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HYPERPHOSPHORYLATION OF TAU BY GSK-3β IN ALZHEIMER'S DISEASE: THE INTERACTION OF Aβ AND SPHINGOLIPID MEDIATORS AS A THERAPEUTIC TARGET

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the extracellular deposits of β amyloid peptides (Aβ) in senile plaques, and intracellular aggregates of hyperphosphorylated tau in neurofibrillary tangles (NFT). Although accumulation of Aβ has been long considered a leading hypothesis in the disease pathology, it is increasingly evident that the role hyperphosphorylation of tau in destabilization of microtubule assembly and disturbance of axonal transport is equally detrimental in the neurodegenerative process. The main kinase involved in phosphorylation of tau is glycogen-synthase kinase 3-beta (GSK-3β). Intracellular accumulation of Aβ also likely induces increase in hyperphosphorylated tau by a mechanism dependent on GSK-3β. In addition, Aβ affects production of ceramides, the major sphingolipids in mammalian cells, by acting on sphingomyelinases, enzymes responsible for the catabolic formation of ceramides from the sphingomyelin. Generated ceramides in turn increase production of Aβ by acting on β-secretase, a key enzyme in the proteolytic processing of the amyloid precursor protein (APP), altogether leading to a ceramide-Aβ-hyperphosphorylated tau cascade that ends in neuronal death. Modulators and inhibitors acting on members of this devastating cascade are considered as potential targets for AD therapy. There is still no adequate treatment for AD patients. Novel therapeutic strategies increasingly consider the combination of multiple targets and interactions among the key members of implicated molecular pathways. This review summarizes recent findings and therapeutic perspectives in the pathology and treatment of AD, with the emphasis on the interplay between hyperphosphorylated tau, amyloid β, and sphingolipid mediators.

Keywords

• Alzheimer's disease • Tau hyperphosphorylation • Glycogen-synthase kinase 3β • Amyloid β • Sphingolipids

Abbreviations

Aβ	- Amyloid β protein
AD	- Alzheimer's disease
AICD	- APP intracellular domain
APP	- Amyloid precursor protein
BACE1	- β-secretase
BDNF	- Brain-derived neurotrophic factor
Cer	- Ceramides
Cdk5	- Cyclin-dependent kinase 5
GSK-3β	- Glycogen synthase kinase-3β
NFT	- Geurofibrillary tangles
PA	- Palmitic acid
PHF	- Paired helical filaments
PI3-K	- Phosphatidylinositide 3-kinase
PP2A	- Protein phosphatase 2A
NFT	- Neurofibrillary tangles
SM	- Sphingomyelinase
SPT	- Serine palmitoyltransferase

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A link between hyperphosphorylated tau, Aβ, and lipids

Alzheimer's disease (AD) is a chronic progressive neurodegenerative disorder and the leading cause of dementia syndrome in elderly people. Existing treatments for AD do not delay or modify progression of the disease, and the number of affected people is estimated to rise to 100 million worldwide by 2050 without new effective therapy [1]. Besides age that is considered the most significant risk factor for AD, epidemiological studies indicate that a high-fat diet might contribute to its development, the degree of saturation of fatty acid being the most important factor determining risk [2,3]. In fact, white matter from AD brain is characterized by increased fatty acid content, although the cholesterol levels are decreased in comparison to healthy subjects [4,5]. Hence, understanding the etiopathological processes of AD, particularly in respect of lipid contributions, is of major importance for the development of novel therapeutic strategies that will reverse or slow down progression of the disease.

The most researched neuropathological hallmarks found in AD brains are extracellular senile plaques containing neurotoxic amyloid β-peptide (Aβ) derived from the amyloid precursor protein (APP), and intracellular aggregates of abnormally phosphorylated tau protein that form paired helical filaments (PHF). They associate and build up densely packed networks of neurofibrillary tangles (NFT) [6-8]. Besides AD, phosphorylation of tau protein is also implicated in the pathogenesis of several related disorders called tauopathies [9,10].

Although formation of insoluble Aβ fragments in an amyloidogenic pathway by proteolytic cleavage of APP with β- and γ-secretases has been considered a key step in the pathogenesis of AD, tau increasingly emerged as an obligatory member of the neurodegenerative process [1]. Moreover, a number of reports found evidence for a relationship between Aβ and tau in AD [11-14]. Soluble Aβ oligomers facilitate wild-type human tau accumulation and hyperphosphorylation, leading to neuritic dystrophy [15], and learning and memory deficits in transgenic mice [16]. In addition, treatments with modulators of γ-secretase can reverse Aβ-associated tau pathology and reduce hyperphosphorylated tau levels in AD mouse models [17,18]. Interactions between tau and lipids also seem important in AD pathology. Phosphorylated tau was found in specific microdomains of plasma membrane called lipid rafts in neuronal cells exposed to Aβ peptides [19], in lipid rafts of an AD mouse model, and in lipid rafts from AD brain [20]. Evidence indicates connections between lipids and Aβ as well. While proteolytic processing of APP is influenced by the lipid composition of cellular membranes, particularly with cholesterol and sphingolipids, Aβ in turn plays an essential role in regulating lipid homeostasis [21].

Tau protein and axonal assembly and stability

Tau, a protein with multiple phosphorylation sites, belongs to the family of microtubule-associated proteins. It is highly expressed in neurons, particularly in axons, with an essential role in the assembly of tubulin monomers into microtubules that are fundamental cellular structures for preservation of neuronal shape and axonal transport [22]. Therefore, tau plays a crucial role in the formation and maintenance of axons, microtubule dynamics, neurite outgrowth, and neuritic stability [23].

Tau is encoded by a single gene, but due to alternative splicing of mRNA, several tau isoforms with differential affinity for microtubules are expressed in human brain in a developmental [24], cell-specific, and pathology-related manner [25,26]. In early developmental stages only the smallest tau isoform is expressed, while all tau isoforms are expressed during adulthood [27]. These isoforms differ by the presence of either three or four semiconserved repeat-regions in the C-terminus part of the molecule that form highly homologous microtubule-binding domain, and by the presence of two, one, or no inserts in the N-terminus part. Through these microtubule-binding repeats at the C-terminus, tau protein exerts its specific function in microtubule assembly and stabilization [28,29]. At the level of both mRNA and protein, a ratio of tau isoforms with 4 and 3 repeats (4R:3R) is approximately equal in healthy adult brain [30]. Alterations in 4R:3R ratio and mutations in the tau gene that cause amino acid substitutions lead to formation of intracellular inclusions consisting of highly phosphorylated tau with abnormal conformation [1,31].

Hyperphosphorylation of tau leads to toxicity and loss of function

Function of tau is modulated by alterations in its phosphorylation pattern, in principle on various threonine (Thr) and serine (Ser) residues. The longest human isoform has 80 Ser and Thr residues and additional 5 tyrosine residues indicating that almost 20% of the molecule are amino acids that can be phosphorylated [32]. The exact contribution of each phosphorylation sites or subsets of phosphorylation sites for normal tau function is still not completely understood. The normal tau contains 2-3 moles of phosphate per mole of protein, while in AD brain this phosphorylation stoichiometry is three to four times higher, with all isoforms being found in abnormally phosphorylated NFT [33]. By mass spectroscopy and antibody staining, the total number of identified phosphorylated residues in insoluble tau from AD brains is 45, which is significantly higher than the number of phosphorylated residues in soluble tau of healthy controls, and represents more than half of residues available for phosphorylation [1,34,35].

Hyperphosphorylation results in dissociation of tau protein from microtubules and impairs its role in microtubule assembly and stabilization, leading to the loss of the normal content of microtubules [36]. Certain residues, either within the microtubule-binding domain or apart from it, such as Thr^{212}, Ser^{214}, Thr^{231}, Ser^{235} and Ser^{262}, have been recognized as the major sites contributing to the segregation of tau protein from the microtubules [37-39]. In addition, hyperphosphorylation deregulates axonal transport of vesicles and other cargoes further affecting axonal length and function. This ends in neuronal degeneration, synaptic loss, and ultimately cell death, a key end-point histopathological hallmark of AD [9,30,32,40].

In addition to phosphorylation-dependent loss of function, aberrant phosphorylation of tau induces tau-tau interactions and self-assembly into tangles of paired helical filaments that build up NFT inside neurons [41]. Development of NFT occurs in a stepwise manner, and the specific pattern of tau phosphorylation correlates with severity of neuronal cytopathology [42]. The appearance of pathological changes can be classified in three morphological stages: pre-neurofibrillary tangle, intra-, and extra neuronal fibrillary tangles [43]. NFT also appear in a stereotypical order, certain regions being affected before others [44-48].

Tau can be phosphorylated by proline-directed and non-proline directed kinases [49,50]. *In vitro* and cell studies revealed that tau is a substrate for at least 17 different kinases with considerable overlap of the phosphorylated residues [1,51]. Among them are glycogen synthase kinase-3β (GSK-3β), cyclin-dependent kinase 5 (cdk5) and its regulators, non-receptor tyrosine kinases, casein kinase 1 delta, extracellular signal regulated kinase-2 (ERK2), and cyclic AMP-dependent protein kinase [52-57]. Despite much advances in identifying phosphorylation sites *in vitro*, the individual contributions of each kinase to *in vivo* physiology and pathology are still unknown. The considerable overlap of multiple residues phosphorylated by different kinases *in vitro* suggests that the overall pattern of increased tau phosphorylation is more important than phosphorylation of each particular residue. On the contrary, diverse phosphatases remove phosphate groups and dephosphorylated tau. Protein phosphatase 2A (PP2A) seems to be the major tau phosphatase in human brain [58]. Hence,

coordinated action of protein kinases and phosphatases determines the phosphorylation status of tau, suggesting that downregulation of phosphatases in the AD brain could also lead to tau hyperphosporylation and the prevalence of abnormal, stable tau form with high aggregation potential [59,60].

GSK-3β as a link between hyperphosphorylated tau and Aβ

GSK-3 is a ubiquitous Ser/Thr kinase that modulates many important processes inside the cell. It has two highly homologous isoforms - GSK-3α and GSK-3β. Activation of GSK-3 has been found in a number of neurodegenerative diseases, including AD. Active GSK-3 localizes to pretangle neurons, dystrophic neurites and NFT in AD brains, and temporal and spatial patterns of GSK-3 expression correlate well with the progression of neurodegeneration [61-63]. Altered activity of both GSK-3 isozymes contributes to key neuropathological hallmarks of AD. While GSK-3α participates in APP processing and deposition of Aβ by regulating the activity of γ-secretase [64], the other isoform GSK-3β is considered as the major tau kinase involved in hyperphosphorylation of tau protein [65,66]. As previously explained, GSK-3β-mediated phosphorylation of tau reduces its affinity for microtubules, and also modulates axonal transport [65,67].

GSK-3β recognizes phosphorylated Ser residues, and phosphorylates Ser and Thr residues in the vicinity of previously phosphorylated Ser residues. It requires prior-phosphorylation by cdk5 *in vitro* [68], while evidence for *in vivo* priming is still missing. Studies on transgenic mice also suggested a crucial role of GSK-3β in AD pathogenesis [69], and a role of cdk5 in elevation of tau hyperphosphorylation and NFT formation [70]. Phosphorylation of tau by GSK-3β may promote formation of tangle-like filament morphology in cell-free models [71]. GSK-3β also mediates tau phosphorylation in cultured cell lines [72], while overactivation of GSK-3 in animal model induces tau hyperphosphorylation together with AD-like cognitive effects such as impairment of spatial memory [73]. Most importantly, it was shown that Aβ oligomers enhance tau phosphorylation (Figure 1) and compromise cell survival through the mechanism mediated by GSK-3β activation [12,14,69], and neurons from tau-knockout mice are resistant to Aβ-induced toxicity [11]. Moreover, it has been reported that GSK-3β facilitate APP processing and generation of Aβ [74]. Interestingly, presenilin 1, a part of γ-secretase, brings tau and GSK-3β in close proximity as both tau and GSK-3β bind to the same region of presenilin 1 [75]. Altogether these observations suggest that GSK-3β plays a central role in the pathological progression of neurodegeneration *in vivo*, and points to its great potential as a target for therapeutic interventions in AD.

Inhibition of tau phosphorylation and pharmacological outcomes

GSK-3β is considered an interesting therapeutic target, because it links senescence, metabolic diseases, inflammation, protein aggregation, and cell death [76,77]. Accordingly, inhibitors of GSK-3β activity have been intensively sought as potential pharmacological agents in AD therapy. Many studies were performed using lithium, the most widely used mood stabilizer for treating bipolar disorder. However, lithium is a specific inhibitor of neither GSK-3, nor GSK-3β. It acts as a competitive inhibitor of Mg^{2+} and increases inhibitory phosphorylation at Ser^{9} in GSK-3β through the activation of Akt kinase [78]. Briefly, studies on transgenic mice have shown that inhibition of GSK-3β effectively reduces tau pathology, including level of tau phosphorylation, formation of insoluble aggregates, and axonal degeneration, particularly if administration started during early stages of NFT development (Table 1). However, despite the overall reduction in phosphorylated tau, treatment with lithium failed to improve memory impairments in some studies [79-81].

Regarding Aβ pathology, treatments with lithium resulted in contradictory outcomes. While some studies showed reduced Aβ level and reduced plaques formation [74], increased Aβ production [64], as well as unchanged Aβ production have been reported [77,80]. A more recent study on a double transgenic

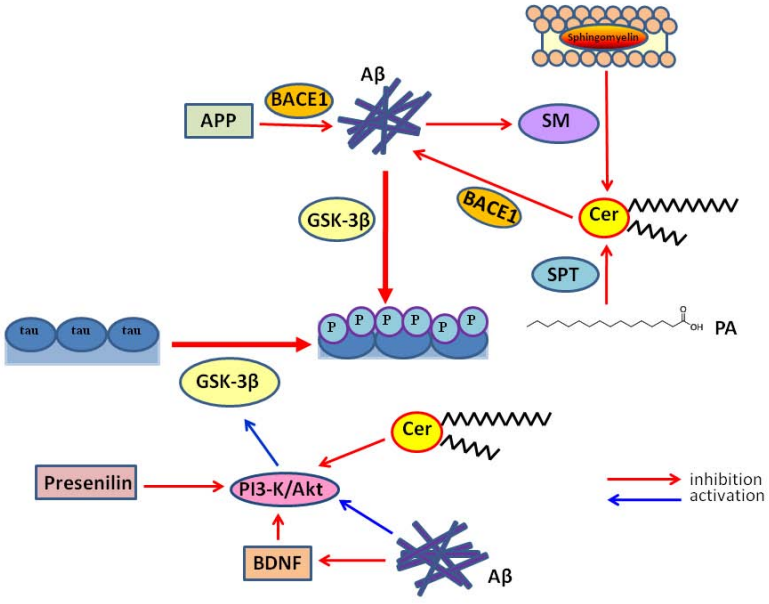

Figure 1. Schematic representation of the interactions between hyperphosphorylated tau, Aβ, and ceramides. GSK-3β is the main kinase involved in phosphorylation of tau. By a mechanism that is dependent on GSK-3β, intracellular accumulation of Aβ may also contribute to tau hyperphosphorylation. In addition, Aβ affects production of ceramides by acting on sphingomyelinases, enzymes responsible for the catabolic degradation of sphingomyelin. Generated ceramides in turn increase production of Aβ acting on β-secretase, a key enzyme in the proteolytic processing of amyloid precursor protein (APP). BDNF and presenilin may modulate these interactions, particularly via the PI3-K/Akt signaling pathway. PI3-K activates Akt/protein kinase B, while activated Akt in turn phosphorylates GSK-3β and induces its inactivation and reduction in tau phosphorylation.

Table 1. Effects of GSK-3 inhibitors on tau and Aβ pathology in diverse transgenic mice models.

Mouse line	Age of mice	GSK-3 inhibitor	Dose	Treatment duration	Effects on AD pathology	Ref
Tau^{vlw}	adult	LiCl	Fed with chow 1.7 g/kg + 2.55 g/kg	4 weeks + 2 weeks	↓ of tau protein in sarkosyl-insoluble fractions ↓phosphorylation and aggregation of mutant tau	[129]
PDAPP	4 weeks	Li_2CO_3	2.4 g/kg chow	7 months	↓ Aβ levels ↓ Aβ plaque deposition	[74]
JNPL3	8-11 months	LiCl	0.6 M (10 ml/g)	30 days	↓ level of aggregated tau in the cortex ↓ axonal degeneration if treatment was started at an early stage	[78]
JNPL3	8-11 months	AR-A014418	30 mmol/kg	Twice daily for 1 month	↓ insoluble tau levels in the brain stem	[78]
PrP T44 Ag	2-6 months	LiCl	2.0 g per kg chow	5 months	↓ tau lesions (promotion of ubiquitination) development of more soluble tau aggregates in spinal cord no obvious effect on tau phosphorylation ↓ number of tau-positive spheroids in spinal cord at 9 months	[130]
PrP T44 Ag	5-9 months	LiCl	2.0 g per kg chow	5 months	limited clearance of tau aggregates	[130]
GSK-3/VLW	12 months	LiCl	1.7 g LiCl/kg chow	7.5 months	no signs of phospho-tau accumulation or aggregation	[131]
GSK-3/VLW	18 months	LiCl	Fed with chow 1.7 g/kg + 2.55 g/kg	4 weeks + 2 weeks	incomplete reversal of tau pathology without effect on amyloid-like pathology	[131]
hAPP tg	3 months	LiCl	20 mg/kg	3 months	Improved water maze performance ↓ Aβ and plaques formation ↓ Aβ phosphorylation of APP	[132]
3xTg-AD	15 months	LiCl	300 µl of 0.6 mol/l	4 weeks	↓ tau phosphorylation unchanged Aβ load without improvement in working memory deficits	[80]
APP^{sw}-tau^{vlw}	9 or 12 months	NP12	200 mg/kg	3 months	↓levels of tau phosphorylation ↓Aβ deposition ↓ astrocytic proliferation preserved neuronal survival in the entorhinal cortex and CA1 prevention of memory deficits	[81]
Tgtau30	3 months	Li_2CO_3	Chronic chow feeding 2.4 g/kg chow	8 months	no changes in the development of NFT	[133]
Tgtau30	9 months	Li_2CO_3	Oral gavage 350 mg/kg	1 month	↓ tau phosphorylation and sarkosyl-insoluble aggregated tau in CNS ↓density of NFT without effect on tau ubiquitination ↑ LC3, marker of autophagic vacuoles motor and working memory deficits not rescued	[133]
3xTg-AD	13 months	MMBO	3 and 10 mg/kg	22 days	↓tau phosphorylation improved cognitive behavioral deficit without changes in Aβ level and Aβ plaque formation	[134]
3xTg-AD	13 months	C-7a	20 and 50 mg/kg	Twice daily for 21 days	↓tau phosphorylation in hippocampus prevention of short-term memory deficitc	[135]

Tau^{vlw}- transgenic for a human 4-repeat tau isoform with 2 N-terminal exons carrying the FTDP-17 mutations G272V, P301L and R406W; PDAPP - overexpressing the human amyloid precursor protein containing the Indiana familial AD mutation (APP_{V717F}); JNPL3 - line overexpressing mutant human tau (P301L, 4R0N); PrP T44 Tg – overexpression of the smallest human tau isoform in the mouse CNS with the mouse prion protein (PrP) promoter; GSK-3/VLW - mice overexpressing GSK-3 and FTDP-17-tau; hAPPtg – tg mice expressing mutant human APP under the Thy1 promoter; 3xTg-AD mice – line expressing human APP_{Swe} and the human tau_{P301L}; APP^{sw}-tau^{vlw} overexpressing human mutant APP (Swedish mutation K670N-M671L) and a triple human tau mutation associated with frontotemporal dementia and parkinsonism linked to chromosome 17 (G272V, P301L and R406W); Tgtau30 express a mutant tau transgene (1N4R human tau isoform) mutated at positions G272V and P301S, under the control of a modified thy-1 promoter; MMBO -2 -methyl-5-(3{4[(S)methylsulfinyl]phenyl}-1-benzofuran-5-yl)-1,3,4-oxadiazole

APP/Tau mouse model with a specific GSK-3 inhibitor reduced tau phosphorylation and Aβ deposition, together with prevention of memory deficits and increase in neuronal survival [81]. Other selective inhibitors of GSK-3α/β also suggested reduction in Aβ production, in particular via an increase of the degradation of APP through the activation of a lysosome/autophagy pathway [77]. Based

on these promising findings, some GSK-3 inhibitors have entered into preclinical and clinical trials [82,83].

Numerous factors may affect tau phosphorylation, and many of them are also assessed for pharmacological application in AD patients. Brain-derived neurotrophic factor (BDNF), an extracellular factor that is down-regulated in AD brains, has been considered as one of the critical contributors to dementia due to its role in cognition, learning, and memory through the modulation of synaptic plasticity in adulthood [84,85]. Regarding GSK-3β, exposure to BDNF induces a rapid decrease in tau phosphorylation at specific phosphorylation sites through a PI3-K/Akt and downstream GSK-3β signaling transduction cascade [86]. PI3-K activates Akt/protein kinase B, while activated Akt in turn phosphorylates GSK-3β and induces its inactivation (Figure 1). Moreover, Aβ at levels insufficient to cause degeneration interferes with BDNF-induced activation of PI3-K/Akt pathway [87], indicating a potential link between an increase in Aβ levels and hyperphosphorylation of tau protein in AD, which may be of importance for novel therapeutic approaches [86]. In contrast, accumulation of Aβ leads to downregulation of Akt, increase in activation of GSK-3β, and apoptosis, while downregulation of Akt paralleled intracellular Aβ accumulation *in vivo* in the Tg2576 AD mouse model [88]. Interestingly, presenilin 1 also induces tau dephosphorylation and promote cell survival due to PI-3K/Akt-mediated inhibition of GSK-3 activity [89].

Sphingolipids in the pathogenesis of AD

Sphingolipids are a heterogeneous class of lipids derived from sphingosine, an aliphatic amino alcohol. The first, rate-limiting step in their synthesis is formation of sphingosine from palmitoyl-CoA and Ser by serine palmitoyltransferase (SPT). Beside important structural role, particularly in lipid rafts, sphingolipids and their metabolites also act as second messengers in diverse signaling pathways involved in signal transduction, cell growth and differentiation, and apoptosis [90,91]. Although sphingolipids represent only a small proportion of the total cellular lipid pool [92], studies suggest that even subtle disturbances in their balance may be involved in the development of neurodegenerative diseases. Diverse methodological approaches have identified altered pathways and products of sphingolipid metabolism that contribute to the pathogenesis of AD, including Aβ pathology, tau formation, and neurodegeneration [93-95]. Hence, preservation of neuronal sphingolipid homeostasis is of major importance for normal brain functioning and potential target for novel therapeutic approaches.

The major bioactive mediators formed by sphingolipid metabolism are multiple ceramides, sphingosine, ceramide-1-phosphate, sphingosine-1-phosphate and glycosphingolipids [92,95]. Several studies indicated that ceramides and sphingosine mediate apoptosis, growth arrest, and senescence, while their phosphorylated derivatives promote proliferative and anti-apoptotic activities [92,96], although it is also observed that endogenous and exogenous ceramides could protect cortical neurons obtained from wild-type mice against Aβ toxicity and Aβ-induced increase in tau phosphorylation by inhibiting cdk5 activity [97]. Irrespective of this controversial finding, it is evident that neuronal survival could be determined with very small shifts in ceramide metabolism [98].

Sphingolipids in AD

Ceramides (Cer), the major molecules of sphingolipid metabolic pathways, are expressed in both neurons and astrocytes. They are composed of sphingosine attached by an amide bond to fatty acids, varying in length from 14 to 26 carbon atoms. On this basic backbone, different moieties can be attached forming complex sphingolipids [91,99]. Hence, glycosphingolipids are formed through Cer glycosylation. Shift in sphingolipid metabolism toward accumulation of Cer and decrease of glycosphingolipids, together with the reduction of anti-apoptotic signaling, has been suggested as a function of the disease progression [100]. On human neuroblastoma SH-SY5Y cells, inhibition of glycosphingolipids biosynthesis markedly reduced secretion of soluble APP and Aβ, indicating one more potential therapeutic avenue in the inhibition of Aβ production [101].

Sphingomyelin, one of the most abundant mammalian sphingolipid especially enriched in the myelin sheet that surrounds axons of neuronal cells, is formed when phosphorylcholine is attached to Cer. Increased levels of sphingomyelin are found in the cerebrospinal fluids of patients in prodromal AD exclusively [102], and increased sphingomyelin/Cer ratio is considered for potential use as sensitive blood-based biomarker for disease progression [103].

Sphingosine, formed in the deacylation of Cer by enzyme ceramidase, can be metabolized to sphingosine-1-phosphate and fine-tuning between concentrations of these metabolites may determine cell fate [95]. As mentioned above, effects of sphingosine-1-phosphate are considered mostly as beneficial [96], although the proteolytic activity of β-secretase may be increased in direct interaction with sphingosine-1-phosphate [104].

Gangliosides, a highly heterogeneous family of glycosphingolipids that contain sialic acid, are also concentrated in lipid rafts, and able to affect neuronal plasticity and cell survival. Composition and amount of some gangliosides change with age, and it is thought that gangliosides also contribute to AD pathology by binding to Aβ that further leads to the formation of large Aβ assemblies [105-107].

Sulfatides, sulfated galactocerebrosides synthesized almost exclusively by oligodendrocytes and present predominantly in the myelin sheath, are also a class of sphingolipids probably involved in AD pathogenesis [107]. It seems that levels of sulfatides are depleted in AD brains, particularly at the stage of very mild dementia [108]. Interestingly, it has been shown that sulfatides may function as effective stimulators for autophosphorylation of GSK-3β [109].

Anabolic and catabolic pathways of sphingolipid metabolism: implications for AD pathology

Sphingomyelin can be hydrolyzed to Cer through the actions of ubiquitous acidic and brain-specific neutral sphingomyelinases

that are activated by oxidative stress and inflammatory processes [98]. In SH-SY5Y cells and primary cortical neurons, the proinflammatory modulator TNF-α stimulates Mg^{2+}-dependent neutral sphingomyelinase activity, and elicits sphingomyelin hydrolysis and Cer increase [98]. Endogenous Cer can also be generated by a *de novo* synthesis pathway. Elevated basal level of particular species of Cer in serum is associated with a higher risk for developing AD [110]. Cer are increased approximately three times in brains from AD patients [108], particularly in vulnerable regions [111]. Besides increased levels of Cer [112], gene expression abnormalities of enzymes that participate in sphingolipid metabolism, including both sphingomyelinases, are also found at different stages of AD progression [113]. A detailed RNA analysis revealed 28 genes involved in Cer homeostasis whose expression was altered through the disease progression. In particular, gene expression of the enzymes involved in *de novo* synthesis of Cer was increased, while downregulation of the enzymes participating in glycospingolipid synthesis occurred [100].

Because Cer exhibit neurotoxic properties acting as pro-apototic molecules, increased activity of sphingomyelinases promotes loss of neuronal cells [112,114,115]. The mechanism leading to cell death may involve, at least in part, a mitochondrial PP2A that dephosphorylates and inactivates the major anti-apoptotic protein Bcl-2 [116]. *In vitro* and *in vivo* studies pointed out to associations between Cer and Aβ. Endogenous pool of Cer strictly regulates Aβ biogenesis, in a way that elevated ceramide levels directly increase Aβ level [117]. Membrane Cer, as the major component of lipid rafts, facilitate Aβ production by increasing the half-life of β-secretase (BACE1) and affecting its molecular stability [117,118]. In turn, oligomeric soluble Aβ and fibrillar $Aβ_{1-42}$ activates neutral and acidic sphingomyelinases [119,120]. Hence, Aβ-dependent increase in ceramide levels leads to further increase in Aβ production (Figure 1). In addition, upregulated sphingomyelinase correlates with increased tau hyperphosphorylation in AD brains [112]. On the other hand, activation of SPT also might increase Cer levels and contribute to AD pathology. SPT consists of SPTLC1 and SPTLC2 subunits, and expression of both has been confirmed in senile plaques [121].

Interaction of astroglia and neurons in sphingolipid metabolism and AD pathology

SPT upregulation in astrocytes is implicated in upregulation of sphingomyelinase activity in neurons. Soluble products released from palmitic acid (PA)-activated human astroglia activates neutral sphyngomyelinase and increases Cer levels and cell death in primary neuronal cultures [122]. In particular, exposure to PA, the most common saturated fatty acid, increases endogenous Cer production in astroglia, which in turn is involved in both increased amyloidogenesis and tau hyperphosphorylation in neurons exposed to conditioned media from PA-treated astrocytes [118,123]. As direct treatment of primary neurons with PA had no effect on the BACE1 activity and Aβ levels [124], such studies emphasized the essential role of abnormal astroglial Cer metabolism in causing pathophysiological changes characteristic of AD. PA-induced SPT activation and *de novo* synthesis of Cer in astrocytes is involved in the production of the cytokines TNF-α and IL-1β. These soluble cytokines, after release into conditioned media activate sphingomyelinases in neurons, and through the catabolic pathway, increase Cer level and upregulate BACE1 and amyloidogenesis [123]. Increased extracellular Aβ could in turn act on both astroglia and neurons, and further enhance Cer levels, reinforcing a Cer-Aβ-Cer cascade [123]. Interestingly, the APP intracellular domain (AICD) decreases the expression of the SPTLC2, the catalytic subunit of the SPT heterodimer, decreasing the SPT activity [125]. Inhibition of SPT in an early-onset mouse model of AD by inhibitor L-cycloserine reduced cortical SPT protein levels, which in turn decreased Cer and induced downregulation of cortical $Aβ_{1-42}$, and also decreased soluble hyperphosphorylated tau level without evident toxic effects [121]. Since L-cycloserine also acts as a partial agonist of *N*-methyl-D-aspartate receptors, cognitive enhancements have been observed following its administration in AD patients [126]. Likewise, treatment of neurons with conditioned media obtained from astroglia exposed to both free fatty acids and L-cycloserine was able to decrease levels of increased Aβ, hyperphosphorylated tau, and activated GSK-3β [118]. Other studies also linked Cer to tau phosphorylation. It was shown that Cer modulate PP2A activity [127], and increase tau phosphorylation by cdk5 at specific sites [128]. Beside direct impact on Aβ pathology, increase in Cer stimulates free radical generation, establishing a link between sphingolipid metabolism and oxidative stress that further exacerbates neuronal degeneration [98]. Hence, therapeutic strategies based on the reduction of Cer accumulation have the potential to prevent tau phosphorylation and further studies *in vivo* are needed to clarify these approaches, particularly in combination with other targets.

Conclusions

In spite of major research efforts, effective treatments for devastating neurodegenerative disorders, such as AD, are still lacking. As AD arise from the combination of different mechanisms (Figure 1), it is generally believed that prevention or reduction of AD pathology should rely on the inhibition of various steps involved in the early stages of disease progression. Several molecular targets represent rational therapeutic approaches for improvement of AD therapy. Among them are GSK-3β and enzymes involved in sphingolipid metabolism. GSK-3β has been identified as a major tau kinase and a signaling link between Aβ and tau pathology, and reduction of its activity certainly has valuable therapeutic potential. However, due to the large number of phosphorylation sites and the many protein kinases implicated in tau pathology, it is argued that valid strategies should be directed to overall reductions in cellular level of tau phosphorylation by targeting kinases with broad inhibitors. In addition, such an approach will probably have minor adverse effects on the physiological roles of targeted enzymes and fewer problems with tolerability and toxicity that are common in elderly [1]. Another prospective avenue in AD therapy is

the modulation of sphingolipids metabolism. Drugs acting along sphingomyelinases and SPT metabolic pathways have recently attracted increasing attention. Beneficial outcomes of pharmacological studies suggest that modulation of Cer-regulated pathways, together with attenuation of kinase activity, may represent a promising strategy to counter pathology development in AD treatment.

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