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AMYLOID-β IN ALZHEIMER DISEASE:

THE NULL VERSUS THE ALTERNATE HYPOTHESES

Hyoung-gon Lee, Xiongwei Zhu, Rudy J. Castellani,

Akihiko Nunomura, George Perry, Mark A. Smith

Department of Pathology, Case Western Reserve University, Cleveland, Ohio, USA (HGL, XZ, GP, MAS)

Department of Pathology, University of Maryland, Baltimore, Maryland, USA (RC)

Department of Psychiatry and Neurology, Asahikawa Medical College, Asahikawa, Japan (AN)

College of Sciences, University of Texas at San Antonio, San Antonio, Texas, USA (GP)

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Correspondence to:

Mark A. Smith

Department of Pathology

Case Western Reserve University

2103 Cornell Road

Cleveland, Ohio 44106

216-368-3670

216-368-8964 (fax)

mark.smith@case.edu

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ABSTRACT

For nearly twenty years, the primary focus for researchers studying Alzheimer disease has been centered on amyloid-β such that the Amyloid Cascade Hypothesis has become the "Null Hypothesis". Indeed, amyloid-β is, by the current definition of the disease, an obligate player in pathophysiology, is toxic to neurons *in vitro*, and, perhaps most compelling, is increased by all of the human genetic influences on the disease. Therefore, targeting amyloid-β is the focus of considerable basic and therapeutic interest. However, an increasingly vocal group of investigators are arriving at an "Alternate Hypothesis" stating that amyloid-β, while certainly involved in the disease, is not an initiating event but, rather, is secondary to other pathogenic events. Additionally, and perhaps most contrary to current thinking, the "Alternate Hypothesis" proposes that the role of amyloid-β is not as a harbinger of death but rather a protective response to neuronal insult. To determine which hypothesis relates best to Alzheimer disease requires a broader view of disease pathogenesis and is discussed herein.

Amyloid-β and Alzheimer Disease: The Null Hypothesis

A wealth of evidence implicates amyloid-β (Aβ) in the pathogenesis of Alzheimer disease (AD) leading to the formulation of the Amyloid Cascade Hypothesis (Figure 1), i.e., the Null Hypothesis (Hardy and Higgins, 1992; Hardy, 2006). Casual observation of postmortem brain tissue of affected individuals makes it quite obvious why Aβ is the primary suspect in disease pathogenesis since Aβ is the major constituent in two of the most distinctive histopathologies, namely senile plaques and cerebral amyloid angiopathy (Glenner and Wong, 1984a; Glenner and Wong, 1984b; Masters et al., 1985). The destruction surrounding Aβ is evident from a close examination of histological preparations in the immediate vicinity of senile plaques and amyloid antiopathy, respectively, where degenerative neuronal processes and smooth muscle cells surrounding and infiltrating the Aβ deposits are found (Geddes et al., 1986; Kawai et al., 1992). Additionally, areas that are severely affected by disease, namely the hippocampal and frontotemporal cortices, show colocalization between Aβ plaques and neuronal cell death (Rogers and Morrison, 1985). Investigators then explored whether Aβ is toxic to neurons in both *in vitro* culture assays and in the intact brain of animals. Initially, results of these experiments seemed extremely contradictory, stemming from variability among commercial preparation of the peptide and the lack of proper control over whether Aβ was aggregated into fibrils of β-sheet conformation (Cotman et al., 1992). However, it is now firmly established that fibrillation of Aβ is needed to obtain neurotoxic effects (Pike et al., 1993; Lorenzo and Yankner, 1996; Yankner, 1996) and that, under many circumstances, it is inherently toxic to both neurons and clonal cell lines in culture (Yankner et al., 1990; Pike et al., 1991). The neurotoxicity of Aβ peptide *in vivo* was likewise measured by infusion of the peptide to various animal models. Notably,

intracortical injection of $A\beta_{1-42}$ or $A\beta_{25-35}$ fragments into aged rats or primates produced lesions that looked like those found in AD patients (Kowall et al., 1992).

The source of Aβ toxicity still remains elusive, regardless of the proliferation of theories advanced. The first theory proposed advocated interactions with cell surface receptors triggering an intracellular signaling cascade but little experimental evidence has supported this hypothesis. On the other hand, studies have long since supported the idea that oxidative event(s) are crucial for Aβ toxicity [reviewed by (Mattson, 1995)] such that Aβ peptide is capable of generating reactive oxygen species (ROS) through the generation of hydrogen peroxide (Hensley et al., 1994; Huang et al., 1999a; Huang et al., 1999b) and of stimulating inflammatory cells (Meda et al., 1995; Butterfield et al., 1996; Van Muiswinkel et al., 1996; Akama et al., 1998). The significance of reactive oxygen in Aβ toxicity is attested by the attenuation of toxicity by administration of antioxidants as well as free radical scavengers, such as vitamin E (Behl et al., 1992). This prooxidant view of Aβ is further supported by *in vivo* evidence where deposits are associated with oxidative damage (Smith et al., 1994; Smith et al., 1998; Atwood et al., 2002) and such damage, like Aβ deposition (Selkoe, 1999), is viewed as an early event in disease pathogenesis (Nunomura et al., 1999; Nunomura et al., 2000). Although it is clear that Aβ directly or indirectly promotes oxidative stress and that such toxicity can be attenuated by antioxidants, the precise mechanism that connects amyloid deposition to increased oxidative stress is not yet resolved. While multiple studies have suggested that the neurotoxicity of aggregated Aβ is mediated by its ability to induce oxidative stress through spontaneous generation of free radicals and ROS (Hensley et al., 1994), this proposition has been called into question on both theoretical and experimental grounds (Sayre et al., 1997; Dikalov et al., 1999; Turnbull et al., 2001). It now appears that the oxidant effects of Aβ are mediated by its

interaction with redox-active metals such as iron and copper since chelation of Aβ significantly attenuates toxicity (Rottkamp et al., 2001). Aβ has an unusually high affinity for both iron and copper (Huang et al., 1997; Atwood et al., 1998; Cuajungco et al., 2000) and can reduce these metals with the subsequent production of H_2O_2 and oxidized amyloid (Huang et al., 1999a; Huang et al., 1999b). The relevance of this mechanism to disease pathogenesis is highlighted by the association of redox active metals with senile plaques in AD (Smith et al., 1997; Sayre et al., 2000). In addition, the deposition of this normally soluble cellular protein promotes a chronic inflammatory response in AD, whereby activated microglia release ROS as part of the respiratory burst [reviewed in (Atwood et al., 2001)].

The strongest evidence for the crucial role played by Aβ in AD pathogenesis has been the characterization of the mutations that underlie familial early onset cases of the disease. All of these inherited mutations directly or indirectly affect both the processing and accumulation of Aβ. Familial Alzheimer disease (FAD) is associated with point mutations in amyloid-β protein precursor (AβPP) in regions that are involved in the proteolytic processing of the peptide (Goate et al., 1991; Lendon et al., 1997). It is thought that these mutations accelerate the onset of AD into the fourth decade by increasing the ratio of $A\beta_{1-42}/A\beta_{1-40}$, thereby increasing the relative amount of the more fibrillogenic form (Suzuki et al., 1994). A double mutation at positions 670/671 (Swedish mutation) increases the production of total Aβ and thereby increases the load of $A\beta_{1-42}$ without changing the relative ratio (Citron et al., 1992; Cai et al., 1993). The fact that an increase in total Aβ load accelerates the deposition of Aβ is supported by the neuropathology seen in patients with Down syndrome, a disorder caused by trisomy of chromosome 21, where the AβPP gene is localized. It is thought that the overexpression of AβPP in these individuals (Greenberg et al., 1996) causes the formation of Aβ plaques very similar to those seen in AD.

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The most common form of FAD is caused by mutations in one of the two presenilin genes (PS1 on chromosome 14 or PS2 on chromosome 1) [reviewed by (Gooch and Stennett, 1996)]. Recently, PS has been identified as one of the component of γ-secretase and FAD mutations increase the activity of γ-secretase although the alternate roles of PS, other than γ-secretase (Haass and Steiner, 2002), in AD pathogenesis also have been suggested (Herms et al., 2003; Tu et al., 2006). Most importantly however, missense mutations in the presenilin genes increase the ratio of $A\beta_{1-42}/A\beta_{1-40}$ (Citron et al., 1997; Tomita et al., 1997). Finally, one allele of the apolipoprotein E gene, namely apolipoprotein ε4 predisposes individuals to the development of late-onset AD (Corder et al., 1993). Of the three alleles (also including apolipoprotein ε2 and apolipoprotein ε3), apolipoprotein ε4 has the greatest affinity for Aβ, is found associated with senile plaques and is thought to accelerate fibrillogenesis (Wisniewski et al., 1994). Interestingly, the apolipoprotein ε2 inhibits fibril formation and is protective against the development of AD (Corder et al., 1994).

The above evidence implicating Aβ in AD pathogenesis led to the supposition that generation of transgenic animals that either overexpress AβPP, or a mutation in AβPP that affects processing of the full length protein thereby leading to an increase in the $A\beta_{1-42}/A\beta_{1-40}$ ratio, may mimic the pathophysiology that is seen in AD. Taken as a group, the various transgenic mice strains that have been produced thus far have demonstrated that overexpression of mutated AβPP but not wild type AβPP or overproduction of the $A\beta_{1-42}$ peptide fragment is alone sufficient to cause deposition of the peptide into senile plaque-like structures [reviewed by (Holscher, 1998)]. Indeed, despite the fact that each of the different constructs yielded somewhat different phenotypes, some aspect of AD pathophysiology is apparent in each of them. For example, Games and colleagues created a transgenic mouse expressing human AβPP with the Val717Phe

mutation at ten times the endogenous level and these animals developed Aβ plaques in the hippocampus, cerebral cortex and corpus collosum by 6-9 months of age and also show synaptic loss and astrocytosis (Rogers and Morrison, 1985). Another popular model is Tg2576 mouse which overexpresses AβPP containing the Swedish double mutation (Lys670Asn/Met671Leu). In addition to a marked increase in levels of Aβ in the CSF and deposition of amyloid plaques, these mice demonstrated marked deficits in spatial learning, as demonstrated by the Morris water maze test, by the age of 9 months (Hsiao et al., 1996). Even though no neurotoxicity was observed in these mice, it is thought that their impaired spatial learning, which is correlated with long-term potentiation (a model for memory), is related to synaptic loss. Interestingly, these mice also displayed oxidative stress in association with the plaques, much like that seen in AD (Smith et al., 1998). Neurodegenerative phenotypes have also been seen accompanied by an increased mortality rate, with 50% of mice dying by 12 months of age compared to an average of 24 months in controls, in mice overexpressing AβPP (LaFerla et al., 1995). Although there are differences in the details of these studies, it is clear that Aβ can independently cause AD-related pathology and some behavioral defects, which mimic AD pathology to a limited extent.

Taken together, the above evidence indicts Aβ as being the pivotal, if not the sole, culprit for causing disease.

Amyloid-β and Alzheimer Disease: The Alternate Hypothesis

The first, and foremost, argument that Aβ is not the initiator of AD that deposition of Aβ into senile plaques is by no means specific to AD patients and is instead a marker of normal aging (Davies et al., 1988). Therefore, the strong association of Aβ in AD may simply mark an acceleration of age-related deterioration. Support for this view can be found with the number of

plaques in cognitively-normal individuals rivaling those seen in advanced disease (Mann et al., 1992; Schmitt et al., 2000). Furthermore, there exists only a weak correlation between the burden of Aβ and neuronal loss or cognitive impairment (Giannakopoulos et al., 2003; Guillozet et al., 2003). Additionally, increased amyloid production and deposition is also seen as a response to injury in the central nervous system, especially following ischemia and head trauma (Gentleman et al., 1993; Roberts et al., 1994; Geddes et al., 1997). Aβ deposition in aging and following injury might be a compensation for the primary insult (Lee et al., 2004b). This is not to imply that in attempting to respond to cellular stresses that Aβ cannot lead to cellular destruction. However, it does require an underlying pre-existing stress, i.e., the presumptive etiology of AD.

A review of the literature would indicate that the underlying stress is of energetic origin, since a shortage of energy supply [and Ca(II) overload] induces an upregulation of AβPP expression. Ischemia, hypoglycemia and traumatic brain injury, a condition that has been shown to put neurons under metabolic stress (Xiong et al., 1997), all upregulate AβPP and its mRNA in animal models and culture systems (Hall et al., 1995; Jendroska et al., 1995; Yokota et al., 1996; Shi et al., 1997; Murakami et al., 1998). Not only does energy deficiency and Ca(II) dysregulation promote AβPP expression, but they also route the metabolism of AβPP from the non-amyloidogenic to the amyloidogenic pathway. Loss of mitochondrial energy metabolism alters the processing of AβPP to generate amyloidogenic derivatives (Gabuzda et al., 1994; Mattson and Pedersen, 1998), and, likewise, oxidative stress increases the generation of Aβ (Frederikse et al., 1996; Neve and Robakis, 1998; Paola et al., 2000). It also goes quite well with the role of AβPP as an acute phase reactant that is upregulated in neurons, astrocytes and microglial cells in response to inflammation and numerous associated cellular stresses including axonal injury (Gentleman et al., 1993; Blumbergs et al., 1995), loss of innervation (Wallace et

al., 1993), excitotoxic stress (Topper et al., 1995; Panegyres, 1998), heat shock (Ciallella et al., 1994), oxidative stress (Yan et al., 1994; Frederikse et al., 1996), aging (Higgins et al., 1990; Nordstedt et al., 1991; van Gool et al., 1994) and inflammatory processes (Brugg et al., 1995). Other pro-inflammatory stimuli that mediate the synthesis and release of AβPP include IL-1β (Goldgaber et al., 1989; Buxbaum et al., 1992) and tumor necrosis factor α converting enzyme (Buxbaum et al., 1998). The increased expression of AβPP by these stresses is likely a result of diminished energy resources. It is interesting that Aβ can regulate glucose metabolism (Ling et al., 2002) and that β islet cells of the pancreas express AβPP and several related enzymes at high levels (Figueroa et al., 2001).

The increased generation of Aβ under conditions of energetic stress may therefore be both a response to oxidative challenge observed in AD and following injury as well as a re-routing of metabolic priorities. In this scenario, Aβ in fact plays a protective role. The Aβ burden of the brain negatively correlates with oxidative stress markers (Nunomura et al., 1999; Cuajungco et al., 2000; Nunomura et al., 2000). This argues against the neurotoxic role of Aβ *in vivo*, as does the observation that cultured neurons can be cultured directly on top of isolated Aβ plaques or immobilizing Aβ with out any notable toxicity (Canning et al., 1993; Carpenter et al., 1993; DeWitt et al., 1998). Perhaps the *in vitro* toxicity that is sporadically shown in culture, and very unreliable to reproduce in animal models, may not be an intrinsic property of the peptide itself (Rottkamp et al., 2001). Notably, Aβ appears to attenuate oxidative stress *in vivo* (Nunomura et al., 2000; Nunomura et al., 2001) by likely acting as an antioxidant (Cuajungco et al., 2000). Furthermore, at nanomolar concentrations, Aβ can serve as a trophic factor withdrawal (Yankner et al., 1990) and metal-induced oxidative damage (Zou et al., 2002). These findings are consistent with the trophic and neuroprotective action of Aβ at physiological concentrations

(Whitson et al., 1989; Whitson et al., 1990; Yankner et al., 1990; Koo et al., 1993; Singh et al., 1994; Luo et al., 1996). Aβ also protects neurons from death following injection with saline or iron (Bishop and Robinson, 2003) and protects lipoproteins from oxidation in cerebrospinal fluid and plasma (may involve metal ion sequestration) (Atwood et al., 1998; Kontush et al., 2001). Moreover, low concentrations of Aβ possess significant anti-oxidant activity in an ascorbate-stimulated-lipid-peroxidation assay (Andorn and Kalaria, 2000). In sum, the physiological explanation for increased generation of Aβ in AD and following head trauma is the need to reduce ROS to prevent neuronal apoptosis (Raina et al., 2001) and promote neuritic repair.

Neurons are especially prone to oxidative stress due to high oxygen consumption, low levels of classic antioxidants, high unsaturated lipid content of neuronal membranes, and lack of mitotic renewal. As a result, the ratio between ROS formation and antioxidant defenses are essential for proper neuronal function. Normally antioxidant defense systems are sufficient to block ROS mediated damage. However, in cases of age related neurodegeneration, where there is considerable redox imbalance (Zhu et al., 2005). Given that Aβ is associated with the production of free radicals *in vitro*, it is essential to consider the *in vivo* temporal relationship between oxidative stress phenomena and Aβ deposition. Notably, oxidative stress, *in vivo*, is found in morphologically normal neurons in AD and seems to be inversely correlated with Aβ deposition (Nunomura et al., 1999; Nunomura et al., 2000). It therefore seems unlikely that Aβ accumulation, *in vivo*, by itself is sufficient to explain altered oxidative balance.

The strongest evidence for the role of Aβ in AD comes from the familial forms of this disease and involves mutations of genes that are directly involved in AβPP processing. Though a tremendous amount of effort has been dedicated to determining the mechanism of disease related to these mutations, this has proven only marginally useful to our understanding of sporadic AD,

which represents the majority of cases. For example, AβPP mutations have been identified in only 20-30 families world wide and represent far less than 0.1% of the 15 million known cases of AD (Josefson, 2002; Lleo et al., 2004; Hardy and Crook, 2005). Mutations in both presenilin 1 and 2, which are the most common genetic determinant of AD, only contributes an additional 120-130 families. While it is clear that mutations in these proteins involved in AβPP processing are capable of inducing amyloid deposition and dementia, no aberrant change is observed usually for many decades, and even then, this is likely a result of exacerbation of age-related deposition of Aβ in these individuals (the joint result of increased Aβ concentration and microenvironmental conditions) leading to the chronic neuroinflammation associated with the disease.

The positive correlation between apolipoprotein E ε4 genotype and incidence of AD in supporting a causative role for Aβ is flawed. While it is true that apolipoprotein E ε4 has the greatest affinity for Aβ, is found associated with senile plaques and is thought to accelerate fibrillogenesis (Wisniewski et al., 1994), this is not the sole or even the major physiological role of apolipoprotein E proteins. Apolipoprotein E helps to regulate the transport and metabolism of lipids. The level of apolipoprotein E is elevated in response to injury in the peripheral and central nervous system (Horsburgh et al., 2000) and, just like Aβ, apolipoprotein E may thus serve a protective role after ischemia or traumatic brain injury by distributing phospholipids and cholesterol to injured neurons (Poirier et al., 1993). Apolipoprotein E may further protect against oxidative injury and prevent the accumulation of lipid peroxidation end products, such as hydroxynonenal, which are prominent features in AD and acute brain injury. In support of this view, various studies show that patients that are homozygous for apolipoprotein ε4 genotype have longer periods of unconsciousness and higher incidence of post-traumatic coma following

severe traumatic brain injury (Sorbi et al., 1995; Friedman et al., 1999). Thus, apolipoprotein ε4 predisposes patients for any number of neurodegenerative processes, not specific for AD. Therefore, as in acute injury, apolipoprotein ε4 may be associated with a higher incidence of AD because it is less efficient in protecting neurons from the causative insult and therefore may have very little to do with its affinity for Aβ.

Recently, the original Amyloid Cascade Hypothesis was subsequently forced into revised to the Oligomeric Amyloid Cascade Hypothesis since a number of discordant findings disprove the Amyloid Cascade Hypothesis as we discussed above. For example, there is a very weak correlation between Aβ and disease state (Giannakopoulos et al., 2003) with very high Aβ loads often seen in cognitively intact old people (Crystal et al., 1988). Moreover, transgenic animal models, including Tg2576, with supraphysiological Aβ levels show little/no neuronal loss (Irizarry et al., 1997a; Irizarry et al., 1997b). Despite a relative paucity of solid experimental evidence, oligomeric Aβ has quickly risen to be the new star in the field, seemingly through the momentum of the original hypothesis. Nonetheless, until recently, the pathophysiological identity of oligomeric Aβ was unclear. In this regard, recently, Lesne and colleagues showed that Aβ*56 (which may be a dodecameric oligomer of Aβ) is found in cognitively impaired Tg2576 animals without Aβ plaques, but not in unimpaired animals (Lesne et al., 2006). Aβ*56 correlates with early declines in memory but not later ones (Lesne et al., 2006), and, when isolated and injected into rats, Aβ*56 leads to reversible cognitive deficits. While this is an interesting study that will definitely add fuel to the oligomeric amyloid hypothesis, before we get ahead of ourselves, a few salient aspects bear remembrance. First, different groups have reported that knockout of PS1 (i.e., no Aβ and surely no Aβ*56, either), while attenuating Aβ pathology in AβPP mutant transgenic mice, does not cure cognitive deficits (Dewachter et al., 2002; Saura

et al., 2005). In fact, these PS1 knockout-AβPP double transgenic animals lacking Aβ fare worse than single AβPP animals with Aβ might even indicate that Aβ is beneficial in certain circumstances as we previously indicated (Nunomura et al., 2001; Rottkamp et al., 2001; Lee et al., 2004a; Lee et al., 2004b). In any event, what is clear from this is that cognitive deficits do not relate to Aβ (in any guise, even Aβ*56). Furthermore, the study by Galvan et al. (Galvan et al., 2006) provides another clear example that Aβ is *not* responsible for the cognitive and pathological changes that stereotypify AD (Lee et al., 2004a; Lee et al., 2004b; Lee et al., 2006). Specifically, by introducing an additional mutation to AβPP mice that prevents the cleavage of AβPP by caspase, rescues cognitive and pathological deficits but does NOT affect Aβ plaques. While these authors did not specifically address whether Aβ*56 was affected, the similarities in other Aβ species would tend to indicate not. Therefore, transgenic manipulations have now clearly demonstrated that cognitive deficits and pathological abnormalities in AβPP transgenic mice bear no relationship to Aβ – both the positive and negative control experiments show this (i.e., cognitive deficits with no Aβ and rescue of cognitive deficits without change of Aβ).

The "Alternate Hypothesis" (Figure 2) is that Aβ simply represents a bystander or a protector rather than the causative factor of disease (Smith et al., 2002; Lee et al., 2003; Lee et al., 2004b). Notably, all therapeutic studies that have an effect on Aβ levels in cells or animals have shown extremely poor or no efficacy in subsequent clinical trials. This includes indomethacin (Weggen et al., 2001), ibuprofen (Lim et al., 2000), sulindac sulphide (Weggen et al., 2001), a nitric oxide-releasing nonsterodial anti-inflammatory drug (Jantzen et al., 2002) and estrogen (Zheng et al., 2002). Clearly, the "Alternate Hypothesis" points to greater therapeutic efficacy by directing efforts to the upstream metabolic and oxidative abnormalities that are what lead to Aβ.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. The Null Hypothesis: Increased Aβ, often through genetic influences, leads to oxidative stress that then leads to neuronal death.

Figure 2. Alternate Hypothesis: Risk factors for AD lead directly to oxidative stress that not only causes neuronal death but also an adaptive response by neurons (amyloid-β) as a protective measure.

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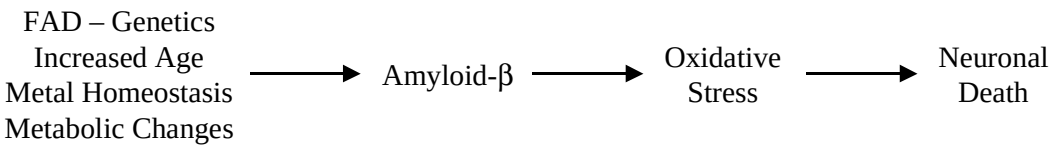

Figure 1

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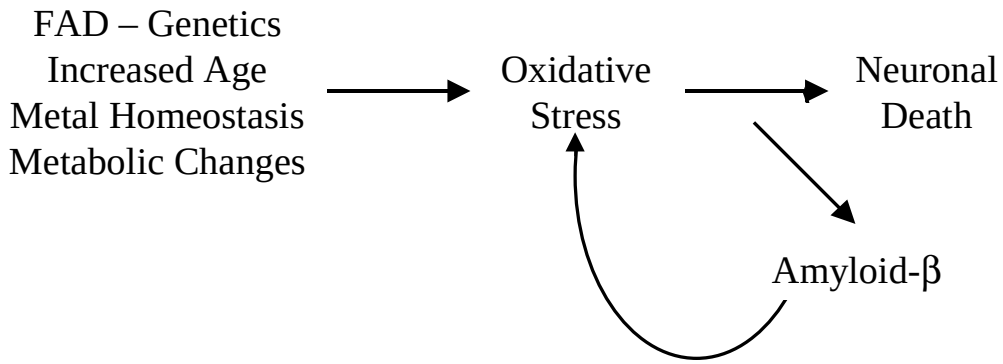

Figure 2