Roles of Glycogen Synthase Kinase-3 in Alzheimer's Disease: From Pathology to Treatment Target

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by progressive and irreversible memory loss and other cognitive impairments. AD patients also suffer from various psychiatric symptoms such as psychosis, hallucinations, depression, anxiety, sleep disorder, and aberrant behavior disturbances. These symptoms have a devastating emotional impact on the patients and their caregivers. There is also a tremendous economic burden on the families and society as a whole; it is estimated that the direct cost of dementia is $148 billion annually in the United States alone.3–5

The medications used to treat the cognitive manifestations of AD include cholinesterase inhibitors (donepezil, rivastigmine, tacrine, and galantamine) and an N-methyl-D-aspartate (NMDA) receptor antagonist (memantine). The effects of these symptomatic treatment agents are limited.6 For psychiatric and behavioral symptoms, psychotropic agents such as second-generation (atypical) antipsychotic drugs, antidepressants, and anticonvulsants are commonly used. In addition to pharmacological treatment, nonpharmacological therapies have shown some clinical beneficial effects.7 However, these current therapies target only the symptoms and have no effect on delaying or halting the neurodegenerative progression of AD. Thus, the search for new treatment strategies for this incurable disease is critical.

The neuropathological hallmarks of AD are the deposition of extracellular amyloid plaques and the formation of intracellular neurofibrillary tangles (NFTs). Studies of autosomal-dominant familial AD patients have implicated mutations in the following three genes in the development of AD: amyloid precursor protein (APP, on chromosome 21), presenilin 1 (PS1, on chromosome 14), and presenilin 2 (PS2, on chromosome 1).8 These mutations occur in genes involved in the proteolysis of APP to form β-amyloid (Aβ). Aβ is the major component of the amyloid plaque, and it is widely accepted under the amyloid hypothesis of AD that Aβ deposition is

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the primary event in the pathogenesis of AD.\textsuperscript{9–11} Strategies to halt Aβ production and/or reduce Aβ deposition in the brain are believed to alter the disease progression and even cure AD.\textsuperscript{12} Although research has provided numerous methods to reduce brain amyloid load, clinical results do not sufficiently support the amyloid hypothesis that reducing amyloid burden is beneficial for AD patients.\textsuperscript{13} The well-noted AN1792 Aβ active immunization study showed that Aβ burden was decreased after immunization, and yet there was no clinically significant beneficial effects observed in the immunized patients.\textsuperscript{14} Despite many explanations that have been offered to address this discrepancy, it is still reasonable to consider factors other than amyloid burden in the pathogenesis of AD.

Although the amyloid hypothesis is dominant in AD research, increasing evidence suggests that amyloid alone does not account for the AD pathology in its entirety and that NFTs, the aggregates of hyperphosphorylated tau protein, should be considered as well. Primarily, it is noted that the amyloid burden is not always correlated with clinical disease severity.\textsuperscript{15,16} Whereas, in contrast, NFT progression based on Braak staging resembles the clinical disease progression and NFT density is correlated with AD severity.\textsuperscript{17,18} These observations provide the alternative tau hypothesis of AD.\textsuperscript{19} This article examines the roles of glycogen synthase kinase-3 (GSK-3) in NFT formation and its potential as a therapeutic target for AD.

2. NFT formation in the AD brain

NFTs are aggregates of hyperphosphorylated tau in the brain. Tau is a microtubule-associated protein (MAP) expressed throughout the central nervous system (CNS), but predominantly in neuronal axons. There are six isoforms of tau protein resulting from combinations of alternative splicing of exons 2, 3, and 10. Since exon 10 constitutes one of the microtubule-binding domains, its alternative splicing would lead to isoforms containing either three or four microtubule-binding repeats (tau-3R or tau-4R, respectively).\textsuperscript{20–23} Partially phosphorylated tau can promote association with tubulin, which leads to microtubule stabilization. However, pathological hyperphosphorylation of tau prevents tubulin binding, thereby resulting in microtubule destabilization.\textsuperscript{24} This type of microtubule disruption is believed to cause neurodegeneration. Studies have also shown that hyperphosphorylated tau can sequester normal tau and other MAPs, MAP 1 and 2.\textsuperscript{25,26}

The level of tau phosphorylation is regulated by a dynamic interplay between tau kinases and tau phosphatases. However, the cause of tau phosphorylation dysregulation in the AD brain is still obscure. Protein phosphatases (PPs) have broad substrate specificities; PP2A is believed to be the major tau phosphatase and is downregulated in AD brains.\textsuperscript{27} About 45 phosphorylated sites have been identified in tau protein. These sites predominantly cluster in the proline-rich domain and in the C-terminal region, with few sites located within the microtubule-binding domain.\textsuperscript{21,28} GSK-3β was the first known tau kinase to phosphorylate tau at paired helical epitopes.\textsuperscript{29} Several other kinases have also been shown to be involved in tau phosphorylation, including cyclin-dependent kinase-5 (cdk5), casein kinase-1 (ck1), and cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA). Each kinase has its preferred serine and threonine residue(s) for tau phosphorylation. Alignment of these phosphorylated residues by each kinase with the hyperphosphorylated epitopes observed in AD brain has shown that GSK-3β is the most important kinase to hyperphosphorylate tau.\textsuperscript{21} Strong evidence for GSK-3β phosphorylation of tau has been shown in an in vivo mouse brain where tau-4R wild type and P301L human tau mutant mice were crossed with GSK-3β mice.\textsuperscript{30,31}

In addition to phosphorylating tau, GSK-3 has the ability to affect tau splicing. GSK-3 has been shown to phosphorylate nuclear SC35, an enhancer of splicing elements that regulate exon 10 splicing in tau.\textsuperscript{32,33} GSK-3 is activated by Aβ and then phosphorylates SC35, which in turn enhances the splicing of tau exon 10, thereby decreasing the expression of tau-4R.\textsuperscript{34} The imbalanced tau-3R/4R ratio enhances tau phosphorylation.\textsuperscript{35} GSK-3-mediated tau splicing is interesting; however, most research investigating GSK-3 regulation of tau function has concentrated on the GSK-3-mediated phosphorylation of tau. It is believed that strategies targeting tau hyperphosphorylation could modify the NFT-induced neurodegeneration that is associated with AD, and GSK-3 is the major target of this hypothesis.\textsuperscript{36–38}

Current work has suggested that aggregated tau is not the toxic factor, but rather that insoluble tau is the lethal component. The proposed toxic, insoluble tau species are thought to be oligomers, dimers, or small tau aggregates, and either remain insoluble or aggregate into larger deposits. Aberrant amyloid peptides can activate GSK-3, which in turn contributes to the hyperphosphorylation of tau. Currently, the tau hypothesis is gaining ground over the amyloid hypothesis with regard to the development of AD and disease progression.\textsuperscript{39}

3. GSK-3 in the CNS

GSK-3 was identified in 1980 as one of several protein kinases that phosphorylate glycogen synthase to regulate glycogen synthesis. In recent years, GSK-3 has been implicated in the phosphorylation of many protein substrates and in the regulation of several cellular events.\textsuperscript{40} Moreover, GSK-3 acts as a central switch to receive many cellular signals including insulin, amino acids, and growth factors.\textsuperscript{41} In the 1990s, GSK-3 was found to play an important role in cell fate determination during embryogenesis. It acts in the WNT signaling pathway to phosphorylate and degrade β-catenin to inhibit gene expression.\textsuperscript{42,43} GSK-3 has also been demonstrated to regulate neurogenesis, neuronal polarization, and axon growth during brain development. Elevated GSK-3 activity is necessary for neuronal migration to the cortical plate, while lower GSK-3 activity is essential for maintaining progenitor neuron adherence to the subventricular zone.\textsuperscript{44} Because of these diverse roles, it is speculated that GSK-3 is involved in many neurodevelopmental and neurodegenerative disorders.\textsuperscript{45}

There are two GSK-3 isoforms, GSK-3α and GSK-3β, which are encoded by different genes on chromosomes 19 and 3, respectively.\textsuperscript{46,47} In contrast to other kinases, which activate their substrate, the GSK-3 isoforms inhibit their substrate activity through phosphorylation. This inhibition via phosphorylation is also seen in GSK-3 itself. Thus, phosphorylation of Ser21 in GSK-3α and of Ser9 in GSK-3β leads to decreased GSK-3 activity by inhibition of the priming phosphate site.\textsuperscript{48,49} But phosphorylation of the kinase domain, i.e., Tyr279 in GSK-3α and Tyr216 in GSK-3β, increases activity.\textsuperscript{50,51} These two GSK-3 isoforms share 84% sequence identity overall, but this increases to 95% in the kinase domain, indicating similar substrate specificities.\textsuperscript{47} However, there are still several differences in these two isoforms. For example, GSK-3β depletion causes embryonic lethality, suggesting that GSK-3α does not compensate for the loss of GSK-3β function.\textsuperscript{42} GSK-3β disruption-induced embryonic lethality is likely due to severe liver degeneration, a phenotype consistent with excessive tumor necrosis factor toxicity, as observed in mice lacking activation genes for the transcription factor NF-κB. This suggests that NF-κB is regulated by GSK-3β, but not GSK-3α, at the level of the transcriptional complex. NF-κB activation has been suggested to be one of the differentiating factors between these two GSK-3 isoforms.\textsuperscript{53} In contrast to GSK-3β deletion, GSK-3α knock-out mice survive with...
only aberrant behavioral abnormalities. \textsuperscript{54} Conditional over-expression of GSK-3β in adult mouse brain increases tau phosphorylation, decreases nuclear β-catenin levels, and induces neuronal death in the hippocampus. \textsuperscript{55} This profile resembles the tau pathology that occurs in AD. In rodents and humans, an alternative splice variant of GSK-3β, GSK-3β2, has been reported. This variant contains a 13 amino acid insertion in an internal loop near the catalytic domain. In contrast to the ubiquitously expressed GSK-3β1, GSK-3β2 is expressed specifically in the nervous system, and the highest levels are found during development. \textsuperscript{56} GSK-3β2 has lower tau phosphorylation activity than GSK-3β1. \textsuperscript{57} In regard to neuron-specific functions, GSK-3β has been reported to mediate two synaptic activities: NMDA receptor-mediated long-term potentiation (LTP) and long-term depression (LTD). GSK-3β Ser9 phosphorylation by the PI3K/AKT signaling, which inhibits GSK-3β, is crucial for the formation of LTP or LTD that regulates synaptic plasticity and cognition. \textsuperscript{58}–\textsuperscript{60} Overall, GSK-3β has been studied more extensively than GSK-3α, and its proposed role in AD pathophysiology has been of greater interest.

### 4. Dysregulation of GSK-3 in the AD brain

Direct evidence from human AD brains provides insight into the role of GSK-3 in the pathophysiology of this disorder. It has been shown that the total amounts of both GSK-3α and GSK-3β are decreased in human AD brain extracts irrespective of their activities. \textsuperscript{61} Studies have demonstrated that there is a significant increase in the level of pTyr216 GSK-3β (active form) in the frontal cortex of AD patients. Moreover, there is broad somatodendritic accumulation of pTyr216 GSK-3β at all stages of neurodegeneration, but no accumulation of the inactive form of pSer9 GSK-3β. \textsuperscript{62} This suggests that activation of GSK-3β is an early event that precedes and accompanies the formation of NFTs. Another study showed that pSer9 GSK-3β (inactive form) is co-localized with NFTs, dystrophic neurites of senile plaques, and neuritopil threads. This sequestration of the phosphorylated form of GSK-3β may be a compensatory process to prevent further tauopathy. \textsuperscript{63} In a transgenic AD animal study, GSK-3 activity was increased prior to amyloid deposition, suggesting that amyloid induces tauopathy through GSK-3 activation. \textsuperscript{31} These human and animal studies support the notion that increased GSK-3β activity may play an important role in AD pathogenesis, although further work is still needed to solidify this notion. It should also be noted that regulation of GSK-3α/β activity through phosphorylation of Ser21/Ser9 (inhibition) and/or Tyr279/ Tyr216 (activation) is still a matter of controversy, not only in AD, but in normal physiology as well.

### 5. Effects of GSK-3 inhibition in experimental AD models

Several GSK-3 inhibition studies in AD animal models have shown promising results for both pathology and behavior. In an intracerebroventricular Aβ infused AD model, co-infusion of SB216763, an ATP-competitive GSK-3 inhibitor, normalizes Aβ-induced elevations of phospho-tau, caspase-3, phospho-c-Jun N-terminal kinase, neuronal DNA fragmentation, and gliosis. \textsuperscript{64} Lithium, an anti-bipolar drug and well-known GSK-3 inhibitor, \textsuperscript{65} has been shown to decrease tau phosphorylation through GSK-3 inhibition in AD animals, \textsuperscript{66}–\textsuperscript{67} and even to downregulate tau transcription in cultured cortical neurons. \textsuperscript{68} Lithium also increases PP2A activity, \textsuperscript{69} while decreasing tau phosphorylation. \textsuperscript{68}

In addition to modulating tau phosphorylation, GSK-3 is also involved in the APP/Aβ processing. In a Drosophila AD model, lithium attenuates Aβ-induced fly locomotor activity changes and prolongs life span of the Aβ42 overexpressing flies. \textsuperscript{70} Axonal transport deficits are also reversed in this model. \textsuperscript{71} In tauopathy mouse models, lithium inhibits GSK-3-mediated phosphorylation of tau and the associated neuronal degeneration, \textsuperscript{72} as well as promotes ubiquitination, which results in a decrease of tau lesions. \textsuperscript{73} Furthermore, lithium was found to prevent the development of NFTs in a mouse model developed to display advanced neurofibrillary pathology, \textsuperscript{74} and to decrease mutant tau protein aggregation in another mouse transgenic model. \textsuperscript{66} An additional interesting finding is that Aβ42 levels are reduced after GSK-3 inhibition, suggesting a direct role of GSK-3 in the regulation of Aβ42 biogenesis. \textsuperscript{70} In transgenic mouse models of AD, lithium also improves cognition and reduces Aβ burden. \textsuperscript{75}–\textsuperscript{76} Additionally, previous studies have demonstrated that GSK-3β phosphorylates the intracellular domain of APP protein, \textsuperscript{77} and GSK-3α interacts with presenilin of the γ-secretase complex to interfere in APP processing. \textsuperscript{78} Lithium has also shown promising results in another AD transgenic mouse model overexpressing APP where the treatment decreases tau hyperphosphorylation and Aβ burden, reduces neurodegeneration in the cortex and hippocampus, and even normalizes memory deficits in a water maze test. \textsuperscript{79} Figure 1 is a schematic illustration of the multiple roles of GSK-3 in mediating tau hyperphosphorylation and Aβ generation, and shows that GSK-3 can serve as a potential target of AD treatment.

There are few studies looking at GSK-3 inhibition in humans. A registry database study in Denmark showed that continuous lithium treatment is beneficial in lowering dementia risk. \textsuperscript{80} In a 10-week trial for AD patients, the lithium treatment group did not show cognitive improvements or changes in cerebrospinal fluid (CSF) biomarkers. \textsuperscript{81} Another lithium trial was tested in mild cognitively impaired patients for a treatment period that was

Figure 1 Proposed roles of GSK-3 in the pathophysiology of AD. GSK-3 is proposed to have multiple actions in the genesis of AD pathology. It has been shown that GSK-3 is involved in the mRNA splicing of Tau protein, producing the tau-3R/4R isoforms. GSK-3 is a major kinase that phosphorylates Tau protein at multiple sites. Hyperactive GSK-3 has been detected in the brain of AD patients, thus resulting in tau hyperphosphorylation, which triggers neurofibrillary tangle formation and microtubule instability. These effects play a major role in the neuronal death associated with AD pathology. On the other hand, APP is processed by the enzymatic reactions of β- and γ-secreases, producing Aβ and ultimately leading to the formation of an amyloid plaque. The APP–Aβ pathway also contributes to the neuronal death in the affected brain areas. Aβ itself can further activate GSK-3, inducing a vicious cycle of tau mRNA splicing and protein phosphorylation. GSK-3 has been shown to facilitate APP processing by regulating the activity of the γ-secretase complex. Lithium and other GSK-3 inhibitors have been shown to reduce tau hyperphosphorylation and Aβ generation as well as to improve learning and memory in transgenic mouse models of AD. They have also been used in clinical trials to treat AD patients. Arrows represent stimulatory connections, and lines with flattened ends represent inhibitory connections. Aβ − β-amyloid; AD − Alzheimer’s disease; APP − amyloid precursor protein; GSK-3 − glycogen synthase kinase-3; tau-3R/4R − tau isoforms containing either three or four microtubule-binding repeats.
extended to 12 months. Lithium treatment is associated with a significant decrease in CSF concentrations of phosphorylated tau and improved cognitive performance.\(^{62}\) This human study suggests that GSK-3 inhibition by lithium may be beneficial if the treatment begins at an earlier stage and is maintained for a longer period. Lithium and other GSK-3 inhibitors are also under Phase II clinical trials for tauopathies. Lithium is undergoing testing at the University of Sao Paulo for AD; Tidagliptin (NP-12) is under testing by Norscia for AD and progressive supranuclear palsy (PSP). Valproic acid, a histone deacetylase inhibitor with GSK-3 inhibitory activity, is undergoing Phase II trials at Nantes University Hospital for PSP.\(^{83}\)

The current trend of AD treatment is to focus on the neuro-pathological hallmarks of AD: Aβ and tau. GSK-3 plays a major role in tau hyperphosphorylation and also mediates APP processing. Although there are few studies investigating GSK-3 inhibition for AD in humans, we still believe in its therapeutic potential, especially if more effective and safer GSK-3 inhibitors are discovered, and a plethora of chemical compounds have already been tested for their GSK-3 inhibitory activities.\(^{64}\) We concede that AD treatment could require other avenues than GSK-3 inhibition; however, we still expect GSK-3 inhibition to be an important aspect for AD treatment.

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