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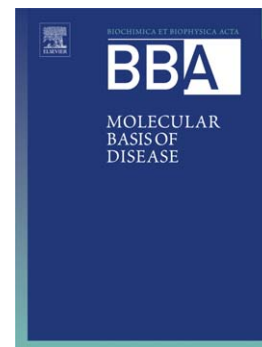
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ALZHEIMER DISEASE, THE TWO HIT HYPOTHESIS: AN UPDATE

Xiongwei Zhu¹, Hyoun-gon Lee¹, George Perry^{1,2}, Mark A. Smith¹

¹Department of Pathology, Case Western Reserve University, Cleveland, Ohio, USA;

²College of Sciences, University of Texas at San Antonio, San Antonio, Texas, USA

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Corresponding Author:

Xiongwei Zhu, Ph.D.

Department of Pathology

Case Western Reserve University

2103 Cornell Road

Cleveland, Ohio 44106 USA

Tel: 216-368-5903

Fax: 216-368-8964

xiongwei.zhu@case.edu

Summary (up to 200 words)

Given the relative modality of single-insult models to accurately reflect Alzheimer disease pathogenesis, based on studies on mitogenic and oxidative stress signaling pathways, we proposed a two hit hypothesis two years ago stating that both oxidative stress and mitogenic dysregulation are necessary and sufficient to cause the disease and suggested that it may be a common mechanism for other neurodegenerative diseases as well [11]. Recent developments in the field confirm some important predictions of the hypothesis and shed new lights on potential mechanisms regarding how steady state may be achieved in sporadic AD cases and therefore, in our opinion, strengthen the hypothesis, which will be the focus of this review.

Introduction

The pathological presentation of Alzheimer disease (AD), the leading cause of senile dementia, involves regionalized neuronal dysfunction/death and an accumulation of intraneuronal and extracellular lesions termed neurofibrillary tangles (NFT) and senile plaques, respectively [1]. To date, despite intensive efforts, the mechanism(s) responsible for selective neuronal death and dysfunction in AD remain unclear which has greatly impacted the development of accurate animal and cellular models and thereby retarded the development of therapeutic modalities. Several independent hypotheses have been proposed to link the pathological lesions and neuronal cytopathology with, among others, apolipoprotein E genotype [2, 3]; hyperphosphorylation of cytoskeletal proteins [4], oxidative stress [5], abnormal cell cycle re-entry [6, 7], inflammation [8] and amyloid- β (A β) metabolism [9]. However, not one of these theories alone is sufficient to explain the diversity of biochemical and pathological abnormalities of AD which involves a multitude of cellular and biochemical changes. Furthermore, attempts to mimic the disease by a perturbation of one of these elements using cell or animal models, including transgenic animals, do not result in the same spectrum of pathological alterations. The most striking example being that while A β plaques are deposited in some transgenic rodent models overexpressing amyloid- β protein precursor (A β PP), there is no neuronal loss [10] - a seminal feature of AD. The relative modality of single-insult models to accurately reflect disease pathogenesis led us to speculate that AD, like cancer, may be the result of serial insults that alone are insufficient to lead to disease and therefore proposed a “two hit hypothesis” stating that both oxidative stress and mitogenic dysregulation are necessary and sufficient to cause the disease and suggested that it may be a common mechanism for other neurodegenerative diseases as well [11]. Two years have passed since we first put forward this hypothesis and exciting developments in

the field confirm many of the predictions and therefore strengthen the hypothesis. Obviously more work is still needed to continue to test and define.

Oxidative Stress and Alzheimer Disease

What many of the theories of AD pathogenesis have failed to incorporate is that AD is a disease of aging [12]. Importantly, this holds true even in individuals with a genetic predisposition, i.e., those individuals with an autosomal dominant inheritance of AD or in individuals with Down's syndrome who develop the pathology of AD. Therefore, age is a clear contributor in 100% of AD cases, whatever the genetic background. Since the aging process is associated with an increase in the adventitious production of oxygen-derived radicals, i.e., reactive oxygen species (ROS), together with a concurrent decrease in the ability to defend against such ROS, not surprisingly, studies over the past ten years have established oxidative stress and damage not only in the lesions of AD but also in neurons at risk of death [13-20]. In AD, in addition to this background level of ROS, there are a number of additional contributory sources that are thought to play an important role in the disease process: (1) Excessive deposits of iron and/or copper catalyze the formation of $\bullet\text{OH}$ from H_2O_2 as well as the formation of advanced glycation end products. (2) Activated microglia, such as those that surround most senile plaques [21], are a source of NO and O_2^- [22] which can react to form peroxynitrite, leaving nitrotyrosine as an identifiable marker [19, 23]. (3) A β itself has been directly implicated in ROS formation through peptidyl radicals [24-26]. (4) Advanced glycation end products in the presence of transition metals (see above) can undergo redox cycling with consequent ROS production [27, 28]. Additionally, advanced glycation end products and A β activate specific receptors such as the receptor for advanced glycation end products (RAGE) and the class A

scavenger-receptor to increase ROS production [29, 30]. (5) Abnormalities in mitochondrial metabolism, such as deficiencies in key enzyme function, resulting in part from detection of the mitochondrial genome, suggest the mitochondria may be the major and possible initiating source of ROS [31]. (6) Abnormalities in proteasomal function or protein degradation systems. An exact determination of the contribution of each source of oxidative stress is complicated if for no other reason than that most sources have positive feedback. Nonetheless, the overall result is damage including advanced glycation end products [32], nitration [19, 23, 33, 34], lipid peroxidation adduction products [35-41] as well as carbonyl-modified neurofilament protein and free carbonyls [14, 16, 17, 32, 41, 42] with the involvement extending beyond the lesions to neurons not displaying obvious degenerative change. Notably, the very earliest neuronal and pathological changes characteristic of AD [43-46] suggest oxidative stress as a very early contributor to the disease. This early role is borne out by clinical management of oxidative stress which appears to reduce the incidence and severity of AD [47, 48]. Indeed, oxidative damage marked by lipid peroxidation, nitration, reactive carbonyls and nucleic acid oxidation is increased in vulnerable neurons whether or not they contain NFT [45, 49] suggesting that increases in neuronal oxidative damage must precede neurofibrillary pathology formation. Moreover, a marked accumulation of oxidative active modification products, i.e., 8-hydroxyguanosine (8OHG) and nitrotyrosine, in the cytoplasm of cerebral neurons from Down's syndrome, who invariably develop AD symptoms in their teens and twenties, temporally precedes amyloid- β deposition often by decades [44, 50]. That oxidative damage is the earliest event preceding the formation of pathologies is also confirmed in AD brains [45]. This is further supported by the findings on Tg2576 A β PP transgenic mice model in which oxidative stress precedes A β deposition [46, 51]. However, given that the field shifts towards the notion that soluble A β oligomers rather than

fibrils may be the culprit, studies seriously assessing the temporal relationship between oxidative stress and soluble A β oligomers are obviously needed but appear to be technically challenging. Nevertheless, recent studies found increased levels of isoprostane, a product of polyunsaturated fatty acid oxidation, in living patients diagnosed with mild cognitive impairment (MCI), a prodromal stage of AD and probable AD, suggesting that lipid peroxidation is present at a very early stage, well before the end-stage of the disease [52-54]. Along this line, individuals with MCI or very mild AD show increased levels of various lipid peroxidation and nucleic acid oxidation in postmortem brain tissues, cerebrospinal fluid, plasma, urine and peripheral leukocytes as well as decreased levels of plasma antioxidants and total plasma antioxidant capacity [55-57]. Heme oxygenase-1, a sensitive marker of oxidative stress, is also upregulated in postmortem brains of AD and MCI [58].

Mitotic Abnormalities and Alzheimer Disease

The cell cycle is a highly regulated process to ensure a homeostatic balance between cell proliferation and cell death in the presence of appropriate environmental signals. In eukaryotic cells, the cell cycle is typically divided into four phases: the S phase of DNA replication and the M phase of mitosis, separated by two gap phases called G₁ and G₂. Cyclin-dependent kinases (CDK), together with cyclins, are the major regulators of cell cycle progression and upon receiving external stimuli, cells up-regulate CDKs and cyclins to orchestrate the numerous process required for cell proliferation [59]. Any perturbation of these regulators will result in the arrest of the cell cycle and/or cell death.

Neurons in the adult CNS have classically been described as being in a terminally differentiated state meaning that they no longer return to the cell cycle. However, around ten

years ago, a rather surprising finding was made, such that in AD, susceptible cortical neurons display an activated cell cycle phenotype including the ectopic presence of various cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors [60-66]. Periodically, we see new markers of cell cycle entry being associated with either neuronal pathology present in AD or present in neurons that are vulnerable to changes that lead to AD [67-74]. This evidence suggests that in AD, susceptible neuronal populations, which are postmitotic and thus restricted in their mitotic competence, actually exit G_0 and re-enter the G_1 phase of the cell cycle during the progression of AD and therefore, helps cement the early and possibly irreversible fate of these vulnerable neurons. In addition to the presence of cell cycle phase dependent kinases, this “re-entrant” phenotype is also supported by activation of selective signal transduction pathways, transcriptional activation that results in cytoskeletal changes such as tau phosphorylation, alterations in mitochondrial activity and DNA replication [75-80]. In this sense, AD neurons resemble those normally seen only during development neurogenesis, in mitotically active cells and in neoplastic cells. In neoplasia, such ectopic mitogenicity is, by definition, due to a successful dysregulated cell cycle, while in the vulnerable neurons of AD it is due to an emergence out of terminal differentiation and attempted re-entry into the cell cycle. However, as yet, there is no evidence suggesting a successful nuclear division in AD implying that the neurons do not complete mitosis (M-phase). In fact, terminally differentiated neurons may lack the ability to complete the cell cycle such that the mitotic alterations (i.e., re-activation of cell cycle machinery) may contribute to neuronal death. Despite the consensus that these vulnerable neurons re-enter cell cycle and no actual mitosis occurs, it is unclear how far they go since G_1 , S and G_2 phase markers are all reported [81]. Indeed, the characteristic profile of cyclin-CDK activity in phase of the each cell cycle in normal dividing cells is lost when these susceptible

neurons attempt to re-enter cell cycle which points to the highly unorganized nature of the cell cycle in these neurons. For example, Cdk4 and p16 are expressed concurrently in these neurons which is not seen in normally dividing cells [64]. Another frequently encountered enigma is that most of these cell cycle markers are re-expressed in cytoplasm rather than in the nucleus where they act [61, 68, 69]. Although the consequence of such a re-entrant phenotype is unclear, it is conceivable that all these abnormalities point to an inadequate or a failed control of cell cycle in these neurons that may contribute to their eventual death in AD. Notably, like oxidative stress, mitotic proteins are not exclusively associated with end stage of neuropathology but rather with the very earliest neuronal changes to occur in the disease [62, 64, 70, 82]. Indeed cell cycle markers occur prior to the appearance of gross cytopathological changes [83] and the proximal nature of mitotic events has been shown in pre-AD patients with MCI which represents a prodromal stage of AD [73].

The Two Hit Hypothesis

It is evident that both oxidative stress and aberrant mitotic signalling play early roles in the pathogenesis of AD, but their temporal relationship to each other is unclear. Based on the studies of oxidative stress signalling and mitotic signalling pathways in vulnerable neuronal populations in AD, we hypothesized that both oxidative stress and aberrant mitotic stimuli can independently initiate, yet both are necessary to propagate disease pathogenesis and progression, which was termed as the “two-hit hypothesis” [11]. Importantly, there are two major assumptions of this hypothesis: 1) the presence of a steady state: after the process being initiated by one of these two hits, neurons recruit permanent adaptive changes and enter a new steady state that can last for decades where they still function normally or at worse, in a slightly compromised fashion; 2) the

depletion of neuronal compensatory potential: the new steady state requires great compensatory adaptations which likely deplete much of the neuronal compensatory potential to fight against insult (i.e, first hit), therefore, neurons at new steady state are uniquely vulnerable to secondary insults that requires additional compensatory changes in other pathways.

It appears that evidence supports the presence of “oxidative steady state” and extensive compensatory adaptations when neurons are subject to an oxidative injury. It is clear that oxidative stress is a pervasive feature in AD at all stages [84, 85]. Although high levels of acute oxidative stress would inflict neuronal death such as those seen in cases of trauma and ischemia, this type of oxidative damage is not the case in AD and indeed neuronal pathology after brain trauma and ischemia is significantly different from AD brain. What is striking is that few neurons (less than 1/10,000 at any given time) exhibit signs of apoptosis [86, 87] as would be expected under conditions of acute and high level of oxidative stress. Thus, oxidative challenges in AD seemingly do not exceed oxidative defenses otherwise rapid apoptotic death will result. In this event, it is clear that relatively low concentrations of oxidants such as hydrogen peroxide induce an adaptive response rather than cell death [88-91]. Therefore, a uniquely chronic, tolerable exposure of neurons to oxidative stress provides an explanation for the low levels of neuronal apoptosis in AD [80, 92-95]. Tolerable levels of oxidative stress provoke compensatory changes that lead to a shift in neuronal homeostasis which is initially reversible if the oxidative stress is acute. However, with persistent oxidative stress such as seen in pre-AD and AD cases which is significant as compared to age-matched controls, it is not only likely, but essential that, after a certain threshold (in terms of severity and chronicity of oxidative stress), the majority of neurons recruit permanent adaptive changes but still function normally or slightly compromised in a pro-oxidant environment. In other words, neurons enter an oxidative steady state. It is of

interest to note that neurons bearing NFT, a potential compensatory adaptation, demonstrate lower level of oxidative damage and can survive for decades [96]. Similarly, increased density of A β plaque deposition, also a likely compensatory adaptation, is associated with decreased levels of neuronal oxidative damage [44, 45, 97]. Although it is debatable whether NFT-bearing neurons exist in an oxidative steady state, obviously they develop extensive compensatory adaptations involving transcriptional changes which likely greatly limit their capability for further compensatory adaptation in the face of additional insults. For example, some recent studies suggested over 225 genes showing progressively increased or decreased expression in AD NFT-bearing neurons compared to AD non-NFT neurons or control non-NFT neurons [98] and many proteins involved in oxidative stress are implicated [99].

A β PP Mutants Cause Neurons Enter Mitotic Steady State

The two-hit hypothesis suggests that the reverse, mitotic followed by oxidative insult, is also possible, in which case neurons that have re-entered into what will become a futile attempt at division (i.e., mitotic steady state), are more vulnerable to changes in oxidative stress that requires further adaptation. The presence of such mitotic steady state, implicating that neuronal cell cycle re-entry is necessary but not sufficient to cause cell death in those postmitotic neurons susceptible to AD, was quite a different view from a more popular idea that initiation of cell cycle is sufficient to cause cell death in postmitotic neurons as observed in a large number of conditions in cell culture and animal models. For example, *in vitro* studies suggested that G₁/S cell cycle blockers and inhibitors of CDKs as well as dominant negative forms of Cdk4/6 prevent neuronal death induced by various conditions such as DNA damaging agents or A β [100-103]; Very recently, it was demonstrated that adenoviral-mediated expression of c-myc and ras

oncogenes drives postmitotic primary cortical neurons into the cell cycle followed by a steady decrease in the overall DNA content and DNA condensation consistent with cell death which provides a more direct link [104]. More importantly, when SV40 T antigen is expressed specifically in maturing Purkinje cells in transgenic mice, the cells replicate their DNA but then subsequently degenerate and die [105]. Similarly, the expression of SV40 T antigen by the rhodopsin promoter causes cell cycle reactivation and DNA synthesis followed by photoreceptor degeneration [106]. Widespread neuronal apoptosis also occurs in the CNS of phosphorylated retinoblastoma (pRb)-deficient mice [107-109]. However, given the widespread cell cycle markers in most susceptible neurons in AD [60, 61], as argued above, it is impractical for all these cells to die acutely as found in these cell culture or animal models. Therefore, it is more likely that these cells achieve a more stable and near normal state and function for relatively a long period of time which we termed “mitotic steady state”. This is supported by very interesting findings in pRb-/- chimeric mice in which the majority of pRb-deficient cells survived and differentiated into neuronal fates despite an obvious ectopic S-phase entry and accumulation of cells with 4n DNA content [110]. Indeed, since there were massive neuronal apoptosis in pRb-deficient mice, this rather unexpected finding in pRb-/- chimeric mice also confirms that other factors (or so-called non-cell-autonomous factors) can determine the final outcome even in the presence of genetic defects directly linked to cell cycle control which has important implications in familial AD. More importantly, conditional deletion of Rb only in the CNS caused cell cycle abnormalities without apoptosis in the CNS and it turned out that hypoxia, which presumably can cause oxidative stress, may be an essential additional insult that is required to induce massive neuronal loss in pRb-deficient mice, therefore, exemplifying a two-hit neuronal degeneration in a mouse model [111].

Mutations in at least three genes, the A β PP and the two presenilin genes, PS1 and PS2, are associated with early-onset AD [112]. We suggested that A β PP, PS1 and PS2 may share a common function in cell cycle control which may be key to the two-hit hypothesis since neurons bearing these mutations may enter a mitotic steady state, depleting their compensatory potential, which renders them more vulnerable to additional insults [11]. The presence of such a mitotic steady state is very likely as discussed above in the case of pRb^{-/-} chimeric mice [110]. Indeed recent development in the field appears to support our proposal of the presence of mitotic steady state in A β PP mutant bearing neurons. Both ectopic expression of cell cycle markers and DNA replication were reported in three different transgenic models of AD carrying A β PP Swedish mutants-R1.40, Tg2576 or Tg2576/PS1 and APP23 [74]. Neuronal cell cycle re-entry in these mice precedes amyloid deposits by several months and occurs in an anatomical pattern that recapitulates the selective neuronal vulnerability observed in AD. Since none of these mouse models develops significant neuronal loss and they only display mild behavioral abnormalities suggesting that these mouse neurons, despite re-entering cell cycle as early as 6 month of age, maintain a near normal state, these findings not only confirm our suggestion that cell cycle re-entry is not sufficient for neuronal death, but also provide an actual example of the presence of mitotic steady state in an AD-relevant model. However, before we can whole-heartedly embrace this notion, it must be noted that there is still controversy regarding A β PP mutant causing cell cycle abnormalities since an earlier study with APP23 mice failed to detect any neuronal cell cycle abnormalities [113] and the reason of such discrepancy is unclear. Recent studies utilizing powerful genetic tools such as Drosophila models convincingly demonstrated tau defects cause neuronal cell cycle activation [114] and the availability of similar Drosophila model overexpressing mutant A β PP [115] may help to answer this important question and shed new

light on the potential mechanism(s). Nevertheless, multiple recent studies demonstrate neurons bearing A β PP mutations show extensive alterations in gene expression profiles including those involved in nucleotide and protein synthesis, neurotransmitter metabolism, neuronal outgrowth and energy metabolism [116-118], underlying the notion of great compensatory adaptations in these neurons which may render them more vulnerable to additional insults. Bearing this in mind, one may then argue why there is no obvious neuronal death in A β PP mutant SOD2 heterozygous knockout mice [119, 120] which presumably have higher oxidative stress along with A β PP mutant. One potential explanation is that neurons bearing these two genetic defects from the very beginning when they possess greatest compensatory potential are very different from those 50-60 years old neurons with stereotyped adaptations facing demands for additional adaptation. The more relevant situation is to subject old A β PP transgenic mice to oxidative stress and indeed these mice demonstrate greater vulnerability to ischemia [121], traumatic brain injury [122] and some physiological stressors [123].

Tau and Two Hit Hypothesis

Tau, as A β PP, is another major protein implicated in AD. Maybe not coincidentally, a recent breakthrough in this field was the demonstration that tau alterations, also like A β PP mutations, lead to cell cycle abnormalities in various animal models. The elegant genetic studies using a fly tauopathy model convincingly demonstrated that tau alterations are causally linked to cell cycle re-entry [114]. Most importantly, this study demonstrated that both wild type and mutant tau can cause cell cycle re-entry which depends upon tau hyperphosphorylation. Complementing these exciting findings in fly model, tau alterations also induce abnormal cell cycle alterations in mouse models. In transgenic mice expressing human P301S tau protein, despite a lack of

activation of various cell cycle activators, there is increased neuronal expression of p21Cip1 and p27Kip1 in spinal cord and cortex and most importantly, just like in AD, they were mislocalized to cytoplasm compared to control mice, a sign of problem in cell cycle control [124]. More clearly, we had found profound nuclear PCNA immunoreactivity in another tauopathy mouse model, JNP301L mice, confirming that disease-causing tau mutations can cause cell cycle abnormalities in mammals (Figure 1 A-D). Perhaps more importantly in relation to sporadic AD, aged htau mice, which express non mutant human tau isoforms but no mouse tau isoforms and develop tau pathology more faithful to AD in terms of age-dependence and distribution [125], display ectopic expression of various cell cycle regulator proteins and DNA synthesis in brain regions where cell death is extensive and most affected in AD, supporting that nonmutant tau also can cause cell cycle re-entry in mammals [126]. The finding that tau alterations, especially the overexpression of wild type tau, can cause cell cycle re-entry in neuronal populations susceptible to AD has profound implications in relation to two hit hypothesis as it applies to sporadic AD and tauopathies since these diseases are associated with abnormal neuronal accumulation of wild type tau as well as hyperphosphorylated tau. Although it is easier to understand in familial AD, as we originally suggested in two hit hypothesis, even in sporadic AD, cases due to mitotic followed by oxidative insult are also possible. That tau alteration (i.e., accumulation and/or hyperphosphorylation) causes cell cycle abnormality may be one of the major mechanisms how such sporadic cases with mitotic steady state followed by additional oxidative hit arise. Indeed, a recent paper describing the presence of several subgroups of AD suggesting different initiation pathways including alterations in tau or ubiquitin (proteasomal dysfunction or oxidative stress?) [127] appears to support this idea. Interestingly, this study also suggested that age effect significantly affects tau which implies that tau alterations-induced cell

cycle re-entry is also age dependent. Nevertheless, one potential drawback is that, unlike those A β PP transgenic mice, these animal models with tau alterations all develop neuronal death [114, 126, 128, 129], seemingly suggesting that tau-induced cell cycle re-entry is sufficient to induce cell death without the necessity of an additional hit. Before reaching such conclusion, one should bear in mind the Rb $^{-/-}$ mouse story [110, 111] to more carefully search for the true reason of neuronal death in these models. Actually, at least in JNP301L tauopathy mouse model, impaired mitochondrial respiration and ATP synthesis together with higher levels of ROS and modified lipid peroxidation levels were noted in aged transgenic mice [130]. Similarly, it was previously reported that the ERK and JNK pathways are activated [131], and we further demonstrated that p38 is also altered in this model (Figure 1 E-F), which makes it very similar to AD where all these three MAPK pathways are activated [80]. Therefore, these findings implicate that not only cell cycle re-entry but also oxidative stress may contribute to the eventual neuronal demise in this model.

Conclusion

In summary, from the perspective of two hit hypothesis, recent developments in the field not only provide strong evidence supporting the presence of mitotic steady state in neurons bearing genetic defects [74, 110, 111], but also shed light on mechanisms regarding how neurons may enter a mitotic steady state in sporadic cases such as through tau alterations [114, 124, 126]. The presence of subgroups of AD suggesting several different initiation pathways in the pathogenesis of this disease [127] may also fit well with this hypothesis. Equally important, the fact that neurodegeneration observed in pRb-deficient mice [107-109] is likely due to both pRb-deficiency induced cell cycle re-entry and hypoxia-induced stress [110-111] underscores our

original hypothesis that the two-hit model may also have more general implications in other neurodegenerative diseases. Depending on the specific brain regions involved and other variations, the hit may not necessarily be oxidative stress or cell cycle abnormalities. As long as it requires compensatory adaptations of different pathways, the first hit makes neurons vulnerable while the second hit triggers the whole degenerative process.

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Figure 1. PCNA is found at significantly higher levels in neuronal nuclei in both the hindbrain (A) and cortex (C) of 8 month old JNP301L tauopathy mouse model when compared with 8 month old wildtype in both the hindbrain (B) and cortex (D). p38 (antibody from Stressgen) is readily detectable in many neuronal cell bodies in 8 month old JNPL301L brain (E). Using double label immunocytochemistry, significant overlap of both p38 (brown) with phosphorylated tau (blue) is seen in the neuronal cell bodies (F). Scale bar A-D= 50 μ m; E-F=50 μ m.

