the regulation of apoptosis. Three phosphorylated serine sites have been identified on Bad, including $serine^{112}$, $serine^{136}$, and $serine^{155}$. Akt preferentially phosphorylates the residue $serine^{136}$ of Bad (Datta, SR *et al*., 1997). A fourth phosphorylation site of Bad has recently been identified at $serine^{170}$ that also results in the blockade of pro-apoptotic activity of Bad (Dramsi, S *et al*., 2002). The endogenous de-phosphorylated Bad is localized in the outer mitochondrial membrane and binds to the anti-apoptotic Bcl-2 family

member Bcl-x_L through its BH3 domain. Subsequent phosphorylation of Bad by Akt leads to the binding of Bad with the cytosolic protein 14−3−3 to release Bcl-x_L and allow it to block apoptosis (Li, Y *et al*., 2001). Bcl-2 and Bcl-x_L prevent Bax translocation to the mitochondria, maintain the mitochondrial membrane potential, and prevent the release of cytochrome c from the mitochondria (Putcha, GV *et al*., 1999).

Interestingly, a number of Bcl-2 family members have been linked to cellular injury during Alzheimer's disease. Apoptotic injury as a result of Aβ (1−42) administration is associated with an increase of Bax and caspase 3 activation (Koriyama, Y *et al*., 2003). Furthermore, work that has infused Aβ (1−40) into the cerebral ventricles of rats results in the up-regulation of Bax as well as the down-regulation of Bcl-2 in cortical and hippocampal regions bordering the lateral ventricle (Wang, R *et al*., 2001). Similarly, in primary human cultured neurons, application of Aβ (1−42) produces sustained induction of Bax and a reduction in Bcl-2 expression (Paradis, E *et al*., 1996). Additional evidence for the ability of Bcl-2 proteins to modulate neuronal survival during Alzheimer's disease is demonstrated with microinjection of a human cDNA expression construct of Bcl-2 that can block cell injury during Aβ exposure (Zhang, Y *et al*., 2002).

Secretases and Caspases during Alzheimer's Disease

Caspases are initially inactive proteins that are proteolytically cleaved into subunits at the onset of apoptosis and function as active caspases after reconstitution to molecular heterodimers. Caspases are composed of three domains including an N-terminal prodomain, a large subunit, and a small subunit. The apoptotic-associated caspases include initiator caspases, such as caspase 2, 8, 9, and 10, that activate downstream executioner caspases, resulting in an amplification of cascade activity. The initiator caspases consist of long N-terminal prodomains that contain caspase recruitment domains (CARDs) in caspase 2 and caspase 9, or death effector domains (DEDs) in caspase 8 and caspase 10. Another set of caspases, termed the executioner caspases, consist of caspase 3, 6, and 7 that function to directly cleave crucial cellular protein substrates that result in cell destruction. The executioner caspases contain short prodomains or have no prodomains (Li, F *et al*., 2004, Maiese, K *et al*., 2004).

Activation of caspases proceeds through an extrinsic pathway and an intrinsic pathway. The extrinsic pathway is initiated by death receptor activation at the cell surface, resulting in the recruitment and activation of the initiator caspase 8 upon apoptotic stimuli (Ashkenazi, A and Dixit, VM, 1998). The intracellular death domain of death receptors, such as the TNF superfamily, CD95/Fas/Apo-1, and the death receptor 3 (DR3), undergoes conformational change upon binding to extracellular ligands and forms an intracellular death-inducing signaling complex (DISC) following recruitment of adaptor molecules, such as the Fas associated death domain (FADD). FADD recruits caspase 8 through its DED domain and leads to caspase 8 activation(Juo, P *et al*., 1998). Caspase 8 can subsequently activate caspase 3. In addition, caspase 8 activation also may result in the cleavage of Bid, a pro-apoptotic member of Bcl-2 family, allowing the truncated Bid (tBid) to translocate to the mitochondria. This leads to cytochrome c release through Bax resulting in the subsequent activation of executioner caspases.

The intrinsic caspase pathway involves mitochondrial dysfunction. The mitochondrial pathway is associated with the release of cytochrome c and subsequent activation of caspase 9 followed by activation of caspase 3 (Liu, X *et al*., 1996). The process is regulated by the Bcl-2 subfamily BH3-only proteins, which are normally located in cellular compartments other than mitochondria, but translocate to the mitochondria in response to apoptotic stimuli (Cosulich, SC *et al*., 1997). The translocation of these proteins delivers an apoptotic signal to mitochondria through the interaction with Bax to induce the release of cytochrome c that then binds to apoptotic protease activating factor-1 (Apaf-1). Apaf-1 consists of three different domains that

include a CARD, repeats of tryptophan and aspartate residues (WD-40 repeats), and a nucleotide-binding domain CED-4. Binding of cytochrome c to Apaf-1 results in the removal of the WD-40 domain, masking the CED-4 and CARD domains, and leads to the oligomerization of Apaf-1 under the assistance of dATP/ATP (Hu, Y *et al*., 1999). The oligomerization of Apaf-1 promotes the allosteric activation of caspase 9 by forming the Apaf-1 apoptosome (Li, P *et al*., 1997). Caspase 9 can subsequently activate caspase 3 as well as caspase 1 through the intermediary caspase 8. Together, caspase 1 and caspase 3 lead to both DNA fragmentation and membrane PS exposure (Chong, ZZ *et al*., 2002, Li, P *et al*., 1997, Maiese, K *et al*., 2000).

Processing of the transmembrane glycoprotein APP to result in β-amyloid deposition is determined by three secretases, termed α, β, and γ. α-Secretases cleave APP within the Aβ region resulting in the release of soluble fragments of α-APPs into the extracellular space along with a C-terminal fragment containing 83 residues (C83) that remains bound to the cellular membrane (Esch, FS *et al*., 1990). β-secretases cleave APP to generate β-APPs and a remaining fragment that contains 99 C-terminal residues (C99) (Seubert, P *et al*., 1993). Both the C83 and C99 fragments are substrates for γ-secretase. Aβ is generated from C99 proteolysis through a transmembrane domain. The Aβ produced includes a 40-residue peptide ($A\beta_{40}$) and a 42-residue peptide ($A\beta_{42}$). Although $A\beta_{42}$ is produced in much smaller quantities than $A\beta_{40}$, it more readily forms fibrils and cerebral plaque than $A\beta_{40}$. As a result, $A\beta_{42}$ is considered to be the β-amyloid product that most directly contributes to the pathogenesis of Alzheimer's disease and apoptotic injury.

In addition to the secretases, caspase activation also is necessary for the processing of APP (Fig. **2**). Caspases cleave APP at three major caspase recognition sites, one at the C-terminus, D720, and two at the N-terminus, D197 and D219. Caspase activation results in the increased production of Aβ. Yet, in some cases, Aβ generation may not be entirely dependent upon the cleavage of APP at its C-terminal (D720) and/or N-terminal caspase sites. For example, during etoposide-induced apoptosis ablation of caspase-dependent cleavage at D720, D197 and D219 (by site-directed mutagenesis) does not prevent enhanced Aβ production (Tesco, G *et al*., 2003). It is conceivable that APP may lead to cell injury through a more direct route that involves the generation of the C-terminal fragment C31. Production of C31 is a result of APP cleavage at the caspase site D720 of the C-terminus. Following caspase 3 activation, caspase 3 generates the carboxyl-terminally truncated fragment C31 from APP, which has been shown to be capable of apoptotic injury independent of caspase 3 (Nishimura, I *et al*., 2002). Furthermore, caspase-dependent APP cleavage at D720 also has been observed in brains of Alzheimer's disease patients through demonstration of C31 expression (Lu, DC *et al*., 2000).

Caspase Activity Regulates Tau and Presenilins

Caspase processing of the tau protein can influence apoptosis and the formation of intracellular neurofibrillary tangles that are composed of the microtubule-associated protein tau (Fig. **2**). Tau can be cleaved at Asp^{421} by caspase 1, 3, 7, and 8 resulting in the generation of a truncated tau protein (1−421) that is more readily assembling into tau filaments (Gamblin, TC *et al*., 2003). The C-terminal peptide (422−441) produced by caspase cleavage at Asp^{421} inhibits polymerization of the tau protein or the truncated tau protein (1−421). Removal of the C-terminal peptide, an inhibitory control element, by caspase cleavage of tau enhances its polymerization and results in the formation of neurofibrillary tangles (Berry, RW *et al*., 2003).

The cleaved fragments of the tau protein also may lead to apoptotic injury. As a neuronal microtubule-associated protein, tau has a central role in the formation of neuronal architecture. During the cleavage of tau in Alzheimer's disease, a soluble dephosphorylated tau fragment of 17 kDa is produced that cannot associate with microtubules. The generation of this fragment

is blocked by caspase inhibitors, and without its accumulation in cells, it is hypothesized that apoptotic injury may not result (Canu, N *et al*., 1998). Several other fragments of the tau protein also have been intimately linked to apoptosis. In an *in vitro* study, a 50 kDa fragment of tau protein produced by caspase 3 promoted cell death in mouse cortical neurons (Chung, CW *et al*., 2001). Overexpression of the tau fragment 152−391 also leads to apoptotic morphological changes, membrane blebbing, and nuclear pyknosis (Fasulo, L *et al*., 2000). Additionally, the tau fragments 1−422 and 22−422 can precipitate apoptosis (Fasulo, L *et al*., 2000).

In Alzheimer's patients, the truncated tau protein has been identified by immunohistochemistry, suggesting that caspase cleavage of tau is involved in the pathology of Alzheimer's disease (Gamblin, TC *et al*., 2003). In cases of frontotemporal dementia, an increase in tau degradation was observed that was histologically associated with DNA fragmentation and caspase 3 activation in neurons (Stanford, PM *et al*., 2003). Taken together with the biochemical data for the caspase processing of tau, these results suggest that tau cleavage may be a significant contributory factor in the pathogenesis of Alzheimer's disease.

The cleavage of presenilins by caspases also has been suggested to contribute to the progression of Alzheimer's disease. Presenilins are proteins that contain eight transmembrane domains. Both presenilin-1 (PS1) and presenilin-2 (PS2) are expressed in neurons throughout the brain. At the subcellular level, PS1 and PS2 are primarily located in the endoplasmic reticulum, Golgi apparatus, nuclear envelope, cell membrane, and the inner membrane of mitochondria (Ankarcrona, M and Hultenby, K, 2002, Kovacs, DM *et al*., 1996, Lah, JJ *et al*., 1997).

Presenilins are proteolytically processed by caspases (Fig. **2**). The endoproteolytical cleavage of PS1 and PS2 yields 27−35 kDa N-terminal fragments (NTFs) and 15−24 kDa C-terminal fragments (CTFs). This cleavage of PS1 or PS2 can be prevented by blockade of caspase 1 or 3 activity or the mutation of caspase cleave sites, such as Asp^{345}/Ser^{346} for PS1 and Asp^{329}/Ser^{330} for PS2 (Loetscher, H *et al*., 1997). It has been demonstrated that multiple caspases possess the ability to cleave presenilins. Caspases 8 and 3 exert proteolytical activity on both PS1 and PS2, whereas caspases 1, 6, and 7 predominantly cleave PS2 (van de Craen, M *et al*., 1999). During apoptosis, cleavage of presenilins, such as PS1, may foster a cell's demise. It is believed that the association of PS1 with β-catenin is necessary to prevent apoptosis by providing stability to β-catenin. Cleavage of β-catenin by caspases can disrupt cellular cytoskeleton components (Brancolini, C *et al*., 1997). In addition, it has been demonstrated that overexpression of CTFs of PS2 promotes apoptosis by promoting Aβ production, caspase 3 activity, cleavage of PARP, and loss of the “anti-apoptotic” protein Bcl-2 (Alves da Costa, C *et al*., 2003).

Calpains are also part of an intracellular family of cysteine proteases that are independent from caspases. At least 15 mammalian calpains have been identified, with two of these calpains, calpain 1 (μ-calpain) and calpain 2 (m-calpain), expressed primarily in the central nervous system. μ-Calpain and m-calpain are heterodimeric proteins with a large 70−80 kDa catalytic subunit and a 29 kDa regulated subunit. In the nervous system, μ-calpain is predominantly distributed in dendrites and the bodies of neurons while m-calpain is expressed in axons and in glia (Onizuka, K *et al*., 1995).

Calpains function not only as key regulators in cytoskeletal remodeling, but also in initiating cell injury. Calpains function through a number of pathways in the apoptotic cascade. Bax, a member of the Bcl-2 family, has been identified as a target of calpain (Wood, DE *et al*., 1998). The generation of pro-apoptotic 18 kDa fragment of Bax by calpain results in the induction of cytochrome c release, caspase 3 activation, and subsequent induction of apoptosis (Gao, G and Dou, QP, 2000). Calpains also can directly modulate the activity of caspases. m-Calpain cleaves caspase 3 producing a 29 kDa fragment, which further facilitates the

subsequent cleavage of caspase 3 into active forms (Blomgren, K *et al*., 2001). In addition, calpain can directly activate caspase 7 and caspase 12 (Nakagawa, T *et al*., 2000, Ruiz-Vela, A *et al*., 1999). Although calpains may enhance caspase activity, calpains also can function to block the activation of caspases. Calpains can cleave caspase 9 rendering it incapable of activating caspase 3 and preventing the subsequent release of cytochrome c (Chua, BT *et al*., 2000).

In light of the known activity of calpains during both the maintenance of cellular cytoskeleton integrity and the induction of cell injury, it has become increasingly evident that calpains have a significant role in the pathogenesis of Alzheimer's disease. For example, calpain activation has been found in clinical brain specimens of Alzheimer's disease (Taniguchi, S *et al*., 2001), calpain 2 was demonstrated to be present in approximately 75% of neurofibrillary tangles (Adamec, E *et al*., 2002), and calpains may promote cell cycle activation, a potential source of cell injury in Alzheimer's disease, through the activation of cyclin-dependent kinase 5 (Taniguchi, S *et al*., 2001). Further work have closely tied calpain activity to the toxicity of Aβ and presenilins. In rat hippocampal neurons, calpain appears to be required for the induction of apoptosis during Aβ application (Jordan, J *et al*., 1997). Over-expression of APP in neurons leads to calpain and caspase 3 activity, but activation of these pathways are lost in neurons that express an APP mutant defective in the Aβ domain (Kuwako, K *et al*., 2002). In regards to presenilin activity, mutations in PS1 have been associated with increased activity of m-calpain and neuronal dysfunction (Chan, SL *et al*., 2002), while m-calpain and μ-calpain have been shown to regulate PS1 activity by cleaving this protein (Maruyama, K *et al*., 2000).

FUTURE CONSIDERATIONS AND STRATEGIES FOR THE DEPLOYMENT OF CELLULAR TARGETS AGAINST ALZHEIMER'S DISEASE

Present treatments for Alzheimer's disease are limited by a few treatment options. For example, these include the potentiation of cholinergic transmission in brain regions such as in the nucleus basalis to compensate for presynaptic cholinergic deficiency (Lazareno, S *et al*., 2003). Although four cholinesterase inhibitors are presently available for the treatment of Alzheimer's disease, more recent entries into the field concentrate on the use of donepezil, rivastigamine, and galantamine which offer a longer duration of action with reduced side effects (Doody, RS, 2003). Prevention of N-methyl-D aspartate (NMDA) receptor activity during cognitive loss is another approach for the treatment of Alzheimer's disease. Memantine, an antagonist of the NMDA receptor, can lead to cognitive improvement in patients with moderate to severe forms of Alzheimer's disease (Reisberg, B *et al*., 2003), but the mechanism of action for this agent is unclear (Religa, D and Winblad, B, 2003). Evidence for the efficacy of non-steroidal anti-inflammatory agents (Aisen, PS *et al*., 2003) or for estrogen replacement therapy (Henderson, VW, 2004) for the treatment of Alzheimer's disease is presently lacking.

Unfortunately, present therapies are limited in nature and may provide only marginal symptomatic relief. New investigative avenues may provide not only attractive alternative therapies, but also viable treatments to either prevent or conceivably reverse the course of the disease. Several unique pathways are now under consideration that involve the modulation of cellular metabolic activity, regulation of attempted cell cycle induction in post-mitotic neurons, manipulation of the metabotropic glutamate system, and cytokine management.

Nicotinamide Adenine Dinucleotide (NAD^+) and its Precursor Nicotinamide

The coenzyme nicotinamide adenine dinucleotide (NAD^+) is closely tied to cellular metabolism and genomic DNA repair. During a cellular insult that affects DNA integrity, PARP catalyses the synthesis of poly(ADP-ribose) from its substrate NAD^+, which stimulates the process of DNA repair (Satoh, MS and Lindahl, T, 1992). Increased activation of PARP

leads to an extensive turnover of NAD^+ and a significant reduction in NAD^+ levels. This can trigger the loss of NAD^+ and ATP, leading to the death of a cell. Furthermore, oxidative stress can trigger the opening of mitochondrial membrane permeability transition pore (Chong, ZZ *et al*., 2003, Di Lisa, F *et al*., 2001, Kang, JQ *et al*., 2003, Lin, SH *et al*., 2000) and subsequently result in the release of NAD^+ from mitochondria (Di Lisa, F *et al*., 2001). During conditions of oxidative stress and energy depletion in neurons, poly(ADP-ribosylation) activation and loss of NAD^+ stores in mitochondria have been shown to lead to apoptotic injury. Restoration of NAD^+ content in mitochondria with liposomal NAD^+ prevents neuronal injury (Du, L *et al*., 2003).

Given the detrimental cellular ramifications of NAD^+ depletion, both acute and chronic neurodegenerative diseases have been linked to the loss of NAD^+ stores (Li, F *et al.*, 2004). In particular, in patients with Alzheimer's disease, PARP and poly(ADP-ribose) can be detected in the frontal and temporal cortex more frequently than in controls, suggesting that increased levels of functional PARP enzyme are present to result in a significant consumption of NAD^+ stores (Love, S *et al*., 1999). Interestingly, a limited pilot study suggested that administration of nicotinamide adenine dinucleotide (NADH) in patients with Alzheimer's disease may show improvement in their cognitive function (Birkmayer, JG, 1996).

However, a darker side may exist for therapy that involves the NAD^+ precursor nicotinamide with cellular aging. Recent work that employs transcriptional profiling of the human frontal cortex in the ageing brain suggests that altered expression of a variety of genes may promote oxidative stress, DNA damage, and impaired mitochondrial function (Lu, T *et al*., 2004). If one examines the aging process on more specific cellular terms, agents such as nicotinamide, an NAD^+ precursor that is intimately tied to cell survival during acute apoptotic injury (Chong, ZZ *et al*., 2004, Li, F *et al*., 2004, Maiese, K *et al*., 2003, Maiese, K *et al*., 2001), may negatively influence the lifespan of cells through the regulation of the *Sir2* gene (Lin, SJ and Guarente, L, 2003). The *Sir2* gene belongs to a family of genes which is a highly conserved group in the genomes of organisms ranging from archaebacteria to eukaryotes (Frye, RA, 2000, Vaziri, H *et al*., 2001). Interestingly, SIRT1 (Sir2α), as a human homologue of Sir2, is intimately linked with the modulation of cellular apoptotic pathways. The Sir 2 protein is associated with nicotinamide and pyrazinamidase/ nicotinamidase 1 (PNC1), an enzyme that deaminates nicotinamide. Nicotinamide appears to be capable of decreasing cell longevity through Sir2. Nicotinamide blocks cellular Sir2 by intercepting an ADP-ribosyl-enzyme-acetyl peptide intermediate with the regeneration of NAD^+ (transglycosidation) (Jackson, MD *et al*., 2003). Physiological concentrations of nicotinamide noncompetitively inhibit both Sir2 and SIRT1 *in vitro*, suggesting that nicotinamide is a physiologically relevant regulator of Sir2 enzymes (Bitterman, KJ *et al*., 2002).

Alternative concepts for the treatment of Alzheimer's disease may focus on the prevention of intracellular nicotinamide accumulation. During nicotinamide depletion, Sir2 is activated and employs PNC1 to regulate cell longevity. Increased expression of PNC1 has been found to be both necessary and sufficient for lifespan extension during calorie restriction (Anderson, RM *et al*., 2003). Nicotinamide and PCN1 are closely linked in controlling cell life span. PNC1 can stimulate Sir2 histone deactylase activity by preventing the accumulation of nicotinamide through its conversion to nicotinic acid in the NAD^+ salvage pathway. Overexpression of PNC1 has been demonstrated to suppress the inhibitory effect of exogenous nicotinamide on silencing, life span, and transcriptional repression of Sir2. As a result, PNC1 can positively regulate Sir2-mediated silencing and longevity by preventing the accumulation of intracellular nicotinamide (Gallo, CM *et al*., 2004).

Cell Cycle Induction in Post-Mitotic Neurons

Attempted cell cycle induction is an important factor in neuronal cell loss during Alzheimer's disease (Arendt, T *et al*., 2000, Busser, J *et al*., 1998, Maiese, K, 2001, Raina, AK *et al*., 2000). In pathological specimens of brains from Alzheimer's patients, the cell cycle regulators P16 and CDK4 have increased expression in regions such as the hippocampus (McShea, A *et al*., 1997). In addition, aberrant expression of other components of the cell cycle, such as cyclin D, Cdk4, proliferating cell nuclear antigen (PCNA), and cyclin B1 have been shown to be present in the hippocampus, subiculum, locus coeruleus, and dorsal raphe nuclei. Staining for cell cycle proteins have been shown to be absent in brain regions without neuronal injury of Alzheimer's patients and in age-matched brains (Busser, J *et al*., 1998). Increased accumulation of cell cycle kinases, such as Cdk5, also has been found in neurons that are developing neurofibrillary tangles (Pei, JJ *et al*., 1998). Interestingly, in patients with mild cognitive impairment, many of which can progress to develop Alzheimer's disease (Bennett, DA *et al*., 2002), cell cycle proteins, such as cyclin D, cyclin B, and PCNA, are significantly increased in the hippocampus and basal nucleus (Yang, Y *et al*., 2003).

Results in experimental models have provided further evidence that cell cycle induction in post-mitotic neurons can activate cellular mechanisms that lead to neuronal apoptosis (El-Khodor, BF *et al*., 2003, Ino, H and Chiba, T, 2001, Konishi, Y and Bonni, A, 2003, Lin, S *et al*., 2001, Rideout, HJ *et al*., 2003, Timsit, S *et al*., 1999). For example, application of Aβ (1−40), Aβ (1−42), and its active fragment Aβ (25−35) in neurons can result in the induction of cyclin D1, cyclin E and A, and the phosphorylation of the retinoblastoma protein. The activation of the upstream cyclin-dependent kinases (Cdk)4/5/6 appears to be required for the induction of apoptosis in neurons by Aβ, since inhibition of Cdks can prevent Aβ induced neuronal apoptosis (Alvarez, A *et al*., 2001, Giovanni, A *et al*., 1999). In addition, expression of familial Alzheimer's disease mutants of APP precipitate apoptotic injury through cell cycle induction and p21 mediated pathways (McPhie, DL *et al*., 2003). Cell cycle proteins also may contribute to neurofibrillary tangle development. Cdk5 has been identified as a critical regulator of tau protein which is a primary component of neurofibrillary tangles. Cdk5 can phosphorylate tau directly (Flaherty, DB *et al*., 2000). Phosphorylation of tau by Aβ can be blocked by treatment with antisense against p35, a protein that yields the potent Cdk5 activator p25 following cleavage, suggesting that Aβ potently activates Cdk5 and subsequent tau phosphorylation that is dependent on the cleavage of p35 (Town, T *et al*., 2002). In addition, the cleavage of p35 to p25 has been shown to occur in Alzheimer's disease patients and the p25/Cdk5 complex has been demonstrated to hyperphosphorylate tau and induce apoptosis in primary neurons (Patrick, GN *et al*., 1999). Taken together, these studies in addition to clinical observations, suggest that prevention of cell cycle deregulation in Alzheimer's disease may block neuronal loss.

The Metabotropic Glutamate System during Neurodegeneration

Metabotropic glutamate receptors (mGluRs) which are coupled to effector systems through guanosine-nucleotide-binding proteins (G-proteins) have been associated with Alzheimer's disease. Early work has reported a down regulation of mGluR binding sites (Dewar, D *et al*., 1991). Current interest has focused on the protective role of the mGluR system in the nervous system. mGluR activation prevents, and in some cases, reverses genomic DNA degradation (Vincent, AM *et al*., 1997), modulates endonuclease activation (Vincent, AM *et al*., 1999), and maintains cellular membrane asymmetry (Vincent, AM and Maiese, K, 2000). Cytoprotection by the mGluR system is believed to act at or below the level of free radical generation and oxidative stress (Maiese, K *et al*., 1996, Sagara, Y and Schubert, D, 1998, Vincent, AM *et al*., 1997). More recent work has suggested that the mGluR offers similar protective capacity to the vascular system by preventing endothelial cell DNA degradation, caspase activity, and

inhibiting a thrombotic state through the maintenance of membrane asymmetry (Lin, S-H *et al*., 2002, Lin, SH *et al*., 2001, Maiese, K and Chong, ZZ, 2004).

Other studies that involve Alzheimer's disease illustrate that group I mGluRs can regulate the metabolism of APP and accelerate the processing of APP into non-amyloidogenic APP (Lee, RK *et al*., 1996). Activation of group I/II mGluRs can enhance the secretion of APP (Jolly-Tornetta, C *et al*., 1998). The process is blocked by the administration of (±)-α-methyl-4-carboxyphenylglycine, a non-selective antagonist of group I/II mGluRs (Ulus, IH and Wurtman, RJ, 1997). Activation of group III mGluRs also has been shown to protect neurons against microglial neurotoxicity during Aβ application (Taylor, DL *et al*., 2003) that may be a result of the regulation of caspase activity (Chong, ZZ *et al*., 2003, Lin, SH *et al*., 2001). Under some circumstances, diminished activity of mGluRs may prove useful for cellular protection. For example, inhibition of group II mGluRs can attenuate microglial activation and subsequent neurotoxicity during toxic stimuli such as chromogranin A (Taylor, DL *et al*., 2003), a protein up-regulated in Alzheimer's disease. mGluRs also are believed to be necessary for the processing of learning and memory (Riedel, G *et al*., 1996). Given the ability of mGluRs to prevent cellular toxicity and to alter memory function, agents that modulate the activation of mGluRs may be viable during Alzheimer's disease.

Cytokines may Open New Doors for Cell Protection

Cytokines may be another viable option that can specifically target cellular pathways in Alzheimer's disease that have been implicated in cell injury. Initially described as hematopoietic growth factor, but now considered to interface with a variety of biological cell functions, the cytokine EPO modulates an array of vital cellular functions that involve progenitor stem cell development, cellular protection, angiogenesis, DNA repair, and cellular longevity (Li, F *et al*., 2004, Maiese, K *et al*., 2004). EPO can offer cellular protection at two distinct levels during apoptosis. Application of EPO during injury can prevent the exposure of membrane PS residues and also inhibit the committed stages of genomic DNA destruction in several experimental models (Campana, WM and Myers, RR, 2003, Chong, ZZ *et al*., 2002, Chong, ZZ *et al*., 2003, Chong, ZZ *et al*., 2003, Digicaylioglu, M *et al*., 2004, Grimm, C *et al*., 2002). Thus, EPO offers early cellular protection by maintaining genomic stability and also provides a more long-term protection by maintaining membrane PS asymmetry to block microglial phagocytosis and prevent the formation of a procoagulant surface in ECs (Kang, JQ *et al*., 2003, Witting, A *et al*., 2000).

As a cytoprotective agent, EPO may have applicability to Alzheimer's disease, since EPO modulates a broad array of cellular pathways and can provide protection against β-amyloid toxicity (Fig. **3**). EPO is dependent upon the activation of phosphoinositide 3 kinase (PI 3-K) and Akt (Bao, H *et al*., 1999). EPO can significantly enhance the activity of Akt during oxidative stress and prevent inflammatory activation of microglia (Chong, ZZ *et al*., 2003,Chong, ZZ *et al*., 2003,Chong, ZZ *et al*., 2003,Marti, HH, 2004). This up-regulation of Akt activity during injury paradigms appears to be vital for EPO protection, since prevention of Akt phosphorylation blocks cellular protection by EPO (Chong, ZZ *et al*., 2003,Chong, ZZ *et al*., 2003,Chong, ZZ *et al*., 2003). Through the regulation of the PI 3-K/Akt dependent pathway, EPO can prevent cellular apoptosis following hypoxia (Chong, ZZ *et al*., 2002) and oxidative stress (Chong, ZZ *et al*., 2003,Chong, ZZ *et al*., 2003,Chong, ZZ *et al*., 2003).

EPO also is linked to Bad through the anti-apoptotic Bcl-2 family member Bcl-x_L. EPO is able to maintain the expression of Bcl-2 and Bcl-x_L and alter the Bcl/Bax ratio towards a net “anti-apoptotic” effect, thereby preventing cellular injury (Vairano, M *et al*., 2002). EPO can require Bcl-x_L expression for cytoprotection, since up-regulation of Bcl-x_L by EPO can be necessary for the prevention of apoptosis in combination with the modulation of apoptotic protease activating factor-1 (Apaf-1) expression (Chong, ZZ *et al*., 2003). Cytoprotection of EPO also

is dependent, in part, upon Akt and the activation of nuclear factor-κB (NF-κB) (Marti, HH, 2004). NF-κB plays a key role in the expression of the EPO gene during HIF-1 induction. Akt can significantly increase NF-κB and HIF-1 activation resulting in the enhancement of EPO expression (Figueroa, YG *et al*., 2002). Through a regulatory loop, EPO also can promote IκB kinase (IKK) activity, resulting in the degradation of IκB and the subsequent liberation of NF-κB. EPO can require NF-κB activation to foster the production of neural stem cells (Shingo, T *et al*., 2001, Wang, L *et al*., 2004) and prevent neuronal apoptosis (Matsushita, H *et al*., 2003).

Modulation of caspase activation by EPO can offer additional levels of protection against cell injury. The ability of EPO to prevent specific caspase 1 and caspase 3 - like activities appears to play a significant role in its cellular protection against genomic DNA cleavage and cellular membrane PS exposure (Chong, ZZ *et al*., 2002, Chong, ZZ *et al*., 2003). EPO prevents PS externalization primarily through the inhibition of caspase 1 - like activity and, to a lesser degree, through other caspases such as 3, 8, and 9 to prevent activation and proliferation of microglia (Chong, ZZ *et al*., 2003, Chong, ZZ *et al*., 2003, Kang, JQ *et al*., 2003, Witting, A *et al*., 2000). EPO further prevents apoptotic injury through parallel pathways that prevent the induction of Apaf-1 and caspase 9 - like activity in conjunction with enhanced Bcl-x_L expression (Chong, ZZ *et al*., 2003).

CONCLUDING REMARKS

The pathways responsible for the development of Alzheimer's disease are complex in nature. Oxidative stress with the generation of free radicals plays an important role during Alzheimer's disease. Both Wnt and Akt oversee tightly regulated cellular pathways that involve components such as inflammatory microglia, Forkhead transcription factors, caspases, and calpains. As our knowledge of these pathways develops to effectively harness them into strategies to potentially treat neurodegenerative disorders, we hopefully will begin to complement much of the early work performed by Alois Alzheimer that initially defined Alzheimer's disease as a devastating illness that eliminates one's ability to enjoy and appreciate the very essentials of everyday life.

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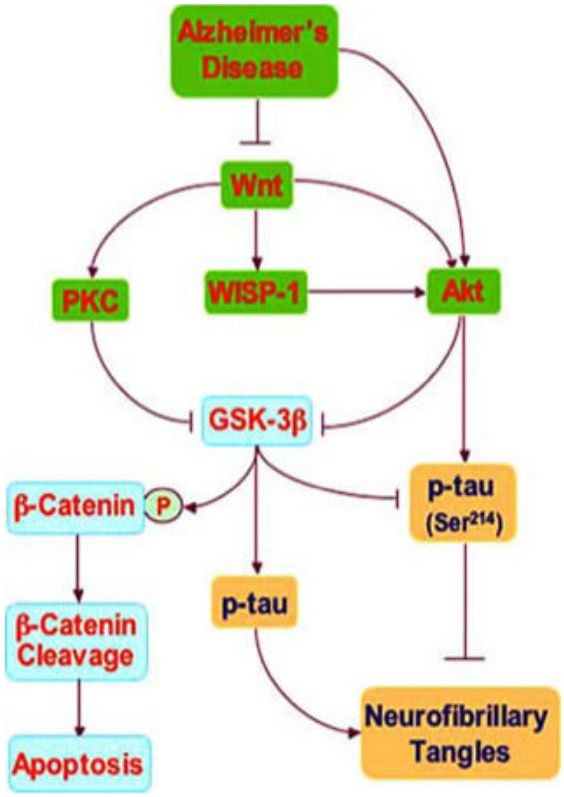

Fig. (1). Pathways that originate with Wnt and Akt in Alzheimer's disease

Wnt and Akt can block activity of glycogen synthase kinase-3 β (GSK-3β). When active, GSK-3β phosphorylates tau protein facilitating the formation of neurofibrillary tangles. GSK-3β also phosphorylates (P) β-catenin leading to its degradation and subsequent induction of apoptosis. Akt inhibits GSK-3β through phosphorylation and also phosphorylates tau protein at the site of Ser^{214} to prevent formation of neurofibrillary tangles. Wnt activates Akt directly or through Wnt-1 induced secreted protein (WISP-1). The phosphorylation and inactivation of GSK-3β by Wnt may occur through protein kinase C (PKC) or through Akt activation.

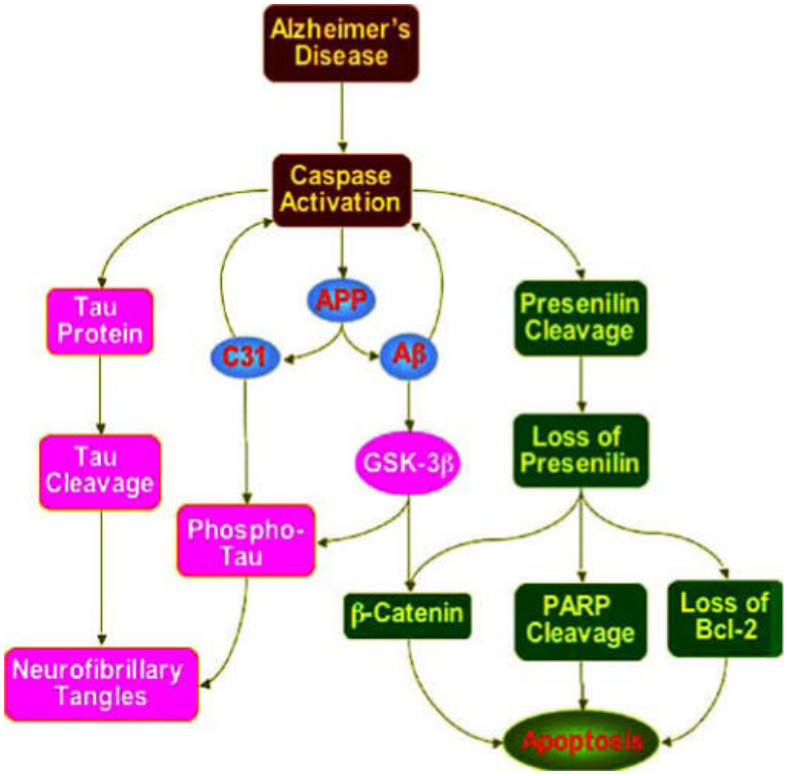

Fig. (2). Pathways of caspase activation during Alzheimer's disease

Caspase activation during Alzheimer's disease results in the cleavage of presenilin leading to apoptosis with loss of β-catenin, poly(ADP-ribose)polymerase (PARP), and Bcl-2. Caspases can cleave amyloid precursor protein (APP) and the resulting C-terminal fragment C31 resulting in hyperphosphorylation of tau protein (p-tau) as well as activation of glycogen synthase kinase-3β (GSK-3β). C31 and β-amyloid (Aβ) promotes the activation of caspases. Caspases also directly cleave tau protein to contribute to the formation of neurofibrillary tangles.

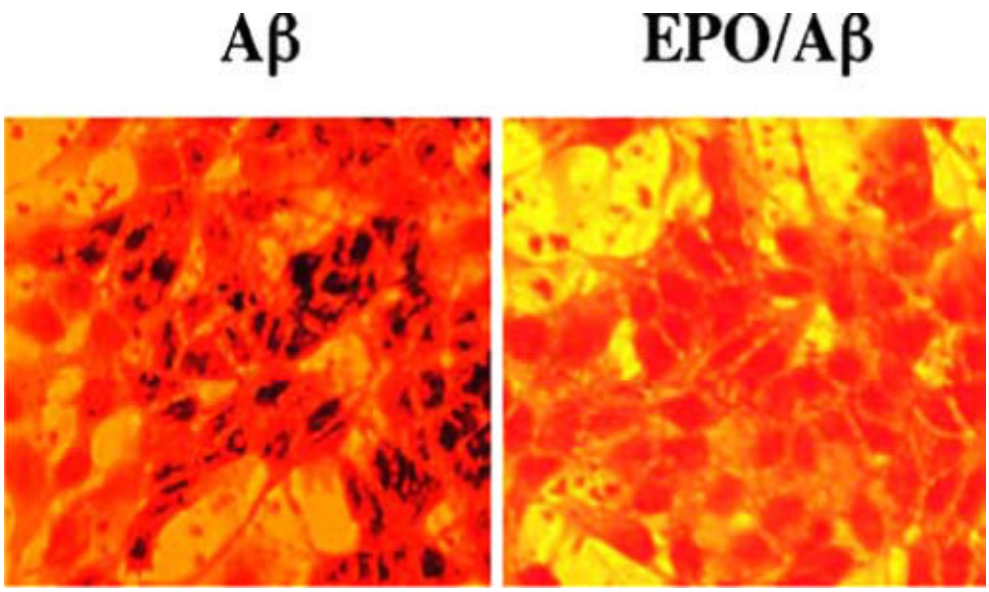

Fig. (3).
Erythropoietin (EPO) prevents DNA fragmentation during β-amyloid (Aβ) application in human neuroblastoma SH-SY5Y cells. Representative images illustrate DNA fragmentation with terminal deoxynucleotidyl transferase nick end labeling (TUNEL) in SH-SY5Y cells 24 hours after administration of Aβ (20 μM). Significant DNA fragmentation in SH-SY5Y cells is illustrated following Aβ treatment. In contrast, application of EPO (10 ng/ml) 1 hour prior to Aβ administration resulted in a significant reduction in DNA fragmentation when assessed 24 hours later.