Exploring sphingolipid function in the pathogenesis of Alzheimer's disease

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Exploring sphingolipid function
in the pathogenesis of Alzheimer's disease

Qian Luo

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Exploring sphingolipid function
in the pathogenesis of Alzheimer's disease

Dissertation

To obtain the degree of Doctor at the Maastricht University,
on the authority of the Rector Magnificus, Prof. dr. Pamela Habibović
in accordance with the decision of the Board of Deans,
to be defended in public
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Chapter 1

General Introduction

Alzheimer's disease (AD) is the most common form of dementia among older adults. It has become the fifth leading cause of death for adults over age 65 years, with an estimated number of 152 million by 2050 worldwide, according to the Alzheimer's Association Report [1]. The increasing healthcare costs and indirect social effects result in a significant economic burden globally, especially in low- and middle-income countries. In the past decades, six drugs were approved by the Food and Drug Administration (FDA) for the treatment of dementia: donepezil, rivastigmine, galantamine, memantine, donepezil and memantine combination, and suvorexant [2-4]. These drugs reduce the cognitive symptoms somewhat or other dementia behaviors, but without slowing down the progression of the disease. This year an antibody against amyloid, aducanumab, has been approved for the treatment of mild and moderate AD, which delays the clinical decline through a reduction of beta-amyloid. The efficacy of the monoclonal antibody, however, is strongly debated [5-7]. Therefore, the development of new therapeutic strategies remains urgent as ever. In this thesis, we propose to bring forward the evidence that maintenance of a steady state of sphingolipids may provide a new strategy for AD treatment.

Pathological features of AD

Hallmarks of AD are the extracellular deposition of plaques with aggregated amyloid- β proteins and the formation of neurofibrillary tangles containing hyper-phosphorylated tau protein [8]. Genetically, AD is divided into two distinct categories, familial and sporadic forms of the disease. Familial AD is rare, accounting for less than 5% of all AD cases. It is caused by autosomal gene mutations of the amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) before the age of 65 [9]. The APP is the precursor of the A β peptide, affecting A β production, cleavage, and aggregation [10]. On the one hand, APP cleaved by α and γ -secretase contributes to forming the non-toxic A β peptide; On the other hand, APP cleavage, initiated by β and γ -secretase, releases mainly toxic A β 42 and less toxic A β 40, which promote the formation of oligomers, fibrils, and plaques. Apart from the APP mutations, PSEN1 and PSEN2 mutations also affect amyloid plaques by altering the proportion of A β 40/A β 42 since they are components of the γ -secretase complex [11-13].

In contrast, the sporadic cases form the majority (90%) in AD, where patients are affected by multiple genetic and environmental factors, which are mostly still unknown. For now, APOE has been recognized as the most important genetic factor for sporadic AD. In humans, the APOE gene has 3 isoforms: APOE2 (cys112, cys158), APOE3 (cys112, arg158), and APOE4 (arg112, arg158), coding for proteins that are abundant in the brain where they modulate lipid transport. Studies demonstrated that APOE is a potential determinant of the aggregation and clearance of A β towards isoforms. APOE2 confers a protective effect by mediating A β aggregation and clearance relative to the APOE3 carriers; in turn, APOE4 increases the risk for developing AD [14]. Evidence shows that both homozygous and heterozygous APOE4 carriers have much worse cognitive performance through modulating the production and aggregation of A β [15]. Although the impact of APOE on A β may be explained, there are still other unexplained pathogenetic phenomena, such as its effect in synaptic plasticity, neuroinflammation, and dysregulated lipid metabolism [16, 17]. More insights on these matters are likely to be equally important for diagnosis and treatment of AD.

Current diagnosis of AD

Efforts are being made in brain imaging, and in the analysis of cerebrospinal fluid (CSF) and of blood biomarkers diagnosis of AD and to identify the typical features, such as A β , P-tau, Tau [18, 19]. Structural magnetic resonance imaging (MRI) and positron emission tomography (PET) are being used since these methods enable us to visualize the pathology directly in patients [20]. Indeed, MRI can recognize the structural changes with 85% sensitivity in mild AD [21]. Fluorodeoxyglucose-PET (FDG-PET) can measure the decrease of glucose metabolism in AD brain with 70% specificity [22]. In addition, several studies suggest that P-tau181, and P-tau217 PET can be used as prognostic monitors of AD [23, 24]. However, the high costs and limited availability of radiotracers unfortunately block the use of PET in the clinic. The biomarkers A β 42, total tau (t-tau) and phosphorylated tau (p-tau) from CSF and the ratio between A β 42 and A β 40, or other combinations have been evaluated in hundreds of clinical studies, reviewed in [20]. But obtaining CSF through lumbar puncture is thought to be too invasive to make the method very useful. In turn, because of the lower cost and the possibility for re-running a test, blood biomarkers are receiving attention. For instance, there is a great diagnostic accuracy of the markers A β 42, A β 42/40 and P-tau by using ultrasensitive immunoassay technique [25]. In addition, several non-protein analytes, including amino acids [26], and lipids [27], have also been shown to be informative for the status of AD. Currently, Mielke, et al found that the plasma ceramide [28] and sphingomyelin [29] are associated with cognitive progression. These findings encourage us to explore how sphingolipid affect the development of AD.

Sphingolipids in AD

Sphingolipids play an essential role in the structure of membranes as well as, via second messengers, in the regulation of cell differentiation and death [30], in blood-brain barrier integrity [31], and also in neuroinflammation in the brain [32]. Alterations of the sphingolipid metabolism, such as those found in ceramide, sphingomyelin, and sphingosine-1-phosphate (S1P), are closely associated with AD progression, revealing that sphingolipids are possibly useful targets for disease treatment [33].

Changes of sphingolipid metabolism in AD

Ceramide is the central component of the sphingolipid metabolism. It is synthesized via three different pathways, the de novo pathway, the sphingomyelin hydrolysis pathway, and the salvage pathway [34, 35]. For the de novo pathway, the reaction starts with the condensation of serine and palmitoyl-CoA by serine palmitoyl-transferase at the endoplasmic reticulum (ER). Subsequently, the product, 3-ketosphinganine, is converted into sphinganine, which promotes ceramide formation by activating the ceramide synthases (CerSs). Of these enzymes, CerS1, 2, 5 and 6 are responsible for the formation of short acyl chain ceramides (C14-C20), while CerS3 and 4 helps long-chain fatty acids (C22-C26) generation [36]. Ceramide levels are found increased in both AD CSF [37, 38] and brain [39]. Currently, Mielke et al. show that plasma Cer16:0 and Cer24:0 levels are increased [40] while they also suggest that the levels of ceramide Cer22:0 could predict memory loss and hippocampal volume loss [28]. Moreover, increased levels of ceramide augmented A β levels by stabilizing the β -secretase Amyloid Precursor Protein-cleaving Enzyme 1 (BACE1) [41].

Once formed, ceramide is immediately transported to the Golgi for the synthesis of more complex sphingolipids, sphingomyelin and glycosphingolipids [42]. Ceramide can reach the Golgi via two pathways: the vesicular pathway (minor) and ceramide transfer proteins (CERTs), the major transporters [43]. CERTs are regarded as main protein which regulate ceramide transport and further influence the production of sphingomyelin and glycosphingolipids. Of these, the best characterized isoforms are GPBP Δ 26/CERT and CERT_L [44] and both can transport ceramide from ER to Golgi [45, 46]. Studies found that the deficiency of CERTs influence normal embryonic development and lead to senescence in primary cells [46, 47]. Recently, our team showed that CERTs modulate the immune system via activation of the classical C pathway and co-localize with A β and bind to serum amyloid P protein (SAP) in the brain [48-50]. Noticeably, CERT_L contains a serine-enriched domain with 26 amino acid residues, which plays an important role in normal brain development [47]. Therefore, the function of CERT_L in AD progression needs to be further explored.

Sphingomyelin and glycosphingolipid can be converted into ceramide again by the degradation pathway. The hydrolytic degradation of ceramide generates sphingosine, which is phosphorylated into S1P by sphingosine kinases (SKs). S1P can be degraded to ethanolamine by S1P lyase and exit the sphingolipid pathway. In turn, sphingosine can enter the ceramide cycle and generate ceramide and sphingomyelin by the salvage pathway [39]. S1P is regarded as a protective factor for AD. It induces cell proliferation and survival in the vascular system during embryogenesis and protects against A β toxicity in cultured cortical neurons [51]. Several studies reported that the levels of S1P were decreased in post-mortem AD brain [52, 53]. In contrast, one study showed that neuronal death was closely associated with elevated S1P levels in cultured neurons [54]. Interestingly, the lack of S1P degrading enzymes (S1PL) causes an accumulation of the full-length APP and amyloidogenic C-terminal fragments (CTFs) [55].

Sphingolipids as therapeutic targets in AD

As aforementioned, sphingolipid metabolism can be regulated mainly by balancing the level of ceramide, sphingomyelin and S1P. Myriocin, L-cycloserine and D-cycloserine are potential inhibitors of serine palmitoyl-transferase and they improve cognition in somewhat way [56, 57]. In our previous study, elevated ceramide and decreased sphingomyelin were found with the administration of HPA-12, a CERT inhibitor, which prevents ceramide transfer into Golgi [58]. CERT was identified to co-localize with serum amyloid P component the major constituent of plaques, suggesting that regulation of CERTs could be a target for AD. FTY720 (Fingolimod) is an analog of sphingosine, approved for relapsing-remitting multiple sclerosis treatment. It is phosphorylated into FTY720-P in the body and binds S1P receptors (S1PR1,3,4,5), and promotes the internalization of lymphocytes and inhibits the over-response during inflammation [59]. Recently, researchers have shown that FTY720 reduces A β levels in neurons [60, 61]. In addition, FTY720 decreases neuroinflammation in microglia in 5xFAD transgenic model [62, 63]. These results suggest an essential role of S1P in AD. Exploring the pharmacological effect of FTY720 in a systematic way might shed some light about its potential for therapy in AD.

Aim of this thesis

This thesis aims to investigate the sphingolipid function in the pathophysiology and treatment of AD.

Chapter 1 reviews the AD pathophysiological features and the role of sphingolipid therein.

Chapter 2 presents the current state of sphingolipids in neurodegenerative diseases and discusses their potential role as diagnostic biomarkers.

Chapter 3 studies CERT_L function in AD via overexpression AAV- CERT_L in the 5xFAD mouse model. We mainly examine how CERT_L influence the sphingolipid metabolism components and determine whether CERT_L affects cognitive impairment and AD pathophysiology.

Chapter 4 assesses the prevention effect of FTY720 on the transgenic EFAD models generated by crossing 5xFAD with the human APOE genes, including APOE3 and APOE4, E3FAD, and E4FAD. Behavioral and pathophysiological effects are analyzed.

Chapter 5 assesses the intervention effect of FTY720 on the EFAD model. This study evaluates cognitive function, sphingolipid levels, and amyloid content.

Chapter 6 summarizes the essential findings and discusses the potential roles of sphingolipids in AD treatment.

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Chapter 2

Sphingolipids as Prognostic Biomarkers of Neurodegeneration, Neuroinflammation, and Psychiatric Diseases and Their Emerging Role in Lipidomic Investigation Methods

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*Authors contributed equally to this paper

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Abstract

Lipids play an important role in neurodegeneration, neuroinflammation, and psychiatric disorders and an imbalance in sphingolipid levels is associated to disease. Although early diagnosis and intervention of these disorders would clearly have favorable long-term outcomes, no diagnostic tests currently exist that can accurately identify people at risk. Reliable prognostic biomarkers that are easily accessible would be beneficial to determine therapy and treatment response in clinical trials. Recent advances in lipidomic investigation methods have greatly progressed the knowledge of sphingolipids in neurodegenerative and psychiatric disorders over the past decades although more longitudinal studies are needed to understand its exact role in these disorders to be used as potential tools in the clinic. In this review, we give an overview of the current knowledge of sphingolipids in neurodegenerative and psychiatric disorders and explore recent advances in investigation methods. Finally, the potential of sphingolipid metabolism products and signaling molecules as potential biomarkers for diagnosis, prognostic, or surrogate markers of treatment response is discussed.

Keywords: Sphingolipids, neurodegeneration, neuroinflammation, psychiatric diseases, lipidomics, biomarkers, surrogate markers.

Introduction

Sphingolipids are a greatly conserved category of lipids, which were discovered in the brain by Johann Ludwig Wilhelm Thudichum more than a century ago [1]. It is therefore not surprising that their denomination as cerebroside, sphingomyelin and ganglioside is derived from the initial sources used for their isolation. The common structural characteristic of this diverse and ubiquitous lipid family is a long chain (sphingoid) base backbone [2]. Sphingolipids are important components of lipid bilayers and contribute uniquely to structural and functional properties of the membrane [3]. Together with cholesterol, they are most important elements of detergent-resistant membrane microdomains, also known as lipid rafts [4, 5]. Dynamics of the membrane, including vesicular trafficking, are closely related to sphingolipid interconversions [6].

Ceramide is the central molecule in the metabolism of all sphingolipids (Fig. 1). Its formation starts with the condensation of two common cellular metabolites, serine and palmitoyl-CoA, in the *endoplasmic reticulum* (ER). The enzyme catalyzing this rate limiting step is serine palmitoyltransferase (SPT) [7]. The reaction product 3-ketosphinganine is then produced to dihydrosphingosine (also known as sphinganine). N-acylation of dihydrosphingosine is catalyzed by a family of 6 different ceramide synthases (CerS), each of which uses a restricted subset of acyl-CoAs [8] to generate the respective dihydroceramide. The final step of *de novo* ceramide formation is introduction of a double bond into the sphingoid base moiety by dihydroceramide desaturase (DES) [9]. Ceramide is then either glycosylated in a stepwise manner leading to the generation of glycosphingolipids (GSLs), including the sialic acid-containing gangliosides and lactosylceramide (LacCer), or gains a phosphocholine head group to form sphingomyelin (Fig. 1). Alternatively, phosphorylation of ceramide can produce ceramide 1-phosphate and further continue to synthesize more complex sphingolipids such as, GSLs and sphingomyelin. Interestingly, the transport of ceramide from the ER to the Golgi apparatus where diverse head groups are added, is rather specific: it reaches the site of glycosylation via vesicular transport by the ceramide transfer protein (CERT) which delivers it to the site of sphingomyelin formation [10].

The degradation of GSLs to ceramide primarily takes place in the lysosomes by the action of specific acid exohydrolases, which start from the hydrophilic group [11]. Small glycoprotein cofactors (sphingolipid activator proteins; SAPs) are needed to assist lysosomal exohydrolases to degrade GSLs with three or less monosaccharide residues [12]. Insufficient degrading hydrolases and/or SAPs causes the respective substrate in the lysosome to accumulate. Since neurons are especially sensitive to the accumulation of lipids, lysosomal lipid storage disorders (LSDs) like sphingolipidoses or gangliosidosis, often affect the brain [13]. Moreover, complex gangliosides are abundant in the brain and particularly in neuronal membranes [14, 15]. Gangliosides are involved in neuronal plasticity and repair, and especially the subtype GM1 has been implicated neurotrophic mechanisms [16]. The degradation of sphingomyelin, and thus recycling of ceramide, is catalyzed by various sphingomyelinases [17]. Since the discovery that membrane sphingolipids could serve in signal transduction pathways and sphingomyelin and ceramide were discovered as a bioactive signaling molecule [18, 19], interest in sphingomyelinases and other enzymes and sphingolipids in this pathway has continuously increased, especially due to their function in central nervous system (CNS) physiology [20-24].

Degradation of ceramide starts with its hydrolytic de-acylation to sphingosine, catalyzed either by a ceramidase (CDase) [25] or by diverse non-lysosomal neutral CDases [26, 27]. Sphingosine is then phosphorylated by two different kinases (SK1 and SK2) [28], generating sphingosine 1-phosphate (S1P). The two isoenzymes differ by their substrate specificity and subcellular localization. Cytosolic SK1 becomes activated by plasma membrane recruitment and is highly specific for sphingosine. The membrane associated SK2 is less specific and constitutively active in the ER/nuclear compartment. Note that S1P is not only a sphingolipid degradation product, but also an evolutionarily conserved bioactive lipid [29]. The final enzyme in sphingolipid degradation is S1P-lyase (Fig. 1). It catalyzes the irreversible cleavage of S1P to phosphoethanolamine and a long-chain aldehyde [30]. Alternatively, S1P is dephosphorylated by S1P-phosphatases (SPPs; two isoenzymes are known so far) [31]. This reaction is considered as the starting point for the sphingolipid salvage pathway [32] (Fig. 1, red arrows), which was shown to be especially important in terminally differentiated cells, such as neurons, to reestablish complex GSLs [33]. Note that S1P-lyase as well as SPPs are located in the ER.

Sphingolipids are highly abundant in the brain [34] where they form cell type-specific profiles [14] that characteristically change during development and aging [35], and with brain pathological alterations. It was initially shown that metabolic modifications of gangliosides are closely connected to neurodegeneration [15, 36-39]. However, during the last decades, the bioactive metabolic intermediates of gangliosides and sphingomyelin, including primarily ceramide and S1P, emerged as critical players in the maintenance of brain health [24, 40-43]. For example, an inborn mutated S1P-lyase was recently associated with brain malformations [44]. Other mutations affecting the same enzyme also result in neurological degeneration in addition to several other abnormalities [45]. Importantly, not only the functionality of an enzyme but also the subcellular site where a bioactive lipid is generated appears to affect its signaling function [46]. In line with this assumption, mislocalization of SK2 has been reported in neurodegenerative diseases [47]. Additionally, the CERT protein has also been implicated in neuroinflammation and neurodegeneration by binding to serum amyloid P-component (SAP) and colocalizes in amyloid plaques in the brain of Alzheimer's disease (AD) patients. [48-51]

Taken together, the role of sphingolipid metabolism and related enzymes in neurological diseases are being increasingly investigated. Additionally, modern research techniques allow higher specificity in measuring sphingolipids in the brain, blood, and CSF. This potentially opens doors towards the development of biomarkers indicating specific neurological diseases that can be used in the clinic to improve treatment options.

Figure 1. Basic scheme of sphingolipid metabolism.

De novo generation of ceramide starts from serine and palmitoylCoA (blue arrows) or through recycling of sphingosine 1-phosphate (S1P) (red arrows). Ceramide is the degradation product, as well as the biosynthetic precursor, of all glycosphingolipids and sphingomyelin. Hydrolytic degradation of ceramide (black arrows) generates a fatty acid (not shown) and sphingosine which can, in turn, be phosphorylated to generate S1P. S1P-lyase irreversibly cleaves S1P into phosphoethanolamine and hexadecenal. Abbreviations used are: CerS, ceramide synthases (CerS1 is widespread in the brain); CDase, ceramidase (acid ceramidase is a major glycoprotein in the brain); DES, dihydroceramide desaturase; KSR, ketosphinganine reductase; SK, sphingosine kinases (SK1 and SK2 isoforms are known); SPP, S1P phosphatases (SPP1 and SPP2 isoforms are known); SMase, sphingomyelinases (acid and neutral forms are known); SMS, sphingomyelin synthase; See text for further explanations.

1.1 Lipid Investigation Methods

Lipidomics as a field has heavily relied upon the high sensitivity of mass spectrometry-based techniques. Recent advances in mass-spectrometry lipidomics now make the detailed lipid composition characterization of a given biological sample possible, which includes different sphingolipid classes and other lipid categories [52]. Initially, experiments were mainly so-called “shotgun” methods, where lipids were extracted from pulverized tissue and injected into the mass spectrometer without further purification or separation [53]. With the advent of electrospray ionization, these “crude extract” methods were useful in determining glycerophospholipids in cellular membranes [54],

bile acids [55], and enzymatic substrates [56]. Further evolution of these techniques incorporated liquid chromatography (LC), improving detection by removing ion suppression, as well as enhancing identification by adding a new criterion [57]. Modifications to the general process in LC- mass spectrometry lipidomics have included normal-phase (non-polar solvent) LC for glycerophospholipid (GPL) headgroup identification [58], reverse-phase (polar solvent) LC for acyl chain separation [59], and has been employed to study a variety of neurodegenerative diseases [60-62]. In this context, lipidomics has played a small, but growing role over the past 2 decades. While these techniques have greatly increased the understanding of lipidomics in both plasma and whole tissue, they unfortunately fail to separate fine-scale phenomenon. Probing the spatial distribution of lipids in tissue lies fully within the wheelhouse of mass spectrometry imaging (MSI). By using the most common MSI technique, matrix-assisted laser desorption/ionization (MALDI), several investigators have made strides in identifying sphingolipids that are tied to neurological dysfunction. This is due to a surge in the analytical methods over the past decade, which have allowed higher sensitivity, greater specificity, and finer resolution than ever before [63-65]. These techniques have been extended to examine lipid biochemistry changes in healthy tissues and have been fundamental for lipidomic studies in neurodegenerative diseases. In this review, current knowledge of sphingolipid alterations in neurodegeneration (AD, Parkinson's disease: PD, and Huntington's disease; HD), neuroinflammation (multiple sclerosis; MS), and psychiatric disorders is discussed. Additionally, current research models and tools to evaluate lipids in these disorders and the potential of sphingolipid metabolism biomarkers in the clinic is evaluated.

2. Sphingolipids in neurodegenerative diseases

2.1 Alzheimer's disease

AD is a neurodegenerative disease resulting in decline in memory and cognitive disability [66]. The major neuropathological hallmarks of AD are the intercellular accumulation of amyloid (A β 42) plaques and the formation of extracellular neurofibrillary tangles of hyper-phosphorylated tau protein, which result in impairment of synaptic function and ultimately neuronal death [66, 67]. In the past decades, various studies have found that an abnormal sphingolipid metabolism is associated with AD pathology. More specifically, increased *de novo* synthesis of ceramide accelerates the production of A β in neurons and higher levels of ceramide in the brain are seen in AD and other neurodegenerative disorders [68, 69]. Additionally, ceramide species are elevated in CSF and brain white matter, which especially peaks in very mild forms of AD dementia [70, 71]. Furthermore, multiple clinical studies suggest a relationship between plasma ceramides and dementia in AD, linking high levels with memory decline among individuals with mild cognitive impairment (MCI) and AD [72-74]. Other studies reported that high plasma ceramides were related to hippocampal volume loss [75-77]. Since these studies additionally suggest that brain and plasma ceramide levels are altered at an early stage of AD, research focusing on the metabolism of ceramide as a target for the development of blood-based biomarkers has been ongoing, although further studies are necessary to make definitive conclusions about the potential of blood ceramides as an indicative tool for AD.

In addition to ceramide, other potential targets in the sphingolipid metabolism (e.g. sphingomyelin, sphingosine, sphingomyelinase and SK1) have also been found (Table 1) [78-80]. Higher levels of sphingomyelin inhibit the activity of γ -secretase in the APP cleaving process, thereby reducing A β accumulation [81]. However, in AD, A β promotes sphingomyelinase activity leading to ceramide increase in the brain. Ceramide, in turn, stabilized

BACE-1 which helps cleave the APP protein to form A β peptides in AD [78, 80]. Although ceramide can be converted into sphingosine, an increase in sphingosine is also seen in the AD brain. Since sphingosine can be phosphorylated to form S1P, which is reduced in AD [80], this pathway may be of interest when researching pharmacological targets for the disease. FTY720 (fingolimod) is an S1P receptor agonist and approved treatment for MS which has shown to modulate neuroinflammatory pathways in an AD mouse model [82, 83]. Since almost all neural cells express S1P receptors [84], fingolimod is considered an interesting target of investigation as a potential AD drug treatment.

Besides ceramides and the S1P pathway, alterations of more complex GSLs have been observed in AD [38]. Six per cent of the total amount of lipids in the brain consists of gangliosides [85]. In AD, GM1 levels are elevated and seem to be particularly associated with A β -plaques [38]. More specifically, a toxic accumulation and aggregation of A β is seen when ganglioside GM1 is enriched in lipid rafts [38, 86]. This observation indicates a strong connection between ganglioside homeostasis and AD. Kaya *et al.* probed the lipid microenvironment of A β plaques, discovering localization of several species of gangliosides and ceramides (specifically GM2, GM3, Cer(d18:1/12:0), and Cer(d18:1/14:0)) [87]. These microenvironments correlate with the largest lipidomics changes throughout the hippocampus and the cortex in AD. In a similar way, Sugiura *et al.* studied the accumulation of gangliosides in developing mouse brain [88]. Gangliosides are typically formed with a long-chain sphingoid base (LCB) of 18 or 20 carbons, with C18 gangliosides being found throughout the nervous system, and C20 gangliosides being localized purely within the CNS [89-91]. However, even within the context of the brain, C20 gangliosides accumulate only within the entorhinal-hippocampus projection during development [88]. As the brains aged, C20 gangliosides in the form of GM1 (GM1(d20:1/C18:0)) accumulated in the outer molecular layer of the dentate gyrus where neuron degradation occurred similar to AD progression [92].

Together, these results suggest that ceramides, sphingomyelin, S1P, as well as more complex GSL, are associated with AD development and progression although further research is needed to enhance the knowledge of underlying mechanisms. Animal models of AD might be a useful tool to investigate lipid metabolism in AD. In a lipidomic analysis where human late-onset AD brain tissue was compared to transgenic familial-AD (FAD) mouse-models with mutations in presenilin 1 (PS1) and APP genes, the authors show that, although many lipid alterations were different between the two models, ganglioside (GM3) and cholesterol esters (CE; components of circulating lipoproteins), in particular, showed that disease-related alterations in brain concentration were comparable between the human and animal model [93]. Another study, showed elevated GM3 levels in the human frontal and parietal cortex [94]. Although differences in AD etiology between the models should be considered and may largely explain variation in lipid alterations, these results indicate that FAD mouse models of AD are useful tools for studying aberrations in lipid metabolism in AD and proof to be particularly relevant models in translational research studies.

2.2 Parkinson's disease

PD is the most common progressive neurodegenerative disease after AD [95]. Symptoms include tremor, bradykinesia, rigidity, and loss of postural reflexes together with behavioral disorders and cognitive decline [96]. Symptoms of PD result from cell loss in the substantia nigra and cognitive and behavioral symptoms among PD patients typically result from over-aggregation of Lewy bodies containing α -synuclein inducing dopaminergic neurons

damage [95, 97, 98]. PD, in contrast to AD, is relatively new to scrutiny within lipidomics. The main works have focused on α -synuclein as the principle agent in PD [99], but more recent studies have examined the downstream effects of α -synuclein toxicity. These have found that α -synuclein impacts lipid homeostasis, leading to PD observed neurotoxicity [100].

Nevertheless, it has been indicated that sphingolipids play an important part in PD development [101-103]. Glucocerebrosidase (GBA, the enzyme breaking down GlcCer into glucose and ceramide) has been implicated in sporadic PD and Lewy Body Dementia since mutation in the gene coding for this enzyme is one of the most established genetic risk factor for these diseases [104]. Additionally, ceramide and sphingomyelin show an abnormal metabolism in PD postmortem brain tissue [101] and in the plasma of PD patients with cognitive impairment, various ceramide species (C16:0, C18:0, C22:0, C24:1) were found to be increased [102]. However, in particular, a loss of GM1 gangliosides is seen in the disease [103]. Consequently, animal models in which GM1 levels are reduced develop PD-like motor symptoms. More traditional models in which α -synuclein overexpression is induced (A53T) or dopaminergic neurons are damaged by administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), have shown SK2 and S1P to promote dopaminergic neuronal survival [105]. Given the effect of fingolimod on S1P receptors, this drug could be a potential modulator for PD patients too. Interestingly, in an A53T transgenic mouse model, fingolimod reduced α -synuclein aggregation and increased brain-derived neurotrophic factor (BDNF) levels in aging mice [106]. More recently, in a PD mouse-model using specific GM2+/- transgenic mice to reduce GM1 gangliosides, the additional beneficial effect of fingolimod administration on motor-behavior was shown [107]. The authors argued that, since normal GM1 levels help to solubilize α -synuclein and that the decreased ganglioside levels in PD could be restored by GM1 treatment, a GM1-deficit mouse model with subsequent motor deficits would make a good animal model for PD. In this model, fingolimod improved movement, sensorimotor function, and urinary tract function in aging GM2+/- mice. Although these effects of fingolimod are promising, other animal studies that used MPTP administration to induce PD-like symptoms did not show neuroprotective effects of fingolimod administration, but found that the increase of BDNF by fingolimod was unsustainable over time after multiple injections [108]. Since the known studies using fingolimod in PD have relied heavily on animal research, clinical studies might give valuable insight to its potential beneficial effects in PD patients. Additionally, in a five year open study, long-term administration of GM1 has been proven safe in humans and clinical benefits for PD patients have been observed [109]. In this controlled randomized double blind placebo study, PD patients who had received five years of GM1 treatment, reported lower Unified Parkinson's Disease Rating Scale (UPDRS) movement ability and Activity of Daily Living grades [109]. However, treatment effects currently remain symptomatic and fundamental research on the pathogenesis of PD and the role of sphingolipids is needed.

2.3 Huntington's disease

HD is an inherited progressive neurodegenerative disorder in which disturbances are seen in motor, psychiatric, and cognitive function that ultimately leads to pneumonia, heart damage and lifespan decline [110-112]. Abnormal expansion of a CAG triplet repeat in the Huntingtin (HTT) gene is causative for HD. The HTT gene has been recognized to play various roles in intracellular functions including protein trafficking, regulating transcription and post-synaptic signaling, as well as proteotoxicity and protein aggregation [113-115]. Although not as abundant as

in AD and PD, over the recent years, various lines of research have been focusing on the sphingolipid metabolism in HD, in which gangliosides and the S1P pathway seem to be primarily involved in the disease [116-123].

In 2017, Di Pardo, et al. showed a significantly altered expression of S1P-metabolizing enzymes in HD. More specifically, S1P Lyase1 (SGPL1) was found to be upregulated and sphingosine kinase 1 (SK1) downregulated in post-mortem striatum and cortex of patients with advanced HD compared to control [118]. Additionally, in a HD transgenic mouse model (R6/2) expressing exon 1 of the human HTT gene carrying 141–157 CAG repeats [124], an overexpression of ceramide species C20:0, C22:0, C24:0 and C24:1 was seen in the striatum, while ceramide C24:0 was found to be upregulated in the cortex of these mice [118]. Conversely, the expression of S1P was decreased. Interestingly, the authors were additionally able to show that ceramide and SGPL1 levels were already increased in an early stage of the disease (6 weeks old mice), while no differences in SK1 and 2 expression was seen. These results suggest that S1P metabolism is affected in HD. Consequently, stimulating S1PRs via fingolimod has been studied as a therapeutic strategy for HD [125]. In 2014, Di Pardo, et al. showed that fingolimod improved motor function and coordination in the open-field test and horizontal ladder task, and activated pro-survival pathways AKT and ERK in R6/2 mice striatum and seemed to reduced brain atrophy [119]. Furthermore, in the same study, fingolimod was able to reduce mutant huntingtin aggregates. More recently, indirect promotion of S1P production by stimulation of SK1 through the hydrophobic “pseudo-ceramide” compound K6PC-5 [126], showed similar favorable effect in R6/2 mice [121]. Importantly, while GM1 levels were decreased in R6/2 mice striatal areas, fingolimod administration restored GM1 expression to non-pathogenic levels [119].

Previous research has also indicated an impaired ganglioside metabolism in HD [116, 123]. A reduction of ganglioside GM1 was found in fibroblasts from patients with HD as well as in HD animal models [123]. In turn, this GM1 reduction leads to HD cells that are more prone to apoptosis. Additionally, in a yeast artificial chromosome (YAC) transgenic HD mouse-model, where the whole human mutant huntingtin protein with 128 CAG repeats is expressed (YAC128) [127], administration of GM1 resulted in lower levels of the mutant huntingtin protein [116]. Furthermore, various other beneficial effects, such as improved dopamine and serotonin metabolism modulation and glutamate, GABA, L-Ser and D-Ser level restore were also observed [116]. Moreover, the same study showed that GM1 treatment in R6/2 mice decelerated neurodegeneration, white matter atrophy and loss of body weight. While no true therapeutic measures are yet available for HD, recent studies have shown that the subventricular zone in the human brain is a region of interest, due to the wholesale loss of lipid architecture within the 4 laminae in HD [128].

	Alzheimer's disease	Parkinson's disease	Huntington's disease
Sphingolipids			
Ceramides	Upregulated (X. Han, et al., 2002; Cutler, et al., 2004)	Downregulated (Abbott, et al., 2014)	Upregulated (Di Pardo, et al., 2017)
Sphingomyelin	Downregulated (He, et al., 2010)	Downregulated (Abbott, et al., 2014)	Not available
Sphingosine	Upregulated (He, et al., 2010)	Not available	Not available
Sphingosine-1-phosphate (S1P)	Downregulated (He, et al., 2010)	Downregulated (Sivasubramanian, et al., 2015)	Downregulated (Di Pardo, et al., 2017)
Ganglioside GM1	Upregulated (Kracun, et al., 1991)	Downregulated (Wu, et al., 2012; Schneider, et al., 2010)	Downregulated (Alpaugh, et al., 2017; Desplats, et al., 2007)
Ganglioside GM3	Upregulated (Kracun, et al., 1991)	Not available	Not available
Enzymes			
Sphingosine kinase-1 (SK1)	Downregulated (Ceccom, et al., 2014)	Downregulated (Motyl, et al., 2018)	Downregulated (Di Pardo, et al., 2017)
Sphingosine kinase-2 (SK2)	Downregulated (Dominguez, et al., 2018)	Downregulated (Sivasubramanian, et al., 2015)	Unchanged (Di Pardo, et al., 2017)
(Acid) Sphingomyelinase (ASM)	Upregulated (He, et al., 2010)	Downregulated (Ysselstein, et al., 2019)	Not available
(Acid) Ceramidase (AC)	Upregulated (He, et al., 2010)	Not available	Not available
Ceramide synthase (CerS)	CerS1 Upregulated, CerS2 and CerS6 Downregulated (Le Stunff et al., 2019; Coutias et al., 2016)	Upregulated (Abbott, et al., 2013)	CerS1 Downregulated (Di Pardo, et al., 2017)

Table 1. Brain sphingolipid level changes in neurodegenerative diseases. Upregulation or downregulation of brain sphingolipids and associated enzymes in Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) [47, 70, 78, 80, 94, 101, 103, 105, 109, 116-118, 120, 129-133].

2.4 Biomarkers for neurodegenerative diseases

Taken together, these results show that sphingolipid metabolism has an important function in neurodegenerative diseases, although the exact role of sphingolipids in these disorders remains elusive. Results from previous studies indicate that GM1 treatment and modulation of the S1P pathway may be interesting targets to pursue in future clinical trials in neurodegenerative diseases. However, concerns about the poor blood-brain barrier (BBB) permeability of GM1 [88] and the limited understanding of the therapeutic effects of S1P-pathway modifying drugs in animals models should be considered and further pre-clinical research is necessary before modulating the sphingolipid metabolism in future clinical trials. Additionally, although gross disruptions in sphingolipid metabolism are seen in these neurological disorders, recent studies propose that a number of neurodegenerative diseases are contributed by more subtle changes in sphingolipid balance. Indeed, plasma ceramides levels have been shown to be altered in MCI and AD dementia [72, 74, 134-137] and plasma ceramide, GlcCer, and ganglioside levels were altered in PD or Lewy Body dementia patients [102, 138, 139]. Thus, the utility and context of use of plasma sphingolipids for clinical purposes including diagnosis, prognosis, and therapeutic aspects is warranted. Additionally, sphingolipids are being investigated for their potential as surrogate markers of treatment response or to predict who is most likely to respond. For example, a Phase 2 clinical trial of ibiglustat for PD GBA mutation carriers is ongoing. Ibiglustat is proved to be a small-molecule inhibitor of GlcCer synthesis. If proven successful, the measurement of GlcCer could be clinically useful in the future for determining adequate response to therapy and whether the dose needs to be increased.

3. Multiple sclerosis

MS is an inflammatory disease, largely influenced by autoimmunity that is characterized by demyelination of the CNS [140, 141]. It is estimated to affect one in 1000 North American and European inhabitants, with a markedly greater prevalence in women [142]. Generally, three stages can be defined in the disease. First, in the preclinical stage, the disease is triggered by various genetic and environmental factors yet symptoms remain below the clinical threshold. In the second, relapsing-remitting (RRMS) stage, clinical manifestations start to emerge, characterized by episodic neurologic dysfunction and remissions. Disabilities and deterioration greatly increase in the third, progressive clinical stage, in which sensory functions and gait are particularly affected [140]. Since disease-modifying drugs that target the progressive form of the disease remain largely ineffective, additional research on its fundamental pathogenic mechanism is needed [140, 143].

Several lines of research have suggested a role for sphingolipids in MS [24, 143-147]. Particularly, the diverse role of ceramides, sphingosine, and S1P are increasingly researched in relation to the process of myelination [24, 144, 145]. While different ceramide species are primarily involved in neurodegenerative diseases, such as AD, sphingosine in particular seems to be associated with MS. Specifically, sphingosine accumulations in the MS brain has been reported [24]. It is suggested that these sphingolipids influence the course of myelination during early brain development. However, in the diseased state of MS, ceramide and sphingosine are involved in CNS demyelinating processes [24]. Conversely, S1P receptors may be important in decreasing demyelination and, at the same time, improving the course of re-myelination in MS [145]. More specifically, it has recently been shown that inactivation of the S1P receptor 2 (S1PR2) reduced demyelination and lowered clinical disability scores after experimental autoimmune encephalitis (EAE) was induced in mice. In general, a hyperactivation of the sphingomyelin-ceramide-sphingosine-S1P pathway has been suggested to be an underlying mechanism resulting in an increased total phospholipid and decreased sphingolipid levels in normal appearing white- and grey matter in MS [147].

One of the key hallmarks of MS is a progressive loss of oligodendrocytes and, consequently, the degradation of neuronal myelin sheets [148]. Since sphingolipids are particularly abundant in myelin sheets, current research efforts focus on investigating the importance of sphingolipids in demyelination. The hyperactive sphingomyelin-ceramide-sphingosine-S1P pathway seen in MS may lead to dysregulation of the delicate sphingolipid balance in CNS white – and grey matter [147]. Following this line of investigation, recent research suggests a sphingosine-induced toxicity in oligodendrocytes [24, 144]. As a case in point, in an experiment to determine cellular toxicity of sphingosine, a significant increase in human oligodendroglioma (HOG) cell death was observed after a high dose of sphingosine was administered to these cells. Interestingly, however, treatment with a low concentration of sphingosine seemed to induce a mild cell growth in HOG cells [24]. Research on the mechanism underlying sphingosine toxicity in MS showed an activation of the de novo sphingolipid biosynthesis. Additionally, inhibiting this pathway by myriocin seemed to block ceramide elevation and protected against oligodendrocyte apoptosis [144].

3.1 *Sphingosine-1-phosphate receptors in multiple sclerosis*

SK1 and SK2 phosphorylate sphingosine to form S1P. Besides the ability to influence cellular properties such as cell-survival, S1P is particularly implicated in regulating the immune system by the ability to bind to S1PR1-

5 [149]. Taking into account the role of inflammation in MS development, S1PRs have been interesting targets of pharmacological potential in MS [150]. Fingolimod is a drug that modulates S1PR1, 3-5 and is able to cross the BBB [143, 151]. Consequently, it was the first FDA approved oral drug for the relapsing form of MS, entering the market in 2010 [83]. There is evidence that fingolimod works by targeting astrocytes that give rise to increased levels of acid sphingomyelinase (ASM) in MS brain lesions where it has an anti-inflammatory effect [146, 152]. Since ASM converts sphingomyelin into ceramide, this leads to an upregulation of ceramide production in the MS brain. It was shown that primary astrocytes that were exposed to tumor necrosis factor- α (TNF- α), a pro-inflammatory cytokine, enhanced ceramide production by increasing the expression of ASM [146]. Interestingly, subsequent treatment of fingolimod on these primary astrocytes reduced this effect. Additionally, fingolimod inhibited SK1 [153] and ceramide production via CerS inhibition [154, 155] (Fig. 2). Furthermore, recent evidence from an EAE secondary progressive MS mouse model suggests that fingolimod decreases neurodegeneration and neurotoxicity by modulating astrocyte activation via S1PRs [143]. Specifically, fingolimod binds to the S1PR1, causing the receptor to internalize and thereby lowering the response of lymphocytes to emigrate from lymph nodes [146].

Fingolimod has been established as a safe and effective drug with great benefits for a broad range of patients with MS. However, its side effects due to nonspecific targeting of S1PRs have to be considered. The FDA recently contraindicated fingolimod for patients with preexisting heart conditions or previous stroke episodes [151, 156]. Although various studies have investigated the role of S1PR1 in MS, S1PR2-5 have received considerably less attention in MS research. However, some recent studies discuss these receptor isoforms and their pharmacological potential in more detail [145, 157-162]. In 2018, Seyedsadr et al. described the role of the S1PR2 [145]. In this study, the authors show that activation of the S1PR2 has a demyelinating effect in MS and, additionally, impairs myelin repair. Moreover, knock-out of the S1PR2 gene, as well as administration of the pharmacological S1PR2 inhibitor JTE-013, reverses these damaging effects. Furthermore, in another study, S1PR2 has been shown to have sex- and strain-specific effects on BBB permeability regulation [158]. Studies that describe S1PR3 and S1PR4 in relation to MS remain limited. However, it has been suggested that the action of fingolimod to protect astrocytes against neuroinflammation depends in part on S1PR3 activity [159]. S1PR4, together with S1PR1 and S1PR2, are believed to be involved in lymphatic CD4 T cell migration [162] and selective S1PR4 agonists and inhibitors with no activity against the other S1PRs are currently emerging [160]. S1PR5 is particularly expressed in the CNS and is involved in early inflammatory processes [163, 164]. A more promising novel S1PR-modulator for MS treatment might be BAF312 (siponimod) which selectively targets S1PR1 and S1PR5 [157]. Importantly, siponimod, in contrast to fingolimod, does not require to be phosphorylated to be effective and exhibits no activity against S1PR3 and S1PR4 [165]. After a successful phase 3 trial, termed the “EXPAND” study, where siponimod was tested against placebo in secondary progressive MS [161], FDA approval was given in 2019, making it the first oral drug treatment for secondary progressive MS with active disease [166].

Figure 2. The effects of FTY720 (fingolimod) on a hyperactive sphingomyelin-ceramide-sphingosine-S1P pathway in MS. The thick arrows indicate the hyperactive part of the pathway in multiple sclerosis (MS) [146, 147]. Abbreviations used are: S1P, sphingosine-1-phosphate; S1PR, S1P receptor; SMS, sphingomyelin synthase; ASM, acid sphingomyelinase; NSM, neutral sphingomyelinase; CDase, ceramidase; CerS, ceramide synthases; SPP, S1P phosphatases; SK, sphingosine kinases (SK1 and SK2).

3.2 Biomarkers for multiple sclerosis

Although various lines of research give further insight into the mechanism driving MS, early diagnosis and subsequent early treatment remains difficult and methods to identify and observe immunological and neurodegenerative processes in MS pathology are lacking [167, 168]. With recent advances in lipidomics research methods, the interest in lipids as biomarkers for MS is growing. Interestingly, although sphingosine and S1P are believed to be crucial molecules in the pathogenesis of MS, few reports have indicated these sphingolipids as potential targets for MS biomarkers.

In 2011, a LC mass spectrometry lipidomic analysis was performed on MS patient serum and compared to healthy controls and patients with other neurological diseases (OND) [169]. Although sphingolipids like galactocerebroside (GalCer, a neutral glycosphingolipid) and sphingomyelin were also measured, the metabolism of phospholipids (the main component of cell membranes), in particular, was altered in the serum of MS patients. Specifically, an altered ratio of glycerophosphatidylcholine (PC) and lyso-PC levels was found [169]. An evaluation of S1P concentrations in the blood and CSF of MS patients revealed that S1P levels in the blood did not differ from the control group, but CSF levels were significantly higher in MS patients [170]. Further analyses demonstrated the toxic effect of CSF from MS patients to neurons [171]. Rat neurons that were exposed to CSF from MS patients showed oxidative stress and a decreased expression of neuroprotective genes. Additionally, levels of ceramide species C16:0 and C24:0 were found to be specifically increased in the CSF of MS patients, leading to the observed toxicity in neurons. More recently, several sphingomyelin species were found to be reduced in MS CSF compared to OND [172]. Importantly, to understand the mechanism underlying this decrease, ASM expression and activity in CSF was further investigated by an enzymatic activity assay to assess its role in the sphingomyelin pathway in MS. This revealed that low levels of sphingomyelin in the CSF results from overexpression of ASM carried by specific exosomes [172]. Further lipidomics and subsequent receiver operating characteristic (ROC) analyses where MS patients were compared to a subgroup of inflammatory neurological diseases, showed that levels of sphingomyelin and overexpression of ASM in the CSF might be considered as interesting biomarkers for MS, independent of other inflammatory and neurological conditions.

Besides S1P, ceramides, and ASM, other lipid species have also been the target of investigation of potential biomarkers for MS. It has been shown that levels of various sulfatide (sulfated GalCer) species are significantly altered in MS. Specifically, total sulfatide levels were found to be reduced by 60% in human MS plaque tissue compared to control tissue [173] and, in the plasma of RRMS patients, sulfatide C18:0 and C24:1 levels positively correlates with a higher disease disability [174]. Recently, in a non-targeted lipidomic approach investigating 9532 lipid species in the CSF of MS and non-MS patients, 47 species, of which three sphingolipids, were identified based on their mass and were significantly altered in MS (glucosylceramide, GlcCer(D42:0) upregulated; C20 sulfatide and ceramide-phosphate (42:2) downregulated) [175]. Although these results contribute to the understanding and potential development of sphingolipid-related biomarkers in MS, CSF collection remains relatively invasive and studies investigating alternative biomarkers that are more easily accessible are ongoing. Interestingly, a recent report discussing the lipidomic and metabolomic analysis of tears as a possible biomarker in MS showed several lipid species to be altered in the lacrimal fluid in MS patients, including multiple sphingomyelin species [167]. Since five of the thirty potential tear biomarkers corresponded to the ones found in CSF, the authors argue that lacrimal fluid could be considered as an intermediate between serum and CSF.

Taken together, the role of sphingolipids in MS is evident and the SM-ceramide-sphingosine-S1P pathway is an interesting target for disease modifying treatment options. However, effective therapy remains limited and a need for reliable biomarkers for MS is evident. Although it has been shown that ASM expression is altered in MS, recent advances investigating ASM as a potential blood-based biomarker revealed no correlation with disease activity, progression or response to treatment [141]. Nevertheless, specific ceramide- and sphingomyelin species require

additional investigation. S1PR modulation shows promising potential but further research investigating S1P receptor specificity in MS remains necessary.

4. Sphingolipids in Psychiatric diseases

The brain is a complex organ organized in tightly regulated circuits and the study of neurodegenerative diseases highlighted the concept that selective brain regions are more vulnerable to pathological processes, such as the atrophy of the medial temporal cortex in AD or substantia nigra in PD [176]. Even though neuropsychiatric disorders do not show such a clear anatomic correlate observed by regional atrophy in humans, our current understanding of these disorders shows that the dysfunction of brain specific regional circuits is at the basis of the characteristic behavioral alterations observed in each disease [177]. Therefore, one hypothesis is that the specific molecular composition of each brain region, namely the lipidomic composition, could be a key contributing factor for either vulnerability or resilience to pathological processes in neuropsychiatric disorders (Fig. 3) [178]. An interesting observation is that patients with mutations in GSL metabolizing enzymes, have been indicated to have characteristic clinical psychiatric presentations, as previously reviewed [179]. However, direct extrapolations from patients with enzymatic deficiencies should be done cautiously. Firstly, these mutations usually affect multiple cell types beyond the CNS, and secondly, they impair in many circumstances the endolysosomal flux, which, as a general disease mechanism, is shown to contribute to neurodegeneration [180]. Therefore, studies addressing the role of sphingolipid metabolizing enzymes beyond its role in neurodegeneration should be carefully performed in order to give insight concerning the complex regulation of regional synaptic functioning, brain circuits and behaviors.

The etiology of neuropsychiatric disorders is multifactorial, with combined genetic and environmental factors, such as the exposure to chronic stress, a known risk factor anxiety and depression development [181]. The use of rodent models that are either exposed to chronic unpredictable stress (CUS) or exogenous administration of corticosterone (one of the biological surrogates of stress exposure), not only show anxious-like behavior, but also present deficits in learning and memory which co-exist with dendritic atrophy in prefrontal cortex (PFC) and the hippocampus [181]. Importantly, it was shown that chronic stress exposure altered the brain lipidomic composition [178, 181]. At the regional level, the PFC was the area showing a higher degree of lipid disturbance and also the hippocampus, to lesser degree [181], in accordance to previous studies that highlighted the susceptibility of these regions to chronic stress induced effects [181]. Interestingly, chronic stress was shown to induce ceramide increase and a sphingomyelin decrease in both the PFC and hippocampus, which suggests that sphingomyelinase activity could be increased in stress conditions [181]. Another lipidomic study evaluating the effects of chronic antidepressant drug treatment using maprotiline and paroxetine, showed a decrease in sphingomyelin and various ceramide species increases selectively in the PFC, while no significant effects were observed with fluoxetine [182]. Moreover, it was shown that both CUS and corticosterone exposure leads to increased hippocampal ceramide levels and that ASM, that synthesizes ceramide from sphingomyelin, is a target for the antidepressant drugs, amitriptyline and fluoxetine [22], further supporting a role for ceramide as a pathological driver of stress effects. Since the PFC presented relative abundant sphingomyelin levels [181], regional brain lipidome could also partially explain the elevated susceptibility to higher ceramide levels upon chronic stress exposure.

Concerning the hippocampus, recent studies showed that it is differentially regulated by dorsal to ventral sub-regions along its longitudinal axis in the rodent, or from posterior to anterior poles in humans [183]. It is generally understood that the dorsal hippocampus, in particular, is implicated in cognitive functions like spatial memory and that emotionally mediated responses are regulated by the ventral hippocampus [183]. Moreover, the dorsal and ventral hippocampus have different intrinsic electrophysiological properties in response to chronic stress [184, 185]. At the level of lipidomic analysis, the rodent dorsal hippocampus shows an enrichment in complex GSLs and lower levels of simpler sphingolipids, such as sphingomyelin and dihydrosphingomyelin (dhSM), comparatively to the ventral hippocampus [186]. Additionally, the ventral hippocampus was more sensitive to chronic corticosterone administration at the lipidomic level, showing not only increased levels of cholesteryl esters, triacylglycerol and phosphatidylserine, but also dhSM [186]. Another study observed a relative increase in ceramide dorsal to ventral hippocampal levels in a relearning extinction paradigm [187], which overall, further supports that the subregional hippocampal lipid composition can be a key contributor to stress susceptibility, potentially affecting circuits predominantly involved in either emotional output or learning and memory [183].

While many advances have been made in the understanding of depression and anxiety through the use of chronic stress animal models, other psychiatric diseases such as schizophrenia, bipolar disorder and addiction present a bigger challenge. A study on schizophrenic patients identified a gene expression profile in the PFC with increased levels of genes involved in sphingolipid metabolism [188]. Studies on brain lipid composition showed ceramide species levels increased in PFC white matter post-mortem samples of both schizophrenic and bipolar patients [189] and sphingomyelin and GalCers reduced levels in the thalamus of schizophrenic patients [190]. On the hand, other studies on schizophrenic patients showed increased GalCer levels in frontal cortex [191] and increased sphingomyelin levels in the caudate [192]. Finally, exposure to the bipolar disorder drug valproate leads to increased ceramide in a yeast model [193] and chronic treatment with antipsychotic drug haloperidol altered the normal metabolome affecting significantly sphingolipid related pathways in the mouse brain [194]. Although it is not clear if a single lipid modulating pathway is at the root of the pathophysiology of these psychiatric disorders, overall these observations support a dysregulation of sphingolipid metabolism.

Concerning addiction, while the study of the brain circuits relevant for addictive-like behaviors is fundamental for its understanding and devising new therapies, there are a number of studies that tackle the direct impact of addictive substances exposure on sphingolipid brain metabolism. For instance, chronic ethanol consumption was shown to upregulate ceramide levels and decrease sphingomyelin levels in adult mouse brain [195] and while bingeing ethanol consumption decreased ceramide levels, ethanol withdrawal increased ceramide levels in the mouse cortex [196]; methamphetamine-sensitized mice had higher degree changes in the hippocampus and PFC lipidome, showing in both, decreased LacCer levels [197]; cocaine conditioned mice have widespread lipidomic alterations in the nucleus accumbens, striatum, PFC and hippocampus, notably showing decreased ceramide levels [198]; and using mass spectrometry imaging, administration of morphine, cocaine and amphetamine in rodents was shown to lead to increased brain sulfatide levels [198].

Upon the identification of altered lipid signaling pathways in psychiatric brain samples and rodent models in lipidomic studies, the use of genetic models that modulate enzymes involved in sphingolipid metabolism highlight not only the susceptibility to altered behaviors, but also validate potential drug targets. As an example of this rationale, since increased ceramide levels was one of the most common metabolic hits, modulation of the enzymes that either synthesize or metabolize ceramide could be tested concerning the impact on relevant behavior or resistance to environmental factors that predispose to neuropsychiatric disorders. In line with that, while ASM overexpression led to depressive-like behavior [22] and increased alcohol consumption [199], ASM heterozygote mice were resistant to chronic stress effects [22] and alcohol consumption associated behaviors [199], which indicates the potential of ASM as a therapeutic target. However, the complex consequences of ceramide modulation are highlighted by various studies that show deleterious effects by both blocking ceramide degradation or synthesis through either ceramidase or CerS, respectively. This is observed upon acid ceramidase deficiency, which leads to compulsive and anxiety-like behaviors, among multiple other behavioral alterations [200]; CerS1 knock-out (KO) mice have decreased levels of both ceramide C18 and gangliosides in general, alterations in neurodevelopment and in various behaviors such as impaired locomotion and impaired spatial working memory [201]; CerS6 KO mice, which present decreased ceramide C16 brain levels, have impaired open field exploration and habituation [202]; or carnitine palmitoyltransferase 1C KO mice that have reduced ceramide levels, increased immature spines in the hippocampus and deficits in spatial learning [203]. Therefore, lipid modulation should be targeted taking into account the enzymatic expression on subcellular organelles, specific cells, brain regions and the impact on specific lipid species and other lipids classes, which could all differentially affect behavioral output.

Interestingly, fingolimod has been also shown to decrease anxiety [204] and improve depressive symptoms in MS treated patients [205]. Studies in mice further supported a protective role for fingolimod upon chronic stress exposure [206] in a neurogenesis dependent way [207], as a facilitator of fear memory extinction [208] and in reversing the anxiety-like behavior induced by the regional expression of α -importin 5 in the ventral hippocampus [209]. These suggest that the effects of fingolimod could go beyond its reported mechanism of action of decreasing lymphocyte recruitment to the CNS and further support for a potential role for sphingolipid modulating drugs in neuropsychiatric disorders.

As with neurodegenerative diseases, sphingolipids could be used in neuropsychiatric disorders as surrogate markers of treatment response. An example is the interaction between sphingomyelinases, anti-depressants, and the clinical course of depression. ASM has been shown to be elevated among a small number of individuals experiencing a major depressive episode [210]. Tricyclic anti-depressants such as amitriptyline and fluoxetine, a selective serotonin reuptake inhibitor, are known to reduce ASM activity and ceramide concentrations and to improve behavior in models of depression induced by stress [22]. Thus, a future possibility would be to measure ASM activity and ceramide levels in the blood in an effort to titrate anti-depressant dose. Although a recent study did not find serum ASM activity was useful for predicting the course of clinical of depression over a period of three weeks [211], additional studies with longer follow-up and measuring levels of other sphingolipids are needed.

Figure 3. Brain regional sphingolipid metabolism impairment in psychiatric disorders. Summary from various studies of alterations observed in sphingolipid metabolism at the brain regional level in human post-mortem psychiatric patients or rodent models of psychiatric diseases such as exposure to chronic stress or to substances of abuse associated with addiction. SM, sphingomyelin; Cer, ceramide; GalCer, galactosylceramide; LacCer, lactosylceramide.

5. Clinical utility of sphingolipids for the diagnosis, prognosis, and treatment of neurodegenerative diseases and neuropsychiatric disorders

As described above, there are several lines of evidence highlighting a potential role of sphingolipid pathways in the etiopathogenesis of both neurodegenerative diseases and neuropsychiatric disorders. Indeed, for normal neuronal function, the right balance of sphingolipids in brain has to be maintained [102], as demonstrated by several severe neurological disorders resulting from enzyme deficiencies in the sphingolipid pathway. Extreme changes in specific sphingolipids, for example GlcCer for Gaucher's disease, are caused by severe pathologic mutations in the sphingolipid pathway. In these situations, it is possible that plasma sphingolipid levels could be useful to aid in diagnosis, in addition to enzyme levels. However, it is unlikely that plasma sphingolipids will be useful for diagnosing sporadic neurodegenerative diseases such as AD dementia, PD, or Lewy Body Dementia. Studies comparing plasma sphingolipid levels among patients with these diseases to cognitively normal individuals or those in prodromal stages of disease (e.g., MCI) show significant overlap between clinical diagnoses, even if the difference in sphingolipids by group are statistically significant [75, 102, 137, 139]. As a result, sphingolipids cannot be currently used as diagnostic

biomarkers for these diseases because they have low diagnostic accuracy and poor sensitivity and/or specificity. Instead, it is more likely that plasma sphingolipids could be used as prognostic markers. Although there are few longitudinal studies, current results consistently suggest that high levels of elevated ceramides indicate a greater rate of cognitive decline among cognitively unimpaired individuals, patients with MCI, dementia, or those undergoing cardiac rehabilitation [72, 134-136, 212]. Thus, measuring plasma ceramides could be clinically useful for identifying individuals who will have the fastest cognitive decline, especially for identifying fast progressors for clinical trials.

Lastly, sphingolipids could be used in neurodegenerative diseases and neuropsychiatric disorders as surrogate markers of treatment response. Sphingolipid metabolism and associated enzymes play significant roles in neuronal diseases and specific alterations of the sphingomyelin/ceramide/S1P pathway, together with an impaired ganglioside metabolism, are particularly seen. Advanced lipidomic tools to investigate sphingolipid metabolism in neurodegenerative and psychiatric diseases are emerging. This may aid the understanding of sphingolipid metabolism and potential development of prognostic biomarkers or surrogate markers of treatment response in these neuronal diseases.

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Chapter 3

CERT_L reduces C16 ceramide, amyloid- β levels and inflammation in a model of Alzheimer's disease

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Abstract

Background: Dysregulation of ceramide and sphingomyelin levels have been suggested to contribute to the pathogenesis of Alzheimer's disease (AD). Ceramide transfer proteins (CERTs) are ceramide carriers, crucial for ceramide and sphingomyelin balance in cells. Extracellular forms of CERTs co-localize with amyloid- β (A β) plaques in AD brains. To date, the significance of these observations for the pathophysiology of AD remains uncertain.

Methods: The plasmid expressing CERT_L, the long isoform of CERTs, was used to study the interaction of CERT_L with amyloid precursor protein (APP) by co-immunoprecipitation and immunofluorescence in HEK cells. The recombinant CERT_L protein was employed to study interaction of CERT_L with amyloid- β (A β), A β aggregation process in presence of CERT_L, and the resulting changes in A β toxicity in neuroblastoma cells. CERT_L was overexpressed in neurons by adeno associated virus (AAV) in a familial mouse model of familial AD (5xFAD). Ten weeks after transduction animal were challenged with behavior tests for memory, anxiety and locomotion. At week twelve brains were investigated for sphingolipid levels by mass spectrometry, plaques and neuroinflammation by immunohistochemistry, gene expression and/or immunoassay.

Results: Here, we report that CERT_L, binds to APP, modifies A β aggregation and reduces A β neurotoxicity *in vitro*. Furthermore, we show that intracortical injection of AAV, mediating the expression of CERT_L, decreases levels of ceramide d18:1/16:0 and increases sphingomyelin levels in the brain of male transgenic mice, modelling familial AD (5xFAD). CERT_L *in vivo* over-expression has a mild effect on animal locomotion and decreases A β formation and modulates microglia by decreasing their pro-inflammatory phenotype.

Conclusion: Our results demonstrate a crucial role of CERT_L in regulating ceramide levels in the brain, in amyloid plaque formation and neuroinflammation, thereby opening research avenues for therapeutic targets of AD and other neurodegenerative diseases.

Keywords: ceramide, sphingomyelin, ceramide transporter protein (CERT), adeno-associated virus (AAV), Alzheimer's disease (AD), 5xFAD, amyloid- β plaques, neuroinflammation, microglia

Background

Key pathological features of Alzheimer's disease (AD) are aggregates of amyloid- β peptides (A β) and neurofibrillary tangles (NFTs), and neurodegeneration, together with blood-brain barrier (BBB) dysfunction, neuroinflammation and lipid disbalance. To date, the molecular mechanism underlying neurodegeneration in AD remains unclear. Elucidation of the deregulated biological mechanisms that lead to the onset and progression of AD is critical to identify new treatment strategies [1-5].

Sphingolipids (SLs) are waxy lipids formed by a sphingosine backbone, important for the cell membrane architecture and for the function of transmembrane proteins. Furthermore, SLs such as ceramides (Cer) and sphingosine-1-phosphate (S1P) are potent second messengers that regulate various important cellular processes, including cell growth and apoptosis [6-9]. Cer are formed by two metabolic pathways: *de novo* synthesis initiated with the precursor palmitoyl-CoA or catabolism of complex SLs such as sphingomyelin (SM) [10-12]. In the cell membrane, SLs are typically organized in microdomains, called lipid rafts, characterized by specific SL species composition.

Several studies have analyzed lipid composition in AD brain tissue, reporting an increase of Cer species [13-16]. Lipid rafts enriched in Cer, isolated from postmortem frontal cortex tissue of AD postmortem tissue, patients showed a reduction of SM levels compared to those isolated from the control brains [17]. Moreover, SM levels in brain regions, particularly vulnerable to A β plaque formation were reduced in AD brains [19, 20]. Strong evidence links A β pathology to SL homeostasis. The enzymes β -secretase and γ -secretase, which cleave the amyloid precursor protein (APP) to generate A β , are stabilized and have an increased half-life in Cer enriched conditions, thus increasing A β biogenesis [18, 19]. In turn, A β can stimulate Cer production by directly activating the phosphodiesterase enzyme sphingomyelinase which converts SM to Cer [20, 21].

Ceramide transfer proteins (CERTs) contain a steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain that confers the ability to transport Cer intracellularly between the endoplasmic reticulum (ER) and the Golgi [22]. CERTs are found in at least two isoforms, which differ for the presence of a 26-amino acid serine-rich domain [23]. CERTs are expressed in the central nervous system, and they are crucial in embryogenesis and brain development [23-25]. When CERTs' activity is blocked pharmacologically or genetically, by compromising the START domain of the protein, SM production decreases significantly [26, 27]. CERT_L can be secreted extracellularly and was found to partially co-localize with serum amyloid P component (SAP) and with amyloid plaques in AD brain [28, 29]. Besides, CERTs are also potent activators of the classical complement pathway that plays an active role in AD pathogenesis [30]. To date, the significance of these observations for the pathophysiology of AD remains uncertain.

In the current study, we investigated the interaction of CERT_L with APP and A β *in vitro*. Next, we explored the effect of CERT_L overexpression on SL composition, amyloid formation, and inflammation *in vivo* using adeno associated virus (AAV)-mediated gene delivery in the 5xFAD mouse model [31]. Our findings showed that an increase of CERT_L modulated SL levels by reducing specific Cer and elevating SM. Notably, CERT_L also affected amyloid plaque formation and brain inflammation, typical pathological hallmarks of AD, supporting the idea that enzymes and transporters of the SL pathways are at the core of the pathophysiological changes observed in AD.

Material and Methods

CERT_L interaction with APP/A β

Immunoprecipitation (IP)

Wild type HEK293 and transgenic HEK293 cells that stably overexpress human APP695 isoform (NP_958817.1) [32], were cultured in DMEM supplemented with fetal bovine serum (FBS), Pen/Strep, and L-glutamine. Stable transfected HEK-APP were maintained in G418 selective medium. Prior to the experiment, cells were seeded in 25 cm² flasks and maintained in serum-free DMEM for 24 hours. For the homogenization, cells were washed two times with PBS, collected in lysis buffer (25mM Tris HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100 and protease inhibitors), centrifuged at 20,000x g for 30 minutes and the resultant supernatants collected for the protein Bradford analysis. Protein extracts (100 μ g) from HEK or HEK-APP were used for immunoprecipitation experiments. Pull down of endogenous CERT_L and APP was performed with 1 μ g mAb anti CERT_L (3A1-C1) [28] and anti-A β mAb 6E10 (Covance), respectively by 1-hour incubation at room temperature. mAb anti syntaxin 6335 (clone 3D10, Abcam) was used as an isotype control. Next, anti-mouse secondary antibodies (Eurogentec) were used to pull down the immune complex. Thereafter, samples were centrifuged at 20,000xg for 30 minutes. Pellets were washed three times in 50 μ L PBS and boiled in reducing sample buffer containing mercaptoethanol to solubilize immunocomplexes. Then, the proteins were separated on a Tris-HCl 4-15% gradient gel (Bio-Rad), and blotted on nitrocellulose membrane (Millipore). Next, the membranes were probed with anti-A β /APP(6E10) or rabbit pAb anti-CERTs (epitope 1-50 of human CERTs, Bethyl Laboratories) antibodies. After 3 washes, membranes were incubated with donkey anti-mouse IRdye 680 and goat anti-rabbit IRdye 800 (Rockland Immunochemicals) and scanned using the Odyssey infrared imaging system.

Immunofluorescent staining

Primary neurons were cultured from 5xFAD neonates P0 as previously described with modifications [33]. After cortical area was dissected, the tissue was digested in 0.25% Trypsin in HBSS (Corning) for 15 minutes. Trypsin activity was stopped with plating medium, DMEM (Gibco, Invitrogen) containing 10% FBS and N2 supplement. Then, the digested tissue was passed through a cell strainer, spun down, and cells resuspended in plating medium. Cells were seeded onto poly-D-lysine-coated coverslips and cultured at 37°C in a 5% CO₂ atmosphere. After 4 hours the plating medium was replaced with Neurobasal medium supplemented with B27 supplement, Penicillin/Streptomycin, and 0.5 mM L-glutamine and kept on for 10-14 days. Every other day supplemented Neurobasal was partially replaced. Neurons were fixed with 4 % PFA in PBS (Thermo Scientific) at 4°C for 10 minutes, permeabilized with 0.25% Triton-X in PBS for 5 minutes, washed three times with PBS and incubated with 3 % BSA for 30 minutes. Cells were stained with rabbit polyclonal anti CERTs (epitope 300-350 of human CERTs, Bethyl Laboratories), goat anti MAP-2 (D-19) (Santa Cruz Biotechnology) and with 6E10 anti APP/ A β [28]. The following secondary antibodies conjugated to fluorophores were used for detection: anti mouse Alexa 647, anti-rabbit cy3 and anti-goat Alexa 488. Fluorescence microscopy was performed using Eclipse Ti2-E inverted microscope system (Nikon). Images were processed using Nikon NIS-Elements software equipped with a 3D deconvolution program.

Microscale thermophoresis binding analysis

Microscale thermophoresis (MST) analysis was performed in the Monolith NT.155 instrument (Nanotemper). In brief, 20 nM of NT647 labeled CERT was incubated for 20 minutes at room temperature in the dark with different concentrations of either A β ₁₋₄₂ (rPeptide Athens) (3-100,000 nM) or control 17kDa Lama antibody fragment (H6) (1-35,000 nM) in PBS Tween20 (0,01%). Afterward, 3-5 μ L of the samples were loaded into glass capillaries (Monolith NT Capillaries, Cat#K002), and the thermophoresis analysis was performed (LED40.51%, IR laser 80%). Statistical analysis was performed with Origin8.5 Software.

A β aggregation assay and cell-based toxicity assay

Transmission Electron Microscopy (TEM)

A β ₁₋₄₂, purchased as the lyophilized salt (Bachem), was dissolved in 0.01M NaOH in ultrapure water to give an A β ₁₋₄₂ stock solution of *ca* 0.20 mM, which was used immediately to prepare each of the treatments. The remaining peptide stock solution was frozen at -20°C until required. Under these highly alkaline conditions, the peptide is fully dissolved and exists only as monomers [34]. Treatments containing A β ₁₋₄₂ and / or CERT_L [28], were prepared in 0.20 μ M filtered modified Krebs-Henseleit (KH) medium (118.5 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.4 mM CaCl₂, 11.0 mM glucose), buffered in 100 mM PIPES at pH 7.4, including 0.05 % w/v sodium azide to inhibit microbial growth. Treatments were incubated at 37 °C until their specified time points prior to being prepared onto TEM grids. For replicate treatments, A β ₁₋₄₂ was thawed thoroughly immediately before use and then vortexed briefly. The stock solution was centrifuged at 15,000 rpm for 5 minutes, and 2.0 μ L was then taken ready for concentration determination by absorbance at 280 nm, utilizing a NanoDrop 1000 spectrophotometer (Thermo). A value of 1390 M⁻¹ cm⁻¹ was taken as the extinction coefficient for A β ₁₋₄₂, and the resultant concentration was calculated by the application of the Beer-Lambert law. CERT_L concentrations were determined in the same manner with a value of 107925 M⁻¹ cm⁻¹ taken as the extinction coefficient using the 72 kDa recombinant CERT_L sequence (hCERT_L, 1875bp NP_005704.1).

All samples for TEM were prepared via a modified TEM staining protocol [35]. Pre-coated S162 200 mesh formvar / carbon coated copper grids (Agar Scientific), were inserted into 20.0 μ L of the treatment beaded onto paraffin film for 60 seconds, then wicked, passed through ultra-pure water, re-wicked and placed into 30 μ L 2 % uranyl acetate (in 70 % ethanol), for 30 s. Following staining with uranyl acetate, grids were removed, wicked, passed through ultra-pure water, re-wicked, and placed into 30 μ L 30 % ethanol for 30 s. Grids were finally re-wicked following this step, covered and allowed to dry for up to 24 h, prior to analysis via TEM. Samples for TEM were viewed on a JEOL 1230 transmission electron microscope operated at 100.0 kV (spot size 1), equipped with a Megaview III digital camera from Soft Imaging Systems (SIS). Images were obtained on the iTEM universal TEM imaging platform software.

Aggregation assay

A β ₁₋₄₂ was purchased from Anaspec. The peptide was solubilized in sterilized PBS, 0.1 % trifluoroacetic acid (TFA) at the concentration 2 mM and frozen in aliquots at -80°C. Aliquots were diluted at the final concentration of 20 μ M in a total volume of 400 μ L containing 1 or 2.5 μ M of affinity-purified recombinant CERT_L [28]. Samples were kept under rotarod shacking for 1, 2, 4, 8, 12 and 24 hours at 37°C before adding 5 μ L to 95 μ L of thioflavin T

(ThT) concentrated 20 μ M, dispensed in 96 well optical plate and measuring fluorescent excitation at 450 nm and emission 486 nm in Victor X3 plate reader (Perkin-Elmer). Two A β antibodies against epitope 1-16 (6E10, Biolegend) and 17-24 (4G8, Biolegend) were used to antagonize aggregation at the dosed 0.1 mg/mL.

Toxicity assay

SH-SY5Y cells were seeded on a 96-well plate at a density of 3×10^4 cells per well in 1:1 DMEM: F12 with phenol red, 4 mM glutamine, 200U/ml penicillin, 200U/ml streptomycin, MEM non-essential amino acids (100 $\times$; Gibco) and 10% FBS, and incubated at 37 °C for 24 hours, reaching up to 100% confluency, with 5% CO₂. After 24 hours, the medium was removed and replaced with 100 μ l/well medium without phenol-red, containing 2% FBS and with 10 μ M A β ₁₋₄₂ oligomers, and/or 1 μ M CERT_L, or alone to control wells and incubated for 24 hours. 10 μ l of MTT (4 mg/ml) was added to each well and incubated at 37°C for 3 hours. MTT solution was decanted and the formazan was extracted with 100 μ l of 4:1 DMSO:EtOH. Plates were read at 570 nm, with a reference filter at 690 nm.

Generation of adeno-associates virus

Human collagen type IV alpha 3 binding protein cDNA sequence (hCERT_L, 1875bp NP_005704.1) was cloned into the plasmid AAV-6P-SEW a kind gift of Prof. S. Kugler Department of Neurology, University of Gottingen. The transgene expression was controlled by a human synapsin-1 promoter (hSYN, 480bp), and an internal ribosome entry site (IRES 566bp) enabled the co-expression of EGFP [36]. The plasmid expressing exclusively EGFP was used as a control (pAAV-EGFP). The AAV-CERT_L plasmid was sequenced by GATC Biotech laboratories and both AAVs plasmids were tested *in vitro* in our laboratories. AAVs particles were produced as explained previously [37]. In brief, the transfer plasmids pAAV-EGFP or pAAV-CERT_L were used to produce AAV2 particles. Eight 15-cm petri dishes each containing 1.25×10^7 HEK 293T cells in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (PS; all GIBCO-Invitrogen Corp., New York, NY, USA) were prepared one day before transfection. The medium was refreshed 1 hour prior to transfection to Iscoves modified Eagle medium (IMEM) containing 10% FCS, 1% PS, and 1% Glutamine. Transfer plasmids were co-transfected using polyethylenimine (PEI, MV25000; Polysciences Inc.) in a ratio of 1:3 with the pAAV-EGFP or pAAV-CERT_L resulting in a total amount of 50 μ g of plasmid DNA per plate. The day after transfection, the medium was replaced with fresh IMEM with 10% FCS, 1% PS, and 1% Glutamine. Two days later (3 days post-transfection), cells were harvested in D-phosphate buffered saline (D-PBS, GIBCO) and lysed with 3 freeze-thaw cycles. Genomic DNA was digested by adding 10 μ g/ml DNaseI (Roche Diagnostics GmbH) into the lysate and incubated for 1 hour at 37 °C. The crude lysate was cleared by ultracentrifugation at 4000rpm for 30 minutes. The virus was purified from the crude lysate using the iodixanol gradient method, diluted in D-PBS/5% sucrose and concentrated using an Amicon 100kDa MWCO Ultra-15 device (Millipore). All AAV vectors were stored at -80 °C until use. Titers (genomic copies/ml) were determined by quantitative PCR on viral DNA primers directed against the EGFP portion (Forward: GTCTATATCATGGCCGACAA; Reverse: CTTGAAGTTCACCTTGATGC). The AAV particles produced with pAAV-CERT_L are referred to in this paper as AAV-CERT_L while the particles produced with pAAV-EGFP are named AAV-control.

Animals

In this study, male mice were used in the present study. To investigate transduction efficiency over time we employed 24 C57BL/6 wild type (WT) animals. B6/SJL WT and 5xFAD animals were obtained from the Jackson Laboratory and bred in house using 5xFAD x non-carriers. This breeding strategy may breed out the retinal degeneration allele *Pde6brd1* from the original strain. The Jackson Lab has observed a less robust amyloid phenotype in this strain. The 5xFAD model carries 5 familial AD mutations, three of them in the human *APP* transgene (Swedish, Florida, and London), and two in the human presenilin-1 (*PS1*) transgene (M146L and L286V mutations). These mutations lead to an increase in A β peptide production [31]. Animals were individually housed under a 12 h light/dark cycle in individually ventilated cages. One week before behavioral tests, animals were adjusted to a reversed day-night cycle. Food and water were provided ad libitum throughout the study. All experiments were approved by the Animal Welfare Committee of Maastricht University (project number DEC2013-056 and DEC2015-002) and followed the laws, rules, and guidelines of the Netherlands.

Stereotactic injection

The animals underwent bilateral stereotactic injections. Mice were placed in a stereotactic head frame, and after midline incision of the skin, two holes were drilled in the skull in the appropriate location using bregma and lambda as references. The layer V of the motor-sensory frontal cortex was targeted; this was verified by light microscopy to observe the dye. Coordinates were determined as follows: anterior-posterior [AP] 0.06; mediolateral [ML] $\pm$ 0.15; dorsoventral [DV] -0.1 [38]. The AAVs were injected at the dose of 1.12×10^8 transducing unit (t.u.) in the anesthetized mice at a rate of 0.2 μ l / minutes with a final volume of 1 μ L for each side.

Behavioral procedures

The Open field (OF) task was performed as described elsewhere [39]. Briefly, locomotion activity was assessed in a square divided into 4 equal arenas. At the start of a trial, the animals were placed in the center of each arena. The total distance traveled was measured under low light conditions by a video camera connected to a video tracking system (Ethovision Pro, Noldus).

The Y-maze spontaneous alternation (AYM) test was conducted to assess spatial working memory. Mice were placed randomly in one of the three arms of the Y-maze and were left free to explore the arena for 6 minutes. The number of arm entries and the number of triads were recorded in order to calculate the percentage of alternations to measure working memory.

The Elevated zero-maze (EZM) was used to measure anxiety. It consists of a circular runway which is divided equally into two opposite open and two opposite enclosed arms. The mice were placed into one of the open arms and allowed to explore the maze over a period of 5 minutes. The total and relative duration (in %) and distance traveled in the open and enclosed arms were measured in the dark via an infrared video camera connected to a video tracking system (Ethovision Pro, Noldus). Percentage (%) of time spent in the open arms was corrected for latency to first closed arm entry.

The Y-maze spatial memory test (SYM) was performed using the same arena as described in AYM above. One arm of the arena was closed by a removable blockade placed in front of it. The mice were placed in one of the open arms, which was randomized over the groups and allowed to explore the 2 open arms of the maze for 5 minutes (pre-test). Afterward, the animal was taken from the arena and put back into its home cage. Five hours later, the mouse was placed back into its corresponding start arm of the arena, now with all three arms accessible (post-test). The previously blocked arm was termed the “novel arm”. Memory was evaluated by calculating the amount of time spent in the novel arm corrected for the latency to move from the start arm to another arm and the amount of time the animal spent in the center of the maze [40].

Immunofluorescence staining

Mice were sacrificed by intracardial perfusion using Tyrode's solution for the first minute, followed by fixation solution 4% paraformaldehyde (PFA) for 10 minutes under deep sodium pentobarbital anesthesia (150 mg/kg). The brains were removed and post-fixed overnight in 4% PFA fixation solution, and subsequently moved every 24 hours in a buffer containing a gradually higher sucrose percentage: 10% and 20% sucrose in 0.1 M phosphate buffer solutions (PBS). Afterwards, brains were quickly frozen using CO₂ and dissected into 16 μ m thick sagittal sections using a cryostat (at -25 °C; Leica). All series of sections were subsequently stored at -80°C until further processing. For the CERTS and neuron co-localization stain, we incubated the antibodies separately to reduce the antibody-antigen interaction. Before the antibodies incubation, the slice sections were fixed with acetone 10 minutes and blocked with 0.3% H₂O₂ for 1h. The sections were incubated with a monoclonal NeuN primary antibody (1:50, chemicon international Inc, Temecula, CA, USA) overnight at 4 °C. Sections were washed 3 times with TBS, TBST, and TBS and streptavidin Alexa 594 (1:500) applied for 1h at room temperature. Then rabbit polyclonal anti-CERTS (epitope 300-350, Bethyl Laboratories) diluted 1:250 was used for recognized CERTS antigen. After overnight incubation, and the corresponding secondary antibody Alexa Fluor- 647(1:100) was applied for 1h at RT. The slices were mounted and stored in 4°C before taking pictures. Next immunofluorescence co-labeling was performed with either anti-Iba1 rabbit (Wako Pure Chemical Corporation) or anti-glia fibrillary acidic protein (GFAP) mouse combined with anti-A β plaques human antibodies [41]. Subsequently, the corresponding anti-rabbit or anti-mouse and anti-human secondary antibodies conjugated to Alexa Fluor-594 or 488 (Jackson ImmunoResearch Laboratories) were added for 2 hours. Washes were performed 3 times for 10 minutes in TBS with 0.2% Triton-X100 (TBS-T), TBS, and TBS-T, respectively in between the antibody incubation steps. Densitometric analysis of the stainings were performed on sagittal brain sections at different lateral depth (6-9 sections per animal) with ImageJ. Microglia ramification and sphericity were analyzed as previously described [42].

Sphingolipid analysis

High pressure liquid chromatography-tandem mass spectrometry (HPLC-MS/MS).

Neuro-2a (N2a) were maintained and prepared for HPLC-MS/MS as described in Supplementary methods. Powder aliquots of cortex, hippocampus, and cerebellum tissue or Neuro-2a (N2a) cell pellet were homogenized in PBS at the concentration of 10 μ L / 1mg. Then, 50 μ L of the brain preparation (or 25 μ L of plasma) were used to measure Cer, sphinganine (SPA), sphingosine (SPH), sphingosine-1-phosphate (S1P) as previously described [43,

44]. Briefly, brain preparation (or plasma) was spiked with internal standards mixture prior to undergoing extraction. Data acquisition was done using select ion monitor (SRM) after chromatographic separation and electrospray ionization on the Thermo TSQ Quantum Ultra mass spectrometer (West Palm Beach) coupled with a Waters Acquity UPLC system (Milford) for Cer, sphinganine (SPA), sphingosine (SPH), sphingosine-1-phosphate (S1P). SM data acquisition was done using multiple reaction monitoring after chromatographic separation and electrospray ionization on the Sciex Qtrap 5500 quadruple mass spectrometer (AB Sciex Inc., Thornhill, Ontario, Canada) coupled with a Shimadzu HPLC system (Shimadzu, Kyoto, Japan). Concentrations of each analyte were calculated against each perspective calibration curve and corrected for internal standard concentrations.

Protein extraction

Mice were terminally anesthetized with sodium pentobarbital, perfused and the brain removed and dissected into cortex, hippocampus and cerebellum. Each brain region was then powdered in iron mortar partly emerged in liquid nitrogen, and aliquoted. Frozen tissue from dissected brains was sonicated in about 15 volumes (w/v) of Tris buffer saline (TBS) with PhosSTOP and protein inhibitors (Roche). Samples were centrifuged, and the TBS-soluble fraction was aliquoted prior to freezing in liquid nitrogen and stored at -80°C . The pellet was re-suspended in about 15 volumes of TBS buffer containing 1% Triton-X 100 and protease inhibitor cocktail with sonicator 1 cycle for 20 seconds. Samples were centrifuged, and the TBS-T-soluble fraction was aliquoted and frozen as described for TBS. The pellet was re-suspended in 70% formic acid to 150 mg/ml based on tissue weight, and mixed by rotation at room temperature for 2 h. Samples were centrifuged, and the formic acid-soluble fraction was neutralized (with 20 volumes of 1 M Tris base), aliquoted, and frozen at -80°C . Total protein content in the TBS and TBS-T extractions was determined with Bio-Rad DC (Life Science Group) protein assay following the manufacturer's instructions.

A β immunoassay and Western blot

Immunoassay

Microplates Microlon/F-shape REF 655092 (Greiner) were coated with 1 $\mu\text{g}/\text{mL}$ of human 3D6 [28] overnight at room temperature in coating buffer (sodium carbonate pH=9.6 0.05 M NaCO_3 in MQ water). After washing plates (washing buffer 0.05% Tween- 20 in PBS were blocked with 4% not fat dry milk and incubated with brain homogenates or with A β to generate the standard curve. Next plates were washed, incubated with 50ng/mL of biotinylated human 20C2 [28] and washed again. Finally, plates were incubated with streptavidin-HRP (Jackson ImmunoResearch Laboratories) diluted 1:8,000 and developed using 3,3',5,5'-Tetramethylbenzidine (TMB). The absorption was measured at 450 nm within 30 minutes of stopping the reaction with 2 M H_2SO_4 using the Perkin Elmer 2030 manager system.

Western blot

TBS and TBS-T samples corresponding to 40 μg of total protein we loaded onto a precast TGX 4-16% gel (Bio-Rad). Then samples were transferred to PDVF membranes, blocked with Odyssey blocking buffer, and probed with 1 μg mAb anti-A β (6E10, Covance). After incubation with donkey anti-mouse IRdye680 (Rockland Immunochemicals) diluted 1:1,000 in Odyssey blocking buffer, the membrane was scanned and analyzed with

Odyssey imager Li-Cor. The intensities of the APP bands detected at 100 kDa, were measured with Odyssey imager Li-Cor.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from 20-50 mg of cortex using Trizol reagent (Invitrogen). One microgram of total RNA was treated with DNase I and transcribed into cDNA (Superscript III, Invitrogen). PCR was performed in duplicate with SensiMix™ SYBR® Low-ROX Kit (Bioline) using the set of primers reported in supplementary table 1. Fold changes of expression relative to control were determined after normalization to GAPDH and Actin. Fold change was calculated by the comparative Ct method [45].

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 23.0 SPSS Inc., Chicago, IL, USA). All graphs were designed in GraphPad Prism (version 5, GraphPad Software, San Diego, CA, USA). All data shown are expressed as mean $\pm$ standard error of the mean (SEM). Behavioral tasks, SLs, RT-PCR and cytokines data were analyzed with two-way analysis of variances (ANOVA) with AAV treatment and genotype as independent factors. Least Significant Difference (LSD) was used for *post-hoc* testing. Comparison of mean values from two groups were performed by an unpaired two-tailed Student's t-test or by Mann-Whitney U test for non-parametric testing. P-Values were considered as significant if $p \leq 0.05$ and marked with (*). Results were marked with (**) if $p \leq 0.01$, or (***) if $p \leq 0.001$.

Results

CERT_L directly affects A β aggregation and toxicity in vitro

It has been demonstrated that specific forms of CERTs can be released extracellularly or found membrane bound [29]. We have shown that CERTs can be found in proximity to A β plaques in AD brain, where they partially co-localize with SAP and with amyloid fibrils [28]. Here, we tested whether the long isoform of CERT, CERT_L, interacts with A β and its precursor protein APP. Cellular extracts of HEK-APP, expressing endogenous CERT_L, and stably expressing APP were incubated with monoclonal antibodies (mAbs) against CERT_L, APP and syntaxin 6 (used as a negative control) for immunoprecipitation (IP). Pull down was performed with anti-mouse antibodies for all 3 conditions as explained in material and method section. Western blot analysis of the samples showed efficient IP of CERT_L and APP by their respective antibodies. APP was detected as a band around 100 kDa whereas CERT_L was detected as a band of 200 kDa. The two respective bands were visualized either when CERT_L or APP were pulled down, but not when syntaxin 6 was used instead. The results, representative of three independent experiments, showed an interaction between CERT_L and APP/A β (Figure 1A). The two proteins show partial colocalization in primary neurons of 5xFAD mice at the plasma membrane and in the perinuclear region (Figure 1B). This was also observed in brain sections of 5xFAD immunolabeled for CERTs and APP/A β (Supplementary Figure 1A) confirming our previous pull down of APP with CERT in the brain lysate of AD animals manifesting severe A β pathology [28]. As shown by microscale thermophoresis (MST) analysis, CERT_L also binds to A β ₁₋₄₂ ($K_d = 2.5\mu M$) (Figure 1C) and by Western blot (Supplementary figure 1B and C). Having shown that CERT_L directly binds to APP and A β , we next tested whether binding of CERT_L to A β ₁₋₄₂ could directly influence the spontaneous fibrilization of A β ₁₋₄₂ by ThT fluorescence spectrometry and TEM. In the absence of CERT_L, the thioflavin T (ThT) fluorescence of A β ₁₋₄₂ peaked after 10-12 hours indicating amyloid formation. With the addition of CERT_L (2.5 μM) A β ₁₋₄₂ maximum ThT fluorescence reduced to 50%. In contrast, at 1 μM concentration, CERT_L was ineffective at preventing A β ₁₋₄₂ fibrilization (Figure 1D). Fibrilization was also blocked by anti-A β antibodies (Supplementary Figure 1D).

In addition to ThT fluorescence assay, we studied A β aggregation in the presence of CERT_L by TEM. The TEM images showed that amyloid-like fibrils were observed in both conditions with and without CERT_L in combination with A β . However, the amyloid structure identified in the presence of CERT_L was not linear in shape and the fibril width varied significantly (Figure 1E-F) when compared to A β ₁₋₄₂ alone. The aggregates observed with CERT_L plus A β ₁₋₄₂ could be precursors to amyloid fibril formation, but not classic straight amyloid fibril formation as previously reported [46].

Furthermore, using SHSY-5Y cells, we examined the effect of CERT_L on cell viability when A β ₁₋₄₂ was present. The addition of oligomeric A β ₁₋₄₂ to the culture medium of SHSY-5Y cells resulted in a 38% decrease in viability after 24 hours, as measured by MTT reduction relative to control conditions ($p < 0.01$). Interestingly, the simultaneous addition of CERT_L significantly ameliorated the toxic effect of A β ₁₋₄₂ (Figure 1G). Our results indicate that CERT_L forms complexes with APP and A β . The interaction of CERT_L with A β affected spontaneous aggregation and toxicity of the peptide.

As aforementioned, CERTs are important regulators of cellular Cer and SM balance. For this reason, we investigated the effect of modulating CERT_L levels on SL composition *in vitro*. After 48-hour cell transfection with pcDNA3.1 carrying CERT_L, SM d18:1/16:0 was significantly increased while CerS were unchanged (Figure 1H). Furthermore, CERT_L overexpression did not affect cell viability (data not shown).

Ceramide species are increased in 5xFAD compared to WT animals depending on brain region and acyl chain length

The 5xFAD model carries 5 familial AD mutations. These mutations lead to a rapid increase of A β peptide production. By 6 weeks of age, mice display elevated levels of A β , amyloid deposits, and age-dependent amyloid pathology accompanied by increase of inflammatory marker levels in the CNS [47-50]. However, it is unknown if this model shows also an increase of Cer level in the brain as it has been reported in the brains of AD patient [13-16]. Sphingolipid species were determined with HPLC-MS/MS in cortex, hippocampus and cerebellum of 5xFAD and wild type (WT) male mice at 25-26 weeks of age (Supplementary Table 1 reports complete analysis). The analysis showed a significant elevation of Cer d18:1/16:0 levels in the cortex and of sphinganine (SPA), S1P, Cer d18:1/16:0, Cer d18:1/18:1, Cer 18:d1/20:0 and Cer d18:1/22:0 in the hippocampus of the 5xFAD animals compared to WT mice (Figure 2A and B). In the cerebellum only S1P levels were found to be significantly higher in the 5xFAD animals. These results indicate that at that specific age and disease stage of the animals the cortex and the hippocampus are more susceptible to increase of Cer levels while the cerebellum is less affected. This suggests that the increase of Cer levels correlate with amyloid burden. In fact, the cortex and hippocampus are the areas where the plaques are first reported to appear in this model [31]. Quantification of CERT levels in the cortex by immunoassay showed a significant reduction of the protein concentration in AD brains compared to controls at 25-26 weeks of age [51]. CERTs reduction was not observed in cerebellum (Figure 2D). Our data suggest that SL metabolism is shifted towards increased production of Cer at different degrees depending on the brain region. Furthermore, CERT concentration is reduced in the cortex of 5xFAD mice compared to WT.

AAV-mediated neuronal expression of CERT_L in mouse brain

To test the hypothesis that increasing CERT_L levels in the cortex counteract the SL disbalance in 5xFAD animals and A β formation, we generated AAV2 particles carrying the CERT_L cDNA sequence controlled by the neuron-specific synapsin promoter (Figure 3A). The AAV- CERT_L was tested *in vitro* on cortical rat primary cell culture, proving to effectively transduce neurons (Supplementary figure 2A). Next, 8 weeks old WT animals were injected in the layer V of the motor cortex with AAV-CERT_L and AAV-control (Supplementary figure 2B). Layer V of the frontal cortex was targeted since the A β accumulation is the most severe [31] and CERT levels are reduced in this mouse model. The transduction efficiency was evaluated 1-, 2-, 6- and 12-weeks post-injection by immunohistochemistry (Supplementary figure 1C-D) and by RT-PCR (Supplementary figure 1E). The AAV-CERT_L was shown to effectively transduce neuronal cells and expressed the CERT_L protein for at least 12 weeks. The AAVs were then injected in 5xFAD mice in a similar fashion. After 12 weeks CERT overexpression was confirmed by immunohistochemistry (Figure 3B) and by Western blot analysis (Figure 3C and D). The immunolabeling of CERT, with neuronal marker NeuN, showed as expected neuronal expression and that AAV-CERT_L was injected in the

targeted area of the cortex. Relative quantification of CERTs levels in cortex homogenates by Western blot illustrated a significant increase of CERTs levels in AAV-CERT_L treated groups (Figure 3D).

5xFAD and WT mice injected with AAV-CERT_L and AAV-WT did not show behavioral abnormalities

Mice underwent stereotactic surgery for CNS administration of AAV particles at 12-13 weeks of age and were monitored for 12 weeks. As mentioned above, AAVs were injected in layer V of the cortex. At week 10 post-injection, mice were challenged with a behavioral test battery in the following sequence: open field (OF) for assessing locomotion activity, Y-maze spontaneous alternation (AYM,) and SYM for assessing spatial memory and elevated zero-maze (EZM) for examining anxiety (Figure 4A-E). It has been reported that 5xFAD mice exhibit changes in hippocampus-dependent spatial working memory by 16 to 24 weeks of age [31]. However, in our hands, no difference was found in the performance of 5xFAD compared to WT in locomotion (Figure 4B), memory (Figure 4C and E), and anxiety (Figure 4D). Our data shows that the WT animals treated with the control virus performed above the 50% chance level in the AYM (Figure 4C). Furthermore, in the OF, the AAV-CERT_L attenuated 5xFAD hyperactivity, even though 5xFAD treated with AAV-control did not perform significantly different from WT animals (Figure 4B, two-way ANOVA, interaction $F=4.170$, $p=0.0463$). Overall, these data suggest that no detrimental behavioral effects in the animals were observed after CERT_L over-expression.

AAV-CERT_L decreased Cer d18:1/16:0 and increased SM levels in the cortex

One of the main functions of CERTs is to shuttle Cer from the ER to the Golgi [22]. It has been reported that toxic increase of Cer species in the muscle can be attenuated by overexpressing hCERT cDNA [52]. HPLC-MS/MS analysis of brain cortex tissue revealed a significant reduction of Cer d18:1/16:0 level due to CERT_L overexpression ($p<0.05$). This effect was not observed in WT animals but only in 5xFAD animals where Cer d18:1/16:0 level was significantly elevated ($p<0.01$). This transport of Cer to the Golgi is crucial for the *de novo* synthesis of more complex SLs such as SM. Previous data from *in vitro* experiments reported that blocking CERTs function SM levels would significantly decrease [26]. Hence, if CERTs activity is enhanced, we expected an increase in SM levels. As reported above, we found that *in vitro* overexpression of CERT_L increased the levels of certain species of SM. Accordingly, SL analysis of the cortex showed that the levels of most of SM species (SM d18:1/16:0 $p<0.001$, SM d18:1/18:0 $p<0.001$, SM d18:1/18:1 $p<0.01$, SM d18:1/20:0 $p<0.05$, SM d18:1/22:0 $p<0.05$ and SM d18:1/24:1 $p<0.01$) were increased in AAV- CERT_L treated animals (Figure 5). The only SM species not found significantly elevated was the SM d18:1/24:0, whose precursor Cer d18:1/24:0 has been reported to be poorly transferred by CERTs [53].

These data suggest that CERT_L overexpression is effective in reducing Cer increase by intensifying the transfer to the Golgi, which leads to an increase of SM. While the Cer attenuation was restricted to a pathological increase of Cer d18:1/16:0 level, SM elevation was consistent in all AAV-CERT_L treated animals. This shift in SL composition did not affect apoptosis markers in the cortex quantified by RT-PCR (Supplementary figure 6A).

AAV-CERT_L reduces A β levels by decreasing APP cleavage in the cortex of 5xFAD mice

Since our previous data indicated that CERT_L could be released to the extracellular milieu and directly affects A β aggregation and toxicity *in vitro*, we investigated the effect of CERT_L overexpression on A β deposition. (Figure 6A). Statistical analysis showed no significant difference in plaque load between the 5xFAD groups at 24-26 weeks

of age (Figure 6B). However, the percentage of small plaques size (10-25 μm) was reduced ($p<0.05$) in AAV-CERT_L treated 5xFAD brains (Figure 6C). Furthermore, A β quantification of brain homogenate in TBS soluble and TBS-T soluble fraction showed that A β levels were reduced in samples treated with AAV-CERT_L ($p=0.04$ and $p=0.03$, respectively), while in the formic acid soluble fraction no change was observed ($p=0.29$) (Figure 6D). Since it has been reported that APP cleavage can be affected by Cer composition [18, 19], we investigated if the reduction of Cer and increase of SM levels mediated by AAV- CERT_L is associated with altered processing of APP. The ratio A β / FL-APP was decreased in CERT_L overexpressing mice implying a reduction of A β biogenesis or increase clearance of A β ($p<0.01$). Since the CTF β is the product of β -secretase cleavage of APP and the immediate precursor of A β formation [54], the ratio of CTF β / FL-APP bands intensities was used to assess APP processing by β - and γ -secretase. The CTF β / FL-APP was found an increase in AAV-CERT_L treated 5xFAD animals ($p<0.05$) (Figure 6E). Meanwhile, the ratio A β / CTF β was reduced in brains of CERT_L overexpressing mice ($p<0.01$) (Figure 6E and Supplementary figure 5). These results indicate that AAV-CERT_L affects the proteolytic processing of APP by β - and/or γ -secretase. These results show that a specific balance of Cer to SM and/or interaction of CERT_L with APP is critical for APP cleavage and A β biogenesis.

Four weeks of administration of CERTs inhibitor (HPA-12) exacerbates Cer and A β pathology in AD transgenic mice

To test if efficient ceramide trafficking from the ER to the Golgi is vital in the regulation of the Cer levels and A β formation, we administered the CERTs inhibitor N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl) dodecanamide (HPA-12) for 4 weeks to AD transgenic mice. As expected, Cer d18:1/16:0, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:1 levels were found an increase in the brain (Figure 7A). Moreover, A β levels were increased by 117% in the TBS soluble fraction ($p<0.05$) and by 47% in the TBS-T soluble fraction of brain homogenates ($*p<0.05$) of CERTs inhibitor-treated AD animals. No significant changes were found in the FA insoluble fraction (Figure 7B). These data suggest that efficient ceramide transfer from ER to *trans*-Golgi, is critical to control Cer levels and thereafter APP processing. Pharmacological interference with CERT activity augments Cer levels and increases substantially A β biogenesis and/or fibrillization.

AAV-CERT_L reduces microglia immunoreactivity as shown by Iba1 labeling and CD86 expression

We have observed that CERTs are associated with A β plaques in AD brain where microglia cells are engaged [28]. Thus, we investigated if microglia cells were affected by CERT_L overexpression in the cortex. To achieve this aim, brain sections were analyzed for Iba1 reactivity. AAV-CERT_L treated brains had a decreased immunoreactivity to Iba1 (percentage of area $p<0.001$) (Figure 8A-C). Iba1 is considered a constitutive marker for microglia, which is highly increased in 5xFAD animals compared to WT [55, 56]. Furthermore, it has been shown to be important for membrane ruffling, a process crucial for macrophages and microglia motility and chemotaxis [57]. We further characterized microglia based on ramifications and sphericity. The analysis showed that microglia of AAV-CERT_L injected mice had longer ramifications and lower sphericity index (Figure 8D and E). To investigate if AAV-CERT_L affected other microglia membrane markers, we run RT-PCR on cortex tissue. We quantified the CD86 membrane marker, which is an indicator for microglia pro-inflammatory polarization. Statistical analysis showed that CD86 was

increased in AAV-control treated 5xFAD animals compared to AAV-control treated WT, and that AAV-CERT_L specifically decreases CD86 in the 5xFAD group (Figure 8F).

Next, we analyzed cytokines levels in brain homogenate using multiplex technology. The assay did not reveal any significant effect of AAV-CERT_L treatment. However, we observed a significant increment of IL-1 β ($p < 0.01$) and a significant reduction of IL-4 ($p < 0.05$) when comparing WT to 5xFAD animals in AAV-control treated groups (Supplementary figure 6A). These results indicate overall that AAV-CERT_L decreases pro-inflammatory processes in microglia.

AAV-CERT_L does not change the immunoreactivity of astrocytes as shown by GFAP labeling in the cortex of 5xFAD mice

Reactive astrocytes have been described as a pathological hallmark that generally occurs in response to neurodegeneration in AD [58]. To determine the extent of astrogliosis in the 5xFAD mouse brain treated with AAV-CERT_L compared to control, we performed immunofluorescence GFAP labeling on sagittal brain sections (Figure 8G). Densitometric and particle analysis performed with ImageJ did show a reduction of 47% astrocytes immunoreactivity, which was not statistically significant (Figure 6H). However, as previously reported we observed a 3-fold increase of GFAP immunoreactivity in the 5xFAD animals compared to WT controls [31, 59].

Discussion

In this work, we provide evidence suggesting that CERT_L plays an important role in characteristic processes of AD by affecting A β production and aggregation, neuroinflammation and SL disbalance typical of AD. Our data show that the pathological increase of Cer d18:1/16:0 can be restored to normal levels by upregulating CERT_L. The reduction of Cer levels also proved to be effective in attenuating A β formation. Furthermore, CERT_L overexpression in neurons revealed that CERT_L can downregulate membrane markers indicating a pro-inflammatory status in microglia.

It has previously been reported that APP can interact with extracellular matrix proteins like collagen I [60, 61]. This interaction is thought to be important in neuronal cell to cell adhesion with APP functioning as an anchor [62]. CERT_L is known to be crucial in stabilizing the basal membrane [63]. We previously reported that CERTs can be found in close location to plaques in AD brains, where they co-localize with amyloid fibrils [28]. In line with this, we found that APP can interact with CERT_L and form complexes that can be immune precipitated. Also A β can bind to a variety of biomolecules, including lipids, proteins and proteoglycans [64]. Here, we show that CERT_L binds directly to A β peptides and that this interaction affects A β fibrilization by organizing A β into less neurotoxic aggregates.

Manipulation of CNS SL metabolism can be challenging. Removal or addition of genes in the SL pathway can be deleterious for brain function [65, 66]. Also, the upregulation of specific enzymes in specific cell types can be harmful for brain function. In a previous report on SL interference by AAV particles delivering human acid sphingomyelinase in the brain of rodents and primates, showed that motor function could be severely affected [67]. On the contrary, in our study, behavioral testing at 9-10 weeks post AAV particle injection revealed no detectable side effects when comparing AAV-CERT_L to AAV-control treated animals in the various locomotor-, memory- and anxiety-related behavioral tasks. Of note though, we found a significant reduction of locomotor activity in the 5xFAD due to AAV-CERT_L treatment. It has been reported that 5xFAD animals exhibit a hyperactive behavior compared to control animals even though this specific behavior is not utterly understood and translation to human symptoms is unclear [68]. However, the question whether AAV-CERT_L could improve memory or other behavioral deficits observed in the 5xFAD model could not be answered by our data. No significant impairments in spatial memory were detected in the AYM and the SYM tests. In the original 5xFAD line (on a hybrid B6SJL background and carrying the Pde6b gene) Jawhar et al., reported deficits of working memory, assessed by a cross-maze test, to appear at 6 months [69]. In contrast, the reports in the 5xFAD line on a C57BL/6J background and without the Pde6b gene, are controversial. Richard et al., reported in this last line of 5xFAD spatial memory deficits were only visible after 7 months of age [70]. Others reported spatial memory impairment at an earlier age when using the water maze but with very small difference between WT and 5xFAD [71, 72]. The water maze is a powerful test to assess the spatial memory in rodents. It is based on a four-day learning period where animals are thought to find a hidden platform. Then, the fifth day a probe trial is given where the time spent in the target quadrant - which is the quadrant where platform was located - is calculated. If the time spent in the target quadrant is equal to the time spent in the other quadrants, this means that animals are not remembering the position of the platform. Interestingly, when looking at the score of the 5xFAD in the water maze test, both in the work of Gu et al., and Wu et al., two comments can be put forward: 1) the

5xFAD mice have normal learning process when looking at the training trials from day 1 to 4 and 2) the 5xFAD group remembers the position of the platform since they spent most of time in the target quadrant (about 40% of the time) implying no memory deficit are present. In the present study, none of the groups tested in the SYM test performed above chance level indicating lack of recognition in all groups examined. However, in the AYM, the WT group treated with AAV-control performed above chance level (mean of 68%), which in the AYM is considered to be 50%. As such, future studies should additionally assess shorter inter-trial intervals in this respect.

Cer generation is abnormal in AD, causing an increase in Cer formation [15, 73]. Furthermore, an accumulating body of evidence consistently reported a global rise in Cer levels in specific brain regions of AD patients [13, 74, 75]. In agreement with these observations, we found that 5xFAD transgenic mice at 6 months of age also showed an increase of Cer d18:1/16:0 in the cortex and of several Cer species in the hippocampus (Cer d18:1/16:0, Cer d18:1/18:1, Cer d18:1/20:0 and Cer d18:1/22:0). Previously, other studies showed increased brain Cer levels in different transgenic AD models (APP, PS1, and PS1-APP mutated mice) [76]. This suggests that there is a causal relationship between amyloid pathology and Cer imbalance.

In a genome-wide gene-expression analysis on several AD models, CERTs expression was found downregulated in the cortex compared to control [51]. The deficient transfer of Cer from the ER to the Golgi implicates that Cer accumulates in the ER, causing a chronic stress-like reaction in the cell that compromises the unfolded protein response (UPR) pathway [10]. In contrast, the enzymes of the SL catabolic pathway like sphingomyelinase are abnormally active contributing to the Cer build-up in other cell compartments [15, 73]. Cer accumulation contributes to amyloid pathology via two mechanisms: first, Cer increases A β production by stabilizing secretases activity; second, A β activates the Cer mediated apoptotic pathway [18, 19].

In this study we report that increasing Cer trafficking from the ER to the Golgi by overexpressing CERT_L, reversed the pathological increase of Cer in the cortex. A similar effect of CERTs overexpression in Cer elevation state has been reported in a lipotoxic mouse model where muscle cells in overload Cer status were rescued by increasing the expression of hCERT [52]. In AD brains, we observed that CERT_L reduced 11% of the total Cer content restoring it close to normal levels. In particular, Cer d18:1/16:0 was the most affected species by AAV-CERT_L being reduced up to 35%. These results demonstrate the importance of physiological ER-to-Golgi Cer traffic in preserving the physiological balance of Cer levels in AD pathology.

The soluble A β forms were reduced in the CERT_L treated animals, whereas the insoluble A β forms were not altered by the treatment. This reduction in the soluble forms could be explained in two ways: i) the 11% reduction of total Cer due CERT_L overexpression and ii) CERT_L interaction with A β . As aforementioned, the amyloidogenic cleavage of APP is favored resulting in more A β formation in Cer enriched conditions [18, 19]. In our study, the total Cer reduction may have affected the secretases activity in the opposite way. We found that the A β / APP ratio, which describes the APP processing to form A β , was lower in AAV-CERT_L animals implying that lesser APP is processed to generate the A β peptide. Importantly, APP processing takes place in different cell compartments not only on the cell surface [77] and the Cer shift is not confined to the ER compartment but can affect the whole cell [78, 79]. This conclusion was further confirmed by pharmacological inhibition of CERT Cer transfer activity with CERT inhibitor

HPA-12. Recently, the pharmacokinetics of HPA-12 was described, and it was proven that the compound reaches the brain intact [24]. Here, we found that after 4 weeks, the treatment of HPA-12 increases Cer and A β levels.

It is now thought that one of the crucial processes in the development and exacerbation of AD is neuroinflammation [80]. Genome-wide association studies reported that variants of genes encoding for immune system proteins, like triggering receptor expressed on myeloid cells 2 (TREM2), were discovered to be associated with AD pathophysiology [81]. Additionally, different inflammatory markers such as activated astrocytes and microglia, increased levels of cytokines and products of complement (C) activation are found in the brain tissue of AD patients [82-85]. This pro-inflammatory environment is thought to be harmful to neurons. As we previously reported, CERT_L also has immune functions. Our lab demonstrated that CERT_L interacts with SAP which belongs to the pentraxin family of the innate immune system [34]. Additionally, we reported that CERT_L can activate the complement system [33]. Here, we found that AAV-CERT_L influenced microglia activation even though CERT_L was specifically expressed in neurons under the control of the synapsin promoter. It has been consistently reported that 5xFAD microglia are polarized towards a more pro-inflammatory status, in response to the extensive plaque formation. Consequently, the Iba1 microglia marker is highly expressed in AD models [52, 53]. Our findings suggest that CERT_L could play a role in the cross-talk between neurons and microglia. Interestingly, neuronal-derived CERT_L activity is exerted only when there is an inflammatory reaction ongoing by reducing membrane markers for the pro/inflammatory status of microglia. Nevertheless, it remains unclear by which mechanism AAV-CERT_L decreases Iba1 and CD86 positive cells. Here, we propose two hypotheses. First, the reduction of Iba1 and CD86 is a direct action of CERT_L on microglia activation status once secreted by neurons. It is known that forms of CERT_L can be released in the extracellular space [58]. The second, Iba1 and CD86 are decreased because of a modified by shifts in Cer and SM composition or other indirect effects like reduction of A β levels. In the CNS, there is an extensive cross-talk ongoing between neurons and microglia, which takes advantage of lipid vesicles. Furthermore, toxic A β has also been reported among the content of exosome and reduction of exosome secretion was correlated to A β reduction [86, 87].

Similarly, to microglia, also astrocyte activation was reduced by AAV-CERT_L, even though not significantly. During inflammation astrocytes are enriched in Cer. They seem to produce the pro-apoptotic Cer d18:1/16:0 [88, 89]. Further, reactive astrocytes release extracellular vesicle enriched in ceramide that carry A β peptides [89]. These specific extracellular vesicles isolated from brains of 5xFAD mice showed to be particularly toxic for neurons.

Limitations

The limitations of this study are three folded. First, we did not detect memory difference between WT and 5xFAD. This could be due to our in-house breeding approach, which is explained in material and methods section. We adopted a breeding scheme where, the Pde6b gene, which is associated to retinal degeneration, was bred out of the genetic background of the 5xFAD transgenic mice. The Pde6b gene is thought to be crucial for early detection of memory impairments. Second limitation is connected to the first. Since no memory impairment was detected, it is unclear if the AAV-CERT_L would protect from memory decline. For this purpose, animals could be tested at older age and the AAV-CERT_L could be injected in the hippocampus. This follow-up study would be of interest also because the hippocampus, as we show for the first time in this study, is particularly affected by the ceramide pathology in

5xFAD males. Third limitation is the effect of AAV-CERT_L on astrocytes. While we had sufficient statistical power to detect the microglia changes, astrocytes denoted a similar trend, which was not statistically significant. These three limitations set the ground for future studies.

Conclusion

In conclusion, by increasing CERT_L expression in neuronal cells, we were able to increase SM production and reduce Cer d18:1/16:0 especially in the CNS. Next, after proving that CERT_L binds and modifies A β aggregation *in vitro*, we observed that administration of AAV-CERT_L in AD animals reduced A β production by at least 2 mechanisms: by altering SL composition and by direct interaction with APP in 5xFAD animals. Moreover, we reported a new immune role of CERT_L. AAV-CERT_L decreased membrane markers important for the pro/inflammatory status of microglia. Overall, our experiments are the first to demonstrate that an increase of CERT_L, modulates SL levels, affects amyloid plaque formation and brain inflammation in AD (see the model in Figure 9). These data open research pathways for therapeutic targets of AD and related neurodegenerative diseases.

List of abbreviations

Alzheimer's disease	AD
Amyloid- β peptides	A β
Neurofibrillary Tangles	NFTs
Blood Brain Barrier	BBB
Sphingolipids	SLs
Ceramide	Cer
sphingosine-1-phosphate	S1P
Sphingomyelin	SM
Ceramide transfer proteins	CERTs
Ceramide transfer proteins long form	CERT _L
Steroidogenic acute regulatory protein (StAR)-related lipid transfer	START
Endoplasmic Reticulum	ER
Adeno associated virus	AAV
Immunoprecipitation	IP
Amyloid precursor protein	APP
Full length Amyloid precursor protein	FL-APP
C-terminal fragment- β	CTF β
Microscale thermophoresis	MST
Transmission Electron Microscopy	TEM
Thioflavin T	ThT
Open field	OF
Y-maze spontaneous alternation	AYM
Elevated zero-maze	EZM
Y-maze spatial memory test	SYM

High pressure liquid chromatography-tandem mass spectrometry	HPLC-MS/MS
Monoclonal antibodies	(mAbs)
N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl) dodecanamide	(HPA-12)

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Figures

Figure 1. CERT_L binds directly to APP and A β , and reduces A β aggregation and toxicity *in vitro*. A) Protein interaction detected using co-IP of APP and CERT_L in HEK-APP. The total cell lysate of HEK-APP cells (L) lane 1 and the total cell lysate of HEK-APP cells IP using APP/A β (lane 2), CERT_L (lane 3) and syntaxin (isotype control)

(lane 4) antibodies were analyzed by Western blot. APP (1) and CERTs proteins were detected. The isotype control syntaxin protein were negative. Molecular weight markers are indicated (kDa). B) Immunofluorescent staining showing co-localization of CERTs and APP/A β in primary neurons isolated from 5xFAD brains. Neuronal marker MAP2 was used to immuno-label neuronal cells. DAPI was used for nuclei staining. Scale bar 10 μ m. C) CERT_L and A β ₁₋₄₂ interaction was measure by microscale thermophoresis. The dissociation constant (K_d) calculated was 2.5+/-0.3 μ M. D) Measurement of Thioflavin T (ThT) fluorescence intensity to detect A β ₁₋₄₂ 20 μ M aggregation in the absence or presence of recombinant CERT_L 1 or 2.5 μ M at different time points. Each data point represents the mean fluorescent intensity of three wells. E and F) TEM analysis of 2 μ M A β ₁₋₄₂ aggregation in the absence and presence of 0.1 μ M CERT_L showed a different aggregation pattern quantified by A β width (student's t-test ***p<0.001). G) Measurement of cell metabolic activity of SH-SY5Y by MTT assay in cells incubated with medium alone (control) or medium containing 10 μ M A β ₁₋₄₂, A β ₁₋₄₂, and 1 μ M CERT_L, or CERT_L alone for 24 hours. Graph bar expressed as means $\pm$ S.E.M % of control N= 5-10 (one-way ANOVA, Bonferroni correction *p< 0.05; **p<0.01). H) SM d18:1/16:0, SM d18:1/18:1, SM d18:1/18:0, SM d18:1/24:1 and SM d18:1/24:0 measured by HPLC-MS/MS in N2a cells after 48 hours transfection with vector control or pcDNA-CERT_L. Graph bar expressed as means $\pm$ S.E.M % of control N= 4 / group (one-way ANOVA, Holm-Sidak's multiple comparisons test ***p<0.001)

Figure 2. Ceramide levels are increased in 5xFAD compared to WT animals depending on brain region and acyl chain length while CERT levels are reduced. Sphingolipids levels were measured in the hippocampus (A), cortex (B), and cerebellum (C) by HPLC-MS/MS of WT and 5xFAD mice. Sphingolipids were classified based on acyl chain number of carbons (Sph, S1P, SPA, Cer d18:1/16:0, Cer d18:1/18:1, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:0). CERT was quantified by ELISA in protein extract of cortex and cerebellum of WT and 5xFAD animals. Bars represent the mean $\pm$ S.E.M per group N=11-12 (student's t-test *p<0.05; **p<0.01).

Figure 3. AAV-mediated neuronal expression of CERT_L in mouse brain. A) The recombinant genomes of the two AAV-2 vectors. → Abbreviations: From left to right, **ITR**, inverted terminal repeats; **hSYN1**, human synapsin 1 gene promoter; **CERT_L**, cDNA sequence coding for ceramide transfer protein long isoform (hCERT_L, 1875bp NP_005704.1) ; **IRES** internal ribosome sequence for translation initiation. **EGFP**, cDNA coding for enhanced green fluorescent protein; GFP; **WPRE**, woodchuck hepatitis virus posttranscriptional control element; **bGH**, bovine growth hormone gene-derived polyadenylation site; **TB**, synthetic transcription blocker. B) Representative images of immunofluorescent staining of cortical brain area from 5xFAD animals treated with AAV-control or AAV-CERT_L. Section was co-stain for CERTs protein (green), neuronal marker NeuN (red). Scale bar 200 μ M and 50 μ M. C) Western blot showing band intensities of CERTs and GAPDH. D) Relative quantification of CERTs levels normalized to GAPDH in cortical protein extract from WT and 5xFAD animals treated with AAV-control or AAV-CERT_L. Bars represent mean one representative experiment with 5-7 samples *per* group (Mann Whitney test, * $p < 0.05$).

Figure 4. No behavioral abnormalities 10 weeks after injection of AAV-CERT_L. A) The effects of CERT_L overexpression were investigated in 30 5xFAD and 30 WT males. Mice were bilaterally injected at 12-13 weeks of age with AAV-CERT_L or AAV-control particles at the dose 1.12*10E8 transducing unit (t.u.). Starting at week 22 of age, animals were challenged with the following behavioral tests: Open Field (OF) for locomotion activity, Alternate Y-Maze (AYM), and Spatial Y-Maze (SYM) for spatial memory and Elevated Zero Maze (EZM) for anxiety. B) Locomotion expressed as distance traveled in OF task. C) The graph shows the results of the working memory in the AYM task as a percentage of correct alternation in the first four triads. Percentage were compared to 50% chance levels (one sample t-test **p<0.01). D) Anxiety was assessed, measuring the percentage of time spent in the closed arm in EZM. E) Memory was measured in SYM expressed as a percentage of time spent in the novel arm. (Bars represent the means $\pm$ S.E.M per group N=10-20 (two-way ANOVA, interaction effect F= 4.170 p= 0.0463, LSD, *p < 0.05).

Figure 5. AAV-CERT_L reduces Cer d18:1/16:0 and increases sphingomyelin species in the cortex. Sphingolipids levels were measured in the cortex by HPLC-MS/MS. Ceramides were classified based on acyl chain number of carbons (Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/18:1, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:1), as well as sphingomyelin (SM d18:1/16:0, SM d18:1/18:0, SM d18:1/18:1, SM d18:1/20:0, SM d18:1/22:0 and SM d18:1/24:1). Ceramides levels were expressed as ng/mg tissue, while sphingomyelins were expressed as pmol/mg tissue. Bars represent the mean $\pm$ S.E.M per group N=5-12 (two-way ANOVA, LSD, significant effects, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 6. Neuronal increase of CERT_L reduces Aβ by decreasing APP cleavage. A) Representative photomicrographs of sagittal brain sections imaging the motor sensory cortex (M1 and M2) stained for nuclei in blue and Aβ plaques in green. All photomicrographs were exposed and processed identically. Scale bar represents 200 and 50 μm (from right to left). B) Immunofluorescent quantification of plaques measured by the percentage of area, plaques counts / mm². C) Frequency distribution of plaques based on size (10-25 μm) (Errors bars represent +/- SEM of 4-6 animals per experimental condition, ANOVA, Bonferroni correction, significant effects, *p<0.05; **p<0.01). D) Aβ quantification in three extraction buffers, BS, TBS-T, and formic acid (FA) by ELISA showed that Aβ was

significantly reduced in the soluble fractions in the cortex but not in the insoluble fraction (student's t-test $*p<0.05$).

E) Western blot analysis of TBS cortex homogenate stained with 6E10 antibody showed that ratios of amyloid $A\beta$ / FL-APP and $A\beta$ / CTF β are reduced while CTF β / FL-APP is increased in AAV-CERT_L treated animals while CTF β / FL-APP is increased. Errors bars represent $\pm$ SEM of 5 animals per experimental condition (student's t-test $*p<0.05$; $**p<0.01$). (Full length amyloid precursor protein = FL-APP; amyloid- β peptide = $A\beta$; C-terminal fragment β = CTF β). Western blot membranes are shown in Supplementary figure 5.

Figure 7. CERTs inhibitor increases Cer and A β levels in the brain of transgenic AD mice. A) Sphingolipids levels were measured in the cortex by HPLC-MS/MS. Ceramides were classified based on acyl chain number of carbons (Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/22:0 and Cer d18:1/24:1) and levels were expressed as pmol / mg tissue. Bars represent the mean $\pm$ S.E.M per group N=10-12 (student's t-test * p <0.05) A β quantification in hippocampus homogenate extracted in three buffers, TBS, TBS-T and formic acid (FA) by enzyme-linked immunoassays. Means of each fraction were compared with unpaired t-test (Control N=10; HPA-12 N=13; * p <0.05)

Figure 8. AAV-CERT_L reduces microglia reactivity to Iba1 and CD86 expression levels but has no significant effect on GFAP immunoreactivity in the cortex of 5xFAD mice. A) Representative photomicrograph of Iba1 staining in the cortical motor sensory region of 5xFAD animals treated with AAV-control or AAV-CERT_L (Scale bar 50 μ m). B) Densitometric analysis of Iba1 staining represented as a percentage of the area (AAV-control n=6 and AAV-CERT_L n=4 for WT and 5xFAD groups). C) Densitometric analysis of Iba1 staining represented as number of positive Iba1 cells / mm² (AAV-control n=6 and AAV-CERT_L n=4 for 5xFAD groups). D) Length of microglia ramification and sphericity per cell in AAV-control or AAV-CERT_L. Morphological analysis was performed on 3-5

pictures/group. E) Illustrations of the microglia morphological analysis applied to a fluorescent photomicrograph captured with 60x objective with a single cell cropped to show details. Scale bar = 20. F) Analysis of gene expression of membrane markers CD86 (4-5 number of animals per group). G) Representative photomicrographs of GFAP staining in the cortical motor sensory region of 5xFAD animals treated with AAV-control or AAV-CERT_L (Scale bar 50 μ m). H) Densitometric analysis of GFAP staining represented as a percentage of the area (AAV-control n=6 and AAV-CERT_L n= 4 for WT and 5xFAD groups). Bars represent the mean $\pm$ S.E.M per group (two-way ANOVA, LSD, significant effects, *p< 0.05; **p<0.01).

Schematic model of CERT_L action in AD. A) CERT_L concentration is decreased in AD neuronal cells. Consequently, the transport of Cer to the Golgi is impaired and Cer accumulates in the cell. Cer elevation stabilizes and favors the secretases activity. The amyloidogenic APP processing is favored and Aβ is produced. The neighboring microglia changes the resting status to activate. B) By overexpressing CERT_L the physiological transfer of Cer from the ER to the Golgi is restored favoring SM synthesis, which is intensified. The reduction of Cer levels in neuronal cells diminished secretases activity, reducing Aβ biogenesis. The interaction between CERT_L and APP may be important in stabilizing APP in the membrane and in protecting APP from secretase activity. Furthermore, CERT_L affects Aβ fibrilization by organizing Aβ into less neurotoxic aggregates that may be cleared from the brain more easily and reduces the number of activated microglia.

Supplementary methods

Co-localization of CERTs and APP/A β in 5xFAD brain

Immunofluorescent staining was performed in 10 μ m thin sagittal brain section of 3 months old males 5xFAD. The sections were fixed with acetone 10 minutes, blocked with 3% BSA for 1h and incubated overnight at 4 °C with rabbit polyclonal anti-CERTS (epitope 300-350, Bethyl Laboratories) diluted 1:500 and anti APP/A β (clone 6E10 Covance) diluted 1:500. Next day sections were washed 3 times and the corresponding secondary antibodies conjugated to Cy2 or Cy3 (1:500) were applied for 1h at RT. After washing, the NeuroTrace 640/660 (ThermoFisher) was used following manufacturer instruction. Hoechst staining was performed for nuclei visualization. The slices were mounted with 80% glycerol and imaged.

Lipidomics in CERT_L transfected neuroblastoma cells

Neuro-2a (N2a) obtained from ATCC (CCL-131™) were cultured in DMEM supplemented with fetal bovine serum (FBS), Pen/Strep, and L-glutamine. Cells were seeded in 75 cm² flasks and maintained in complete DMEM for 24 hours prior to the transfection. Cells were transfected with 2 μ g pcDNA3.1-CERT_L (NM_005713.3) by Effective Reagent (Qiagen) following the manufacturer's instructions. Cells transfected with 2 μ g of plasmid expressing GFP were used as a control. After 48 hours, cells were washed three times with PBS, trypsinized, and centrifuged at 300 x g for 5 min. Pellet was stored at -80 before the lipid analysis. Sphingolipid analysis was performed as explained in Material and Methods section.

Far Western blot

For Far Western experiments, human recombinant CERT_L (hCERT_L, 1875bp NP_005704.1) (80ng), produced as previously described [28], and A β peptide 1-42 (80ng) (Anaspec) were analyzed by SDS-PAGE under reducing conditions. A 17kDa Lama antibody fragment (H6) with myc and hist6 tags (a kind gift of Dr A.J. Groot), was used as a negative control (80ng). Proteins were transferred to nitrocellulose membranes renatured in Tris-buffered saline (TBS) in the presence of Tween20 (0.05%) and probed for 1 hour at 37°C with 30 μ g/mL of CERT_L or A β peptide in the same buffer. Bound complexes were detected using polyclonal rabbit anti-CERTs (epitope 1-50 of human CERTs, Bethyl Laboratories), mouse anti-A β mAb 6E10 (Covance) and mouse mAb anti-human c-Myc (clone 9E10, Invitrogen). Finally, incubation with secondary goat anti-rabbit IRdye 800 and donkey anti-mouse IRdye 680 (Rockland Immunochemicals) was followed as described above.

Neuronal primary cell culture

Rat hippocampal neuronal cultures were prepared as reported elsewhere [90]. In brief, embryos from pregnant Wistar rats (Charles River) were removed on embryonic day 18. After removing the meninges, the hippocampi were isolated under the lupa preserved in the Hibernate medium. Subsequently, they were incubated with trypsin for 20 minutes at 37 °C. Hippocampus were then transferred to a tube with DMEM complete medium. With a sterile glass pipette, the cells were disbanded doing up and down until the tissue was disaggregated/disintegrated and homogeneous. Cells were plated at ~80,000 cell/ 35mm Ø plate containing coverslips and incubated in Neurobasal

medium (supplemented with B27) (Thermo Fisher Scientific) for 2-3 weeks. The medium was partially exchanged 24 hours after and then once a week.

Immunoassay for CERTs detection

CERTs were quantified by enzyme-linked immunoassay or Western blot. In brief, microplates were coated with 100 μ L of polyclonal rabbit 01 (2,46 mg/mL) diluted 1:1000 using coating buffer (50 mM carbonate pH 9.6), sealed with a plastic sticker and incubated for 1 h at 37°C. To build up the standard curve serial dilutions of recombinant CERTs, produced as previously explained [28], were diluted in 10% BSA, 0.02% Tween-20 in PBS. Biotinylated polyclonal rabbit anti-CERTs 02, was used as a detection antibody diluted 1:500 in 1% BSA and 0.02% Tween-20 in PBS and incubated for 1 h at 37 °C. Blocking, washes, and absorbance were performed as described in method sections immunoassays. Cortex sample were lysed in composed by 0.1% SDS, 0.1% Triton X-100, 1% glycerol, 1 mM EDTA, 1 mM EGTA, PhosSTOP and protein inhibitors (Roche). 40 μ g of protein extracted separated in SDS-PAGE electrophoresis and blotted onto nitrocellulose membrane. The membrane was probed with anti-GAPDH antibody (10R-G109A, Fitzgerald) as internal control and anti CERTs (epitope 300-350 of human CERTs, Bethyl Laboratories). After incubation with donkey anti-mouse, IRdye680, and anti-rabbit IRdye800 (Rockland Immunochemicals) diluted 1:10,000 in Odyssey blocking buffer, the membrane was scanned and analyzed with Odyssey imager Li-Cor. The intensities were measured with Odyssey imager Li-Cor.

CERTs inhibitor administration

HPA-12 was prepared in control solution PEG400/PBS 1:4 (V/V). Before re-suspension vehicle was sterilized by filtration and pH was adjusted to 7.4. PEG400 / PBS was warmed up to 37°C and vortexed to completely dissolve the compound. HPA-12 was administered subcutaneously (SC) for 4-weeks at the dose 4 μ g / g animals every 48 hours. The volume injected per animal was of 0.15 mL with insulin syringes. HPA-12 was tested on 8-14 months old mice. The AD transgenic (E4FAD and E3FAD) mice were purchased from Dr. Mary Jo LaDu (University of Illinois at Chicago) and bred in house as described elsewhere [91]. The mice were housed socially on a 12-hour day-night cycle and had *ad libitum* access to food and water. All experiments were approved by the Animal Welfare Committee of Maastricht University and followed the laws, rules, and guidelines of the Netherlands.

Cytokines analysis

Cytokines were measured in total protein homogenate extracted from the cortex in lysis buffer containing 0.1% SDS, 0.1% Triton X-100, 1% glycerol, 1 mM EDTA, 1 mM EGTA, phosphatase and protease inhibitors. Samples were diluted to 0.1mg / mL total protein and cytokines (IFN- γ , IL-1 β , TNF- α , IL-6, IL-4, IL-10, and IL-33) were measured on Meso Scale Discovery V-PLEX custom mice (K152A0H-1) and read on a Meso Scale Discovery SECTOR 600 (Meso Scale Discovery).

Supplementary figures

Supplementary figure 1. Immunostaining of CERT_L in the brain and analysis of CERT_L Aβ interaction by far Western blot. A) Photomicrographs of 5xFAD brain section showing CERT and APP/Aβ colocalization intra and extra neurons. CERT is shown in red, APP/Aβ in green, neurons in magenta and nuclei in blue. Arrows point to intra and extra-neuronal colocalization B) Image showing detection of recombinant CERT_L separated by SDS-PAGE, transferred to membrane and detected with anti-CERT_L antibody without Aβ₁₋₄₂ peptide preincubation. CERT_L was detected with anti-Aβ antibody after Aβ₁₋₄₂ peptide preincubation. IgG remained negative control. C) Image showing detection of Aβ₁₋₄₂ peptide separated by SDS-PAGE, transferred to membrane and detected with anti-Aβ antibody (6E10). After recombinant CERT_L incubation, Aβ₁₋₄₂ peptide was immunolabeled with anti-CERT_L antibody but not a 17 kDa Lama antibody fragment (H6) (negative control). D) Measurement of Thioflavin T (ThT) fluorescence intensity to detect aggregation of Aβ₁₋₄₂, concentrated 20 μM, alone or in presence of recombinant CERT_L 2.5 μM, anti- Aβ (epitope 1-16) or 17-24 (dosed 0.1mg/mL) at 20 hours. Each data point represents the mean fluorescent intensity of three wells.

Supplementary figure 2. CERTs concentration in WT compared to 5xFAD and evidence of CERT_L transduction *in vitro* and *in vivo*. A) AAVs transduction on neuronal primary cell culture. Neonatal rat neuronal cells were isolated from the cortex of pups at embryonic day 18. After 2-3 weeks in Neurobasal medium were incubated with AAVs particles at the dose of 100 multiplicity of infection (MOI) for 6 days. CERT_L was visualized in red and nuclei in blue. B) A timeline describing the experimental design to assess transduction efficiency *in vivo*. C) AAVs transduction in WT animals 1, 2, 6- and 12-weeks post-injection by immunofluorescence. CERT_s were visualized in red and nuclei in blue (scale bar 50 μ m). D) Immunofluorescence staining on brain sections showing injections site (CERT_L in red and nuclei in blue). Scale bars 200 and 50 μ m. **M1**: motor sensory cortex 1; **M2**: motor sensory cortex 2; **LV**: lateral ventricle; **I, II, III, IV, and V** cortical layers. E) AAVs were tested for brain transduction in WT animals 1, 2, 6- and 12-weeks post-injection by qPCR. Bars represent the mean $\pm$ S.E.M 3/per group. A significant increase in CERTL transduction was measured independently from time point (ANOVA; * p < 0.05).

Supplementary figure 3. Sphingolipid changes in the hippocampus region of AAV-control or AAV-CERTL treated WT and 5xFAD mice. Sphingolipids levels were measured in the hippocampus by HPLC-MS/MS. Ceramides were classified based on acyl chain number of carbons (Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/18:1, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:1). as well as sphingomyelin (SM d18:1/16:0, SM d18:1/18:0, SM d18:1/18:1, SM d18:1/20:0, SM d18:1/22:0 and SM d18:1/24:1). Ceramides levels were expressed as pg / mg tissue, while sphingomyelins were expressed as pmol/mg tissue. Bars represent the mean $\pm$ S.E.M per group (Two-way ANOVA, LSD, significant effects, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Supplementary figure 4. Sphingolipid changes in plasma of AAV-control or AAV-CERTL treated WT and 5xFAD mice. Plasma was collected 1 week before AAV injection (time1), 2 (time 2), and 6 (time 3) weeks after injection, and when the experiment was ended (time 4). Sphingolipids levels were measured at each time point by HPLC-MS/MS. Ceramides were classified based on acyl chain number of carbons (Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/18:1, Cer d18:1/20:0, Cer d18:1/22:0 and Cer d18:1/24:1). Ceramides concentration is expressed in μ Molarity. Bars represent the mean $\pm$ S.E.M per group (Two-way ANOVA, Bonferroni correction, significant effects, WT AAV-control vs 5xFAD AAV-CERT_L §p<0.05; 5xFAD AAV-control vs 5xFAD AAV-CERT_L *p<0.05).

Supplementary figure 5. Western blot analysis of APP fragments to study APP processing. TBS cortex homogenate was analyzed by Western blot FL-APP, CTF β , and A β bands were detected with 6E10 antibody at the band corresponding to respectively of 100, 15, and 6-4kDa.

Supplementary figure 6. Cytokines and apoptotic markers Bcl-2 and Caspase 3 were unaffected by AAV-CERT_L treatment. A) Cytokines measurements in brain homogenate with multiplex system IFN- γ , IL-1 β , TNF- α , IL-6, IL-33, IL-4, and IL-10 (5-10 number of animals per group). **B)** Analysis of gene expression of apoptotic markers Bcl-2 and Caspase-3 (4-5 number of animals per group, two-way ANOVA, Bonferroni correction).

Sphingoli	Cortex					Hippocampus					Cerebellum				
	WT		5xFAD			WT		5xFAD			WT		5xFAD		
	Me	ST	Me	ST	p-	Me	ST	Me	ST	p-	M	ST	M	ST	p-
SPH	0.3	0.2	0.3	0.1	<i>0.94</i>	0.2	0.1	0.3	0.1	<i>0.23</i>	0.4	0.1	0.3	0.1	<i>0.182</i>
SPA	0.5	0.2	0.5	0.2	<i>0.50</i>	0.3	0.1	0.5	0.2	↑0.0	0.4	0.2	0.4	0.1	<i>0.920</i>
S1P	2.8	1.6	3.0	1.6	<i>0.80</i>	2.0	0.8	3.0	1.8	↑0.0	3.2	1.5	3.6	2.3	↑0.01
Cer	0.1	0.1	0.1	0.0	<i>0.89</i>	0.0	0.0	0.0	0.0	<i>0.40</i>	0.0	0.0	0.0	0.0	<i>0.588</i>
Cer	0.8	0.3	1.1	0.3	↑0.0	0.6	0.3	0.8	0.2	↑0.0	0.5	0.3	0.6	0.2	<i>0.681</i>
Cer	0.2	0.1	0.2	0.1	<i>0.05</i>	0.1	0.1	0.2	0.1	↑0.0	0.1	0.0	0.1	0.0	<i>0.930</i>
Cer	40.	14.	43.	10.	<i>0.48</i>	36.	10.	43.	15.	<i>0.09</i>	14.	4.7	14.	3.9	<i>0.884</i>
Cer	1.4	0.4	1.7	0.4	<i>0.13</i>	1.0	0.3	1.4	0.3	↑0.0	0.8	0.2	0.9	0.2	<i>0.659</i>
Cer	0.6	0.2	0.7	0.2	<i>0.18</i>	0.4	0.2	0.6	0.2	↑0.0	0.6	0.2	0.6	0.2	<i>0.950</i>
Cer	50.	25.	55.	20.	<i>0.57</i>	31.	11.	32.	9.6	<i>0.78</i>	10	57.	90.	36.	<i>0.588</i>
Cer	0.4	0.2	0.5	0.2	<i>0.52</i>	0.2	0.1	0.4	0.2	<i>0.06</i>	0.5	0.2	0.5	0.2	<i>0.982</i>
Total SM	25	122	28	888	<i>0.26</i>	40	192	50	373	<i>0.43</i>	-	-	-	-	-

Supplementary Table 1 Sphingolipid levels on cortex, hippocampus, and cerebellum of WT and 5xFAD treated with AAV-control (student's t-test*p<0.05)

↑ 5xFAD mean significantly bigger than WT animals

Gene	GenBank Accession No.	Sequence (5'→3')	
Bax	XM_011250780.2	CACCTGAGCTGACCTTGGAG	Forward
		CCACGTCAGCAATCATCCTCT	Reverse
Caspase 3	NM_009810	TGCAGCATGCTAAGCTGTA	Forward
		GAGCATGGACAATACACG	Reverse
Bcl-2	NM_009741	TGGGATGCCTTTGTGGAAC	Forward
		GAGACAGCCAGGAGAAATCA	Reverse
CERT_L	NM_023420.2	ATGTTACAGATTCAGCTCCC	Forward
		CTTCTTCAACAACCAGTTGCC	Reverse
CERT	XM_011244694.2	CAGGATGTAGGTGGTGATGC	Forward
		CACCTTTAACTGCATGAGTAGC	Reverse
GAPDH	XM_017321385.1	CTCATGACCACAGTCCATGC	Forward
		TTCAGCTCTGGGATGACCTT	Reverse
Actin	NM_007393.5	CTCTCAGCTGTGGTGGTGAA	Forward
		AGCCATGTACGTAGCCATCC	Reverse
CD86	NM_019388.3	GGGCTTGGCAATCCTTATCT	Forward
		ACCAACTTTTGCTGGTCCTG	Reverse
Fizz-1	NM_020509.3	GGAAGTCTTGCCAATCCAG	Forward
		ACACCCAGTAGCAGTCATCCC	Reverse

Supplementary Table 2. RT-PCR primer sequences

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Chapter 4

FTY720 decreases ceramides levels in the brain and prevents memory impairment in a mouse model of familial Alzheimer's disease expressing APOE4

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Chapter5

The treatment effect of FTY720 on sphingolipid disbalance and cognitive decline in aged EFAD transgenic mice

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Chapter 6

General discussion and summary

The overall aim of this thesis was to investigate sphingolipid metabolism as an underlying factor in the pathogenesis of Alzheimer's disease and to modulate sphingolipid levels by gene therapy and pharmacological intervention. In my work, we found that CERT_L, the long isoform of the ceramide transporter protein, could be an effective target to inhibit the progression of AD by direct binding to A β and reducing neuroinflammation directly or indirectly. Secondly, our aim was to investigate whether FTY720, a sphingosine-1-phosphate (S1P) analog, could rebalance sphingolipid levels and change cognition and pathophysiology in an AD mouse model. Our results showed that FTY720 reduced pathophysiological changes and prevented cognitive decline in early stages of AD in mice, but unfortunately, it did not have treatment efficacy when administered for short time at late stages of AD. Overall, this thesis shows that sphingolipids rebalance is potentially a good treatment strategy, which could interfere with the progression of AD.

Sphingolipid disbalance in the progression of Alzheimer's disease

Bioactive sphingolipid including ceramide (Cer), sphingomyelin (SM), sphingosine and sphingosine-1-phosphate (S1P), play an important role in AD development [1, 2]. Abnormal ceramides levels have been found both in brain and plasma of AD patients [3-6] and it has been shown to enhance β and γ secretase activity in AD [7, 8]. Interestingly, the elevation of sphingomyelin levels was associated with cognitive progression [9], and was influenced by sex and APOE genotype [10]. Since the ratio change of ceramide and sphingomyelin could predict the progression of AD, we investigated the sphingolipid metabolism in mice with different sex and genetic background in **chapter 3**, 3 to 4 months old 5XFAD male mice were used as experimental model and the brain tissue was collected at 6 to 7 months of age. This model normally presents with early amyloid aggregation starting at the age of 2 months and neuronal loss from 6 months of age. The results showed that long chain ceramides including Cer d18:1/16:0, Cer d18:1/18:1, and very long chain ceramides including Cer d18:1/20:0, and Cer d18:1/22:0 were increased in the cortex, while sphingomyelin did not show any significant change. These observations confirmed previous reports showing a global rise in ceramide levels in specific AD brain regions [11].

CERT_L as a potential target for AD treatment

Ceramide transporter proteins (CERT_s) transfer ceramide from the endoplasmic reticulum (ER) to the Golgi apparatus to generate sphingomyelin [12]. CERT_L, the long isoform of CERT, has an important function during embryogenesis [13, 14] and lack of ceramide transport during embryogenesis could induce ceramide accumulation in the ER, leading to embryonic death and tissue loss in the brain [14-16]. In our previous study, we found the CERT_s are ubiquitous proteins present in all cell types and co-localize with serum amyloid P complement (SAP) that bind to amyloid deposit [17, 18]. In **chapter 3**, the effect of CERT_L on AD pathogenesis was explored in the 5XFAD mouse model. Firstly, we found that ceramides include Cer d18:1/16:0 were increased in the cortex as mentioned above, whereas CERT_s levels were lower in the AD cortex compared to control brain. After injection of CERT_L into the frontal cortex, only Cer d18:1/16:0 was significantly reduced in 5XFAD mice. However, ceramide levels were reversely elevated again along with increased amyloid plaques numbers when animals were treated with HPA-12, a CERT inhibitor which blocks ceramide trafficking [19]. These results suggested that CERT_L probably helped the trafficking of Cer d18:1/16:0 and further prevent the worsening of the pathophysiology of AD. As next step, we

investigated the function of CERT_L in amyloid plaques formation and in amyloid precursor protein (APP) processing. Results showed that CERT_L reduced A β production and plaque size.

As innate immune cells in the central nervous system (CNS), glia cells protect the brain from injury and disease. In the AD brain, microglia is excessively activated by releasing pro-inflammatory markers, such as IL-1, IL-6, and CD86, subsequently induced neuronal damage [20]. Therefore, we also focused on whether CERT_L produced neuroinflammatory effects in this model. We performed several techniques including immunohistochemistry (IHC) and RT-PCR in the cortex to clarify it. The analysis revealed reduced numbers of activated microglia cells and showed reduced expression levels of CD86, a pro-inflammation marker, in the 5xFAD CERT_L injected group compared to the untreated group. Thus, CERT_L also has a potential anti-inflammatory protective effect in this AD model. Even if these data represent a great advancement in this field, further investigations are needed to better understand the upstream and downstream effect of CERT_L modulation in AD mouse models. These outcomes could be potentially used to develop new pharmacological treatments for AD.

APOE as a main factor for sphingolipid metabolism in AD progression

In **chapter 3**, we did not take the influence of the APOE genotype for the pathogenesis of AD into consideration. Since the APOE gene is a major risk factor for non-familial AD [21], we further investigated the influence of the APOE genes on the sphingolipid metabolism in the 5XFAD mouse model. 5XFAD was crossed with human APOE3 or APOE4 genes respectively (APOE4, E4FAD and APOE3 and E3FAD) and used as experimental model in **chapter 4 and 5**. Firstly, we explored it in a female model of 6 to 7 months of age (**chapter 4**). When we focused on the comparison between APOE3 and APOE4 genes, we found that APOE4 carriers from both control and AD groups had higher sphingomyelin levels in the cortex compared to APOE3 carriers, whereas ceramide did not show a large difference. This was inconsistent with the sphingolipid changes we had found in **Chapter 3**. Together, our results indicated that the change of sphingolipid levels was more dependent on APOE genes than AD status (the gender difference in these 2 studies should be also kept in mind). In **chapter 5**, we used the same gene background but male mice of 11 to 12 months of age were used. We found that the majority of ceramides were increased compared to sphingomyelin in APOE4 carriers. It is interesting to see the opposite changes of these sphingolipids between younger and older age. Understanding the sex, age, and APOE association will help to better diagnose and define possible personalized treatments for AD. More continuous studies are needed to explore the underlying mechanisms.

FTY720 as a potential target for AD prevention

FTY720, a sphingosine-1-phosphate receptor (S1PR) modulator, which binds to the S1PRs, promoting a protective effect by mediating the lymphocyte egress [22]. In **chapter 4**, we focused on the question whether FTY720 would prevent the pathophysiology of AD using the young EFAD transgenic model. Regarding to the memory tests, water maze was used for the spatial memory evaluation. The results showed that FTY720 improved the time that the experimental mice spent in the target quadrant and more mice successfully found the platform, particularly in the low dose treated E4FAD group, compared to untreated group. Besides, the alternative memory performance showed in the Y maze test that treated mice presented with a higher correct alternation. Next, we examined the drug effect on amyloid levels. The low dose of FTY720 reduced by almost half the levels of insoluble A β in the cortex. These results were in

line with previous studies [23, 24]. Regarding the sphingolipid metabolism, the main classes including ceramide, sphingomyelin, dihydroceramide, and hexosyl-ceramide were measured. FTY720 reduced ceramide and sphingomyelin levels, particularly, SM d18:1/24:1 was reduced when the low dose FTY720 was administered. Our study shows that FTY720 has the ability to prevent cognitive impairment via reducing the A β level in the brain, while this process is potentially correlate to reduced levels of sphingolipids.

Because of these promising findings in **Chapter 4**, we further investigated whether FTY720 could reverse AD in aged EFAD mice by affecting sphingolipid metabolism in **chapter 5**. However, we did not find improvement in the behavioral tasks nor changes in A β levels. But FT720 remarkably reduced ceramide levels in this model, suggesting that sphingolipids are prognostic markers of AD [25]. The short-term treatment or severe AD status should be taken into account as the reasons why less protective effect on behavioral tasks and A β level are observed. Taken the results of chapter 4 and 5 together, FTY720 provide a potential possibility for modulation of AD.

Conclusions

Our research aim was to explore the role of sphingolipids in AD pathophysiology. Given this, we explored sphingolipid metabolism via gene therapy and drug administration. We revealed that CERT_L could be a novel target for AD by mediating sphingolipid balance and modulating the neuroinflammation in the brain. Furthermore, APOE gene, sex, and brain region are important factors which impact on the changes of sphingolipid levels. Regarding the drug prevention and treatment studies, our results show that FTY720 could be a potential preventive drug for early stages of AD, but more studies are needed to investigate if it can also be useful to treat more advanced stages of the disease. Overall, our study emphasizes the important of the sphingolipid metabolism in AD but also reveals the complexity of this pathway. Therefore, more specific studies would be very helpful to understand the underlying mechanism.

Chapter 7

Valorization

According to the world Alzheimer report 2021, more than 60 million people live with Alzheimer's disease (AD), 1/3 seniors dies with dementia and as estimated 130 million individuals will suffer from this disease in 2050, which corresponds with growing economic burden globally [26]. In the past decades, thousands of studies were mainly focused on amyloid and tau cascades to explore potential diagnostic and treatment methods for it. In fact, there remains no efficient cure for the disease although drugs have been approved for reducing symptomatic characteristics with a short-term benefit [27]. In this thesis, we have new evidence that the sphingolipid metabolism plays an important role in pathophysiology of AD, and we show evidence on the promise of the development of novel diagnostic tools and intervention strategies based on lipids. Our study revealed the critical role of sphingolipid in AD. We found that the change in sphingolipid levels depend on age, sex and APOE gene background. Of interest is that the APOE gene showed a much more pronounced effect on sphingolipid levels than the AD status in the EFAD mice model.

In **chapter 3**, we showed the long form of ceramide transporter protein (CERT_L) played an important role in AD pathophysiology. Higher levels of CERT_L injected into the cortical layer of 5XFAD mice, lower amyloid, and ceramide levels were observed. However, these changes were abolished after HPA-12 administration, which inhibited the trafficking of ceramide. We also found that CERT_L induced less microglia activation. These suggested that CERT_L may have a potential role in AD treatment by down modulating the amyloid and neuro-inflammation pathways. However, we did not find improvement in working memory in chapter 3 with Y maze or spatial Y maze [28]. We used an adapted 5xFAD model (C57BL/6J background and without the Pde6b gene) in this study, only small deficits in spatial memory were visible at 7 months of age [29]. In our study, we did not see differences in the behavioral tests among the studied groups, except for a very modest improvement in the SYM novel arm-exploring test. These results encouraged us to select more informative and sensitive methods for further confirmation of these results.

In **chapter 4**, to investigate the role of the APOE4 isoform on AD pathophysiology, the EFAD model was used in the study [30, 31]. We found that sphingolipid levels were more depended on APOE gene rather than AD status, and higher ceramide levels were found in E4FAD animals. After FTY720 treatment, ceramide levels were reduced in the EFAD group. Interestingly, FTY720 also decreased A β levels and CD86 mRNA expression levels in the E4FAD treated group, although the underlying mechanism is unclear. The results motivated us to explore the intervention effect of FTY720 in older aged mice. Thus, 6 weeks of FTY720 was given to 9.5 to 10.5 months old of EFAD mice in **chapter 5**. We found that FTY720 decreased the levels of ceramide in the majority of ceramide species but did not affect amyloid levels or improve behavioral outcomes.

There are a few limitations in these studies. Regarding the behaviors tests, the results did not show a significant change between EFAD mice and corresponding APOE carriers' mice in both chapters. These experimental mice model was originated from 5XFAD and crossed with human APOE gene, which induced the delay of A β aggregation and of behavior deficits. Until now, there are not many publications showing specific APOE associated behavioral deficits. Ladu's group has shown some difference in the Morris water maze and Y maze on this model in the EFAD mice and the corresponding APOE carriers [31]. In contrast, Felecia et al, did not find differences between EFAD and APOE carriers [32]. These conflicting results might be due to discrepancy in the methodology between

laboratories. Therefore, optimizing the current test protocols or selecting more precise and sensitive experimental methods would be very helpful to investigate the behavior deficits.

Regarding the effects of FTY720, it is important to mention that it showed protective effects in 6 to 7 months old female mice, which received 3 months treatment. However, when FTY720 was given for 6 weeks to 9.5 - 10.5 months old male mice in chapter 5, we did not see significant differences in amyloid levels and their cognitive ability did not improve. These mice were severely ill at this age, and the pathophysiological damage might be too severe and becomes irreversible. Besides, according to the literature, FTY720 generated protective effects after at least 2-months treatment. Therefore, a short-term treatment may be causative for the unchanged AD pathology as well.

Even though the abovementioned limitations, our studies demonstrate that changes of sphingolipids rely more on the APOE gene rather than AD status. Sex and age are also important factors, which induce differences in the levels of sphingolipids in AD mice. Changes in sphingolipid metabolism using ceramide transporter proteins modulators could reduce A β levels, neuroinflammation and cognitive impairment, suggesting that sphingolipids could be a potential target in AD treatment. Overall, these findings also encourage us to explore sphingolipid metabolism in more specific conditions, which would help to clarify the underlying mechanisms.

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Appendix

Acknowledgements
List of publications
Curriculum Vitae

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List of publications

1. **Qian Luo***, Simone Mwenda Crivelli*, Jo Stevens, Caterina Giovagnoni, Daan van Kruining, Gerard Bode, Sandra den Hoedt, Barbara Hobo, Anna-Lena Scheithauer, Jochen Walter, Monique T Mulder, Christopher Exley, Michelle M. Mielke, Helga E De Vries, Kristiaan Wouters, Daniel L.A. van den Hove, Dusan Berkes, María Dolores Ledesma, Joost Verhaagen, Mario Losen, Erhard Bieberich, Pilar Martinez-Martinez *CERTL Reduces C16 Ceramide, Amyloid- β Levels and Inflammation in a Model of Alzheimer's Disease*, ***Alzheimer disease & therapy***, 2021, (IF:6.116)
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Curriculum Vitae

Qian Luo was born on December 20th1991 in Shandong, China. In 2010, she was admitted to Linyi University to start her bachelor's study with the major in Pharmacy. After obtaining her degree in 2014, she continued her study in Shandong university in faculty of Pharmacy. She got 3 times of university scholarships during her master process. When she completed her master study in 2017, and subsequently moved to Maastricht university to start her PhD research. Her study was supported by CSC scholarship and supervised by Prof. Pilar Martinez. Her PhD is about sphingolipid function in Alzheimer's disease, which aims to investigate the potential pathophysiological effects of sphingolipids and pharmacological intervention targets.