LIPIDS IN THE BRAIN

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Maria Dolores Ledesma and Fanny M. Elahi
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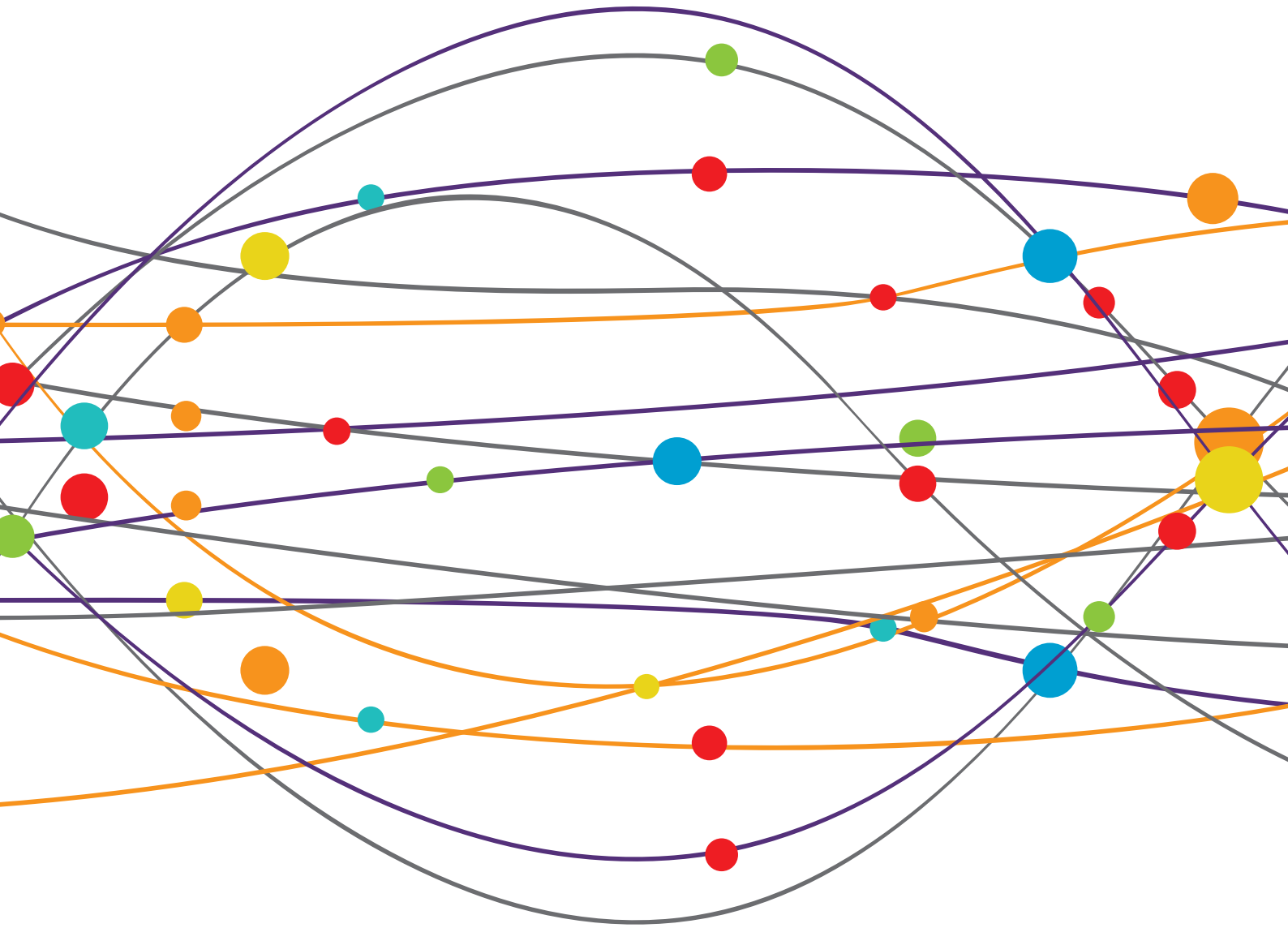

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LIPIDS IN THE BRAIN

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EDITORIAL
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Editorial: Lipids in the Brain

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Keywords: Alzheimer, lipid, sphingolipid, cholesterol, neurodegeneration

Editorial on the Research Topic

Lipids in the Brain

Until about 30 years ago, lipids were thought to only play a structural role in cell membranes. Over time they have acquired a new face and today represent a new frontier research aimed at studying them as biologically active molecules essential for cell fate, especially in the central nervous system (CNS).

This special issue includes nine papers, five reviews, and four research articles, covering some of the more recent and exciting findings about the role in the brain physiopathology.

The first paper of the special issue is a review written by Corraliza-Gomez et al. "Lipid-Binding Proteins in Brain Health and Disease" that summarizes a systematic literature search to identify lipid interacting proteins and the biological processes in which they are involved related to nervous system function and dysfunction. Lipid-binding scaffolding proteins, membrane transporters and receptors, and transcription regulators contribute to blood brain barrier crossing and maintenance, control of oxidative stress and inflammation, organelle function, vesicle trafficking, myelin management, and amyloid dynamics. When altered, they may become therapeutic options.

The minireview by Torres et al. "Mitochondrial Cholesterol in Alzheimer's Disease and Niemann–Pick Type C Disease" illustrates the case of the aberrant accumulation of cholesterol in mitochondria as a common pathological event in Alzheimer disease (AD) and Niemann Pick type C. Mitochondrial cholesterol accumulation alters membrane physical properties and impairs the transport of glutathione into this organelle disrupting the antioxidant defense. The potential role of the up- regulation of a family of lipid transporting proteins containing StAR-related lipid transfer domains (StARD), by different mechanisms in the two diseases, is discussed.

The minireview of Isacson et al. "Novel Results and Concepts Emerging From Lipid Cell Biology Relevant to Degenerative Brain Aging and Disease" describes biochemical and clinical evidence of an interdependence of lipids and proteinpathy in neurodegenerative diseases (NDD). The authors illustrate that Parkinson's disease (PD) can be triggered by lipid disturbances caused by lysosomal genetic or similar age-induced enzymatic loss of function. The lipid-carrying apolipoprotein E4 variant is associated with increased risk for dementias and α-synuclein may even have a cooperative role with apolipoproteins and lipid transport.

Given the biological links between sphingolipids and brain injury, Azizkhanian et al. in "Plasma Lipid Profiling Identifies Biomarkers of Cerebral Microvascular Disease" investigate the biomarker value of plasma sphingolipids as a non-invasive marker of chronic subclinical cerebral small vessel disease. To this end they quantify the association of plasma sphingolipid (SphLs) levels with white matter hyperintensity volumes on brain magnetic resonance imaging, the current imaging gold-standard for cerebral small vessel disease. They find that levels of certain plasma SphLs are highly associated with white matter hyperintensities.

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As the relevance of lipid alterations is confirmed for the pathology of numerous brain diseases the use of lipid levels as diagnostic tool gains interest. Wai Kin Wong et al., in "Comparison of Single Phase and Biphasic Extraction Protocols for Lipidomic Studies Using Human Plasma" compare the efficacy of different lipid extraction protocols for lipidomic studies. Using human plasma from normolipidomic adult volunteers they show that the recently developed single phase Alshehry method, which also has the advantage of avoiding the use of chloroform, is more effective than established biphasic methods particularly in the extraction of polar lipids.

The following paper spans the association between lipid dysmetabolism with complex psychiatric symptomatology. Taking an innovative approach to the study of acid sphingomyelinase pathways in comorbid psychiatric symptoms of depression-alcoholism, Kalinichenko et al. in "Enhanced Alcohol Preference and Anxiolytic Alcohol Effects in Niemann-Pick Disease Model in Mice" use acid sphingomyelin-knockout mice to show that forced alcohol consumption decreases anxiety while increasing depression in these models.

Martin-Segura et al. in "Aging Increases Hippocampal DUSP2 by a Membrane Cholesterol Loss-Mediated RTK/p38MAPK Activation Mechanism" study a mechanism that would link age-associated cholesterol changes to the chronic sterile inflammation when homeostatic control of kinases is lost. The authors provide compelling findings on the involvement of p38MAPK activity and its downstream targets in the hippocampus of old mice. The work presented builds on this to show that age-dependent loss of membrane cholesterol takes part in a negative-feedback loop that keeps p38MAPK activity levels within physiological range.

The next review "Yin-Yang Mechanisms Regulating Lipid Peroxidation of Docosahexaenoic Acid and Arachidonic Acid in the Central Nervous System" by Yang et al. provide information on the role of Yin-Yang mechanism in the release of arachidonic acid and docosahexaenoic acid due to different phospholipases A_2 and in the regulation on their lipid peroxidation. The products of lipid peroxidation may be detected in body organs and fluids and may confer cytotoxic or protective effects depending on the conditions for production. The review reports in detail the involvement of these lipids in neurological and inflammatory pain, cerebral ischemia and brain injury, several neurodegenerative and neuropsychiatric disorders.

The review "Exploring Sphingolipid Implications in Neurodegeneration" by Alessenko and Albi provides evidence of participation of SphLs in the pathogenesis of AD, Parkinson's disease (PD) and, Amyotrophic Lateral Sclerosis (ALS). Recent studies have shown that SphLs play a decisive role in the neuronal function due to regulation of cell growth, differentiation, and death in the CNS. The review discusses results obtained *in vitro* and *in vivo*, such as brain tissue from both animals in which diseases were induced and humans in autopsy samples, liquor, and blood. It was highlighted that SphL species might be diagnostic markers and/or new targets for innovative therapeutic strategies.

Thus, the special issue gives an overview about the current knowledge and highlight interesting new insights into the roles of the complex world of lipids in the brain and on its involvement in NDD.

AUTHOR CONTRIBUTIONS

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REVIEW
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Yin-Yang Mechanisms Regulating Lipid Peroxidation of Docosahexaenoic Acid and Arachidonic Acid in the Central Nervous System

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Phospholipids in the central nervous system (CNS) are rich in polyunsaturated fatty acids (PUFAs), particularly arachidonic acid (ARA) and docosahexaenoic acid (DHA). Besides providing physical properties to cell membranes, these PUFAs are metabolically active and undergo turnover through the "deacylation-reacylation (Land's) cycle". Recent studies suggest a Yin-Yang mechanism for metabolism of ARA and DHA, largely due to different phospholipases A_2 (PLA_2s) mediating their release. ARA and DHA are substrates of cyclooxygenases and lipoxygenases resulting in an array of lipid mediators, which are pro-inflammatory and pro-resolving. The PUFAs are susceptible to peroxidation by oxygen free radicals, resulting in the production of 4-hydroxynonenal (4-HNE) from ARA and 4-hydroxyhexenal (4-HHE) from DHA. These alkenal electrophiles are reactive and capable of forming adducts with proteins, phospholipids and nucleic acids. The perceived cytotoxic and hormetic effects of these hydroxyl-alkenals have impacted cell signaling pathways, glucose metabolism and mitochondrial functions in chronic and inflammatory diseases. Due to the high levels of DHA and ARA in brain phospholipids, this review is aimed at providing information on the Yin-Yang mechanisms for regulating these PUFAs and their lipid peroxidation products in the CNS, and implications of their roles in neurological disorders.

Keywords: arachidonic acid, docosahexaenoic acid, $cPLA_2$, $iPLA_2$, lipid peroxidation, 4-hydroxyhexenal, 4-hydroxynonenal, neurodegeneration

PHOSPHOLIPIDS IN THE CENTRAL NERVOUS SYSTEM ARE ENRICHED IN ARA AND DHA

The mammalian brain is rich in lipids, with phospholipids playing important roles in biological functions. Changes in composition of membrane phospholipids can lead to alteration of cell functions, including signal transduction, cell-cell recognition, DNA replication, and protein trafficking (1), and with implications in neurological diseases, such as stroke, traumatic brain

injury (TBI), depression (2), as well as Alzheimer's and Parkinson's diseases (AD and PD) (3, 4). Major phospholipids in brain include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylethanolamine plasmalogen (PEpl), together with smaller amounts of phosphatidylserine (PS), phosphatidylinositol (PI), and cardiolipin (CL) (5) (**Figure 1**). While these phospholipids are present in different types of cell membranes, CL is specifically present in the inner layer of mitochondrial membrane (6). Phospholipids have fatty acids (FAs) that are largely saturated or monoenes in the *sn-1* position of the glycerol moiety, whereas the *sn-2* position contains mainly polyunsaturated fatty acids (PUFAs). A characteristic feature for PE in brain is the large proportion of PEpl with alkenyl group in the *sn-1* position. These PEpl are abundant in the myelin sheaths (7). The PUFAs in PE are enriched in docosahexaenoic acid (22:6 n-3, DHA), whereas the PUFAs in PC have both DHA and arachidonic acid (20:4 n-3, ARA). PS is an anionic phospholipid with high levels of palmitic acid (16:0) and DHA, and translocation of this phospholipid from the inner to outer membrane surface through the flippases and scramblases can serve as an initiator for apoptotic processes through binding with annexin V (8, 9). PI is comprised of high levels of stearic acid (18:0) and ARA, and the inositol head group can be phosphorylated to form PIP and PIP2. Hydrolysis of PIP2 by phospholipase C results in the production of diacylglycerols and inositol phosphates (5), which are second messengers for activation of protein kinase C (PKC) and for mobilization of calcium from intracellular stores, respectively (10). An obvious difference between the PUFAs in the central nervous system (CNS) and the peripheral system is the low levels of linoleic acid (18:2 n-6) in CNS (11).

In the mammalian brain membranes, the PUFAs in the phospholipids (mainly PC and PE) are metabolically active and undergo turnover through the "deacylation-reacylation cycle", also known as the "Land's cycle" (12, 13) (**Figure 1**). This cycle enables PUFAs to be released from membrane phospholipids through phospholipases A_2 (PLA_2s) and subsequently return to the membrane phospholipids through the lysophospholipid acyltransferases. In the CNS, different PLA_2s are responsible for the release of DHA and ARA from phospholipids, thus suggesting a Yin-Yang mechanism for their metabolic functions (14). Besides production of eicosanoids and docosanoids, which are lipid mediators, these PUFAs are also substrates of oxygen free radicals, resulting in alkenal products that are metabolically active. In this review, attention is focused on factors regulating metabolism of ARA and DHA through different PLA_2s, and the role of their peroxidation products in health and disease.

ARA Release by $cPLA_2$

As reviewed by Sun et al., release of ARA from phospholipids is catalyzed mainly by the Group IV calcium-dependent cytosolic $PLA_2\alpha$ ($cPLA_2$), a ubiquitous enzyme present in all cells in the CNS (15). Besides the requirement for calcium which binds to the C2 domain, a characteristic property of the $cPLA_2$ is its susceptible to phosphorylation and activation by protein kinases, including the mitogen activated protein kinases (MAPKs) and PKC (16). A study with primary neurons demonstrated ability for NMDA (an excitatory glutamate receptor agonist) to stimulate phosphorylation of $cPLA_2$ through activation of ERK1/2 (17). Studies with microglial cells also indicated the ability of lipopolysaccharides (LPS) to stimulate p-$cPLA_2$ through p-ERK1/2 (18, 19).

Activation of $cPLA_2$ and release of ARA have been implicated in a number of neurologic disorders and brain injury. Subjects with traumatic brain injury (TBI) showed a significant increase in PUFAs, including ARA, in the cerebrospinal fluid as compared with non-TBI controls (20, 21). In a study with acute stroke subjects, the decrease in DHA/ARA ratios in serum was attributed to the increase in $cPLA_2$ activity and release of ARA (22, 23). Indeed, increases in p-$cPLA_2$ were observed in animal models of cerebral ischemia (15). Furthermore, the transient increase in p-$cPLA_2$ suggests tight regulation of this enzyme under pathological conditions (24).

A number of studies with animal models have demonstrated the involvement of $cPLA_2$ in the pathophysiology of AD (25). Evidence included increases in ARA as well as its metabolites in the brain tissue of AD mutant mice (26). Studies with cultured neurons also demonstrated ability for the cytotoxic amyloid beta (Aβ) to stimulate phosphorylation of ERK1/2 and p-$cPLA_2$ (17). Since action of $cPLA_2$ not only releases PUFAs but also lysophospholipids, the increase in lysophosphatidylcholine (LPC) in AD mouse models was marked with a progressive decline in behavior (13). The changes in FAs as well as LPC are in agreement with perturbation of the "Land's cycle." Using mass spectrometry (MS)-based shotgun lipidomics, analysis indicated increases in MAPK, PLA_2, unesterified PUFAs, as well as LPC in the brains of AD transgenic mouse model (14). Using antisense nucleotide against $cPLA_2$, studies by Levy's group demonstrated ability for aggregated Aβ to up-regulate $cPLA_2$ in neurons and microglia (27, 28). Taken together, these studies demonstrate an important role for $cPLA_2$ and the release of ARA in response to brain injury as well as in different neurologic disorders.

The Release of DHA by $iPLA_2$

The group VI calcium-independent PLA_2s ($iPLA_2$) are comprised of a number of isoforms and can release PUFAs (including ARA) from phospholipids. However, several studies suggested preference for the release of DHA through $iPLA_2\beta$ (29–32). As reviewed by Farooqui and Horrocks, $iPLA_2\beta$ is abundant in the brain, and is able to influence a number of CNS functions (33, 34). $iPLA_2$-knockout mice showed a decrease in DHA metabolism, further demonstrating the association between DHA and $iPLA_2$ (35). Recently, there is suggestion for a genetic link for this $iPLA_2$ to PD (36). Mutation of $iPLA_2$ was also linked to an infantile autosomal recessive gene disorder with brain iron accumulation, a neurologic disorder showing severe psychomotor and cognitive dysfunction (37, 38). The study by Shinzawa et al. showed neurologic impairments as well as accumulation of spheroids containing tubulovesicular membranes in a mouse model depleting this group of $iPLA_2$ (39).

A study by Gattaz et al. indicated a significant decrease in activity of $iPLA_2$ in platelets from AD patients and subjects with mild cognitive impairment (MCI) compared to normal controls (40). In fact, subjects with MCI also showed alterations

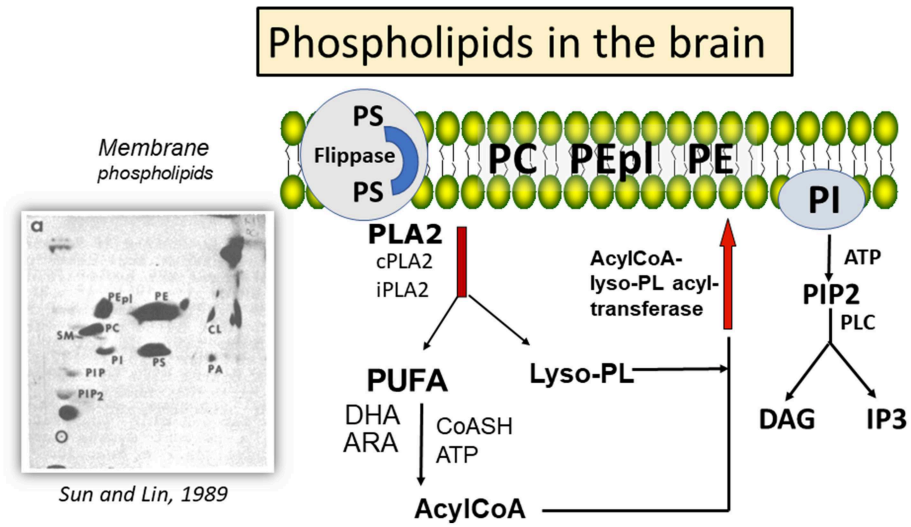

FIGURE 1 | Mechanism for deacylation-reacylation of polyunsaturated fatty acids in phospholipids, and relative amount of phospholipids in the brain. [a]High performance thin layer chromatography (HPTLC) separation of phospholipids in mouse cortex and detection by charring with cupric acetate; PE, phosphatidylethanolamine; PEpl, PE plasmalogen; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; CI, cardiolipin; PIP, phosphatidylinositol-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate. HPTLC chromatograph was reprinted from Sun and Lin (5), with permission from Elsevier.

of other types of PLA_2, including $cPLA_2$ and $sPLA_2$. Chew et al. provided evidence for $iPLA_2$ to play a role on synaptic plasticity and nociception, and these neuronal abnormalities could be reversed by suppressing $iPLA_2$ through antisense oligonucleotide (41). Studies using hippocampal slices indicated involvement of $iPLA_2$ in induction of long-term potentiation (42), and in agreement with the release of DHA through $iPLA_2$, impairments in long-term potentiation could be reversed upon treatment with DHA (43, 44). Taken together, these studies provide support for the role of $iPLA_2$ and DHA in modulating brain function.

LIPID MEDIATORS DERIVED FROM ARA AND DHA

PUFAs are substrates for a number of oxygen enzymes including cyclooxygenases (COX), lipoxygenases (LOX), cytochrome P_{450} ($CytP_{450}$), soluble epoxide hydrolase, and prostaglandin dehydrogenase, resulting in the production of a wild array of lipid mediators (**Figure 2**). Depending on the cell types and conditions, these lipid mediators have shown protective and detrimental effects. ARA is converted to prostaglandin H2 (PGH2) through COX1/2, and in turn, PGH2 is further converted to prostanoids (prostaglandins and thromboxanes) with both inflammatory and anti-inflammatory properties (18, 45). ARA also serves as substrate for LOX 5/12/15 to produce leukotrienes and lipoxins, and these lipid mediators work through specific receptors to mediate a variety of responses (46, 47). Interestingly, in contrary to the leukotrienes which exert cytotoxic and inflammatory effects in blood cells, lipoxin A2 and lipoxin B4 (synthesized through 5- and 15-lipoxygenases) are mediators with anti-inflammatory and immunomodulatory properties (see overview by Parkinson) (48). In a study by Livne-Bar et al, metabolomics screening for neuroprotective signals in astrocyte conditioned medium indicated the ability for lipoxin A4 and B4 to suppress injury-induced damage in retina (49). In another study on an intracerebral hemorrhage model in rat, lipoxin A4 was shown to offer anti-inflammatory effects and ameliorated the ischemic injury (50).

DHA is metabolized by a specific LOX (e.g., 15-lipoxygenase, Alox15) to form oxylipin intermediates leading to synthesis of resolvin D1 (RvD1), maresins, and neuroprotectin D1 (NPD1) (**Figure 2**). These structurally distinct molecules possess pro-resolving and pro-homeostatic properties (51–56). RT-PCR and Western blot analysis indicated high Alox 15 expression in the prefrontal cortex, olfactory bulb, and the hippocampal brain area (51). A study with human neuroblastoma cells demonstrated upregulation of Alox15 in association with histone deacetylases (HDAC), thus suggesting an epigenetic link (57). Aspirin-triggered RvD1 was shown to mitigate inflammation by enhancing the pro-resolution status as well as protecting the brain from synaptic dysfunction (58). Systemic prophylaxis treated with aspirin-triggered RvD1 was shown to improve surgery-induced cognitive decline and abolish synaptic dysfunction (59). Studies by Bazan's group indicated ability for NPD1to rescue inflammatory responses by inhibiting production of pro-inflammatory cytokines such as interleukin-1β (60). In a laser-induced mouse model, intraperitoneal injection of NPD1 could inhibit choroidal neovascularization, further confirming the mechanism for NPD1 to upregulate the resolution phase of the inflammatory response (61). Taken together, despite a Yin-Yang mechanism for metabolism of ARA and DHA, considerable cross-talks may occur among the lipid mediators in conferring detrimental and pro-resolving actions in different cell types and systems.

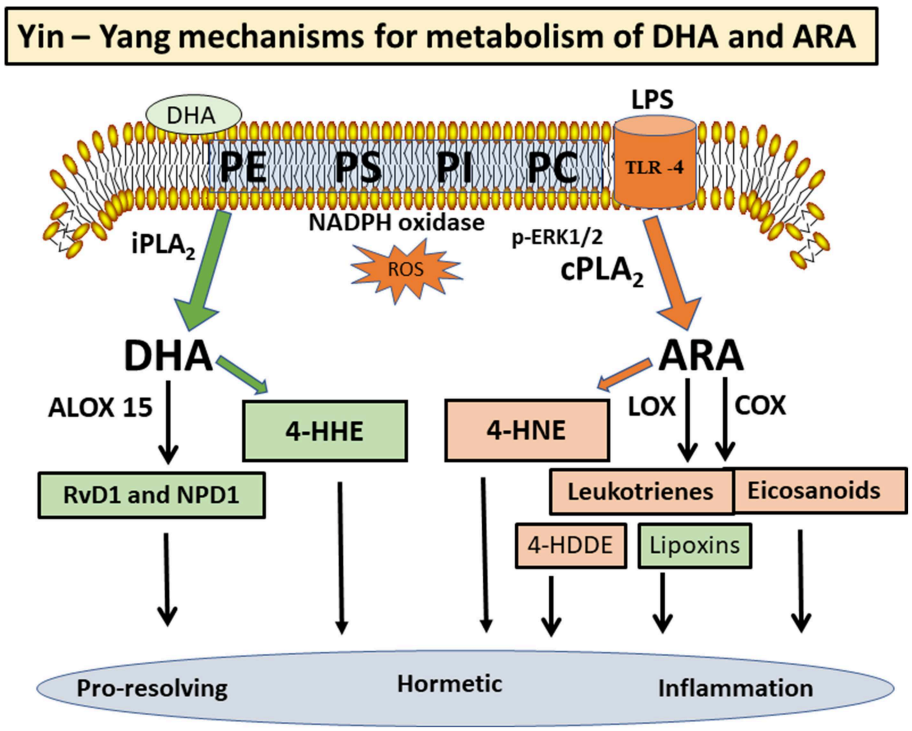

FIGURE 2 | Yin-Yang mechanisms for metabolism of DHA, ARA, and their metabolites.

LIPID PEROXIDATION OF ARA AND DHA

4-Hydroxy-alkenals

PUFAs, including DHA, ARA, linoleic acid, and linolenic acid, are susceptible to lipid peroxidation mediated by oxygen free radicals generated from a number of factors, including radiation, cellular response to xenobiotics, cytokines, bacterial and viral invasions, and mitochondrial oxidative stress (62, 63). A growing body of research has demonstrated the important role of lipid peroxidation in disease pathology, including diabetes, atherosclerosis, cardiovascular diseases, as well as neuroinflammation and neurodegenerative diseases (64–68). Oxygen free radicals can target the esterified PUFAs in membrane phospholipids as well as the free PUFAs in the cell cytoplasm. Peroxidation of these PUFAs is generally comprised of three main steps: initiation, propagation, and termination (69). These lipid peroxides are highly reactive, and upon interaction with iron, they can induce ferroptosis, which is a form of cell death different from that mediated by caspases (70, 71).

Depending on the position of the double bond, lipid peroxidation of n-3 and n-6 PUFAs can undergo fragmentation at different sites to give rise to a large number of carbonyl-containing lipid oxidation compounds including α, β-hydroxy aldehydes, acrolein, glyoxal, and malondialdehyde (72–74). The most prominent products are 4-hydroxyhexenal (4-HHE) from DHA and 4-hydroxynonenal (4-HNE) from ARA (75). In addition, ARA can react with 12-lipoxygenase to produce 12-hydroperoxy-eicosatetraenoate (12-HpETE), and in turn, undergoes peroxidation to form reactive 4-hydroxydodecadienal (4-HDDE) (69, 76, 77). These reactive aldehydes can readily undergo oxidation and reduction, and converted to acids or alcohols, which are more stable metabolites (78). Due to the high reactivity, these alkenal compounds are regarded as "second messengers of free radical reactions" (79), and biomarker of oxidative stress in inflammatory diseases [see review by Ito et al. (68)].

An important property of the 4-hydox-alkenal products is their ability to form adducts with proteins, phospholipids, and nucleic acids through Michael addition and Schiff base reactions (73, 74, 77, 78, 80–83). The phospholipid adducts are formed largely with phosphatidylethanolamine (PE), especially the alkenylacyl PE subtype (77). These alkenal compounds are present widely in the body organs, in different cell types (84), and in body fluids including plasma and urine (76). Studies by Sasson's group provided evidence for binding of 4-HNE and 4-HDDE to peroxisome proliferator-activated receptors (PPARs). The activation of PPARs leads to a mechanism for their hormetic and regulatory effects against the pathology of metabolic and vascular diseases such as diabetes and atherosclerosis (69, 85–88) (**Figure 3**). Other studies demonstrated formation of protein adducts with 4-HNE in other metabolic and inflammatory diseases (78), such as rheumatoid arthritis (89), neuroborrelious (90), and in age-related disorders and low back pain (91, 92).

The involvement of 4-HNE and 4-HDDE in inflammatory diseases has led to studies searching for mechanisms regulating the release of ARA. Studies with microglial cells demonstrated

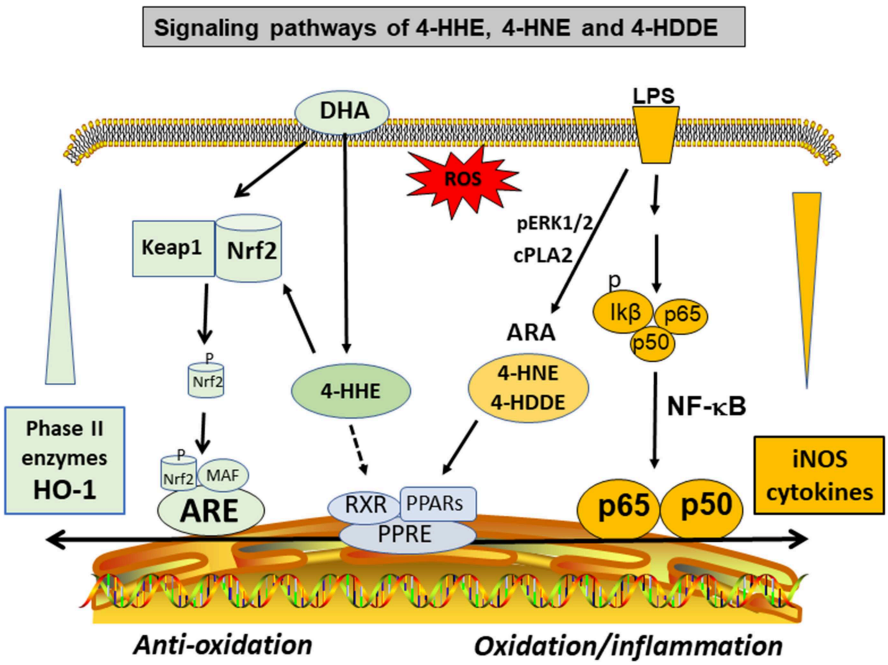

FIGURE 3 | Hormetic and signaling effects of 4-hydoxy alkenals involving inhibition of NF-κB and $cPLA_2$/ARA pathways, and activation of PPARs and Nrf2/HO-1 pathways.

the ability for lipopolysaccharides (LPS) to stimulate ARA release through phosphorylation of ERK1/2 and $cPLA_2$ (15, 18), and in turn, stimulation of this pathway leads to an increased production of 4-HNE (84). Activation of Toll-like receptors by LPS also induces COX-2, which acts on ARA to produce prostanoids. A study by Uchida demonstrated the involvement of 4-HNE in the induction of COX-2 expression (through the p38MAPK pathway), implicating the role for 4-HNE in a feed-forward mechanism to enhance inflammation (93). The acyl groups in cardiolipin (CL) are comprised with high levels of PUFAs (especially linoleic acid), and is thus a good source for production of 4-HNE. Therefore, peroxidation of CL and production of 4-HNE could account for oxidative disturbances of mitochondrial function (6, 94).

The high levels of DHA in the brain and retina have alerted studies on the pathophysiological role of its peroxidation product, mainly 4-HHE [see review by Long et al. (95)]. However, despite similar properties for both 4-HNE and 4-HHE, subtle differences were observed. For example, in a study with primary neurons in culture, application *in vitro* of both 4-HHE and 4-HNE showed differences in toxicity with LD_{50}'s of 23 and 18 μmol/L, respectively (96). In this study, cytotoxicity mediated by both alkenals could be suppressed by adding thiol scavengers such as glutathione (GSH) and N-acetyl cysteine, suggesting their ability to form adducts with these compounds. The study by Soulage et al. observed increase in circulating 4-HHE levels in type 2 diabetic human and in the diabetic Zucker rats (97). In this same study, exogenous 4-HHE was shown to reduce the glutathione concentration in L6 muscle cells (97). In a review by Long and Picklo, 4-HHE was 5- to 20-fold more potent in forming adducts with glutathione as compared with 4-HNE (95). Besides interaction with the GSH pathways, recent studies provided evidence for 4-HHE to exert hormetic effects by stimulating the stress response pathway involving nuclear factor-like 2 (Nrf2) and synthesis of anti-oxidant enzymes such as heme oxygenase-1 (HO-1) (95, 98–100) (**Figure 3**). In fact, our recent study with microglial cells also indicated ability for 4-HHE to induce higher levels of HO-1 as compared to 4-HNE (84).

Despite of perceived hormetic effects, the alkenal compounds may have deleterious and toxic effects, especially when tested at high concentrations and at *in vitro* settings. In a study by Awada et al. increase in 4-HHE in plasma was observed in mice consuming a diet with oxidized lipids, and the increase in 4-HHE was linked to oxidation and inflammation in the small intestine (101). In this study, the damaging effect on intestinal absorption was attributed to ability for 4-HHE to form protein adducts and alter glutathione metabolism (101). However, in a review by Mauerhofer et al., oxidized phospholipids could offer protective effects, including activation of PPARs, suppression of the toll-like receptors, and up-regulation of the Nrf2 anti-oxidant pathway (102). These diverse results clearly suggest the importance of future studies to further examine oxidized phospholipids and specific protein adducts formed by the reactive hydroxyl-alkenal compounds under different pathological conditions (103).

METHODS FOR ANALYSIS OF 4-HHE AND 4-HNE

Immunohistochemistry Assay of Protein Adducts

The reactivity of 4-HHE and 4-HNE to form adducts with biomolecules suggests the possibility to detect the adducts by antibody-based methods (73). An immunoblot assay was used to detect 4-HNE in human blood (104). In a study in which plasma proteins were separated by two dimension electrophoresis, increases in HNE-protein adducts were observed in autism subjects as compared to controls (105). Using a mouse monoclonal antibody that specifically recognizes the 4-HNE-histidine epitope, an ELISA method was developed for assaying 4-HNE in cell lysates (106), and increased levels of the 4-HNE-histidine adduct were observed in the middle frontal gyrus of AD patients as compared with controls (107). Using an anti-4-HNE adducted protein, increases in 4-HNE were observed in the hippocampus and entorhinal cortex in rat brain after binge ethanol administration (108). Due to the increasing interest to study the role of 4-HDDE, an oxidative by-product of ARA, a monoclonal antibody was recently developed against the 4-HDDE adduct (109). However, many of these antibody assays lack sensitivity and specificity, and commercial availability is limited (110). Therefore, future studies using advance techniques to detect specific protein adducts will enhance understanding the role of these peroxidation products in different disease conditions.

Efforts to develop immunohistochemical techniques to detect localization of 4-HNE in tissues and cells have also become important tools to understand the effects of oxidative damage in tissues and cells (111). In the study by Mamalis et al., increases in immunohistochemistry staining of 4-HNE together with other oxidant markers such as 8-hydroxydeoxyguanosine and advanced glycation end products, were detected in damaged human epidermis due to excessive exposure to UV light (112). In another study, age differences in 4-HNE immunoreactivity were observed in the mouse hippocampal CA1 area comparing groups at 40–42 to 50–59 weeks (113). In addition with an increase in oxidative stress with age, the increase in 4-HNE was marked by a decrease in mitochondrial dysfunction. Interestingly, administration of epigallocatechin-gallate, a polyphenol abundant in green tea extract, could attenuate 4-HNE immunoreactivity in the aged brain (114).

Relatively few studies have developed effective immune assay for 4-HHE. Using a mouse monoclonal IgG1 antibody, mAbHHE53, which is specific for protein-histidine bound HHE, increases in 4-HHE in autopsy samples of spinal cords were observed in amyotrophic lateral sclerosis patients (115). However, despite that the mouse monoclonal IgG1 antibody was developed to target 4-HHE, there is evidence that both 4-HNE and 4-HHE were detected (116). Considering that different PLA_2s are responsible for the release of ARA and DHA and subsequently their peroxidation products, future studies for production of more specific immune products for the alkenals will help to advance understanding of their metabolism.

LC-MS/MS Analysis for Simultaneous Measurement of 4-HHE and 4-HNE

Methods for accurate and precise analysis of free 4-HHE and 4-HNE present in biological samples include gas or liquid chromatography (GC or LC) and sometimes coupled with MS (73, 95). Depending on the chromophore, these peroxidation products could be quantified through LC with UV detection at 220 nm (117). However, due to lack of sensitivity and precision, GC-MS and LC-MS methods were developed as an alternative to the spectrophotometric detection. The classic multiple reaction monitoring assay performed on a triple quadrupole mass spectrometer has long been used for simultaneous quantification of 4-hydroxyalkenals, and this technique is capable of monitoring characteristic transitions for each targeted compound. Without derivatization, 4-hydroxyalkenals in human T cell leukemia extracts or mouse liver samples can be detected by selected reaction monitoring as $[M+H]^+$ (118, 119), but the sensitivity of this analysis is not sufficient to detect the levels of 4-hydroxyalkenals in small biological samples. Subsequently, different approaches were used to derivatize 4-hydroxyalkenals in order to increase detection sensitivity (120–124). Wang et al. enhanced sensitivity with a shotgun lipidomics-base method using the Michael adduct of 4-HNE with carnosine (122). Another agent is 1,3-cyclohexanedione (CHD), which takes advantage of the intrinsic reactivity of aldehydes, allowing for more specific and sensitive detection in different biological matrices (84, 100, 125–127). Because the alkenyl aldehydes are rather unstable, derivatization can help to stabilize these molecules. Recently, we applied the CHD-derivatization strategy to quantify the levels of 4-hydroxyalkenal species in different biological matrices over a wide range of concentrations (84, 128). Since phospholipids are common interfering species in reverse-phase LC, and can cause significant ionization suppression in the mass spectrometer, we employed a solid phase extraction (SPE) strategy to remove phospholipids while preserving the concentrations of the analytes (129). Our results for analysis of 4-HNE in biological samples, including plasma and brain tissue, fall into the range from 0.1 to 1.5 nmol/mg protein, depending on the samples and conditions (63, 120, 130, 131).

YIN-YANG MECHANISMS REGULATING THE PRODUCTION OF 4-HHE AND 4-HNE

With an established LC-MS/MS protocol to simultaneously measure 4-HNE and 4-HHE, an experiment was carried out to examine factors regulating levels of the hydroxyl-alkenals in BV-2 microglial cells stimulated with LPS and/or supplemented with DHA (84). Results showed that treating cells with exogenous DHA led to a dose-dependent increase in 4-HHE but not 4-HNE, whereas stimulation of cells with LPS resulted in an increase in 4-HNE but not 4-HHE (84). Since LPS is known to stimulate $cPLA_2$ and release of ARA in microglial cells (18), an association between 4-HNE and the $cPLA_2$/ARA pathway was confirmed using inhibitors for $cPLA_2$, which readily suppressed the LPS-induced increase in 4-HNE in these cells (84). Quercetin is a botanical polyphenol known to suppress LPS-induced $cPLA_2$

and NF-κB related pro-inflammatory responses in microglial cells (132). Along this line, treatment of cells with quercetin also suppressed LPS-induced 4-HNE (133). Taken together, these studies demonstrated a link between stimulation of the $cPLA_2$/ARA pathway and production of 4-HNE, and thus assay of 4-HNE can serve as a marker for the inflammatory pathway.

Our study with microglial cells indicated an increase in levels of 4-HHE upon treating microglial cells with DHA within a relatively short period of time of 6 hr (84). These results suggest that despite of uptake of DHA and incorporation into membrane phospholipids, some DHA may directly enter the cells and make available for lipid peroxidation to form 4-HHE. Studies by Ishikado and Nagayama with endothelial cells also attributed ability for DHA to exert anti-inflammatory effects through increased production of 4-HHE, and in turn, ability for 4-HHE to upregulate the antioxidant stress pathway with increased synthesis of HO-1 (125, 126). Consequently, the ability for DHA to suppress inflammatory responses through its oxidative product is a novel phenomenon worthy of future studies with animal models (**Figure 3**).

N-3 PUFAS SUPPLEMENTATION AND LIPID PEROXIDATION PRODUCTS: IMPLICATIONS ON NEUROLOGICAL DISORDERS

To-date, n-3 PUFAs in the form of fish oil are one of the most highly consumed dietary supplements by humans (134). Although fish oil contains substantial amounts of eicosapentaenoic acid (EPA) together with DHA, EPA is an intermediate for biosynthesis of DHA, and is present in relatively low levels in brain. In recent years, there is enormous interest to examine effects of dietary fish oil and DHA supplement on health and diseases [see review by Sun et al. (135)]. However, few studies have focused on the hormetic and deleterious effects of the peroxidative products. In a recent study, human subjects consuming Atlantic salmon showed increases in n-3 PUFAs in the phospholipids (e.g. PC) and triacylglycerols in plasma (136). In another study, healthy male subjects given different levels of DHA supplement (up to 1600 mg of DHA per day) showed a dose-dependent increase in levels of 4-HHE in the plasma (137). In agreement with the Yin-Yang mechanism for production of 4-HHE and 4-HNE, results of Calzada's study showed that DHA supplementation did not alter levels of 4-HNE. In this study, DHA supplementation not only did not alter redox homeostasis, but also prevented low-density lipoproteins from oxidation (137).

In a review by Trepanier et al. there is evidence for dietary n-3 PUFA to exert anti-inflammatory effects and neuroinflammatory outcomes in a number of animal models with neurological disorders (138). Studies with animal and cell models also pointed to the role of microglial cells in mediating the neuroinflammatory responses (139). Treating DHA to cells *in vitro* could mitigate LPS-induced pro-inflammatory responses through interaction with the toll-like receptor 4 (84, 140). Nevertheless, these effects *in vitro* are dependent on the treatment conditions, as high levels of DHA was shown to cause profound cell swelling and induce pyroptotic cell death (141). In a study with human microglial cells, both EPA and DHA were effective in decreasing the pro-inflammatory M1 markers and stimulating the anti-inflammatory M2 markers (142).

Lipid Peroxidation in Cerebral Ischemia and Brain Injury

Although increase in oxidative stress has been implicated in many forms of brain injuries and ischemic stroke, relatively few studies have successfully assessed the peroxidation products under these conditions (143–145). In a study by Zhang et al., mice fed a fish oil diet showed resistance to insult due to focal cerebral ischemia. The protective effects of n-3 PUFA supplements were attributed to ability of 4-HHE to upregulate the antioxidant pathway involving Nrf2 and synthesis of HO-1 (146). Considering the important role for the Nrf2 pathway to promote transcription of a number of antioxidant genes for regulating the body defense systems, recognition of a metabolic link between DHA and/or 4-HHE with the Nrf2 pathway may provide therapeutic strategy against oxidative damage due to cerebral ischemia and other brain injuries (147, 148).

Lipid Peroxidation in Neurological and Inflammatory Pain

There is emerging evidence for a connection between activated immune cells with oxidative stress, inflammation, and nociceptive responses (149–151). Low back pain is among the highest prevalence neurologic disorders in humans (152–154). Acrolein, a reactive aldehyde derived from a number of environmental factors, has been regarded as an important factor contributing to the hyperalgesia following traumatic injury. Besides acrolein, there is indication that other oxidant stressors such as 4-HNE and H_2O_2, may also augment the transient receptor potential channels (TRPA1 and TRPV1) in mediation of inflammatory responses and pain (155–157). More importantly, 4-HNE was shown to activate these channels through binding specific sites on the channel protein (158). In agreement with this phenomenon, inhibitors to acrolein and antibodies to oxidized phospholipids were able to mitigate the channel activity as well as diminish pain sensation (159). Future studies to further understand how the channel proteins are activated by these oxidant stressors will undoubted be an important path to advance therapy to mitigate neuropathic and inflammatory pain.

Lipid Peroxidation Associated With Binge Alcohol Consumption

Indications that chronic alcohol consumption is linked to increases in oxidative stress in the brain have led to studies searching for the mechanism whereby ethanol may induce lipid peroxidation products (160). Studies by Collins' group used an animal model as well as an organotypic slice culture model to demonstrated a link between binge alcohol administration with increases in PLA_2s together with PARP-1 and aquaporin-4 (AQP4) (108, 161, 162). Using a commercial antibody against 4-HNE protein adduct, binge ethanol administration was shown

to induce increases in 4-HNE in specific brain regions, including the hippocampus and entorhinal cortex (108). In agreement with the increase in PLA_2 activity, a time-dependent increase in levels of 4-HNE adduct was also observed in the brain slice culture experiment exposed to ethanol (161). This experiment further showed that supplementation of DHA (25 μM) to the slice culture could readily abrogate the oxidative markers due to the binge ethanol treatment (108). Consequently, future studies are needed to elucidate the mechanism for DHA to inhibit oxidative stress due to binge alcohol administration.

Lipid Peroxidation Associated With Alzheimer's Disease (AD)

Oxidative stress is known to play an important role in AD pathogenesis; lipid peroxidation together with the increases in production of 4-HNE as well as 4-HHE have been reported in the progression of this disease (163–166). Oligomeric Aβ could target lipid peroxidation resulting in increase in 4-HNE which in turn, form adducts with cysteine, histidine, and lysine residues (167, 168). Accumulation of HNE-adducts may lead to irreversible changes and thus impairing metabolic functions in the development of AD pathology (169). In an AD transgenic mouse model, administration of oligomeric Aβ resulted in an increase of free PUFAs together with levels of 4-HNE in the brain, suggesting increase in lipid peroxidation activity (14). Since lipid peroxidation is a relatively early event of oxidative stress, increases in alkenal compounds were detected in the hippocampal gyrus in individuals in the preclinical phase (165, 170). In addition, increased levels of 4-HNE and acrolein were observed in the brains of mild cognitive impairment and in early AD patients (131). In the late AD stage, propagation and amplification of oxidative stress were marked by increases in malondialdehyde (MDA), acrolein, and 4-HNE (165).

In a group of AD patients showing age-related decline, DHA supplements appeared to show improvement in learning and memory in this group of patients (171). Dietary supplement of DHA also protected AD-model mice from amyloid and dendritic pathology (172–174). In a study to examine effects of DHA supplementation on different phospholipids in brain regions of AD mouse model, results indicated significant increases in PC and PE levels in cortex, hippocampus and cerebellum (175). However, further studies are needed to examine whether the changes in brain lipids due to dietary DHA supplementation are associated with altered lipid peroxidation activity in the brain.

DHA and Lipid Peroxidation Associated With Autism Spectrum Disorder (ASD)

As reviewed by Romano et al. lipid peroxidation appears to play a role in several neuropsychiatric disorders, including bipolar diorder, depression, schizophrenia and Huntington's disease (176). Autism spectrum disorder (ASD) is a neurodevelopmental disorder that occurs in the early stage of life. The pathogenesis of ASD remains controversial; nevertheless, interactions between multiple genes and environmental risk factors are strong candidates (177). Early studies showed alterations in FA profiling in plasma of ASD patients with specific reduction of DHA (178). A study on proteomes in plasma of ASD and control subjects indicated significant increase in proteins involved in acute inflammatory response together with protein adducts to 4-HNE, thus suggesting a link between oxidative stress and lipid peroxidation in the pathophysiology of ASD (105). In particular, younger children with autism seem to be more vulnerable to oxidative stress and thus showing a greater increase in lipid peroxidation products. In a study on Egyptian autistic children, levels of malondialdehyde (MDA) were significantly higher in children with autism <6 years of age, whereas this difference diminished with increasing age of the children (179). In another study with older patients, significantly higher levels of 4-HNE protein adducts were found in erythrocyte membranes and plasma from autistic patients as compared with controls (180).

In a study with a gene/stress mouse model, maternal DHA supplementation was shown to mitigate autistic behaviors in offspring pups (181). In order to investigate mechanism(s) whereby DHA supplementation could suppress autistic behavior, Yang et al. examined FAs and peroxidation products in mouse pups from mothers given a control diet or a diet containing 1% DHA (128). Results showed increases in DHA and decreases in ARA levels in all regions of the pup brain. However, DHA-supplemented diet increased 4-HHE levels mainly in the cerebral cortex and hippocampal regions, whereas levels of 4-HNE were not changed (128). Considering the important role of neurons in mediating memory and cognitive functions in the cerebral cortex and hippocampus, the specific increase in 4-HHE in these brain regions upon supplementation with DHA suggests greater peroxidation activity associated with these brain regions. Further investigations are needed to examine the physiologic role of 4-HHE in brain development. This dietary regimen also altered DHA, ARA, and 4-HHE in mouse heart and plasma, and interestingly, analysis of these alkenals in plasma indicated not only an increase in 4-HHE but also a decrease in 4-HNE. A study by Nakagawa et al. showed a similar increases in DHA and decreases in ARA as well as elevated levels of 4-HHE but not 4-HNE in adult mice fed dietary fish oil in multiple peripheral organs of adult mice (100). Consequently, more studies are needed to examine whether increase in 4-HHE due to maternal DHA supplement can enhance the hormetic effects during brain development (88).

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

The abundance of DHA and ARA in phospholipids in the CNS remains a subject of interest for understanding the physiologic role of their metabolites. The current review demonstrated a Yin-Yang mechanism for metabolism of DHA and ARA due to their release by different PLA_2s, and these mechanisms led to diverse down-stream pathways for production of lipid mediators that are pro-inflammatory and pro-resolving.

Peroxidation of DHA and ARA by oxygen free radicals results in production of 4-HHE, 4-HNE, 4-HDDE, and other carbonyl products, which can be detected in body organs and

body fluids. These reactive aldehydes are electrophiles, and can confer cytotoxic and protective effects depending on the conditions for production. There is increasing evidence for the hormetic and anti-inflammatory effects of these alkenals through interaction with PPARs, suppression of NF-κB inflammation, and upregulation of the stress pathway involving Nrf2 and synthesis of HO-1. However, more efforts are needed to identify binding of these alkenals to specific proteins in pathological conditions. Many neurological disorders are accompanied by oxidative stress, neuroinflammation and apoptotic cell death. Although it is not possible to elucidate individual mechanism(s) for the pathogenesis of these disorders, this review provided an emphasis on an increase in 4-HHE due to supplementation of DHA, and the increase in 4-HNE associated with the inflammatory pathway involving activation of $cPLA_2$ and release of ARA. Due to the complexity of membrane lipids, advance techniques for lipidomics are needed to better understand mechanisms of oxidative stress and source of lipid peroxidation products in disease processes.

AUTHOR CONTRIBUTIONS

BY and GS initiated writing this review. KF, DB, ZG, JL, WF, and CG contributed substantially to the concept and editing of the review.

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Aging Increases Hippocampal DUSP2 by a Membrane Cholesterol Loss-Mediated RTK/p38MAPK Activation Mechanism

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Numerous studies suggest that the increased activity of p38MAPK plays an important role in the abnormal immune and inflammatory response observed in the course of neurodegenerative diseases such as Alzheimer's disease. On the other hand, high levels of p38MAPK are present in the brain during normal aging, suggesting the existence of mechanisms that keep the p38MAPK-regulated pro-inflammatory activity within physiological limits. In this study, we show that high p38MAPK activity in the hippocampus of old mice is in part due to the reduction in membrane cholesterol that constitutively occurs in the aging brain. Mechanistically, membrane cholesterol reduction increases p38MAPK activity through the stimulation of a subset of tyrosine kinase receptors (RTKs). In turn, activated p38MAPK increases the expression and activity of the phosphatase DUSP2, which is known to reduce the activity of different MAPKs, including p38MAPK. These results suggest that the loss of membrane cholesterol that constitutively occurs with age takes part in a negative-feedback loop that keeps p38MAPK activity levels within physiological range. Thus, conditions that increase p38MAPK activity such as cellular stressors or that inhibit DUSP2 will amplify inflammatory activity with its consequent deleterious functional changes.

Keywords: cholesterol, RTKs, p38MAPK, aging, DUSP2

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INTRODUCTION

Brain inflammation is frequently related to several diseases and it has been described to be a conspicuous component of Alzheimer's disease (AD), Parkinson's Disease (PD) and multiple sclerosis (MS) (1) and also of acute situations such as stroke and head trauma (2, 3). In all these conditions the final outcome is usually the loss of neuronal cells. However, different physiological events lead along lifespan to the development of inflammatory processes (4). In this regard, brain inflammation is also evident in the brain during non-pathological aging (5–8), where the loss of neurons is not usual (9, 10). These last observations are consistent with the view that during aging precise mechanisms must be developed to keep the level of activity of the different mediators of the inflammatory process within physiological range.

The inflammation onset is characterized by the increment of inflammatory cytokines (11) together with the activation of several key elements, such as MAPKs (mitogen-activated protein kinases) and other signaling proteins, that allow the progression of the inflammation generating a positive feedback between those two elements. Although there are several signaling proteins that regulate inflammation, one of the main players is the p38MAPK pathway (12). This pathway is a crucial regulator of inflammatory events through several mechanisms including changes in gene expression (12). Furthermore, p38MAPK activation has been related to several neurodegenerative diseases (13, 14). As an important signal integrator pathway, p38MAPK has also been linked to other processes different from inflammation such as development, cell cycle or even memory processes (15). Considering its importance in gene expression modulation, the increased p38MAPK activity observed in physiological brain aging (16) suggests that this pathway could be part of the age-associated mechanisms responsible for maintaining brain inflammation within a physiological range.

In previous studies, we demonstrated that the gradual loss of cholesterol from the neuronal plasma membrane during aging contributes to neuronal survival thanks to the increased activity of pro-survival kinase AKT1 due, among other causes, to the increase in basal activity of tyrosine kinase receptors (RTKs) (17, 18). In addition, our previous works suggested that the constitutive reduction in neuronal plasma membrane cholesterol during aging may be, at least in part, a consequence of increased activation and plasma membrane translocation of the cholesterol catabolic enzyme Cyp46A1 (17, 19). Hence, the recent demonstration that RTK activation favors survival in the developing brain via the p38MAPK pathway (20), moved us to test the hypothesis that reduced membrane cholesterol, via RTKs' stimulation, could contribute to the increase in p38MAPK activation in the old brain.

RESULTS

Age Increases p38MAPK Activity Levels in the Hippocampus, in Part Due to Cholesterol Loss

Previous work has shown that p38MAPK activity increases with age in the mouse hippocampus (8). Analysis of hippocampal extracts from mice of different ages confirmed that there was a significant increase in active p38MAPK levels between 2–3 and 7–9 months of age, and these remain elevated in 22–24 months-old mice (**Figure 1A**).

There are also several examples where brain inflammation, in which p38MAPK has a preponderant role, has been associated to the loss of neuronal cholesterol that occurs both in conditions of acute (e.g., stroke) and chronic (aging) inflammation (21, 22). Therefore, we decided to investigate the relationship between p38MAPK increase and neuronal cholesterol loss. As a first approximation, we reduced cholesterol levels in hippocampal slices from young mice by cholesterol oxidase (Choox) treatment (see Materials and Methods). We used Choox at a concentration 10 IU/ml, which based on our previous works is a dose that induces a mild (∼20%) reduction of plasma membrane cholesterol, without affecting cell viability (Palomer et al., 2016) (23). **Figure 1B** shows that a cholesterol decrease of this magnitude increases the levels of the phosphorylated (active) form of p38MAPK in hippocampal slices from young mice. A similar treatment in cultured hippocampal neurons also resulted in a significant increase in p38MAPK activity (**Figure 1C**), altogether indicating that cholesterol loss can be sufficient for p38MAPK activation.

In order to determine if cholesterol loss is necessary for p38MAPK increase with age (see **Figure 1A**), we raised the levels of this lipid to hippocampal slices of old mice by adding a solution of cholesterol-methyl-beta-cyclodextrin (MβCD-Ch, referred in figures as Ch). It has been previously shown that the high affinity of methyl-beta-cyclodextrin (MβCD) for cholesterol can be used to generate inclusion complexes that increase membrane cholesterol levels (24, 25). Hippocampal slices from old mice were incubated with MβCD-Ch following protocols used in previous studies in which we evaluated that this treatment restores cholesterol content to levels similar to those of young mice (25, 26). **Figure 1D** shows that MβCD-Ch significantly reduces the levels of phosphorylated p38MAPK in the old hippocampal slices. Further supporting that cholesterol loss can account for the increased p38MAPK activity in the old slices, the increase due to Choox was restored when the Choox-treated slices from young mice were re-incubated with the MβCD-Ch complex (**Figure 1E**). Altogether, the results are consistent with the possibility that conditions that lead to a reduction of neuronal cholesterol, acute or chronic, increase p38MAPK activity. The next question we asked was: how does cholesterol loss lead to the activation of p38MAPK?

RTK Activation Plays a Role in Cholesterol Loss-Mediated p38MAPK Activity Increase

Considering that an acute loss of cholesterol could generate cellular stress, a well-known p38MAPK activator, we checked if the activation of p38MAPK upon cholesterol removal was due to an increase in oxidative stress. To investigate this possibility, hippocampal neurons in culture were incubated with an antioxidant cocktail at the time of the Choox-induced cholesterol reduction (see Materials and Methods). The antioxidant treatment partially prevented the Choox-induced increase in p38MAPK activity (**Figure S1A**), indicating that still a significant fraction of the p38MAPK activated by cholesterol loss occurs independently from oxidative stress, although this is also induced by cholesterol loss (27).

The loss of cholesterol from the plasma membrane, even if <20% as in our conditions, will necessarily lead to substantial structural changes in the plasma membrane causing a panoply of functional alterations. Therefore, we investigated whether activation of p38MAPK in the neurons with reduced cholesterol was the consequence of a particular type of membrane signaling alteration or, on the contrary, the consequence of multiple altered pathways. In particular, we checked the possibility that different known p38MAPK activators could become active upon cholesterol loss. Incubation of cortical neurons with a cell

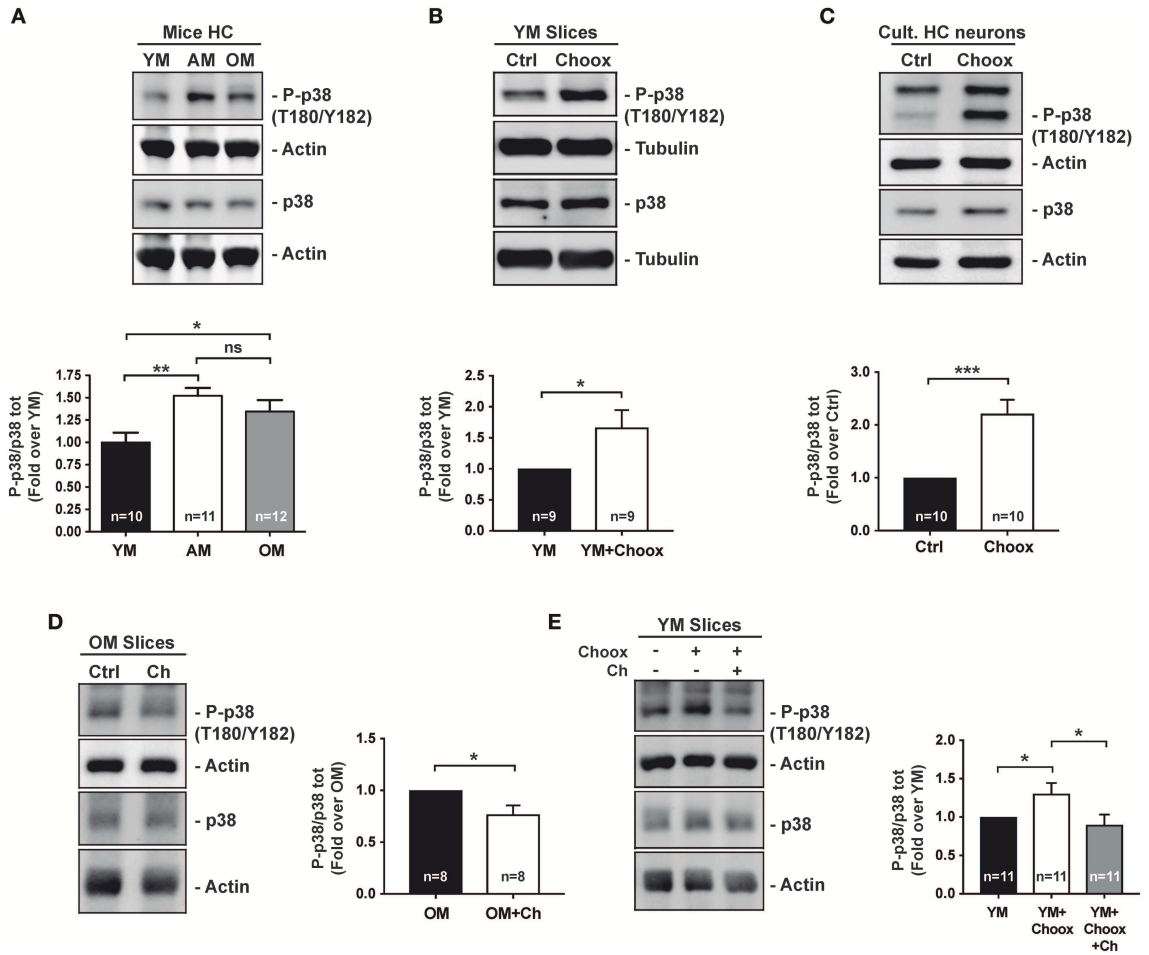


FIGURE 1 | Age increases p38MAPK activity in mice hippocampus in a cholesterol-dependent manner. **(A)** Age-associated increase in the phosphorylation of p38MAPK (p38) activating residues (T180/Y182) in mice hippocampus (HC): young (2–4 months; YM), adult (7–12 months, AM), old (20–24 months OM). **(B)** Increased p38MAPK activating marks (phosphorylation on T180/Y182) in hippocampal slices from 2 month-old mice with reduced cholesterol (treated with cholesterol oxidase, Choox, for 30 min). **(C)** Increased p38MAPK activating marks in hippocampal neurons in culture treated as hippocampal slices in **(B)**. **(D)** Reduced p38MAPK activity in old mice hippocampal slices incubated with a cholesterol replenishment solution (cholesterol-methyl-beta-cyclodextrin complex, referred as Ch). **(E)** Increased p38MAPK activity induced by the cholesterol extracting enzyme Choox become reduced when the same slices are subsequently incubated with the cholesterol (referred as Ch) rich solution. Numbers in bars reflect number of independent experiments. Data are represented as mean ± SEM. The asterisks indicate the *p*-values (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. ns, not significant).

permeable calcium chelator (BAPTA-AM) did not significantly reduce the p38MAPK increase induced by cholesterol loss (**Figure S1B**), ruling out Ca^{2+} levels alterations as promoter of p38MAPK activation in this experimental situation. Similarly, incubation of the neurons with H-89, a protein kinase inhibitor with preference for protein kinase A (PKA) (28, 29), or with Chelerythrine-chloride, an inhibitor of PKC isoforms A and B (30), also failed to interfere with the cholesterol loss-induced p38MAPK activation (**Figure S1C**). These results show that the loss of cholesterol induced by Choox treatment does not have, in itself, such a pleiotropic effect. Given that in previous studies we showed that the loss of cholesterol increases the activity of the RTKs TrkB and insulin receptor (IR) (17, 31), we analyzed next if RTKs were involved in cholesterol loss-mediated p38MAPK activation. As a first approach, we incubated cell lysates of Choox-treated and un-treated cultured hippocampal neurons, with a membrane-based antibody array to determine the relative phosphorylation levels of several mouse RTKs (see Materials and Methods). This study revealed that cholesterol loss was able to increase the phosphorylation levels of different RTKs, most notably SCFR (Stem Cell Factor Receptor) also known and referred here as c-Kit, VEGFR2 (Vascular Endotelial Growth Factor receptor 2), and IGF-1 (Insulin Growth Factor receptor 1) (**Figure S2**). On the other hand, the phosphorylation levels of several other RTK receptors were not affected by the cholesterol loss (**Figure S2**), again implying that membrane cholesterol reduction has a limited series of targets, at least at the low levels of reduction induced in our experimental model.

In order to validate the antibody array results, we performed western blotting with extracts from cultured hippocampal

neurons that had been exposed to the Choox treatment. This experiment confirmed that cholesterol reduction increased VEGFR2 and c-Kit activity (**Figures 2A,B**). To directly assess the existence of a functional link between cholesterol loss, RTK activation and activation of p38MAPK, we incubated Choox-treated cultured neurons with the broad-spectrum RTK inhibitor K252a (32–34). This treatment significantly prevented p38MAPK activation induced by cholesterol loss (**Figure 2C**). A similar inhibition of p38MAPK activity was observed using the RTK activity inhibitor Cabozantinib (XL184), a small-molecule kinase inhibitor with potent activity toward c-Kit and VEGFR2 (35, 36), (**Figure 2D**).

The demonstration of a mechanistic link between cholesterol loss, activation of particular RTKs and activation of p38MAPK in cultured neurons, moved us to ask whether the RTK activity increase was also observed *in vivo*, in the old adult brain, as for p38MAPK activity (see **Figure 1**). To test this possibility, we analyzed the activity levels of the two RTKs activated by cholesterol loss whose inhibition reduced p38MAPK activation (**Figure 2D**), namely VEGFR2 and c-Kit. **Figures 2E,F** show that while the VEGFR2 activity increased significantly from the young age (2–3 months) to adulthood (7–9 months of age), remaining high in the old mice (22–24 months old), the c-Kit increased gradually with age, with the change being most significant between young and old mice. As a whole, these experiments show that the activation of p38MAPK in the hippocampus of old mice could be due to a cholesterol loss-mediated activation of particular RTKs. Hence, we next aimed to identify the downstream targets of p38MAPK.

Cholesterol Loss-Mediated p38MAPK Activation Increases *Dusp2* Gene Expression

p38MAPK is a well-known modulator of gene expression (37, 38), and its activation in the old mouse brain could be leading to the expression of different genes involved in aging progression. To determine which genes are regulated by the activity of p38MAPK induced by cholesterol loss, we performed a RNA sequencing (RNAseq) study using cultured hippocampal neurons either untreated or treated with Choox to reduce cholesterol, and incubated with or without the p38MAPK inhibitor SB203580 (Choox+SB20) (39). We reasoned that the expression of the downstream targets of p38MAPK induced by cholesterol loss should be altered by Choox but unchanged in Choox+SB20 conditions. Non-Choox treated neurons were used as controls. The statistical comparison of the mRNA expression levels reported 38 differentially expressed genes, 36 upregulated and 2 downregulated in response to Choox treatment (q-value <0.05) (**Figure 3**). On the other hand, 58 genes were differentially expressed when comparing Choox+SB20 with control neurons, 23 upregulated and 35 downregulated in Choox+SB20 treated neurons (see **Figure 3A**). It is not surprising that more genes are affected in this second analysis, as the p38MAPK inhibitor can have effects independently from cholesterol loss-induced p38MAPK activation. Irrespectively, the analysis of these two lists of genes unveiled a set of 25 genes whose expression was modified by the loss of cholesterol through a p38MAPK dependent mechanism, i.e., changing with Choox treatment and the change being suppressed in the Choox+SB20 treatment (**Figure 3B**).

Next, the above RNAseq data was subjected to a bio-informatic Artificial Neural Network (ANN) score analysis, a strategy that estimates the functional relationship of a gene/protein with a biological process by analyzing mathematical models aimed to simulate the molecular activity of such process (see Materials and Methods). This approach allowed us to identify genes with a potential role in apoptosis and survival. **Figure 3B** shows that a well-known target of p38MAPK activity, *Interleukin 1* (see Introduction), has the highest predicted value (functional clustering), confirming the accuracy of this bio-informatics tool. In addition, dual specificity protein phosphatase 2 gene (*Dusp2*) also presented high-predicted value. This result was of interest to us as DUSP phosphatases dephosphorylate MAPKs, and therefore have a potential role limiting the extent of the p38MAPK activation that occurs in the old brain (see **Figure 1A**). Interestingly, the gene expression of another phosphatase of the same family, *Dusp1*, appeared to be altered by cholesterol removal but in a MAPK-independent manner (see **Table S1**) thus revealing a specific pattern of gene expression in the context of age-related cholesterol loss.

To validate the RNAseq data, we carried out a qPCR study using as template mRNA from 15 days *in vitro* (DIV) hippocampal neurons subjected to Choox treatment in the presence or absence of the p38MAPK inhibitor SB203580. We focused on *Dusp2*, as this gene showed increased levels by cholesterol reduction in a p38MAPK activity-dependent manner and has a high ANN predicted value as survival-apoptosis related genes (see above). These experiments confirmed that cholesterol loss induced the up-regulation of *Dusp2* mRNA in a p38MAPK-dependent manner (**Figure 4A**).

To investigate if the increase of these mRNAs was the result of the acute loss of cholesterol induced by Choox or can also occur in conditions of gradual cholesterol loss, as during physiological aging, we repeated the qPCR analysis using as template mRNA from the hippocampus of young (2–3 months-old), adult (7–9 months-old) and old (22–24 months-old) mice. **Figure 4B** shows that *Dusp2* mRNA levels gradually increase with age, reaching significance between young and old mice. This indicates that *Dusp2* upregulation may be the consequence of the gradual changes produced in the plasma membrane during aging. Then, we investigated if the increase with age of *Dusp2* was dependent on the activity of p38MAPK. For this, we incubated hippocampal slices of old mice with the more potent and specific p38MAPK inhibitor PH797804 (40). This experiment resulted in a reduction in the levels of *Dusp2* mRNA (**Figure 4C**).

To confirm the increased expression of DUSP2 in the old brain and in cholesterol loss conditions at the protein level, we immunoblotted cell lysates of Choox-treated neurons that had been pre-incubated with the p38MAPK inhibitor PH797804. This experiment confirmed the cholesterol loss and p38MAPK activity-dependent activation of DUSP2 protein (**Figure 5A**). To test if the increase in DUSP2 observed following cholesterol extraction of cultured neurons was also

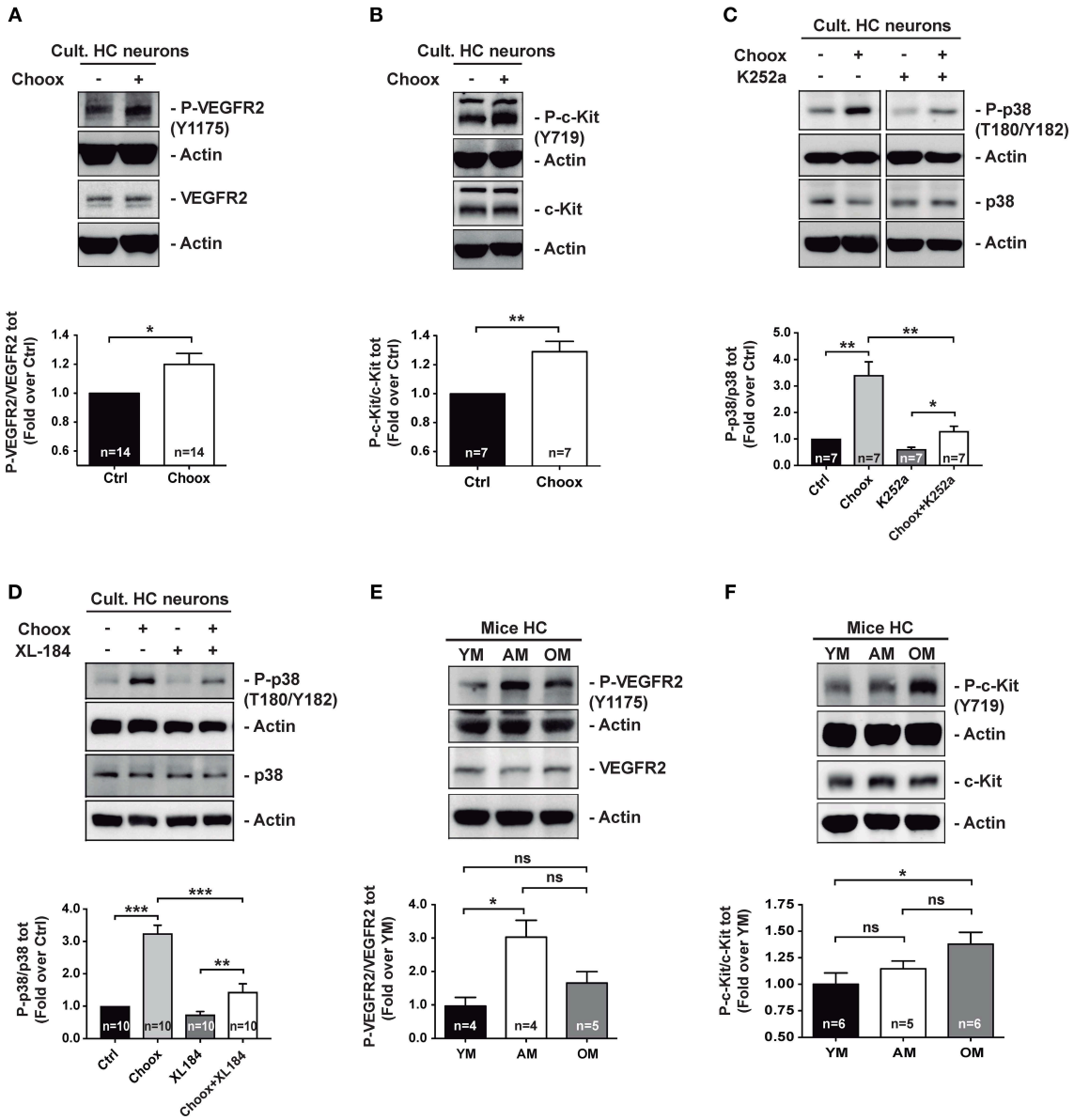


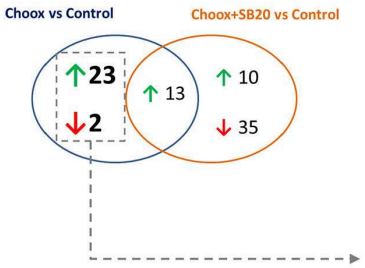
FIGURE 2 | Age-dependent cholesterol loss leads to increased p38MAPK activity through RTKs signaling. **(A)** Increased VEGFR2 activating phosphorylation Y1175 in cultured hippocampal neurons after treatment with cholesterol oxidase (Choox). **(B)** Increased c-Kit receptor activating phosphorylation Y718 in neurons in culture treated with Choox for cholesterol removal. **(C)** Incubation of hippocampal neurons in culture with K252a, a broad RTK inhibitor, significantly prevents Choox-induced p38MAPK (p38) activation. **(D)** Incubation of hippocampal neurons in culture with XL-184, an inhibitor of VEGFR2 and c-Kit receptors, prevents Choox-induced p38MAPK phosphorylation. **(E,F)** Western blots showing activating phosphorylations of RTK receptors, VEGFR2 Y1175 **(E)** and c-Kit Y718 **(F)**, in hippocampus of young (YM), adult (AM), and old mice (OM). The values inside the bars indicate the number of independent experiments. Data are represented as mean ± SEM. The asterisks indicate the *p*-values (ns, not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

present *in vivo,* we performed a western blotting analysis using hippocampal extracts from mice of different ages, young, adult and old. In agreement with the mRNA results (see **Figure 3B**), DUSP2 protein was also found elevated in the hippocampus of old mice compared to young mice (**Figure 5B**). Immunofluorescence microscopy experiment in hippocampal sections of mice of different ages confirmed this result (**Figures 5C,D**).

DISCUSSION

The results presented here have several biological implications. On one hand, they extend previous reports showing that age increases brain p38MAPK activity (8). Mechanistically, our data suggest that during aging the gradual loss of cholesterol leads to the activation of RTKs, which in turn activate p38MAPK signaling. On the other hand, we showed that the cholesterol

A



B

Uniprot ID	Gene name	Choox vs Control	Choox+SB 20 vs Control	Choox vs Choox+SB 20	ANN score-Apoptosis and survival
P01584	**IL1B**	1	0	1	90,87%
P01583	**IL1A**	1	0	1	71,14%
Q05923	**DUSP2**	1	0	1	68,4%
Q96P20	NLRP3	1	0	0	56,19%
P21580	TNFAIP3	1	0	1	45,2%
Q9Y662	HS3ST3B1	-1	0	0	29,32%
Q8N5N7	MRPL50	1	0	0	20,59%
P13500	CCL2	1	0	1	20,55%
Q08116	RGS1	1	0	1	20,55%
P11161	EGR2	1	0	0	19,15%
Q8TDS4	HCAR2	1	0	0	18,22%
O00622	CYR61	1	0	1	18,05%
Q8N2Q7	NLGN1	1	0	0	17,84%
Q8NI38	NFKBID	1	0	0	15,84%
Q16548	BCL2A1	1	0	0	14,6%
O00421	CCRL2	1	0	1	13,87%
P15018	LIF HILDA	1	0	0	12,16%
O76094	SRP72	1	0	1	9,21%
Q9UBC3	DNMT3B	-1	0	0	7,52%
P41273	TNFSF9	1	0	1	6,81%
O95992	CH25H	1	0	1	5,33%
Q01151	CD83	1	0	1	5,21%
P13236	CCL4	1	0	1	5,06%
Q6ZNA5	FRRS1	1	0	0	-
Q99988	GDF15	1	0	0	-

FIGURE 3 | Gene expression changes induced by cholesterol loss in neurons. **(A)** Venn diagram showing the number of genes whose differential expression is statistically significant ($p < 0.05$) upon cholesterol loss (Choox vs. Control) or upon cholesterol loss in the absence of p38MAPK activity (Choox+SB20 vs. Control). Green arrows stand for upregulation (higher expression of the genes in the Choox or Choox +SB20 neurons) and red arrows for downregulation. **(B)** Shows the list of 25 genes whose expression is modified by the loss of cholesterol through a p38MAPK dependent mechanism. It is also indicated if these genes are differentially expressed when statistically comparing the Choox vs. Choox+SB20 cohorts. In the columns referring to the three comparisons, it is indicated if the genes were statistically upregulated (1), downregulated (−1) or if there was no statistically significant change on their expression (0). The column in the right displays the Artificial Neural Network (ANNs) score obtained by each gene when calculating their functional relationship with apoptosis and survival through the analysis of mathematical models.

loss-mediated p38MAPK activation results in the up-regulation of pro-inflammatory genes and also of phosphatases such as *Dusp2* that can potentially limit p38MAPK activity.

DUSP2, originally named phosphatase of activated cells 1 (PAC-1), is one of the members of the dual-specificity phosphatases (DUSPs) that act as negative regulators of MAPKs by dephosphorylating both phosphotyrosine and phosphoserine/threonine residues (41). Since DUSP2 was originally identified in stimulated human peripheral T cells, most of our current knowledge on this phosphatase is on its role in the immune response and inflammation (41, 42). In addition, it has also been proposed that DUSP2 plays a role in apoptosis and cancer (43–45). There are also a few studies on DUSP2 in the central nervous system, for example it has been reported that *Dusp2* mRNA expression is increased in forebrain neurons resistant to ischemia, but not in the vulnerable neurons, suggesting that DUSP2 may be protecting against this type of stress (46). A similar neuroprotective role for DUSP2 has been reported in granule neurons treated with apoptotic stimuli such as cisplatin (47). Thus, the increased expression of DUSP2 in response to cholesterol loss/redistribution, as it occurs in the old brain, could have a dual role in brain physiology: it would maintain p38MAPK activity at physiological levels, so that this pathway can perform its usual functions in synaptic plasticity and cytoskeletal stability, while at the same time it would ensure that the extent of p38MAPK activation is not too exaggerated, which could lead to neuronal damage, such as in stress conditions. The different roles of p38MAPK in physiological vs. pathological situations, might be the consequence of a qualitative process, due to the existence of "pools" of p38MAPK receiving input from different signaling pathways. Alternatively, physiological or pathological responses could be the consequence of a quantitative process and rely on the intensity of the pathway activity induced by different stimuli. Thus, the activity of p38MAPK that leads to the transcription of *Dusp2* could be due to a low intensity stimulus, such as the one elicited by the physiological loss of membrane cholesterol, while in response to stronger stress stimuli the negative regulation by DUSP2 phosphatase could be overcome by higher levels of upstream p38MAPK activators or by other mechanisms leading to enhanced p38MAPK activity and neuronal death. As a matter of fact, although the activity of p38MAPK is significantly higher

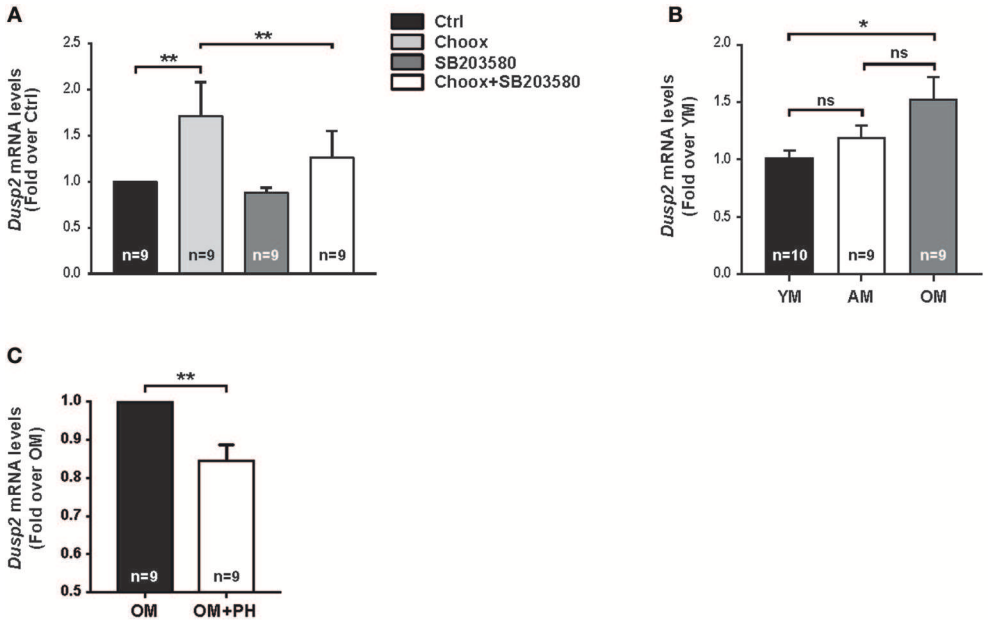


FIGURE 4 | Cholesterol loss-dependent p38MAPK activation increases *Dusp2* mRNA levels in old mouse hippocampus. **(A)** Graphic shows *Dusp2* mRNA levels after cholesterol loss. Hippocampal neurons in culture where treated or not with p38MAPK inhibitor, SB203580 (20 μM), 1 h previous to treatment with Choox for cholesterol removal to address p38MAPK involvement in gene transcription. **(B)** Plot reflects *Dusp2* mRNA levels in the hippocampus of young (YM), adult (AM) and old mice (OM). **(C)** Graphic shows *Dusp2* mRNA levels in hippocampal slices of old mice treated for 1 h with a potent p38MAPK inhibitor, PH787904 (referred as PH; 2 μM). Bar graphs: Numbers inside indicate the number of independent experiments. Data are represented as mean ± SEM. The asterisks indicate de *p*-values (ns, not significant; $*p < 0.05$; $**p < 0.01$).

in the old brain, it is not enough to induce neuronal death nor are there signs of pathological inflammation, suggesting that p38MAPK activity levels may have not reached the disease-producing threshold during normal aging. In addition to the negative regulation of p38MAPK activity, the cholesterol loss-induced upregulation of DUSP2 may also protect old neurons by dephosphorylating ERK1/2, a pathway known to be less active during aging (48, 49). Although a number of studies have shown the ERK1/2 pathway to have an anti-apoptotic role in neurons, pro-apoptosis induced by ERK1/2 signaling has also been observed. Thus, aberrant activation of MEK/ERK signaling induced by β-amyloid peptide promotes the apoptosis of rat embryonic cortical neurons by regulating the entry of neurons into the cell cycle (50). Furthermore, neuronal apoptosis mediated by the Ras/Raf-1/MEK/ERK signaling pathway was also reported in conditions of mitochondrial dysfunction (51) and zinc depletion (52), or when ERK signaling is activated together with JNK (53), when glutamate receptors (NMDA) are activated by tumor necrosis factor (54), and in conditions of sustained ERK activity (55).

In conclusion, we propose that cholesterol loss-mediated DUSP2 expression during physiological aging may be part of a protective signaling mechanism to regulate p38MAPK over-activation in neurons. In this hypothetical scenario, impaired expression of DUSP2 could facilitate the exacerbation of p38MAPK-mediated responses, thus contributing to the development of pathologies like AD, PD or MS. Future studies will be required to explore this working hypothesis.

MATERIALS AND METHODS

Animal Handling

Male C57BL/6J mice were used in this study: young 2–3 month-old, adult 7–12 month-old and old 20–24 month-old. All the animals were kept in the Centro de Biología Molecular Severo Ochoa's (CBMSO) animal facility. The mice and manipulations presented in this work count with the approval of the Dirección General de Medio Ambiente de la Comunidad Autónoma de Madrid (Ref. PROEX 066/15) and the CBMSO's Ethical Committee. All the experiments were performed in accordance with European Union guidelines (2010/63/UE) regarding the use of laboratory animals.

Cell Cultures

Primary hippocampal neurons were extracted from Wistar rat embryos at embryonic day 18 (E18), seeded in culture conditions as previously described (56) and kept in culture for 15 days *in vitro* (DIV). All cells were incubated at 37°C, humidity conditions and 5% CO_2.

Hippocampal Slices

Hippocampal slices were obtained from C57BL/6J mice. Hippocampi were extracted and placed in dissection solution (10 mM D-glucose, 4 mM KCl, 26 mM $NaHCO_3$, 233.7 mM sucrose, 5 mM $MgCl_2$, 1:1000 Phenol red) oxygen saturated with carbogen (95% O_2/5% CO_2), and sliced using an automatic tissue chopper (McIlwain Tissue Chopper, Standard Table, 220 V,

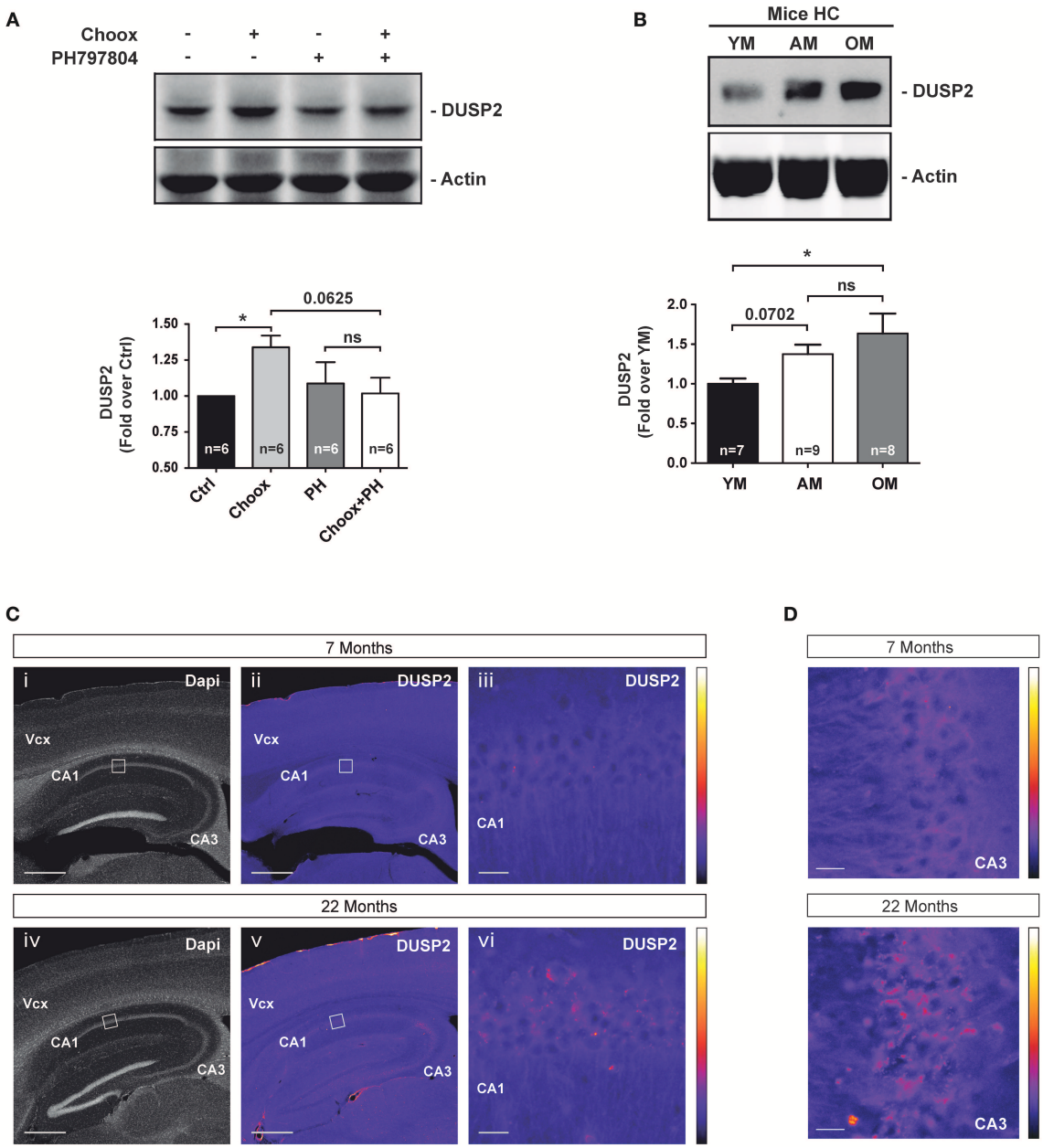


FIGURE 5 | DUSP2 protein levels are upregulated in the hippocampus of old mice. **(A)** DUSP2 levels increase in a cholesterol loss and p38MAPK activity dependent manner. Hippocampal neurons in culture were or were not treated with the p38MAPK inhibitor PH797804 (2 μM, also referred as PH) prior to cholesterol oxidase (Choox). **(B)** DUSP2 protein levels increase with aging in the mouse hippocampus. **(C)** Representative pictures show the increased expression of DUSP2 in the cortex and in the hippocampus with age. Top pictures (i-iii) show expression of DUSP2 in adult mice (7 months); lower pictures (iv-vi) show DUSP2 expression in old mice (22 months). DUSP2 is expressed in CA1 layer (iii; vi). Panels (iii) and (vi) show higher magnifications of the regions boxed in (ii) and (v) respectively. Vcx, Visual cortex; CA1, Cornu Ammonis of the hippocampus layer 1; CA3, Cornu Ammonis layer 3. Scale Bar in i, ii, iv, v, 500 μm; Scale bar in iii, vi, 20 μm. Colored bars on the right show the Look-Up-Table used to color-code the intensity of DUSP2 labeling. **(D)** Pictures show a magnification of CA3 layer. Scale bar represents 20 μm. Bar graphs: Values inside indicate the number of independent experiments. Data are represented as mean ± SEM. The asterisks indicate the *p*-values (ns, not significant; $*p < 0.05$).

Ted Pella Inc.) to obtain 400 μm hippocampal slices. Then slices were kept in artificial cerebrospinal fluid (ACSF: 119 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4, 11 mM glucose, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, osmolarity adjusted to 290 Osm) oxygen saturated with carbogen for 1 hour. Experiments were performed in ACSF oxygen saturated.

Cell and Slices Treatments

The following compounds were added to cell medium of hippocampal neurons: Cholesterol oxidase (Choox; Calbiochem ref.: 228250; 10 IU/ml); K252a (Tocris ref.: 1683; 1 μM); SB203580 (Shelleckchem ref.: S1076; 20 μM); PH797804 (Axon Medchem ref.: 1837; 2 μM); H89 (Tocris ref.: 2910;

50 μM); Chelerythrine (Tocris ref.: 1330; 10 μM); XL-184 (Tocris ref.: 5422; 1 μM); BAPTA-AM (Invitrogen ref.: B-6769, 10 μM). Experiments with the antioxidants cocktail in cultured hippocampal neurons used: N-Acetyl-L-Cysteine (NAC, Sigma-Aldrich ref.: A7250, 5 mM) and L-Glutathione reduced (GSH, Sigma-Aldrich ref.: G4251, 5 mM). Hippocampal slices were treated with Cholesterol oxidase (Choox; Calbiochem ref.: 228250; 10 IU/ml) for cholesterol removal. Experiments for cholesterol addition conducted in hippocampal slices were performed at 25°C. Methyl-β-cyclodextrin-cholesterol (MβCD-Ch) solution was prepared freshly at use concentration in ACSF, containing 30 μM Cholesterol Water-soluble (Sigma-Aldrich ref.: C4951) and 5 μM Cholesterol (Sigma-Aldrich ref.: C3045).

Antibodies

The following antibodies were used for western blot (WB) and immunofluorescence (IF): anti-α-Tubulin (WB 1:10000, Abcam ref.: ab7291), anti-β-Actin (WB 1:20000, Sigma-Aldrich ref.: A5441), anti-Phospho p38MAPK (T180/Y182) (WB 1:1000, Cell Signaling ref.: #4511), anti-p38MAPK (WB 1:1000, Abcam ref.: ab170099), anti-Phospho VEGFR2 (Y1175) (WB 1:1000, Cell Signaling ref.: #2478), anti-VEGFR2 (WB 1:1000, Cell Signaling ref.: #3770), anti-Phospho c-Kit (Y719) (WB 1:1000, Cell Signaling ref.: #3391), anti-c-Kit (WB 1:1000, Cell Signaling ref.: #3074 and WB 1:1000, Santa Cruz ref.: sc-13508), anti-DUSP2 (WB 1:1000, IF 1:100, Sigma-Aldrich ref.: SAB4300841), PathScan® RTK Signaling Antibody Array Kit (Chemiluminescent Readout, Cell Signaling ref.: #7982).

Relative RT-PCR

Cultured hippocampal neurons or mice hippocampi were homogenized in Trizol Reagent (Life Technologies ref.: 15596018) and the RNA was extracted using Direct-zolTM RNA minipreps (Zimo research ref.: R2052). RNA was quantified at 260 nm absorbance using a Nanodrop ND-100 (Themo Fisher Scientific). First strand cDNA was obtained using RevertAid H Minus First Strand cDNA Synthesis kit (Themo Fisher Scientific ref.: K1631). 5 ng of synthesized cDNA were used to perform the qPCR using GoTaq® qPCR Master Mix (Promega ref.: A6002) in ABI PRISM 7900HT SDS (Applied Biosystems; Life Technologies). Primers obtained from Sigma-Aldrich were used at 0.5 μM final concentration (see list below). Three housekeeping genes *Gapdh*, *Gus-B* and *Pgk-1* were used as endogenous controls.

Primers

Rat primers used for qPCR in neurons in culture:

Gapdh forward: 5′- ATGACTCTACCCACGGCAAG -3′
Gapdh reverse: 5′- GATCTCGCTCCTGGAAGATG -3′
Gus-B forward: 5′- GCCAATGAGCCTGTCTCTTC -3
Gus-B reverse: 5′- TCCAGTTCTTGGGGAATCTG -3′
Pgk-1 forward: 5′- AATGATGCTTTTGGGACTGC -3′
Pgk-1 reverse: 5′- TCAAAAATCCACCAGCCTTC -3′
Dusp2 forward: 5′- CCCGAGGGTTCCTATCTATG -3′
Dusp2 reverse: 5′- AGGGCAAGATTTCCACAGG -3′

Mouse primers used for qPCR in hippocampal samples:

Gapdh forward: 5′- CTCCCACTCTTCCACCTTCG -3′
Gapdh reverse: 5′- CATACCAGGAAATGAGCTTGACAA -3′
Gus-B forward: 5′- AGCCGCTACGGGCGTCG -3′
Gus-B reverse: 5′- GCTGCTTCTTGGGTGATGTCA -3′
Pgk-1 forward: 5′- TACCTGCTGGCTGGATGG -3′
Pgk-1 reverse: 5′- CACAGCCTCGGCATATTTCT -3′
Dusp2 forward: 5′- CCGAGGGTTCCGATCTATGA -3′
Dusp2 reverse: 5′- TAGGGCAAGATTTCCACAGG -3′

mRNA Sequencing Data

Hippocampal neurons where treated or not 30 min with Cholesterol oxidase (10 IU/ml) after 1 h treatment either with DMSO or the broad p38MAPK inhibitor SB203580 (20 μM). Total RNA was extracted as described for Relative RT-PCR. A differential gene expression analysis of the RNA extracted was performed by GATC Biotech (InView Transcriptome Advance; GATC Biotech) on a Genome Sequencer Illumina HiSeq2500 (HiSeq Rapid Run, 50 bp paired end). Gene expression was analyzed using the Bowtie, TopHat, Cufflinks, Cuffmerge, Cuffdiff software suite.

For the subsequent biocomputational analyses, the differentially expressed rat genes were converted into the corresponding human equivalent UniProt reviewed protein according to the following steps: (i) UniProt ID automatic crossing of the rat proteins with human proteome with corresponding databases (57), (ii) gene name automatic crossing of the rat genes with human genes (Gene Name in UniprotKB database) and (iii) Manual Blast (58), selecting the best reviewed match presenting at least an identity value ≥70% and E-value 10^{-6}.

Artificial Neural Network (ANN) Score Analysis

The possible molecular relationship between the differentially expressed genes and apoptosis and survival was evaluated by means of artificial neuronal networks (ANNs), following TPMS technology protocols (59, 60). This approach involves the generation of mathematical models of the biological processes through the use of artificial intelligence techniques, a methodology involving three steps: (i) the molecular characterization of apoptosis and survival according to bibliography to identify key effector proteins currently associated with these processes (databases: PubMed, ScienceDirect and Scopus), (ii) the generation of a protein-protein map (physical interactions or functional relationships) around these key effectors using information stored in public databases (e.g., Reactome, MINT, BioGrid) and (iii) the transformation of the protein map into mathematical models by training it with a collection of known input-output physiological signals was used obtained from literature mining and a compendium of databases that accumulates biological and clinical data (61).

Then, mathematical models of apoptosis and survival were solved by ANNs, which are supervised algorithms that identify relationships between the different nodes in the network. ANN analysis yields a score for each differential gene based on the validations of the prediction capacity of the mathematical models toward known drugs and diseases, as described in databases.

The higher the score, the stronger is the predicted mechanistic relationship between the evaluated protein and the biological process. Each score is associated with a *p*-value that describes the probability of the result being a true positive one.

Immunofluorescences

Immunofluorescences were performed according to standard procedure. Briefly, brains were perfused with PBS and postfixed in 4%PFA-PBS, cryoprotected and cut sagittal at 40 μm at the cryostat. Sections were incubated with rabbit anti-DUSP2 antibody (SAB4300841, Sigma-Aldrich) diluted 1/100 in 2%BSA-0.1%TritonX100-PBS at 4 degrees for 48 h; next, with a donkey anti-rabbit antibody conjugated with Alexa555 (ThermoFisher, A-31572) diluted 1/500 and DAPI 1/2000 (Merck, 268298) in 2%BSA-0.1%TritonX100-PBS. Pictures were taken in identical conditions for the various samples on a microscope Zeiss Cell Observer, with a camera ORCA-Flash4.0 LT sCMOS (C11440-42U) (Hamamatsu). For low magnification we used a 5X/0.15 Plan-Neofluar dry; for high magnification, 25X/0.8 Plan-Neofluar Oil. Images were processed with ImageJ software to adjust luminosity with identical parameters for control and experimental conditions. The look-up-table "Fire" of ImageJ was used for color coding.

Statistical Analyses

Statistical analyses were performed with Graphpad Prism 5 (Graphpad Software Inc.). All values of the independent experiments are presented as mean ± S.E.M. (standard error of the mean). The numbers of biological replicates are indicated in each figure. Data normality and variances were tested by Shapiro-Wilk test. Student's *t*-test was used for statistical analysis of parametric data. Mann-Whitney *U*-test was used for non-parametric data. Asterisks in the figures indicate *p*-values as follows: * <0.05; ** <0.01; *** <0.001.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and the **Supplementary Files**.

ETHICS STATEMENT

The mice and manipulations presented in this work count with the approval of the Dirección General de Medio Ambiente de la Comunidad Autónoma de Madrid (Ref. PROEX 066/15) and the CBMSO's Ethical Committee: Dr. José Fernández Piqueras, Dr. José Javier Lucas, Dra. Elena Hevia, Dra. Carmen Fernández and D. Fernándo Núñez. All the experiments were performed in accordance with European Union guidelines (2010/63/UE) regarding the use of laboratory animals.

AUTHOR CONTRIBUTIONS

AM-S and CD contributed to the design of the different experiments. AM-S, ÁC-P, and PF performed the experimental work. JM, LA, and RV performed the RNA seq analysis. AM-S did the statistical analysis. AN contributed with experimental suggestions and data interpretation. CD, AM-S, and AN prepared the manuscript. CD is the guarantor of this study.

FUNDING

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We want to especially thank Irene Palomares for the primary cultures of neurons and hippocampal slices.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00675/full#supplementary-material

Figure S1 | Regulation of cholesterol loss-dependent p38MAPK activation in hippocampal neurons. **(A)** Western blots showing p38MAPK (p38) activation (phosphorylation of residues T180/Y182) in hippocampal neurons in culture after cholesterol removal in presence or absence of antioxidants (referred as Antiox.; N-Acetyl-L-Cysteine 5 mM and Glutathione reduced 5 mM) incubated 15 min previous to Choox treatment. **(B)** Western blots of hippocampal neurons in culture show p38MAPK phosphorylation in residues T180/Y182 upon cholesterol depletion in presence or absence of calcium chelator BAPTA-AM (10 μM) 1 h incubation before Choox treatment. **(C)** Blot analysis, in cultured hippocampal neurons, of p38MAPK activating marks (phosphorylated T180/Y182) using PKC (H89 50 μM, left images) or PKA (Chelerythrine 10 μM, referred as Chelery., right images) inhibitors 1 h previous to cholesterol removal treatment. Numbers in bars reflect number of independent experiments. Data are represented as mean ± SEM. The asterisks indicate the *p*-values (*$p < 0.05$; ** $p < 0.01$; ns, not significant).

Figure S2 | Identification of RTKs activated by cholesterol loss in hippocampal neurons. Detail of the RTKs protein array (Cells signaling ref.: #7982) top part left, showing an example of RTKs whose activity state is being modified by cholesterol loss (after incubation with Choox) in hippocampal neurons in culture. Magnification of some representative examples are shown on the top-right part of the figure. The graphics at the bottom of the figure show how the activity state of the representative RTKs change upon cholesterol depletion in hippocampal neurons in culture.

Table S1 | The list of the genes differentially expressed in the comparisons Ctrl vs. Choox, Ctrl vs. Choox+SB203580 and Choox vs. Choox+SB203580, according to the analysis of the RNA sequencing experiment in hippocampal neurons in culture. Gene ID, fold change, *p*-value and *q*-value of the comparisons are included in the tables. The RNA sequencing experiment was performed in cells treated for 30 min with Choox for cholesterol removal, as previously described. Cells were treated or not 1 h before with a known p38MAPK inhibitor (SB203580, 20 μM) in order to determine the effect of p38MAPK cholesterol loss-dependent upregulated activity on the changes observed in genes expression.

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Enhanced Alcohol Preference and Anxiolytic Alcohol Effects in Niemann-Pick Disease Model in Mice

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Major depression and alcohol use disorder are severe psychiatric diseases affecting the world's population with high comorbidity level. However, the pathogenesis of this comorbidity remains unclear, and no selective treatment for this condition is available. A pathogenic pathway and a possible therapeutic target for the treatment of depression-alcoholism comorbidity based on the hyperfunction of acid sphingomyelinase (Asm) were recently suggested. Here we analyzed the effects of alcohol on the depression/anxiety state of homozygous Asm-knockout mice (Asm −/−), which can be considered as a model of an early stage of Niemann-Pick disease, as well as their drinking pattern under normal and stress conditions. It was observed that forced treatment with alcohol (2 g/kg, i.p.) reduces the anxiety level of Asm−/− mice as measured in the elevated plus maze (EPM) test, but enhances the depression level in the forced swim test (FST). The analysis of drinking pattern of these animals in a free-choice alcohol drinking paradigm revealed higher alcohol intake and preference in Asm−/− mice compared to wild type (wt) littermates. However, this difference was overwritten by the stress exposure. Stronger sedating effects of alcohol were observed in Asm−/− mice compared to wt animals in the loss of righting reflex test after single and repeated alcohol injections (3 g/kg, i.p.). Altogether, the present findings might indicate an Asm involvement in the mechanisms of comorbidity between alcoholism and anxiety/depression.

Keywords: acid sphingomyelinase, alcohol, anxiety, depression, stress

INTRODUCTION

Alcoholism is a chronic relapsing brain disorder with high medical and social importance (1, 2). In accordance to WHO data from 2016, more than 2.3 billion people worldwide, which refers to more than one third of the population, are current drinkers of alcohol (3). Epidemiological studies indicate the significance of the problem of alcohol dependence and abuse, as globally an estimated

237 million men and 46 million women have alcohol use disorders leading to about 3 millions yearly deaths worldwide (3).

The harmful effects of alcohol are one of the leading risk factors for mental diseases. Alcohol use disorders increase the risk for depression development by a factor of almost five (4). About 80% of patients diagnosed with alcohol dependence develop depression at some periods of their lives (5). On the other hand, alcohol dependence develops in more than 15% of patients with major depressive disorder (5). The time course of alcohol use disorder is aggravated by depression, as this interaction enhances the probability of alcohol relapse in patients with these comorbid disorders (6–9). However, even though the comorbidity between depression and alcohol consumption is of high importance, little is known about the potentially common mechanisms determining this dangerous interaction. Moreover, personalized treatment strategies for these comorbid disorders have not been developed yet.

Recent studies have observed that ceramide, one of the crucial sphingolipids in biological membranes, might be link between depression and alcohol use disorder. Clinical studies showed a significant increase in the level of lysosomal and secretory acid sphingomyelinase (ASM) in peripheral blood cells of patients with acute alcohol intoxication (10–13). Similarly, a rise in the concentrations of total ceramide as well as the products of ceramide metabolism, sphingosine and sphinganine, was observed in the liver of alcohol-fed mice (14).The levels of ceramide precursor sphingomyelin is found to be reduced in the blood serum of rats exposed to liquid alcohol-containing diet as well as in alcohol dependent patients (15). A study by Roux et al (16) showed a significant elevation of ceramide, and especially Cer16:0, level in the brain of rodents exposed to long-term alcohol drinking. Altogether, these data indicate that a significant shift in ceramide pattern occurs in peripheral tissues during alcohol treatment (17, 18).

On another hand, ceramide was shown to be involved in the pathogenesis of depressive disorder. Clinical studies revealed an increase in ASM activity in the peripheral blood mononuclear cells of patients with major depressive disorder, which positively correlated with symptom severity (19). Similarly, post-traumatic stress disorder is associated with elevated ASM activity and ceramide levels in the blood (20). Animal studies have also shown depression-like behavior as well as a reduction in neurogenesis typical for depression in mice with Asm overexpression (tgAsm) (21). Interestingly, several antidepressants from various chemical groups serve as functional inhibitors of ASM (22–24) suggesting that this enzyme might be crucial for the antidepressant effects (25, 26).

In a recent study in mice, paradoxical antidepressant effects of alcohol were found to depend on Asm activity and the regulation of the sphingolipid rheostat in the brain (27, 28). Here we asked how a complete reduction of Asm activity, which is the main mechanism of type A and B Niemann-Pick disease, affects the interaction of depression and alcohol in Asm deficient (Asm $-/-$) mice. We investigated the behavioral and drinking phenotype of these animals as well as the effects of forced treatment with alcohol on the emotional state.

METHODS

Animals

Asm$-/-$ mice (*Smpd1*$-/-$) are characterized by age-dependent sphingomyelin accumulation and serve as a model of type A or B Niemann-Pick disease (29). Male and female Asm$-/-$ mice and respective wild type (wt) littermates (8–12 weeks old) were studied in gender-balanced designs. Animals were housed in groups in standard cages, or individually housed, when the experiment required it. They were provided with food and water *ad libitum*, and kept on a 12:12 h light: dark cycle (lights on at 7.00 am). Behavioral tests were performed during the light cycle between 09:00 and 16:00 h. Room temperature was maintained between 19 and 22°C at a humidity of 55% (±10%). All experiments were carried out in accordance with the National Institutes of Health guidelines for the humane treatment of animals and the European Communities Council Directive (86/609/EEC) and were approved by the local governmental commission for animal health (Regierung von Unterfranken, Peterplatz 9, 97070 Würzburg). The analysis of data was performed by an investigator blind to the genotype of the tested animals.

Free-Choice Alcohol Drinking Paradigm

Alcohol drinking was tested in naïve 8–9-weeks-old Asm$-/-$ ($n = 15$) and wt ($n = 16$) mice using a two-bottle free-choice drinking paradigm. Each cage was equipped with two constantly available bottles, one of which contained tap water and another bottle contained alcohol (Carl Roth). After an acclimatization period to establish a water-drinking baseline, animals received alcohol at increasing concentrations of 2, 4, 8, 12, and 16 vol. %. Mice were exposed to each concentration of alcohol for 4 days. The bottles were weighed and their positions were changed every second day. Thereafter, alcohol concentration was maintained at 16 vol. % for a total of 13 days. At day 9 of the exposure to 16 vol. % alcohol the animals were exposed to 3-days' forced swim stress. For this purpose, each mouse was placed into a glass transparent cylinder (17 cm diameter, 18 cm height) filled with water (12 cm, 25°C) for 15 min. The consumed amount of alcohol relative to body weight and the preference vs. water were measured and corrected for fluid loss (27, 30).

Taste Preference Test

Alcohol experienced Asm$-/-$ animals (29 days of exposure to free-choice drinking, as described above) were used for this test. Sucrose (0.5 and 5%; Merc Chemicals) and quinine (2 and 20 mg/dl; Merc Chemicals) preference was measured in a two-bottle free-choice test vs. water, 3 days after the last alcohol exposure. Each dose was offered for 2 days with the position of the bottles being changed and weighed daily with 1 day wash out between sucrose and quinine testing (27, 30).

Loss of Righting Reflex

Alcohol naïve 8-weeks-old animals (Asm$-/-$, $n = 23$; wt, $n = 21$) were used for this test. Animals were administered with an alcohol injection of 3.5 g/kg (i.p., 20 ml/kg) for 7 consecutive days at the same time. Testing of loss of righting reflex (LORR) was performed at days 1 and 8 of the treatment. After an alcohol

injection, the animals were immediately placed in an empty cage. LORR was observed when the animals became ataxic and stopped moving for at least 30 s. Each animal was then placed on its back. Recovery from LORR was defined as the animal being able to right itself three times within a minute. Time taken for the animals to lose its righting reflex, and time to recovery from the alcohol effect was recorded (27, 30).

Blood Alcohol Concentration

Alcohol naïve 8–10-weeks-old animals (Asm−/−, *n*=8; wt, *n*=7) were used for this test. Mice received alcohol injections (3 g/kg, 20 ml/kg, i.p.) and 20 μl blood samples were obtained from the submandibular vein 1, 2, and 3 h after alcohol injection. The blood samples were directly mixed with 80 μl 6.25 % (w/v) trichloroacetic acid (Sigma). After centrifugation 15 μl of the supernatant were subjected to enzymatic alcohol determination using the alcohol dehydrogenase method as described elsewhere (31, 32).

Behavioral Testing

Alcohol naïve 8–10-week-old mice were tested in a battery of behavioral tests in the following order: open field (OF), elevated plus maze (EPM), novelty suppressed feeding (NSF), and forced swim test (FST). Animals received injections of alcohol (2 g/kg, 10 ml/kg; i.p.; Carl Roth) or physiological saline 30 min before each test. Group sizes were the same for all tests (Asm−/− receiving alcohol: $n = 9$; Asm−/− receiving saline: $n = 8$; wt receiving alcohol: $n = 14$; wt receiving saline: $n = 12$). All tests were performed on separate days between 09:00 and 15:00 h with at least 2 days' interval between them. Mice were moved to the behavioral test room 1 h before testing and were tested in a pseudorandom order. Each test apparatus was cleaned with 70% alcohol between subjects.

Open Field

Each mouse was placed in a square white acrylic arena (50 × 50 cm), facing a wall, for 20 min and allowed to freely explore the arena. The area was lighted with white light (100 lx) (33). Video recordings were taken and analyzed using Biobserve Viewer III (Biobserve GmbH, Germany). A virtual square of equal distance from the periphery (25 × 25 cm) was defined as the "central zone," and the outer part of the arena was defined as "peripheral zone." Distance moved in the peripheral and central zones, number of entries, latent period of the first entrance, and time spent in the central zone were registered (27, 34).

Elevated Plus Maze

The EPM (35) was constructed from black opaque acrylic with white lining on the floor, each arm measuring 30 × 5 cm and the central platform 5 × 5 cm. Two opposite arms were enclosed by a 15-cm wall of opaque acrylic, while the other two arms were open with a ledge of 0.5 cm on the sides. The maze was elevated 50 cm from the ground. Each mouse was placed on the central platform, facing toward a closed arm, and allowed to freely explore the maze for 5 min. Biobserve Viewer III tracking software (Biobserve GmbH, Germany) was used to record the distance moved in the open and closed arms, and the number of entries into the closed and open arms and time spent in them (27, 34).

Novelty Suppressed Feeding

Animals were deprived from food for 24 h before the test. After deprivation each mouse was put in the corner of a square white acrylic arena (50 × 50 × 50 cm, 100 lx), facing a wall. A piece of food was placed in the center of the arena. Video recordings were taken and analyzed using Biobserve Viewer III (Biobserve GmbH, Germany). The time (s) before a mouse began eating after placing to the arena and the distance moved before eating were registered (27, 34).

Forced Swim Test

At the day 1 of the test, each mouse was placed into a glass transparent cylinder (18 cm diameter, 19 cm height) filled with water (13 cm, 25°C) for 15 min (36). After 24 h mice were again placed in this cylinder with water for 5 min. The latency of first floating, and total floating time during the day 2 were recorded (Biobserve Viewer III, Biobserve GmbH, Germany) and analyzed manually (27, 34).

Enzyme Activity Measurement

Asm activity was measured in the dorsal hippocampus (DH) of Asm−/− mice after forced treatment with alcohol and following behavioral testing. In addition, the activity of Asm, neutral sphingomyelinase (Nsm), neutral ceramidase (Nc), and acid ceramidase (Ac) were measured in the DH of Asm−/− mice exposed to alcohol treatment on the model of two-bottle free-choice alcohol drinking. After the behavioral testing the animals were sacrificed, brain tissue was harvested and snap frozen with dry ice. The DH was isolated from the brains for enzyme analysis.

The Asm and Nsm activity was determined using the fluorescent substrate BODIPY-FL-C12-SM (N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza-s-indacene-3-dodecanoyl)sphingosyl phosphocholine, Invitrogen/Life Technologies), with four replicates for each sample. NBD-C12-Cer fluorescent substrate was used for the analysis of Ac and Nc activities. Tissues were homogenized in lysis buffer (250 mM sucrose, 1 mM EDTA, 0.2% Triton X-100, 1 × Roche protease inhibitor cocktail, 400 μl/10 mg tissue) using a TissueLyser LT bead mill (Qiagen) with steal beads followed by freezing at −80°C to enhance lysis. Supernatants obtained after centrifugation at 13,000 × g and 4°C for 10 min were diluted 1:3 in lysis buffer and used for activity assays and for protein determination (Bradford/Coomassie kit, ThermoFisher). A standard enzyme reaction contained 58 pmol BODIPY-FL-C12-SM (for Asm and Nsm) or 50 pmol NBD-C12-Cer (for Ac and Nc) as a substrate and 2 μl tissue lysate in a total volume of 50 μl reaction buffer of the following composition: 200 mM sodium acetate buffer (pH 5.0), 500 mM NaCl, 0.2% Nonidet P-40 detergent for Asm, 200 mM HEPES buffer (pH 7.0), 200 mM $MgCl_2$, 0.05% Nonidet P-40 for Nsm; 200 mM sodium acetate buffer (pH 4.5), 100 mM NaCl, 0.03% Nonidet P-40 for Ac and 200 mM HEPES (pH 7.0), 100 mM NaCl, 0.03% Nonidet P-40 for Nc. Further analysis using thin layer chromatography was performed as described previously (27, 37).

Statistical Analysis

All data are presented as means ± standard error of the mean (S.E.M.). The data were compared using a two-way analysis of variance (ANOVA; IBM SPSS Statistics 21). Following a significant ANOVA, the independent pre-planned comparisons were made. Since the specific scientific hypotheses was investigated and all comparisons among means were considered to be of substantive interest a priori, the Fisher's LSD test was used for pre-planned comparisons between individual groups. Pair-wise comparisons using two tailed *t*-tests (IBM SPSS Statistics 21) were performed between trials for each genotype group (38). Although sex differences are well-known in the studies on addiction and depression (39, 40), we did not see significant sex differences in the major parameters of this study, and thus the data were collapsed for analysis. A significance level of $p < 0.05$ was used for statistical significance.

RESULTS

Free-Choice Alcohol Drinking

Previous data indicate the contribution of Asm in alcohol sensitivity and pathogenesis of alcohol use disorder (10, 11, 14, 15, 27). Here, we report the higher preference of alcohol on the model of free-choice alcohol drinking in Asm−/− mice compared to wt littermates. A two-way ANOVA for alcohol preference did not reveal a significant effect for genotype [$F_{(1,14)} = 0.659$, $p = 0.431$] and dose [$F_{(4,56)} = 2.408$, $p = 0.060$], as well as interaction of these factors [$F_{(4,56)} = 2.114$, $p = 0.091$; **Figure 1A**]. However, the pre-planned comparison showed a significantly higher alcohol preference at 16 vol. % alcohol in Asm−/− mice compared to wt animals ($p = 0.042$). The ANOVA analysis for alcohol consumption also did not show a genotype effect [$F_{(1,14)} = 0.326$, $p = 0.577$], but revealed a dose effect [$F_{(1,14)} = 81.809$, $p < 0.001$]. A genotype × dose interaction for alcohol consumption was not significant [$F_{(1,14)=}$ 1.583, $p = 0.229$; **Figure 1B**]. Single day comparison showed slightly higher alcohol consumption at 16 vol. % alcohol concentration in Asm−/− mice, which, however, did not reach statistical significance ($p = 0.160$). A two-way ANOVA for total fluid intake showed no significant effect for the genotype [$F_{(1,14)} = 0.107$, $p = 0.748$], but a significant dose effect [$F_{(4,56)} = 4.443$, $p = 0.003$; **Figure 1D**]. A genotype × dose interaction for this parameter was not significant [$F_{(4,56)} = 0.838$, $p = 0.507$]. Similarly, ANOVA for water intake revealed no significant effect for the genotype [$F_{(1,14)} = 0.401$, $p = 0.537$] or genotype × dose interaction [$F_{(4,56)} = 2.394$, $p = 0.061$], but a significant dose effect [$F_{(4,56)} = 3.356$, $p = 0.016$; **Figure 1C**]. However, pre-planned comparison did not reveal significant differences between genotypes at various alcohol concentrations ($p > 0.05$).

The analysis of alcohol drinking after 3-days' swim stress showed no genotype effect [$F_{(1,14)} = 1.626$, $p = 1.223$], but a significant effect of stress [$F_{(5,70)} = 31.697$, $p < 0.001$; **Figure 1E**]. The genotype × stress interaction was not significant [$F_{(5,70)} = 1.475$, $p = 0.209$]. Pre-planned comparison revealed a significantly lower post-stress alcohol consumption both in Asm−/− and wt mice at all test days, respectively (day 1: $p < 0.001$, $p = 0.025$; day 2: $p < 0.001$, $p = 0.035$; day 3: $p < 0.001$, $p = 0.002$; day 4: $p < 0.001$, $p < 0.001$; day 5: $p < 0.001$, $p < 0.001$). Alcohol consumption did not differ between Asm−/− and wt mice during and after stress except for the first post-stress day ($p = 0.041$). These data suggest that a complete lack of Asm is associated with the higher susceptibility of mice to alcohol consumption at high doses. This effect is overwritten by stress exposure.

Taste Preference

Statistical analysis did not reveal any genotype differences in the preference of 0.5% sucrose ($t = -0.741$, $p = 0.471$; **Figure 2A**). However, the preference of 5% sucrose was significantly higher in wt mice compared to Asm−/− animals ($t = -3.168$, $p = 0.007$). Similarly, no differences were observed in the preference of quinine in the dose of 2 mg/dl ($t = -0.647$, $p = 0.529$), but the preference of quinine in the dose of 20 mg/dl was higher in wt mice ($t = -2.450$, $p = 0.030$; **Figure 2B**). These data indicate that a complete lack of Asm mildly reduces hedonic preference, but enhances avoidance of aversive stimuli.

Loss of Righting Reflex

No significant genotype [$F_{(1,36)} = 2.298$, $p = 0.138$] or time effect [$F_{(1,36)} = 2.918$, $p = 0.096$], as well as no genotype × time interaction [$F_{(1,36)} = 0.229$, $p = 0.635$] were observed for LORR latency (**Figure 2C**). Pre-planned comparison did also not yield genotype-specific and time-induced differences in this parameter ($p > 0.05$).

A two-way ANOVA for the duration of LORR revealed a genotype effect [$F_{(1,22)} = 12.609$, $p = 0.002$] as well as time effect [$F_{(1,22)} = 9.936$, $p = 0.005$], but no genotype × time interaction [$F_{(1,22)} = 1.073$, $p = 0.312$; **Figure 2D**]. Pre-planned comparison showed that the duration of LORR is significantly higher in Asm−/− mice compared to wt animals both at day 1 ($p = 0.016$) and 8 ($p = 0.016$). The duration of LORR in wt animals significantly declined from day 1 to day 8 ($p = 0.015$). This effect was also observed in Asm−/− mice, but did not reach statistical significance ($p = 0.098$). These findings suggest that a complete lack of Asm makes animals more susceptible to sedative effects of alcohol.

Blood Alcohol Concentration

A two-way ANOVA for blood alcohol concentration revealed a genotype effect [$F_{(1,12)} = 5.551$, $p = 0.036$] as well as time effect [$F_{(2,24)} = 44.537$, $p < 0.001$], but no genotype × time interaction [$F_{(2,24)} = 0.544$, $p = 0.587$; **Figure 2E**]. Pre-planned comparison did not show significant genotype differences in the blood alcohol concentration within 1 h ($p = 0.671$), 2 h ($p = 0.165$), but a tendency after 3 h ($p = 0.057$) after the injection. These finding suggests a preserved bioavailability of alcohol in Asm−/− mice.

The Activity of Enzymes Mediating Ceramide Metabolism After Free-Choice Alcohol Drinking

Our previous data indicate that the effects of alcohol on the depression/anxiety-like behavior of mice are mediated by the alterations in brain Asm activity as well as the state

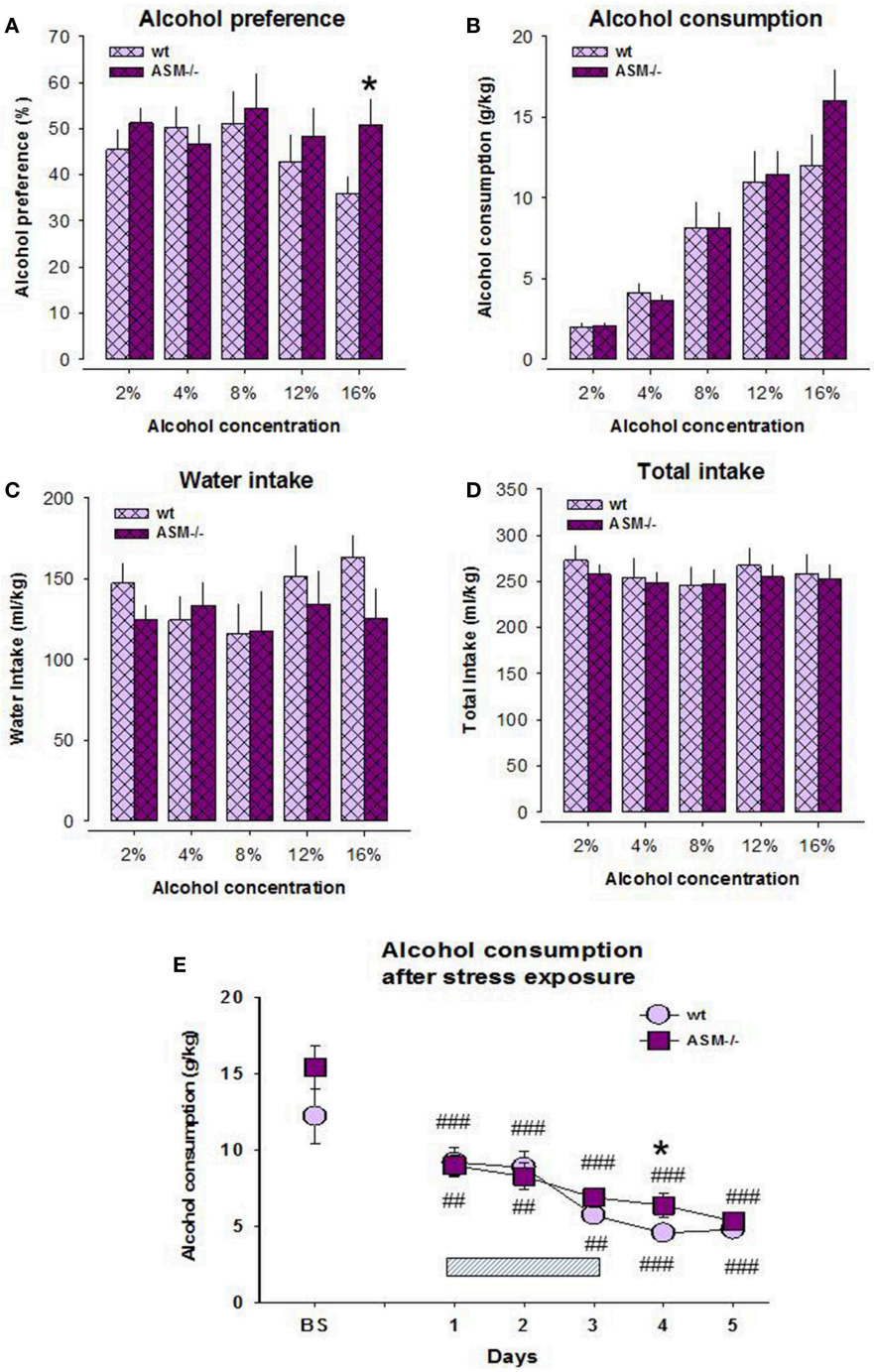

FIGURE 1 | Alcohol preference **(A)** and alcohol consumption **(B)** on the model of two-bottle free-choice drinking is enhanced in homozygous mice with acid sphingomyelinase deficiency (ASM−/−) compared to wild type animals (wt). The levels of water intake **(C)** and total fluid intake **(D)** do not significantly differ between ASM−/− and wt mice. Stress **(E)** overwrites the differences in the drinking pattern of these animals. The hatched box indicates the time of stress exposure. The error bars show the mean ± SEM (*$p < 0.05$ vs. wt animals; ###$p < 0.001$, ##$p < 0.01$ vs. baseline, BS).

of sphingolipid rheostat (27). Here we showed that other enzymes of the sphingomyelinase pathway are not involved in the effects of alcohol. A two-way ANOVA revealed a strong genotype effect for Asm activity in the DH of mice exposed to the free-choice alcohol drinking paradigm [$F_{(1,37)} = 215.091$, $p < 0.001$], but no treatment effect [$F_{(1,37)} = 0.549$, $p = 0.463$] or genotype × treatment interaction [$F_{(1,37)} = 0.590$, $p = 0.447$; **Figure 3A**]. Pre-planned comparison did also not reveal alcohol effects on the Asm activity in Asm−/− ($p = 0.986$) or wt mice ($p = 0.248$). As

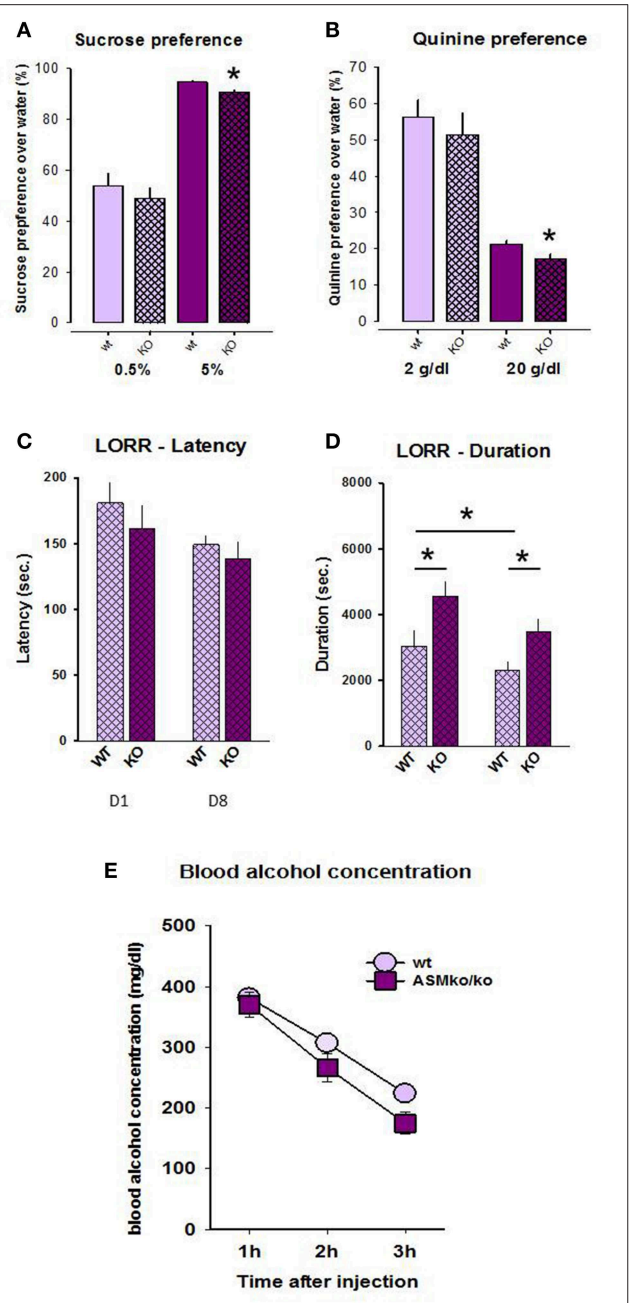

FIGURE 2 | Sucrose **(A)** and quinine **(B)** preference is lower in mice with acid sphingomyelinase deficiency (KO) compared to wt littermates. The duration **(D)**, but not the latency **(C)** of loss of righting reflex is significantly higher in mice with acid sphingomyelinase deficiency both at day 1 and 8 of alcohol treatment. Blood alcohol concentration **(E)** does not significantly differ between mice with acid sphingomyelinase deficiency and wt mice. The error bars show the mean ± SEM (*$p < 0.05$ vs. wt animals).

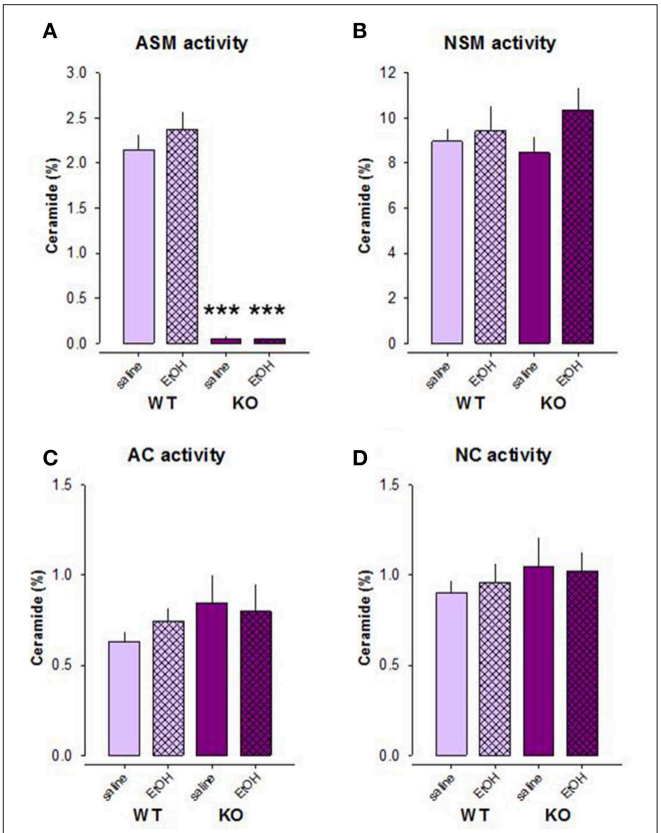

FIGURE 3 | The activity of Asm **(A)** is significantly lower in the dorsal hippocampus of mice with acid sphingomyelinase deficiency (KO) exposed and not exposed to alcohol treatment on the model of two-bottle free-choice drinking. No significant differences in the activities of neutral sphingomyelinase **(B)**, acid ceramidase **(C)**, and neutral ceramidase **(D)** are observed under these conditions. The error bars show the mean ± SEM (***$p < 0.001$ vs. wt animals).

expected, Asm activity was significantly lower in Asm−/− mice drinking both water and alcohol compared to wt mice ($p < 0.001$).

No significant genotype [$F_{(1,37)} = 0.062$, $p = 0.805$] or treatment effects [$F_{(1,37)} = 1.770$, $p = 0.192$] on Nsm activity or a genotype × treatment interaction [$F_{(1,37)} = 0.659$, $p = 0.422$] were observed (**Figure 3B**). Nsm activity did not differ between Asm−/− and wt mice receiving water ($p = 0.697$) or alcohol ($p = 0.450$). Moreover, alcohol had no effect on this parameter in wt ($p = 0.689$) and Asm−/− ($p = 0.170$) mice.

Similarly, the activity of Ac and Nc was also not altered by alcohol treatment in Asm−/− or wt mice (**Figures 3C,D**). A two-way ANOVA did not reveal an effect of genotype [Ac: $F_{(1,37)} = 1.658$, $p = 0.206$; Nc: $F_{(1,37)} = 0.953$, $p = 0.335$] or the treatment [Ac: $F_{(1,37)} = 0.105$, $p = 0.747$; Nc: $F_{(1,37)} = 0.025$, $p = 0.876$], as well as no effects of genotype × treatment interaction [Ac: $F_{(1,37)} = 0.597$, $p = 0.444$; Nc: $F_{(1,37)} = 0.150$, $p = 0.701$]. Pre-planned comparison also did not reveal any alcohol-induced alterations in the activity of Nc or Ac in the animals ($p > 0.05$). Altogether, these findings confirm the efficiency of Asm−/− model and a lack of counteracting mechanisms in the enzymes of the ceramide rheostat. They also show that alcohol consumption has no effects on Asm, Nsm, Ac, and Nc activity in the DH of animals.

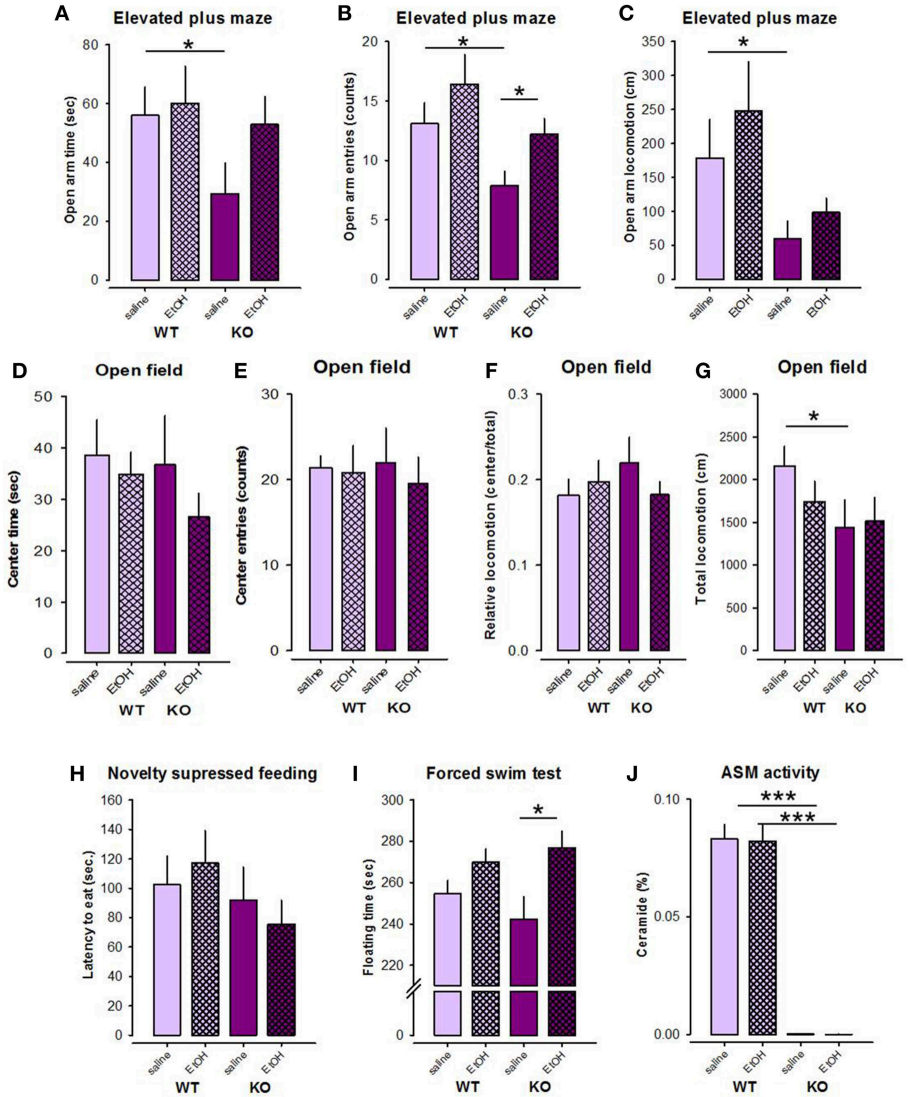

FIGURE 4 | Forced treatment with alcohol modeled by intraperitoneal injection with alcohol reduces anxiety level selectively in mice with acid sphingomyelinase deficiency (KO) as observed in the elevated plus maze **(A–C)**, but not in the open field test **(D–G)**. Alcohol injections do not affect the behavior of mice in the novelty suppressed feeding test **(H)**, but has depressogenic effect in KO mice in the forced swim test **(I)**. Forced treatment with alcohol does not affect acid sphingomyelinase (Asm) activity in the dorsal hippocampus of KO and wild type (wt) mice **(J)**. The error bars show the mean ± SEM (***$p < 0.001$, *$p < 0.05$ vs. wt animals).

Forced Treatment With Alcohol

Elevated Plus Maze

Previous data showed the alterations in depression/anxiety-like behavior in mice with altered Asm activity (21, 27), which in the animals with Asm overexpression can be specifically altered by alcohol treatment (27). Here we observed higher anxiety level in Asm−/− mice in the EPM test. An ANOVA revealed significant genotype differences (Asm−/− vs. wt) between the number of entries to the open arms and locomotion in the open arms of the EPM [$F_{(1,37)} = 5.781$, $p = 0.021$; $F_{(1,37)} = 6.018$, $p = 0.019$], respectively, but not in the time spent in the open arms [$F_{(1,37)} = 2.304$, $p = 0.138$; **Figures 4A–C**]. However, no treatment effects [$F_{(1,37)} = 3.803$, $p = 0.059$; $F_{(1,37)} = 0.975$, $p = 0.330$; $F_{(1,37)} = 1.524$, $p = 0.225$] or genotype × treatment interactions [$F_{(1,37)} = 0.065$, $p = 0.800$; $F_{(1,37)} = 0.073$, $p = 0.788$; $F_{(1,37)} = 0.761$, $p = 0.389$] were observed for these parameters. Pre-planned comparisons showed that Asm−/− mice receiving saline were characterized by lower number of entries to the open arms ($p = 0.014$), reduced locomotion in the open arms ($p = 0.038$), and also lower time spent in the open arms ($p = 0.041$) of the EPM compared to wt littermates (**Figures 4A–C**). These data indicate higher level of anxiety in alcohol-naïve Asm−/− mice. Asm−/− mice receiving alcohol were characterized by the significantly higher number of entries to the open arms comparing to the Asm−/− animals treated with saline ($p = 0.015$). Similar tendencies were found for the

time spent in the open arms ($p = 0.060$) and locomotion in the open arms ($p = 0.128$). However, no significant changes in the study parameters were observed in wt animals injected with alcohol ($p > 0.05$). Thus, these data indicate that the high anxiety observed in Asm−/− mice was reversed by the forced alcohol treatment.

Open Field

An ANOVA for the time spent in the center of the OF did not reveal significant genotype [$F_{(1,38)} = 0.667$, $p = 0.419$] or treatment effects [$F_{(1,38)} = 1.257$, $p = 0.269$], as well as no genotype × treatment interaction [$F_{(1,38)} = 0.269$, $p = 0.607$; **Figure 4D**]. No effects of alcohol on this parameter were observed in wt and Asm−/− mice ($p > 0.05$). Similarly, no genotype effect [$F_{(1,38)} = 0.010$, $p = 0.921$], treatment effect [$F_{(1,38)} = 0.231$, $p = 0.634$], or genotype × treatment interaction [$F_{(1,38)} = 0.096$, $p = 0.758$] was found for the analysis of the number of entries to the center of the OF (**Figure 4E**). Pre-planned comparison did not show any effects of alcohol on the number of entries to the center of the OF in Asm−/− or wt animals ($p > 0.05$). Similarly, the relative locomotion of the study animals was also not altered by alcohol [genotype effect: $F_{(1,38)} = 0.244$, $p = 0.624$; treatment effect: $F_{(1,38)} = 0.202$, $p = 0.656$; genotype × treatment interaction: $F_{(1,38)} = 1.329$, $p = 0.256$; **Figure 4F**]. Pre-planned comparison did not reveal alcohol-induced changes in the relative locomotion of Asm−/− or wt animals ($p > 0.05$). Total locomotion tended to decrease in Asm−/− mice compared to wt littermates [genotype effect: $F_{(1,38)} = 3.170$, $p = 0.083$; treatment effects: $F_{(1,38)} = 0.436$, $p = 0.513$; genotype × treatment interaction: $F_{(1,38)} = 0.873$, $p = 0.356$; **Figure 4G**]. Pre-planned comparison confirmed a significant reduction in total locomotion in alcohol-naïve Asm−/− mice receiving vehicle compared to wt mice ($p = 0.023$).

Novelty Suppressed Feeding

ANOVA revealed no genotype or treatment effects on the latency of first eating in the NSF test [$F_{(1,38)} = 1.599$, $p = 0.214$; $F_{(1,38)} = 0.002$, $p = 0.969$; **Figure 4H**]. Genotype × treatment interaction was also not significant [$F_{(1,38)} = 0.568$, $p = 0.456$]. Pre-planned comparisons also did not yield significant between-group differences ($p > 0.05$).

Forced Swim Test

An ANOVA did not show a genotype effect [$F_{(1,38)} = 0.117$, $p = 0.734$], but a significant treatment effect on time of floating in the FST [$F_{(1,38)} = 9.907$, $p = 0.003$; **Figure 4I**]. The genotype × treatment interaction was not significant [$F_{(1,38)} = 1.508$, $p = 0.227$]. Pre-planned comparison showed that alcohol treatment induced a significant increase in floating time in Asm−/− mice ($p = 0.013$), and a tendency to increase in wt animals ($p = 0.056$). These data suggest that forced alcohol administration has depressogenic effect, which are elevated in mice with lack of Asm.

Asm Activity After Forced Alcohol Treatment

An ANOVA revealed a significant genotype effect on the Asm activity in the DH [$F_{(1,39)} = 299.415$, $p < 0.001$], but not treatment effect [$F_{(1,39)} = 0.053$, $p = 0.820$] or genotype × treatment interaction [$F_{(1,39)} = 0.045$, $p = 0.833$; **Figure 4J**]. Asm activity in the DH of Asm−/− mice receiving saline was significantly lower than in wt specimens ($p < 0.001$). Subchronic treatment with alcohol (2 g/kg) did not affect Asm activity in the DH neither in Asm−/− ($p = 0.213$), nor in wt animals ($p = 0.391$). These findings suggest that the forced administration of alcohol does not affect Asm activity in the DH.

DISCUSSION

Ceramide is an important lipid molecule shown to contribute to the interaction between alcohol use disorder and depression via an Asm-mediated rebalancing of the sphingomyelinase rheostat (27). Here we report that Asm deficiency in mice is associated with enhanced alcohol preference in a two-bottle free-choice drinking paradigm. However, stress exposure overwrites the differences in the drinking phenotype of Asm−/− and wt mice. Forced alcohol treatment modeled by intraperitoneal alcohol injections reverses the high anxiety-like behavior in Asm−/− mice, but does not affect anxiety-related behavior in wt animals. However, acute alcohol treatment induced depression-like behavior specifically in Asm−/− mice. Interestingly, the effects of alcohol on the emotional state of the mice were not mediated by alteration/inhibition of ceramide synthesis. Thus, these data expand the specific involvement of Asm in the interconnection between alcoholism and anxiety/depression in mice.

The analysis of drinking pattern in Asm−/− mice showed a higher alcohol preference on the two-bottle free-choice alcohol drinking compared to wt animals. The total fluid intake did not differ between Asm−/− and wt mice reflecting the specificity of the observed pattern of alcohol consumption. Interestingly, the BAC study has observed slightly reduced alcohol availability 3 h after alcohol administration in Asm−/− mice. This might indicate higher metabolic rate of alcohol in these animals compared to wt mice. These results might possibly explain the higher consumption of alcohol in the free-choice alcohol drinking paradigm in Asm−/− mice. In order to reach the same blood alcohol concentration (BAC) over time, Asm−/− mice may have to consume higher amounts of alcohol compared to wt animals. These data are in line with the data obtained in patients with chronic alcoholism. They show greater tolerance to alcohol due to an increase in the alcohol elimination rate and metabolic tolerance (41). Previous animal studies also confirm this hypothesis as alcohol-preferring rats are characterized by a significantly lower blood alcohol concentration compared to non-preferring controls (42), probably due to more rapid alcohol metabolism and higher activity of alcohol dehydrogenase (43).

The analysis of taste preference in Asm−/− mice showed a slightly lower preference of sucrose and lower avoidance to quinine in these animals compared to wt littermates, which reached significance only at high doses of both substances (20 g/dl and 5%). These data might reflect a higher aversion developing in Asm−/− mice and a reduced hedonic perception. Thus, the higher preference of alcohol in these animals compared to wt mice might indicate the specificity of alcohol drinking

phenotype, which is unlikely due to the changes in the hedonic and aversion avoidance.

The exposure to stress on the model of forced swimming induced a decrease in alcohol consumption in wt and Asm−/− mice and overwrote the genotype-specific difference in the alcohol drinking pattern. The consumption of alcohol was reduced after stress in both Asm−/− and wt mice. These data are in line with previous studies. It is well-known that stress is a factor triggering alcohol consumption in humans as well as in animal models. However, human studies have observed ambiguous effects of stress on the pattern of drinking in both females and males. Even though the frequency of heavy drinking has positive correlation with the stress level, the frequency of moderate drinking decreased with an increase in stress levels (44, 45). The model of two-bottle free-choice alcohol drinking allows animals to self-titrate with alcohol and to maintain a moderate level of drinking as confirmed by BAC studies (46). Moreover, our data suggested that alcohol treatment enhances depression level in wt and particularly Asm−/− mice. Taking into account that FST may induce a depressive state (47), it may be suggested that Asm−/− mice reduce alcohol consumption after stress to possibly reduce the depression level.

It was proposed that re-balancing of brain sphingolipid composition after stress exposure might play a protective role against a stress-induced increase in alcohol consumption. Previous studies revealed a significant dysregulation of sphingolipid and phospholipid metabolism in rats after chronic unpredictable stress (48). In the prefrontal cortex, chronic unpredictable stress was followed by an increase in ceramide, lysophosphatidylethanolamine, and 38 carbon (38C)-lipid levels, and a reduction in sphingomyelin and dihydrosphingomyelin concentrations, phosphatidylethanolamine, ether phosphatidylcholine, and 36C-lipid levels. Similarly, a decrease in phosphatidylcholine and sphingomyelin, and an increase in phosphatidylserine, ceramide, and dihydrosphingomyelin levels were observed in the hippocampus of rats exposed to chronic unpredictable stress (48). As it was shown, various lipid species contribute to the development of addiction, and particularly alcohol dependence (18), and thus the reestablishment of lipid balance after stress exposure might determine the absence of increase in alcohol consumption in Asm−/− mice.

The analysis of LORR showed higher sedative effects of alcohol in Asm−/− mice compared to wt animals at day 1 and 8 reflecting the higher sensitivity of the CNS to alcohol. These data are in line with previous studies showing that mice with higher alcohol preference display higher initial sensitivity to alcohol, as measured by the static dowel task (49). Similarly, human studies also suggested a higher sensitivity to the subjective effects of alcohol in patients with a family history of alcohol use disorder (50). Interestingly, the latency and duration of LORR significantly decreased in wt animals and tended to decrease in ASM−/− mice at day 8 of the experiment, reflecting the development of alcohol tolerance.

Interestingly, the observed drinking phenotype of Asm−/− mice resembles the phenotype of mice with Asm overexpression, but not heterozygous Asm knockout mice (Asm+/−) as it was shown in our previous study (27). It might be proposed that the complete knockout of the ASM coding gene induces possible alterations in other pathways, particularly other mechanisms of ceramide synthesis, such as *de novo* pathway, in Asm−/− mice, which may independently contribute to elevated alcohol drinking levels. The *de novo* pathway was shown to mediate the elevation in ceramide synthesis in the liver during alcohol consumption reflecting that both, sphingomyelinase and *de novo* pathways, contribute to the ceramide response to alcohol treatment (51–53). Particularly, Tong et al. (54) showed that an inhibitor of the *de novo* ceramide synthesis, myriocin, inhibits a rise in liver ceramide level and the severity of alcohol-related steatohepatitis in rats exposed to chronic treatment with alcohol. However, single treatment of animals with myriocin does not completely block the observed alcohol-induced changes (51–53). Thus, it might be speculated that the *de novo* pathway of ceramide synthesis determines the similarity in the drinking pattern of Asm−/− mice and animals with Asm overexpression. Moreover, recent data showed that pharmacological inhibition of Asm in mice by antidepressants amitriptyline and fluoxetine is followed by the reduction of lysosomal ceramide level, but the accumulation of ceramide in the endoplasmic reticulum (21, 55). It can be proposed that an increase in endoplasmic reticulum ceramide might serve as a compensatory mechanism balancing the ceramide level in ASM−/− mice. However, a variety of additional lipidomic mechanisms determining alcohol consumption, which include several interconnected lipid species besides ceramide (17, 18), might be altered in Asm−/− mice. Thus, further analysis of possible compensatory pathways is needed to clarify the exact pathways.

The analysis of anxiety/depression in Asm−/− mice showed higher anxiety of these animals in the EPM, but not in the OF test. These differences might be related to the specific features of behavior investigated in these tests. Thus, the behavioral pattern of animals investigated in the OF test is mostly based on the exploratory behavior and locomotor activity. In contrast, cue-related behavior is mostly investigated in the EPM (56). It has been proposed that emotionality is a multi-dimensional parameter, which can be explored from various perspectives. Thus, different kinds of environments, e.g., open spaces, illuminated or elevated areas, might yield different kinds of behavioral responses (57).

Our behavioral data do not replicate the previous findings showing lower anxiety and depression levels in Asm−/− mice manifested in the higher center time in the OF test and time spent in the light compartment of the light-dark box, as well as shorter latency of first eating in the NSF test and shorter immobility time in the FST (21). However, the study by Gulbins et al. (21) was performed on the animals aging up to 7 weeks, which do not develop any behavioral manifestations of Niemann-Pick disease and are not characterized by the excessive sphingolipid accumulation in the tissues. In the present study we used 8–10-weeks old Asm−/− mice, which might be considered as a model of an early stage Niemann-Pick disease. Investigation of these animals showed that they do not possess full symptom expression yet, but have a slight impairment of locomotor activity in the OF test. However, the difference in the age might determine sphingolipid accumulation in the tissues, which might reverse the low depression and anxiety phenotype observed in younger

ASM−/− mice. Moreover, the reason of this dissociation might be also based on the differences in the experimental conditions. In our experiment the animals received alcohol/saline injections 30 min before all tests, which can be considered as a mild stress. However, the present investigation also revealed that stress might affect animal behavior and even overwrite the Asm genotype-driven differences (21).

The data on the anxiety/depression state in Asm−/− mice are in line with the clinical studies on patients with Niemann-Pick disease. Niemann-Pick disease is a rare neurovisceral lysosomal storage disorder with a heterogeneous phenotype. The pathogenesis of types A and B of this disorder are based on Asm deficiency followed by sphingomyelin accumulation in the large, lipid-laden foam cells, which are present in the liver, spleen, lymph nodes, adrenal cortex, lung airways, and bone marrow (58). To our knowledge, no data on the anxiety and depression state of these patients are available. However, enhanced anxiety is observed in the patients with Niemann-Pick diseases, type C (59), which is characterized by sphingolipid accumulation in biological tissues and organs due to the mutations in the *NPC1* or *NPC2* genes (60). It was shown that ASM activity in the cells of these patients is also reduced (61). Moreover, BALB/cJ *Npc1* ^{nih} mice with *NPC1* haploinsufficiency, an animal model of type C Niemann-Pick disease, are characterized by increased anxiety-like behavior in early maturity. However, to our knowledge, the level of ASM activity in these animals was not measured (62). Thus, our data on the anxiety/depression state of Asm−/− mice, which might be considered as a model of Niemann-Pick disease type A and B, might add on to the current knowledge of the pathogenesis of this disorder.

The higher anxiety pattern in Asm−/− mice is in line with higher alcohol preference observed in these animals. Previous clinical data show that higher basal anxiety level might result in greater alcohol intake, which can promote alcohol abuse in humans (63). Moreover, a shared genetic risk for both anxiety and alcohol use disorder was observed in family studies (64, 65). Animal data also confirm these observations. For example, Spanagel et al. (66) showed that a high anxiety level in the EPM is associated with higher voluntary drinking in rats.

Interestingly, forced treatment with alcohol in a moderate dose (2 g/kg) had anxiolytic and depressogenic properties in Asm−/− mice. No such effects were observed in wt littermates. Anxiolytic effects of alcohol were manifested in an increase in the time spent, entries and locomotion in the open arms of the EPM. Depressogenic effects became evident by an increase in the floating time in the FST in Asm−/− mice. These data resemble our previous results on Asm+/- mice, which show only partial reduction of Asm activity in the brain. Similar anxiolytic and depressogenic effects of alcohol were observed in these animals (27). A reduction in anxiety level observed in homo- and heterozygous mice with reduced Asm activity are in line with literature. It was shown that rats preselected for high anxiety in the EPM demonstrate more pronounced anxiolytic effects of an alcohol injection compared to low anxiety animals (67). And vice versa, a rodent line preselected for high alcohol consumption showed stronger anxiolytic effects of alcohol in a battery of emotional tests (68, 69).

Similar depressogenic effects of alcohol, as it was shown in Asm−/− mice line, were also shown before (70, 71). A similar dissociation in the effects of alcohol on the emotional behavior was observed in our previous studies on mice with Asm overexpression (27). It should be noted that despite of the high comorbidity of anxiety and depression, certain pathways and neurological mechanisms of these disorders differ in the brain (72). Thus, our findings may suggest that Asm-mediated mechanisms might at least partly contribute to the pathways determining this difference, particularly after alcohol treatment. Moreover, the schedule of alcohol administration might mediate this dissociation. The EPM test was performed after two injections of alcohol, and, thus, the anxiolytic effects of alcohol might refer to its acute action. However, the FST was performed after a series of five alcohol injections referring to a rather subchronic alcohol treatment that caused depressogenic effects. Thus, it might be proposed that the duration of treatment might also affect alcohol action in Asm−/− mice.

Our previous data showed that a depressed state in animals with Asm overexpression can be partially reversed by voluntary alcohol consumption (27). Naïve Asm−/− mice are also characterized by higher alcohol preference and anxiety level. As alcohol treatment in these animals has anxiolytic effects, it might be speculated that Asm−/− mice might consume more alcohol compared to wt littermates in order to reduce their anxiety level. It is well-known that individuals use alcohol to enhance coping with stress and to reduce manifestations of mood disorders, especially depression, and anxiety. Chronic moderate alcohol drinking might reduce physiological and behavioral signs of anxiety and depression in humans (73). On another hand, anxiety and depression might enhance alcohol consumption and higher alcohol intake is associated with lower levels of nervousness (74). Thus, our data show that alcohol might be voluntarily used by ASM−/− mice to control their anxiety level. We believe that our study provide an additional confirmation of the hypothesis on the contribution of Asm to alcohol instrumentalization of emotional state, showing that alcohol might be used voluntarily to control possible emotional effects of the Niemann-Pick disorder.

Previously we observed that the reversion of a depressed state in mice with Asm overexpression with alcohol was mediated by partial normalization of Asm activity and downstream monoaminergic deficiency in the nucleus accumbens of these mice (27). Thus, it was proposed that alterations in sphingomyelin pathway of ceramide synthesis might mediate the effects of alcohol on emotional state (28). In the present study we checked if other enzymes of the sphingomyelinase pathway of ceramide metabolism contribute to the mechanisms of alcohol-induced decrease in anxiety and an increase in depression level in Asm−/− animals. The activities of Nsm, a sister enzyme of Asm involved in ceramide production, and Ac and Nc, which catalyze ceramide degradation to sphingosine, were analyzed. It was observed that voluntary alcohol consumption does not affect the activities of these enzymes neither in Asm−/− animals, nor in wt littermates. Thus, it can be suggested that no additional compensatory changes in the sphingomyelinase pathway of ceramide synthesis develop in these animals, confirming the specific contribution of Asm in the observed phenotypes.

Altogether, the present findings might add on to our hypothesis of an involvement of Asm in the mechanisms of comorbidity between alcoholism and anxiety and depression. We suggest that the lack of Asm might facilitate the anxiolytic and depressogenic effects of alcohol as well as an enhanced drinking pattern in mice. Further analysis of specific mechanisms of this comorbidity in Asm−/− mice is needed as the understanding of particular pathways might allow developing a new therapeutic strategy for comorbid psychiatric disorders as well as pathogenetic mechanisms of type A and B Niemann-Pick disease.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All experiments were carried out in accordance with the National Institutes of Health guidelines for the humane treatment of animals and the European Communities Council Directive (86/609/EEC) and were approved by the local governmental commission for animal health (Regierung von Mittelfranken).

AUTHOR CONTRIBUTIONS

LK, JK, EG, MR, and CPM initiated the study. LK, CM, VE, and MP performed the experiments. LK, VE, CPM, and CM analyzed the data. MR provided the animals. LK and CPM wrote the manuscript. All authors discussed the manuscript.

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Comparison of Single Phase and Biphasic Extraction Protocols for Lipidomic Studies Using Human Plasma

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Lipidomic profiling of plasma is an emerging field, given the importance of lipids in major cellular pathways, and is dependent on efficient lipid extraction protocols. Recent attention has turned to plasma lipidomics as a means to identify potential diagnostic and prognostic biomarkers related to dementia, neuropsychiatric health and disease. Although several solvent-based lipid extraction protocols have been developed and are currently in use, novel and more efficient methods could greatly simplify lipid analysis in plasma and warrant investigation. Human plasma from normolipidemic adult volunteers was collected to evaluate three different solvent extraction protocols, including the classical Folch method, the methanol/tert-butyl methyl ether (MTBE) (Matyash) method, and a recent single-phase methanol/1-butanol (Alshehry) method. Extracted lipids were analyzed using liquid chromatography mass spectrometry (LC-MS) in positive and negative ion mode. Overall, more than 500 different lipids were identified in positive and negative ion mode combined. Our data show that the single phase Alshehry method was as effective as the Folch and Matyash methods in extracting most lipid classes and was more effective in extraction of polar lipids. Normalized peak areas of the Alshehry method were highly and positively correlated with both the Folch and Matyash methods ($r^2 = 0.99$ and 0.97, respectively). Within- and between- subject correlations were $r = 0.99$ and 0.96, respectively. Median intra-assay coefficient of variation (CV%) in positive mode was 14.1, 15.1, and 21.8 for the Alshehry, Folch and Matyash methods, respectively. Median Alshehry inter-assay CV (collected over 5 separate days) was 14.4%. In conclusion, the novel Alshehry method was at least as good as, if not better than the established biphasic extraction methods in detecting a wide range of lipid classes, using as little as 10 μL of plasma, and was highly reproducible, safer and more environmentally-friendly as it doesn't require chloroform.

Keywords: lipidomics, lipid extraction, mass spectrometry, plasma lipids, chromatography

INTRODUCTION

It is well-documented that lipids have multiple structural and functional roles, including signaling (1, 2), maintenance of membrane structure (3, 4), myelin sheath formation (5, 6), neurotransmission (7), and protein interactions in both plasma and organs including the brain (8, 9). Physiological processes such as synaptic and mitochondrial function, and lipid raft formation are critically dependent on lipid composition (10–12). By contrast, lipid by-products, particularly metabolites of arachidonic acid and lipid products of oxidative stress are drivers of inflammation (13). Consequently, the plasma lipidome has a considerable impact on the cellular lipid environment, vasculature function, and inflammatory and oxidative processes. The plasma lipid profile therefore also represents a lifestyle modifiable factor which can play a decisive role in the health state and maintenance of cognitive function during aging (14).

The field of lipidomics is constantly expanding, enabling high throughput analysis of lipid analytes in crude extracts for the study of health and disease. Recent advances in mass spectrometry have resulted in greater sensitivity, increased mass accuracy and faster scan speeds (15, 16). This has enabled greater sensitivity and better characterization of lipid changes in bodily fluids, cells, and tissue extracts, leading to renewed understanding of the role of different lipid classes in the pathobiology of diseases. Lipidomics has recently been applied to the study of dementia, where brain, CSF and plasma lipids have been identified as potential diagnostic and prognostic biomarkers for Alzheimer's disease (17, 18), frontotemporal dementia (19), as well as other common neuropsychiatric disorders, such as schizophrenia and bipolar disorder (20, 21).

The future of lipidomics lies in clinical application. As such, blood-based biomarkers are ideal since venepuncture is already routinely used in the clinic and is less invasive compared to CSF extraction via lumbar puncture. Additionally, it is much simpler to take repeat measurements of blood which may be useful for longitudinal analysis. We will discuss further the relationship of centrally derived biological signals with the periphery and vice versa in this paper. Although interest in lipidomics especially in the study of health and disease is expanding, there is still much room for improvement in lipidomic methodologies for research and clinical use. Since lipidomics often involves the study of 100 to 1,000 of individual biological samples within a single experiment, any improvement to the cost or efficiency of sample processing could have a considerable impact on streamlining plasma lipidomics research.

One key area in which improvement is possible is in the extraction process, whereby total lipids within plasma, tissue, and other biological extracts are isolated prior to further analysis. Solvent-based extraction systems must efficiently extract lipids present in biological samples while minimizing bias, lipid degradation/oxidation, or contamination from non-lipid components, such as sugars, peptides and amino acids. Therefore, effective and reliable identification and profiling of lipids in plasma is dependent on the efficiency of the lipid extraction protocol (22). The traditional Folch method (2:1 chloroform/methanol) (23), developed in 1957 is still used as a benchmark extraction process in many laboratories for a wide range of biological fluids, including, blood, tears, urine, saliva, cerebrospinal fluid, human milk, bronchoalveolar lavage fluid and sperm (22). The Folch method is based on the partitioning of lipids in a biphasic mixture of chloroform and methanol. Methanol disrupts hydrogen bonds between lipids and protein following addition of an organic solvent such as chloroform.

Alternative methods, such as the Matyash (tert-butyl methyl ether (MTBE)/methanol) (24), have gained popularity, in particular for the extraction of sphingolipids. Replacement of chloroform with MTBE demonstrated similar extraction of major lipid classes (22, 24). Recently, a single phase (1:1 1-butanol/methanol) extraction developed by Alshehry et al. (25, 26) has also been published. Both newer methods eliminate the need to draw lipids from the lower phase, thereby saving time compared to the Folch method, and are safer and more environmentally friendly as they do not use toxic chloroform. Moreover, the Alshehry method does not involve any biphasic solvent separation and could therefore be even more convenient, and potentially yield better lipid recoveries, than the Matyash method. The Matyash method has already been shown by independent laboratories to be as good, if not better, at extracting lipids as the Folch method (22, 24, 27). A recent comparative study of multiphasic methods has been published (28), however the single phase Alshehry method was only recently published and has not been compared with established biphasic methods.

In this paper, we examine the performance of the single-phase Alshehry extraction system and compare the results against the two biphasic extraction methods, the Matyash method and the traditional Folch method (**Figure 1**) in terms of coverage of lipids extracted and reproducibility from pooled plasma of healthy volunteers. Liquid chromatography mass spectrometry (LC-MS/MS) was used to identify the lipids extracted.

METHODS

Reagents and Chemicals

HPLC grade methanol was purchased from Thermo Fisher Scientific (Sydney, Australia). Analytical reagent grade 1-butanol was purchased from Asia Pacific Specialty Chemicals (APS) (Sydney, Australia). Other high grade solvents and reagents were used, including MTBE (Sigma Aldrich, Israel), chloroform (Honeywell, USA), acetonitrile (Honeywell, Korea), formic acid (Chem-supply, Australia), ammonium formate (Honeywell, Germany). Isopropanol was of LC-MS grade (Honeywell, Michigan, USA). SPLASH Lipidomix stable isotope labeled internal standards were purchased from Avanti (Alabaster, Alabama, USA). All other reagents were analytical reagent grade. MilliQ water was used for buffer preparation and had a minimum resistivity of 18mΩ.

Internal Standards

Internal standards were purchased from *Avanti* (Splash Lipidomix, Alabaster, United States) and included phosphatidylcholine 15:0–18:1(d7) PC, phosphatidylethanolamine 15:0–18:1(d7) PE, phosphatidylserine 15:0–18:1(d7) PS, phosphatidylglycerol 15:0–18:1(d7) PG,

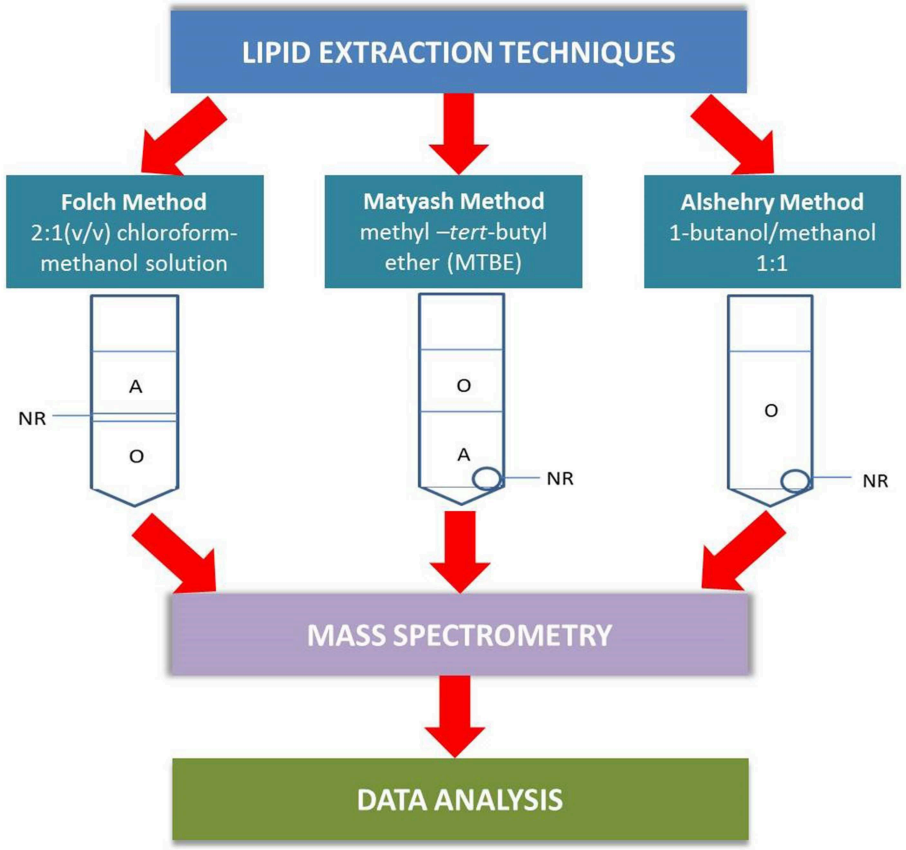

FIGURE 1 | A schematic outlining the differences between the plasma extraction methods in the analytical pathway. In the Folch method, the lipid-containing organic phase (O) lies beneath a non-extractable residue (NR) and must be collected by passing a micropipette through the aqueous phase (A) and NR. The Matyash MTBE extraction simplifies the extraction process by having the lipids collected in the upper phase, though care must be taken not to take up the aqueous phase. The Alshehry 1-butanol/methanol extraction collects all lipids within a single-phase supernatant, with NR as a white pellet at the bottom of the tube.

phosphatidylinositol 15:0–18:1(d7), PI, phosphatidic acid 15:0–18:1(d7) PA, lysophosphatidylcholine 18:1(d7) LPC, lysophosphatidylethanolamine 18:1(d7) LPE, cholesteryl ester 18:1(d7) CE, monoacylglycerol 18:1(d7) MG, diacylglycerol 15:0–18:1(d7) DG, triacylglycerol 15:0–18:1(d7)-15:0 TG, sphingomyelin 18:1(d9) SM and Cholesterol (d7). The Splash Lipidomix standards are deuterium labeled and not present endogenously, but with concentrations similar to physiological plasma concentrations of lipids for each class and thus are ideal for use as internal standards. A constant amount of internal standards was added to all samples and controls prior to lipid extraction (10 μL of a 1:10 dilution of SPLASH Lipidomix to 10 μL neat plasma) for normalization of raw peak areas and to correct for differences in extraction and ionization efficiencies and matrix effects (29).

Blood Collection

Our study utilized fasting EDTA plasma collected from 10 cognitively "healthy" subjects aged between 65 and 85 years with informed consent, as part of a larger population-based study known as Sydney Memory and Aging Study (MAS) (30, 31). Subjects did not have any forms of mild cognitive impairment or dementia, as assessed by a panel of neuropsychiatrists according to consensus diagnosis criteria (30, 32), All subjects had mini-mental state examination (MMSE) scores >24 (33) and no history of cardiovascular complications or diabetes mellitus. Plasma taken from the 10 subjects (five males and five females) were then pooled and aliquoted (10 μL). Extractions from 10 pooled plasma aliquots were used to assess the intra-assay coefficient of variation (CV%) of each extraction method detailed below, while extractions taken on 5 separate days were used to assess inter-assay CV% of the Alshehry method. Blood collection, processing and storage were performed under strict conditions to minimize preanalytical variability (18, 34), which included restricting plasma collection and aliquot to freezer time to a maximum of 2 h, using EDTA plasma, and minimizing the number of freeze-thaw cycles. In particular, freezer storage of plasma and extracted lipids at −80°C prior to analysis and limiting the number of freeze thaw cycles, is now part of international standardization guidelines in order to maximize stability of lipids over prolonged periods of time (18, 35, 36).

The study was approved by the Ethics Committees of the University of New South Wales and the South Eastern Sydney and Illawarra Area Health Service (ethics approval HC12313 and HC14327, respectively). All work involving human subjects conformed to the principles of the Declaration of Helsinki of the World Medical Association.

Lipid Extraction Protocols

Single Phase 1-Butanol/Methanol 1:1 (v/v) Alshehry Extraction Method

Lipids were extracted as previously described (25, 26). Briefly, we added 10 μL of internal lipid standards (*Avanti* SPLASH Lipidomix) that had been first diluted 1:10 in 1-butanol/methanol, to 10 μL aliquots of plasma in 0.5 mL polypropylene tubes (Eppendorf). 1-Butanol/methanol (100 mL, 1:1 v/v) containing 5 mM ammonium formate was then added to the sample, vortexed (10 s), then sonicated (1 h). Tubes were centrifuged (13,000 ×g, 10 min) and the supernatant removed into a clean polypropylene tube. A further 100 μl of 1-butanol/methanol (1:1 v/v) was added to the pellet to re-extract any remaining lipids. The combined supernatant was evaporated by vacuum centrifugation and stored at −80° C prior to analysis by LC-ESI MS/MS.

Traditional Biphasic 2:1 Chloroform/Methanol (v/v) Folch Extraction Method

Lipids were extracted as previously described (23), but using a 20-fold scale down of volumes used in order to produce comparable results against the same volume of plasma as used in the Alshehry method. Briefly, 10 μL aliquots of plasma and 10 μL of internal lipid standards (*Avanti* SPLASH Lipidomix) that had been first diluted 1:10 in 1-butanol/methanol were added to 160 μl of ice-cold methanol followed by addition of 320 μl of ice-cold chloroform and vortexed (10 s), then sonicated (1 h). Tubes were centrifuged at 10,000 ×g (10 min). The upper aqueous phase was transferred into a fresh polypropylene tube and re-extracted by addition of 250 μl ice-cold chloroform/methanol as described above. The upper phase was discarded, and the organic phase combined from both extractions, which was then evaporated by vacuum centrifugation and stored at −80°C prior to analysis by LC-ESI MS/MS.

Methanol-Tert-Butyl Methyl Ether Matyash Extraction Method

Lipids were extracted as previously described (24) with a 20-fold scale down of volumes; 10 μL aliquots of plasma and 10 μL of internal lipid standards (*Avanti* SPLASH Lipidomix) that had been first diluted 1:10 in 1-butanol/methanol were added to 400 μl of ice-cold methanol followed by addition of 500 μl of MTBE, vortexed (10 s), then sonicated (1 h). Afterwards, 500 μl of MilliQ water was added to induce phase-separation. Tubes were centrifuged (10,000 ×g, 10 min). The upper aqueous phase was transferred into a clean polypropylene tube and re-extracted by addition of 200 μl MTBE as described above. The upper phase was discarded, and the organic phase was evaporated by vacuum centrifugation and stored at −80° C prior to analysis.

LC-MS Analysis

Extracted lipid samples and lipid internal standards (*Avanti* SPLASH Lipidomix) were removed from the −80° C freezer and resuspended in 100 μl of 1-butanol/methanol (1:1 v/v) containing 5 mM ammonium formate and transferred into Chromacol autosampler vials containing a 300 μl glass insert. Lipid analysis was performed by LC ESI-MS/MS (Dionex LC system in-line to a Thermo QExactive Plus Orbitrap mass spectrometer; ThermoFisher Scientific; Waltham, Massachusetts). A Waters ACQUITY UPLC CSHTM C18 1.7 μm, 2.1 × 100 mm column was used at a flow rate of 260 μL/min, using the following gradient: 32 to 100% solvent B over 25 min, a return to 32% B and finally isocratic 32% B (5 min) prior to the next injection. Solvents A and B consisted of acetonitrile: MilliQ water (6:4 v/v) and isopropanol: acetonitrile (9:1 v/v) respectively, both containing 10 mM ammonium formate and 0.1% formic acid. The first 3 min of eluent, containing sample salts, was diverted to waste. Product ion scans in positive and negative ion mode were performed on each sample, to maximize numbers of lipid species identified. Sampling order was randomized prior to analysis. LC-MS grade isopropanol was used to minimize intensity of the background signal.

Alignment and Peak Detection Using LipidSearch 4.2.2

The raw data was aligned, and chromatographic peaks selected using LipidSearch software version 4.2.2 (Thermo Scientific, Tokyo, Japan), the database which now includes masses of the stable isotope labeled SPLASH Lipidomix internal standards. We performed search on raw files using the databases "General" and "labeled standards." For peak detection, recalc isotope was set to "ON," RT interval = 0.0 min. We used product search for LC-MS method and the precursor and product tolerances were set at 5.0 and 8.0 ppm, respectively. The intensity threshold was 1% parent ion, and the m-score threshold was set to 2.0. For quantitation, m/z tolerance was set at −5.0 to 5.0 ppm, and the retention time range was set at −0.5 to 0.5 min. The m-score threshold was 5.0, and all lipid classes were selected for inclusion. Ion adducts included +H, +NH4 for positive ion mode and –H, +HCOO for negative ion mode.

Data Analysis

The results from LipidSearch 4.2.2 were exported as a.csv file and opened in Excel software for further data processing and analysis. Peak areas of lipids in samples were normalized by dividing with peak area of the corresponding internal standard for that lipid class (i.e., peak area ratios). Correlations between normalized peak areas from the three extraction methods were calculated using the SPSS statistics software. The intra-assay coefficient of variation (CV) was calculated by dividing the standard deviation of the normalized abundances of each lipid (for each method) by the mean across lipid species. Lipid ion identifications were filtered using the LipidSearch parameters rej=0 and average peak quality>0.75. Recovery was calculated as the average raw intensity of internal standards spiked before extraction and divided by the average raw intensity of internal standards spiked post extraction. The inter-assay CV% for the Alshehry

method was also determined by comparing measurements from extractions taken across 5 separate days within a month.

RESULTS

Concordance Between Methods

When we compared the coverage of the different lipid classes based on extracted lipid internal standard peak areas, normalized peak areas in both positive and negative modes were highly and significantly correlated with the biphasic Matyash and Folch methods (r = 0.97 and 0.99 respectively, $p < 0.0001$, **Figures 2A,B**). This concordance was also reflected when comparing lipid measurements between duplicate extractions of plasma lipids prepared using the Alshehry protocol (r^2 = 0.995), and in two different subjects, which is indicative of high reproducibility (**Figures 2C,D**). Additionally, Bland-Altman analyses revealed that the Alshehry method largely agreed with the Folch and Matyash methods (bias ratio=1.09 and 1.247, respectively), except more for some lowly abundant peak areas, which were higher using the Alshehry method. All three methods produced similar number of LipidSearch molecular ion IDs prior to filtering (381, 400, and 390 IDs in positive ion mode for the Matyash, Folch and Alshehry methods respectively, and 122, 127, and 119 IDs in negative ion mode, similarly).

Comparison of Lipid Abundances

One-way ANOVA was used to test whether the raw peak areas for internal standards using the Alshehry method were significantly different from the Matyash or Folch methods. There were no significant differences in peak areas between extraction methods for DG, PE and SM species ($p > 0.05$). Peak areas of LPC, LPE, PI, PC and PG were higher in the Alshehry method than the Matyash method ($p < 0.05$, Tukey's *post-hoc* test), while the peak area of PG and PI were also greater in the Alshehry method relative to the Folch extraction. The Alshehry method was more efficient in extracting the highly polar lipids, such as LPC and LPE internal standard relative to the Matyash method ($p < 0.05$, one-way ANOVA, Tukey's *post-hoc* test) (**Figure 3A**).

Interestingly, TG was significantly higher in the Folch method compared to Matyash ($p < 0.05$, Tukey's *post-hoc* test), but yielded comparable, or slightly higher peak areas compared to Alshehry method, which did not reach significance ($p = 0.08$). Similarly, Alshehry peak areas of internal standards were larger than or equal to the Folch, and larger than the Matyash method in negative ion mode (**Figure 3B**, all $p < 0.05$, Tukey's *post-hoc* test). Overall, the Alshehry method produces similar, if not greater yields than either the Folch or Matyash method.

Recovery of Internal Standards

Recoveries for each method were assessed by comparing the ratio of peak areas of internal standards spiked prior to and after extraction (**Figure 4**). The average recoveries were 99, 86, and 73% for the Alshehry, Folch and Matyash extractions, respectively. The highest recoveries for the Alshehry method were achieved with the phospholipids (>95%) and lowest for the less polar TG, DG lipids (<80%).

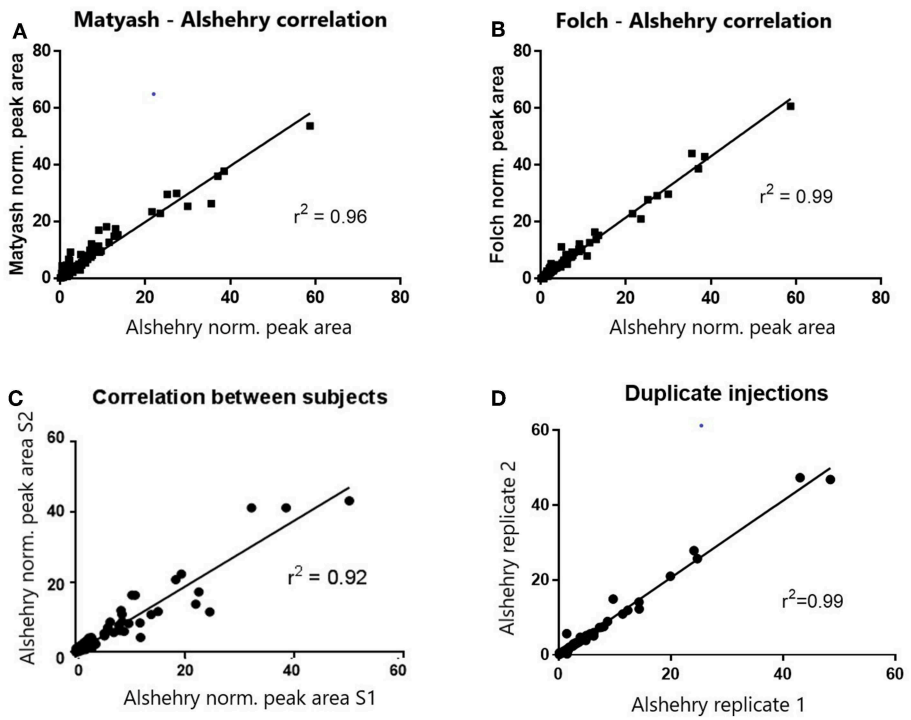

FIGURE 2 | Correlations of normalized peak areas between methods **(A,B)**, subjects **(C)** and runs **(D)**. Normalized peak areas of the Alshehry method were highly and positively correlated with both the Folch and Matyash methods. Within-subject correlation across separate runs was also high, though between- subject correlation was lower.

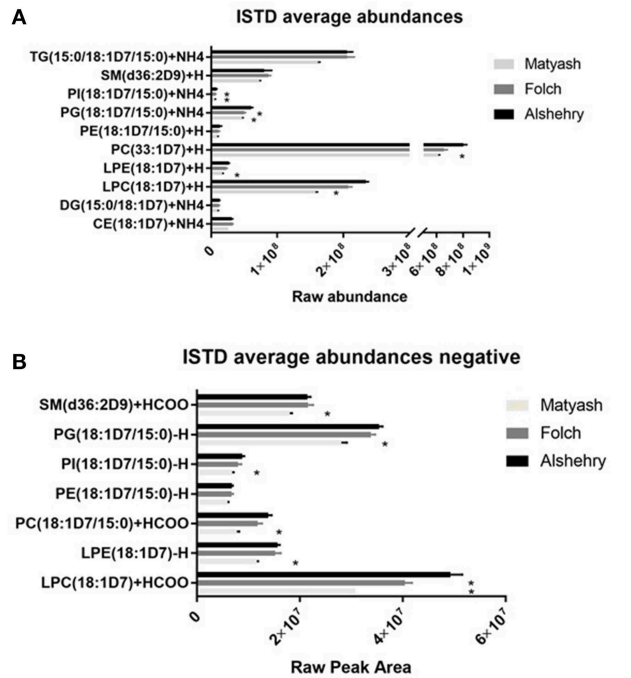

FIGURE 3 | ISTD raw abundances across the three extraction methods in **(A)** positive ion mode and **(B)** negative ion mode. The Alshehry method was as effective as the Folch and Matyash method in extracting most lipid classes and was more effective in extraction of more polar lipids (LPC, LPE and PI). Error bars represent SE (*$p < 0.05$ relative to single phase method). CE, cholesterol ester; D5DG, deuterated diacylglycerol; DG, diacylglycerol; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol, PI, phosphatidylinositol, PS, phosphatidylserine, SM, sphingomyelin; TG, triacylglycerol.

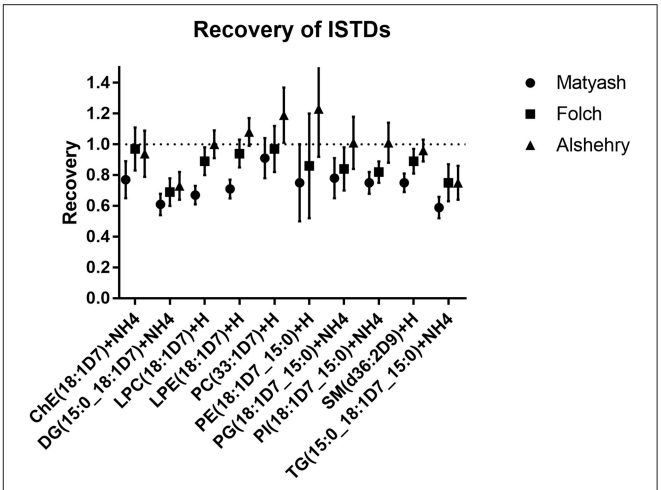

FIGURE 4 | Recoveries of internal standards for extraction methods. The values represent ratios of peak areas ± SD (error bars) relative to unextracted internal standards (i.e., spiked after solvents were added).

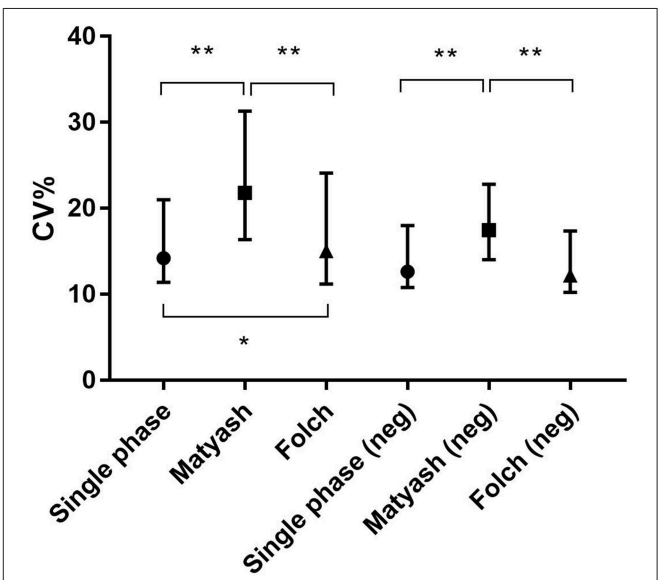

FIGURE 5 | Intra-assay CV was lower in single phase extraction relative to the Folch and Matyash methods for all lipids in positive ion mode and negative ion mode. Error bars represent median ± interquartile range. *$p < 0.05$, **$p < 0.001$, Mann-Whitney *U*-test.

Reproducibility of Methods

We also compared intra-assay coefficients of variation (**Figure 5**), defined as the standard deviation of the peak areas across several samples in a single experimental batch divided by the mean of the peak area, expressed as a percentage. A lower CV% then corresponds to consistency of values obtained from extraction of technical replicates. The median intra-assay (repeat injections within a single experiment) and inter-assay (injections from samples processed across separate days) coefficients of variation (CV%) for all lipids in positive ion mode originally reported by Alshehry were 12 and 14% respectively (37). Here, we report median intra-assay and inter-assay CV of 14.2 and 14.4%, respectively for the Alshehry extraction, which is consistent with this previous report. The median intra-assay CV% we obtained using the Alshehry method was lower compared to that obtained for the Matyash and Folch methods (21.8 and 15.1% respectively, $p < 0.0001$ and $p = 0.014$ respectively, Mann-Whitney *U* test). Seventy-five percent of lipids in the Alshehry method had a CV of 21% or lower, and the median CV for internal standards was 8.5%. Median CV for lipids in negative mode were 12.7, 17.5, and 12.2% for the Alshehry, Matyash and Folch recipes respectively ($p < 0.0001$ for Folch and Alshehry relative to Matyash, Mann-Whitney *U*-test). The comparative data collected in this experiment is summarized in **Table 1**. Typical representative total ion chromatograms of internal standards (**Figure 6A**), alongside extracted ion chromatograms for each standard (**Figure 6B**), as well as the total ion chromatogram for plasma lipids (**Figure 6C**) are shown.

DISCUSSION

In the original study by Alshehry et al. (25) the single phase 1-butanol/methanol 1:1 (v/v) method was reported to be as

good an extraction as similar single phase extraction protocols with lipid measurements (based on peak areas normalized against internal standards). In particular, results between the Alshehry method and a single phase 2:1 methanol/chloroform extraction method were strongly correlated ($r^2 = 0.976$). In the present study, both the Matyash and Folch methods were also strongly correlated with Alshehry method ($r = 0.97$ and 0.99, respectively) which suggests strong agreeance between the methods, also supported by Bland-Altman bias ratios. Overall, this suggests that methods correspond well, though the Alshehry method may be superior in detection of more lowly abundant lipid species. Number of extracted lipids were also similar between the methods indicating similar coverage of lipids for all three extractions.

TABLE 1 | Results summary.

	Matyash	Folch	Alshehry
Volume of plasma per sample (this study)	10 μL	10 μL	10 μL
Volume of plasma per sample (original)	200 μL	200 μL	10 μL
Volume of solvents per sample (this study)	900 μL	480 μL	100 μL
Volume of solvents per sample (original)	18,000 μL	9,600 μL	-
Numbers of individual lipids in positive ion mode	381	400	390
Numbers of individual lipids in negative ion mode	122	127	119
Median intra-assay CV% in positive ion mode	21.8	15.1	14.1
Median intra-assay CV% in negative ion mode	17.5	12.2	12.7
Average total recovery %	73	86	99

Although most lipids classes were extracted similarly between methods, certain lipid classes were more abundantly extracted using the Alshehry method as opposed to the Matyash or Folch methods. This included the lysophospholipids LPC and LPE, as well as some glycerophospholipids containing a polar head group, such as PC, PI and PG. This is consistent with reports that the Alshehry method is more amenable to extraction of highly polar lipids compared to other methods (25), since the hydrophilic phases are discarded in these biphasic methods. A previous pilot analysis also showed markedly greater extraction of the highly polar lipid standards, phosphatidylserine, PS(17:0/17:0) and sphingosine phosphate, SoP(d17:1) in the Alshehry method compared to either the Matyash or Folch methods. The ability of the Alshehry method to capture more polar lipids may be particularly useful in analysis of

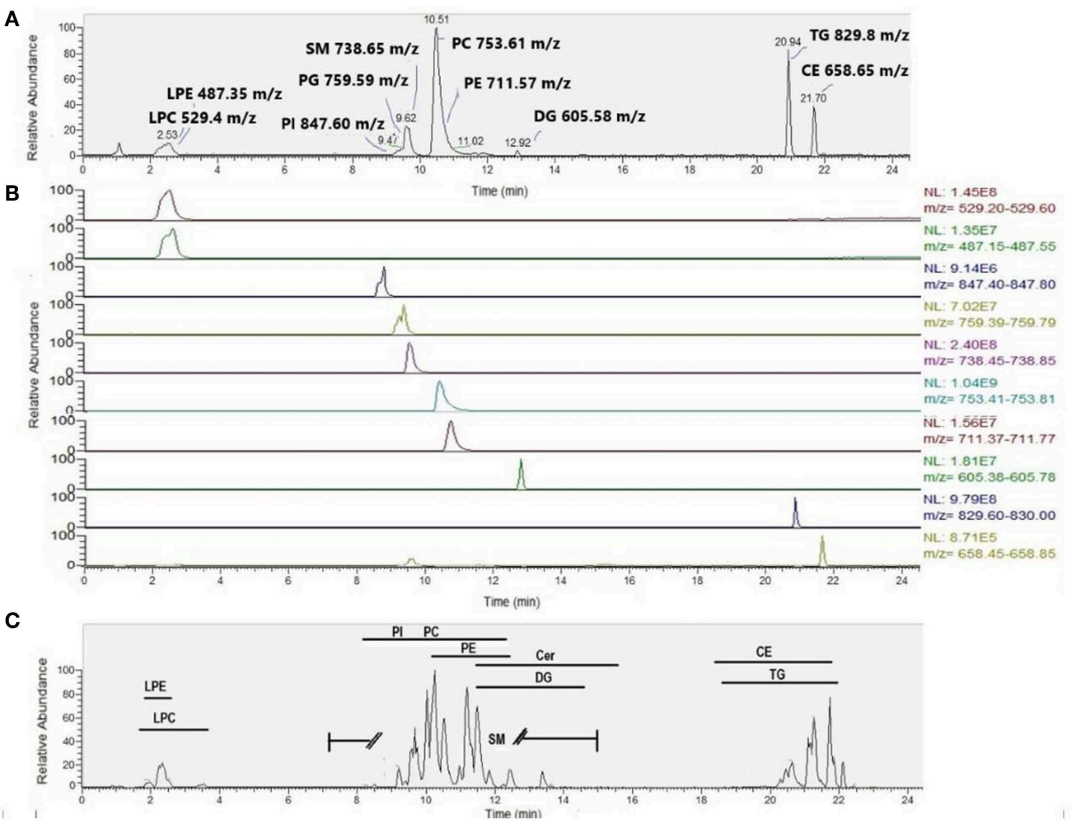

FIGURE 6 | Typical chromatogram featuring **(A)** SPLASH Lipidomix internal standards in positive ion mode (total ion chromatogram), **(B)** SPLASH Lipidomix internal standards (extracted ion chromatograms for each standard narrowed to ±0.2 Da of the theoretical *m/z*), presented from top to bottom in order of increasing retention time, and **(C)** corresponding plasma sample in positive ion mode. Typical retention time ranges of elution (minutes) are provided for various lipid classes.

neuropsychiatric disorders and dementia where these lipids have been implicated in these conditions (17, 38, 39).

Although the Alshehry recipe is ideal for extracting more polar lipids, the method is adaptable for extraction of other lipid classes. For example, a similar study of lipid extraction in cow's milk applied a modified version of the Alshehry single phase method in order to extract higher levels of non-polar lipids (40). This modified version used a single phase mixture of 1-butanol/methanol/chloroform (3:5:4 v:v:v) in order to broaden the coverage of highly lipophilic species, especially triglycerides. Other similar single-phase extraction procedures using different solvent ratios have been described for lipid extraction, such as a 3:1 butanol/methanol extraction for plasma (41) and animal tissue (42), as well as a methanol/MTBE/chloroform (1.33/1/1 v:v:v) extraction (43, 44). All these cases, report comparable, if not greater yields against the Matyash and Folch methods suggesting good feasibility of single-phase extraction methods against traditional biphasic methods, as well as adaptability to capture a wide range of lipids of interest.

Overall, there was strong recovery for a large majority of lipids with the single-phase method, at 99%, especially for polar lipids. The high recovery of the Alshehry method near 100% is consistent with the fact that this method does not experience the risk of dripping losses that occurs with the other two biphasic methods as the bulk of lipids are present in a single phase to handle and readily removable for lipid analysis.

The less than optimal recovery (73%) for the Matyash method compared to previously reported is likely related to the relatively low volume of solvent used during the 20-fold volume scale down procedure, as well as the tendency of MTBE to evaporate quickly, affecting extraction reproducibility (41), which is also reflected in the higher CV% (see below).

Analysis of reproducibility based on CV% revealed the Alshehry method had a median CV% of 14.2%, lower than or equal to the Folch method, and even better than the Matyash method (at 15.1 and 21.8% respectively, in positive ion mode). Thus, the Alshehry protocol produces reliable lipid measurements over technical replicates within experimental batches as well as, or even better than the established Folch and Matyash approaches, which is expected considering reduced dripping losses in a monophasic extraction. Additionally, the inter-assay CV% is very similar to that of the intra-assay CV% for the Alshehry method which suggests that the method produces reliable measurements across experimental batches and any additional variation to this method due to batch differences would be minimal.

Furthermore, the single phase Alshehry method is advantageous in that lipids are drawn into a single phase and can be easily removed from the sample as supernatant without picking up non-extractable matrix. This makes the method easier and potentially faster compared to conventional two-phase solvent partition systems where there is also an increased risk of contamination from unwanted analytes, and the risk of losses during transfer between phases. Additionally, the method can be applied to as little as 10 μl of plasma, and using just 100 μl of solvent per sample, and therefore greatly minimizes the amount of sample and solvent required for extraction. By contrast, the original Matyash extraction reports extractions using 200 μl of plasma, and requires 900 μl of MTBE and methanol combined pre-scale down, which translates to 18 ml of solvent required per plasma sample in the original Matyash method. Considering the broad range of lipids and reduced losses reflected in the high degree of recovery and reproducibility obtainable using a relatively small volume of sample and solvent in the Alshehry method, this represents a strong advantage of the method over existing biphasic methods. The volume savings can be particularly important for high value plasma samples from expensive clinical and population-based studies, since such samples are a finite resource and conservative usage is necessary, to maximize potential experiments and makes extraction of many samples much simpler to handle.

This new method has already been applied for lipid extraction of plasma from large populations for the study of health and disease (37, 45). Nevertheless, we highlight one important limitation of the Alshehry method as reported by the original authors (25): due to the monophasic nature of extraction, ionic contaminants are not readily removed during extraction, leading to an increased risk of ion suppression effects. The Alshehry method is therefore unsuitable for shotgun/direct infusion lipidomics techniques, but is suitable for use with reverse phase LC-ESI MS/MS.

It needs acknowledging that in context of neuropsychiatric disorders and dementia, it is important to establish the connection between putative changes in peripheral signals with that of signals generated by the central nervous system (CNS). Despite all the advantages associated with peripheral biomarkers, a limitation of plasma-based lipidomics research is that the blood brain barrier (BBB) restricts passage of metabolites between the CSF and blood compartments. Therefore, there is not always a strong correspondence between blood and centrally derived metabolites. Nevertheless, many CSF metabolites are selectively reabsorbed back into the vascular system, and essential lipids such as polyunsaturated fatty acids are transported to the brain from blood as free fatty acids (46) or as part of phospholipids, such as lysophosphatidylcholine (47), a lipid class well-detected using the novel method. The BBB may have increased permeability in response to inflammation or disease states (48, 49). Moreover, there is substantial overlap in metabolic changes identified between matched CSF and plasma (50, 51), and some blood lipids may be correlated with CSF tau and amyloid load (52), as well as other imaging measures such as brain atrophy (53).

Other studies have also identified relationships between plasma/serum lipids, apolipoproteins and cognition (54, 55). A recent study identified differential impacts of apolipoprotein E in the CNS and periphery, finding deficits in apolipoprotein E in the CNS induced synaptic loss, whereas a deficit in the periphery lead to impaired cognition (56), though synaptic loss could partially be rescued by increasing plasma apolipoprotein E levels. The potential of plasma lipids to predict memory performance and cognitive decline (or protection) several years in advance (57–59) presents evidence that these lipids may have a substantial influence on the CNS, or that plasma lipids may reflect some underlying pathological process

occurring in the CNS. Many of the metabolites reported in the above studies are robustly extracted using the novel Alshehry method, such as sphingolipids, PC, PE and their corresponding lysophospholipids, and have also similarly been found to be altered in *post-mortem* brain tissue or CSF in patients with Alzheimer's disease (38, 50, 60). We have previously reviewed plasma lipids thought to be altered in Alzheimer's disease (18), which includes plasmalogen phospholipids, sphingomyelins and ceramides. Further, a recent untargeted lipidomics study in plasma found substantial differences in the lipidomic profile between healthy controls, Alzheimer's disease and behavioral variant frontotemporal dementia (bvFTD) (19). Of note, the authors were able to confirm hypertriglyceridemia in bvFTD at the lipid species level, and also detected altered levels of dietary lipids thought to characterize binge eating behaviors among these patients. This study, among others demonstrates the utility of exploring plasma lipids as potential biomarkers for neuropsychiatric and neurological disorders. Even with such a wealth of studies, independent validation and replication remains a high priority concern in the lipid biomarker research community (18). Further, the natural variation in lipids within and between individuals independent of disease is still being characterized (45, 61, 62), which is an important factor to consider before applying comparative lipidomics in disease. Although this pilot study does not focus on inter-individual differences in lipids, we have recently applied this particular method in a larger study of 100 individuals to identify the natural variation of the plasma lipidome in cognitively healthy individuals by age, sex and BMI (63). For future studies, we intend to investigate the influence of *APOE* genotype on the plasma lipidome, as well as identify lipidomic changes associated with vascular dementia. We believe that a consistent and simple method for plasma lipid extraction, such as explored in this paper, will go a long way toward expanding blood lipidomic studies, give due insight into neurological processes and minimize some of the variance in literature of reported biomarkers inherent in extraction methodology.

CONCLUSION

Overall, we confirm the findings of the originally published Alshehry single-phase protocol, but also show that it is good as, if not better than the commonly used biphasic Matyash and Folch methods in the general extraction of plasma lipids, based on its ability to cover a broad range of lipid classes, including more polar lipids that may not be extracted readily using the Folch recipe. The Alshehry method also provides reliable and reproducible measurements when lipids are analyzed by LC-MS/MS above that of the Folch and Matyash methods. The single-phase method offers a safe, environmentally friendly (chloroform-free) and economical method for extraction of plasma lipids with high (nearly 100%) recovery that is a good substitute for the traditional approaches. Notably, all methods produce highly correlated results, indicating that reliable data can be obtained with all these protocols. However, the simplicity involved in having most lipids extractable in a single phase and low plasma and solvent volumes required by this Alshehry method may be particularly useful where sample volumes are limited, for projects where high sample numbers need to be assayed for lipidomic analysis, or where plasma needs to be conserved to maximize experiments.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study was approved by the Ethics Committees of the University of New South Wales and the South Eastern Sydney and Illawarra Area Health Service (ethics approval HC12313 and HC14327, respectively). All work involving human subjects conformed to the principles of the Declaration of Helsinki of the World Medical Association and both informed and written consent was obtained.

AUTHOR CONTRIBUTIONS

MW, NB, and AP wrote and drafted the manuscript. NB, RP, AP, and PS conceived of the project. MW performed the experiments and analyzed the data. RP provided technical expertise and training for use of the QExactive Plus mass spectrometer and LipidSearch software.

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Plasma Lipid Profiling Identifies Biomarkers of Cerebral Microvascular Disease

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Brain-specific sphingolipids (SLs) may serve as effective biomarkers of white matter hyperintensities (WMH). Here, we investigate the efficacy of SLs as a novel fluid-based biomarker to identify WMH reflective of chronic ischemia. Patients presenting to our stroke center for evaluation of acute neurological deficits were enrolled in the Advanced Serum Profiling in Recent Stroke (ASPIRE) study. From this cohort of 202 individuals, 58 patients who underwent an MRI and did not have a clinical stroke event were included in this study. Plasma samples were collected at the time of MRI, and targeted SL profiling was performed by HPLC/tandem mass spectrometry. T_2 FLAIR imaging was evaluated for WMH and scored according to the Fazekas scoring (FS) method and manually quantified. Twenty two SLs were definitively identified, consisting of ceramide (Cer) and sphingomyelin (SM) species. Of these, two sphingolipids, SM 38:1 and Cer 34:1, significantly correlated with high FS ($r = 0.287$, $p = 0.029$, and $r = 0.356$, $p = 0.006$, respectively) and were used in subsequent analysis. SM 38:1 (OR 1.129, 95% CI 1.032, 1.236, $p = 0.008$) and Cer 34:1 (OR 1.118, 95% CI 1.031, 1.212, $p = 0.007$), accurately differentiated between FS 0–2 vs. 2.5–6 in regression analysis. A combined lipid score demonstrated fair discrimination in ROC analysis (AUC = 0.729, $p = 0.003$) and was cross-validated using leave-one-out analysis. Plasma levels of brain-specific SLs may serve as effective biomarkers of subacute white matter disease.

Keywords: sphingolipids, stroke, white matter (WM), biomarker, ceramide (CER), sphingomyelin (SM)

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INTRODUCTION

White matter hyperintensities (WMH) detected on T_2 weighted images are associated with white matter damage and can be identified in 11% of adults over 60 and in 94% of adults over 82 (1). While age is the most consistent risk factor for WMH; diabetes, smoking, and hypertension are also independent risk factors, suggesting that WMHs are pathological rather than a part of the normal aging process (2). A meta-analysis of 22 imaging studies evaluating WMH shows that these lesions are associated with increased risk of stroke (hazard ratio 3.3) and dementia (hazard ratio 1.9) (3). Increased WMH load is also associated with increased severity of cognitive dysfunction (4). WMH are associated with poor recovery after stroke and hemorrhagic transformation after thrombolysis (3, 5–9). Other evidence supports an association with gait disturbances and impaired balance,

putting those with significant WMH load at risk for falls and hospitalizations (2). Populations with limited access to healthcare and MRI studies may have underreported WMH and unrecognized cognitive impairment mandating alternative detection methods for WMH in these populations to promote screening, early intervention and appropriate management to prevent complications and hospitalizations.

Existing blood biomarkers for cerebral vascular disease are not specific to WMH and are not reliably detected in subclinical, or early stages of disease (10). While BNP and CRP are elevated in acute stroke and have shown modest ability to predict risk of future stroke, these biomarkers fail to reflect current extent of cerebral injury (11, 12). Non-specific inflammatory markers such as CRP, TNF, and IL-6, have not been shown to relate to WMH load (13). While some markers of endothelial damage, such as von Willebrand factor and intercellular adhesion molecule 1 are not associated with increasing WMH volume, others, including circulating vascular adhesion molecule-1, are (13, 14). Therefore, the role of different inflammatory pathways in the progression of WMH remains unclear. Serum neurofilament light chain (sNfL) has been recently shown to be a brain-specific biomarker for acute ischemic stroke (15, 16). While sNfL levels are reported to increase with acute ischemic damage and WMH, levels also increase in other neurological disorders including motor neuron disease (MND), multiple sclerosis (MS), and Alzheimer's disease (AD) (17–20). Because AD, MND, and WMH are all more prevalent in the older patient population, sNfL can only be used as a non-specific biomarker for WMH.

Tissue pathology from regions of WMH reveals demyelination, axonal damage, and mild gliosis (21), arising from chronic microvascular dysfunction. Oligodendrocyte vacuolization around venules in perivascular spaces contributes to loss of myelination, axonal damage and white matter fiber disruption in regions of WMH. Both histological and diffusion tensor imaging evidence suggest that white matter changes extend into a penumbra surrounding the region of WMH detected by routine MRI (22–24). Therefore, MRI may underestimate extent of brain injury and can fail to detect early changes. Plasma biomarkers that reflect direct damage to white matter may serve a critical role in identifying early white matter injury.

We previously demonstrated that sphingolipids (SLs) are highly enriched in the brain and that plasma SLs are sensitive markers of acute ischemic cerebral injury. SLs, and in particular one subset of SLs known as sphingomyelins, are known to be key components of cerebral white matter, as major constituents of the cerebral myelin sheaths that contribute to membrane fluidity and structural integrity (25). In this study, we determine the ability of circulating SLs to serve as accurate biomarkers of WMH.

SUBJECT/MATERIALS AND METHODS

Ethics Statement

Research involving human subjects was approved by the Institutional Review Board of the University of California, Los Angeles (IRB # 14-001798) and was conducted in compliance with the Health Information Portability and Accountability Act. A board-certified Neurologist interviewed all patients to determine the capacity of each individual participant to provide informed consent. If in the opinion of the physician the patient suffered from a compromised ability to provide consent, a surrogate consent procedure was instituted whereby the next of kin or legally authorized representative was given the opportunity to consent on the patient's behalf. Formal written consent was obtained for all participants prior to the collection of blood samples.

Study Design

This cohort study was prospectively designed to build from the results of our previous discovery-phase study, to validate findings on lipid biomarkers of acute ischemic stroke in a larger, real-world clinical cohort. Consecutive participants were patients presenting to the Ronald Reagan UCLA Emergency Department with symptoms concerning for stroke between December 2014 and June 2016 and who were offered the opportunity to participate in the study. Patients were included in this study if they had onset of stroke symptoms within 8 h of presentation (or within 2 h of presentation if symptoms were present upon awakening from sleep and the time of symptom onset was unknown); were >18 years of age and were able to offer informed consent or had a suitable surrogate individual who could consent on their behalf. The final clinical diagnosis was based on the results of a detailed evaluation of each patient by the Neurovascular Neurology service. Blood samples were collected by peripheral vein venipuncture into heparin-containing tubes. Samples were kept on ice and then centrifuged immediately at 13,000x g for 5 min at 4°C. The plasma was collected and aliquoted into freezer vials for storage at −80°C. Subjects with evidence of CNS infection, known CNS malignancy, or recent head trauma as a potential cause of neurologic symptoms were excluded. There were no other neurological conditions that met criteria for exclusion. In this pre-specified *post hoc* subset analysis, patients were included if they underwent MRI at the time of blood collection, and were not ultimately diagnosed as having an acute ischemic stroke or TIA. Descriptive statistics are presented on the cohort as a whole, and then for the purposes of model derivation to identify potential biomarkers of subacute cerebral microvascular disease, only stroke mimic cases were used and a leave-one-out validation was performed. Patients with stroke mimics had a range of pathologies including migraines, brain tumors, and syncope (**Table 1**).

Sphingolipid Extraction

Lipid extraction and detection were performed in an identical manner as previously reported (26). Briefly, 20 μL of plasma was placed in 13 × 100 mm screw-capped borosilicate glass test tubes with Teflon caps (Fisher Scientific Catalog Number 14-933A, New Jersey, USA). 0.5 mL of methanol followed by 0.25 mL of chloroform (Fisher Scientific, New Jersey, USA) and 10 μL of internal standards (Sph/Cer Mixture I, Catalog Number LM-6002, Avanti Polar Lipids, Alabaster, AL) were added. Samples were then sonicated in an ultrasonic bath until they appeared evenly dispersed and then incubated for 2 h at 48°C in a heating block. Tubes were then cooled to room temperature and 75 μL of

TABLE 1 | Stroke mimic pathologies.

Stroke mimic	Number (%)
Migraine	12 (20.7)
Seizure	7 (12)
Anxiety	3 (5.2)
Bell's palsy	2 (3.4)
Dementia	2 (3.4)
Tumor	2 (3.4)
Hypertension	4 (6.9)
Acute coronary syndrome	2 (3.4)
Syncope	2 (3.4)
Vestibular	3 (5.2)
Intoxication	1 (1.7)
Multiple sclerosis	1 (1.7)
Other/unknown	6 (10.3)

1M KOH was added. This mixture was sonicated briefly and then incubated for 30 min at 37°C. Samples were then cooled to room temperature and neutralized by addition of 16 μL of glacial acetic acid (Fisher Scientific, New Jersey, USA). pH was checked with test strips to verify near return to neutral pH 7.0. One milliliter of chloroform and 2 mL of water were added to each tube. The solution was mixed gently and centrifuged at 300 x g for 5 min to separate the phases. A Pasteur pipette was rinsed with chloroform and then used to remove the lower layer into another glass test tube, and the solvent was removed using vacuum centrifugation. The lipid residue was then re-dissolved in 75 μL methanol. The upper phase was re-extracted by adding 1 mL chloroform, mixing gently, and centrifuging as above. The lower layer was again transferred to another glass test tube using a Pasteur pipette that had been rinsed with chloroform, and the solvent was removed using vacuum centrifugation. The lipid residue was re-dissolved in 75 μL methanol. These two extracts were then pooled for a total of 150 μL, vortexed, and then centrifuged at 13,000 x g to clarify. The supernatant was then transferred to an autosampler vial (Catalog number 225180, Wheaton, New Jersey, USA). Vials were stored at −80°C until ready for LC-MS analysis.

High Performance Liquid Chromatography-Mass Spectrometry

Methanol (Optima grade, Fisher Scientific, New Jersey, USA), formic acid (99+%, Thermo Fisher Scientific, Waltham, MA), ammonium formate (99%, Alfa Aesar, Ward Hill, MA), and water purified to a resistivity of 18.2 MΩ·cm (at 25°C) using a Milli-Q Gradient ultrapure water purification system (Millipore, Billerica, MA), were used to prepare mobile phase solvents for liquid chromatography-mass spectrometry. Lipid extracts were analyzed using an Agilent 1200 liquid chromatography system (Agilent, Santa Clara, CA) that was connected in-line with an LTQ Orbitrap XL mass spectrometer equipped with an electrospray ionization source (ESI; Thermo Fisher Scientific, Waltham, MA). The LC was equipped with a C4 analytical column (Viva C4, length: 150 mm, inner diameter: 1.0 mm, particle size: 5 μm, pore size: 300 Å, Restek, Bellefonte, PA), and a 100-μL sample loop. Solvent A was 99.8% water/0.2% formic acid and solvent B was 99.8% methanol/0.2% formic acid (volume/volume). Solvents A and B both contained 5 mM ammonium formate. Lipid extract samples in autosampler vials sealed with septa caps were loaded into the autosampler compartment prior to analysis. The sample injection volume was 100 μL. The elution program consisted of isocratic flow at 30% B for 3 min, a linear gradient to 50% B over 0.5 min, a linear gradient to 100% B over 11.5 min, isocratic flow at 100% B for 5 min, a linear gradient to 30% B over 0.5 min, and isocratic flow at 30% B for 19.5 min, at a flow rate of 150 μL per min. The column and sample compartments were maintained at 40 and 4°C, respectively. The injection needle was rinsed with a 1:1 methanol:water (volume/volume) solution after each injection to avoid cross-contamination between samples.

The column exit was connected to the ESI probe of the mass spectrometer using PEEK tubing (0.005″ inner diameter × 1/16″ outer diameter, Agilent, Santa Clara, CA). Mass spectra were acquired in the positive ion mode over the range $m/z = 270$–1,150 using the Orbitrap mass analyzer, in profile format, with a mass resolution setting of 100,000 (at $m/z = 400$, measured at full width at half-maximum peak height). In the data-dependent mode, the five most intense ions exceeding an intensity threshold of 30,000 counts were selected from each full-scan mass spectrum for tandem mass spectrometry (MS/MS) analysis using collision-induced dissociation (CID) or pulsed-Q dissociation (PQD). MS/MS spectra were acquired in the positive ion mode using the linear ion trap, in centroid format, with the following parameters: isolation width 3 *m/z* units, normalized collision energy 30%, activation time 30 ms, activation Q 0.25 for CID, activation Q 0.50 for PQD, and default charge state 1+. A parent mass list was used to preferentially select ions of interest for MS/MS analysis. To avoid the occurrence of redundant MS/MS measurements, real-time dynamic exclusion was enabled to preclude re-selection of previously analyzed precursor ions, using the following parameters: repeat count 2, repeat duration 30 s, exclusion list size 500, exclusion duration 60 s, and exclusion width 0.1 *m/z* unit.

Sphingolipid Identification

Raw Thermo MS data files were converted into centroided data and into the mzXML format using the MS Convert tool in the ProteoWizard 3.0 package (27). Retention time correction and automated peak picking were then performed using XCMS online (28). Feature detection was performed using the centWave method at 2 parts per million (ppm) *m/z* deviation, minimum peak width of 20 s and maximum peak width of 80 s. A 20 s retention time deviation (bw) was used with a width of 0.01 s for overlapping *m/z* slices (mzwid). Peaks were annotated by searching for isotopes plus selected adducts (H^+ and Na^+) with an *m/z* absolute error of 0.015 and relative error of 5 ppm. Parameters were iteratively optimized by manual review of the selective peaks.

From the automatically selected peaks, target lipids were identified from the METLIN database with the annotation parameters described above (Scripps Research Institute, La Jolla, CA). Peaks were then filtered to only include SLs, and then

manually reviewed to ensure accuracy. Peaks that were felt to be inappropriately selected were excluded. Thus, the final list of included SLs consisted of automatically selected peaks that were manually curated.

Imaging Analysis

MRI was performed on either Siemens Avanto 1.5T or Siemens Trio 3T machines. Axial T_2-weighted images were obtained continuously in 5-mm-thick sections with repetition time of 3,800 ms and time to echo of 116 ms. The field of view was 220 cm, and the matrix was 384 × 384. Axial FLAIR images were obtained continuously in 5-mm-thick sections with repetition time of 9,000 ms and time to echo of 89 ms. The field of view was 220 cm, and the matrix was 320 × 216. Axial diffusion-weighted images were obtained continuously in 5-mm-thick sections with repetition time of 5,600 ms and time to echo of 106 ms. The field of view was 255 cm, and the matrix was 192 × 192.

Two authors (I.A. and V.K.), who were blinded to clinical data, evaluated WMH on axial T_2-weighted FLAIR images. The raters used the Fazekas subjective scoring method to assign a score from 0 to 3 in periventricular white matter (PVWM) and deep white mater (DWM) regions of the brain (29, 30). PVWM hyperintensities were scored 0, no hyperintensities; 1, caps or pencil thin lining; 2, smooth halo; 3, irregular periventricular signal extending into the DWM. The DWM was scored 0, no hyperintensities; 1, punctate foci; 2, beginning confluence; 3, large confluent areas. The total Fazekas score (FS) was obtained by summing the scores from periventricular and deep white matter regions. Interrater reliability as measured by quadratic weighted Cohen's Kappa was excellent with $\kappa = 0.81$. The average of the scores assigned by the two authors was used in subsequent analysis. This endpoint was dichotomized to separate low WMH load (FS 0–2) from high WMH load (FS > 2). Other features of cerebral microvascular disease including microbleeds and perivascular spaces were also evaluated using axial gradient-echo sequences and axial T_2-weighted sequences.

White Matter Hyperintensity Segmentation

WMH area was quantified using manual ROI segmentation in OsiriX Lite (Pixmeo SARL). WMH areas on individual T_2-Weighted FLAIR MRI slices were carefully outlined using the wand tool and the total area of each lesion measured. Lesion areas were summed across slices, and classified as deep or periventricular. Deep and periventricular areas were calculated and then correlated with Fazekas scores and lipid species.

Statistical Analysis

Statistical analysis was performed using SPSS, Graphpad v 7.0, and Matlab v2018a software. Continuous variables with a normal distribution were described as mean ± SD, and non-normally distributed variables were described as median and interquartile range. For univariate analysis, categorical variables were compared by χ^2 statistics. A Spearman's correlation was used to measure the association between lipid species, FS, and measured WMH areas. Logistic regression analyses were performed to distinguish independent predictors of heavy white matter damage defined as previously described FS cut-offs. Leave-one-out cross-validation was performed in Matlab using defined code. Statistical significance was defined as $p < 0.05$.

RESULTS

Subjects

Demographic, clinical, radiographic, and plasma specimens were collected as part of the Advanced Serum Profiling in Recent (ASPIRE) Stroke study, a previously conducted UCLA IRB approved, single-center observational study that aimed to identify lipid biomarkers of acute stroke. Data for the ASPIRE trial were collected from all consenting patients over the age of 18 years old presenting to the emergency department (ED) between December 2014 and June 2015 with acute neurological symptoms within the prior 24 h. Subjects with evidence of CNS infection, known CNS malignancy, or recent head trauma as a potential cause of neurologic symptoms were excluded. Demographic data and neurodiagnostic studies are available for all enrolled patients.

To identify only those subjects with subacute white matter disease, the total study population was selectively reduced to exclude those presenting with imaging or clinically confirmed TIA/stroke and those without MRI imaging. Of the 202 patients enrolled in the ASPIRE Stroke study, 34 were excluded as they had not undergone MRI, and an additional 110 were excluded for being diagnosed ultimately with acute ischemic stroke or TIA (**Figure 1**). Among the 58 patients who presented with a stroke mimic syndrome and were included in this analysis, median age was 69.5 and 51.7% were female (**Table 2**). Higher FS was associated with advanced age (29 vs. 70% over 70 yo, FS 0–2 vs. 2.5–6, $p = 0.002$). While sex did not have an effect on FS in χ^2 analysis ($p = 0.32$), age did ($p = 0.0041$). Fazekas positive status was given to 46.5% of the cohort for having FS > 2. Rates of other stigmata of cerebral microvascular disease in the stroke mimic cohort were low with only 5.5% having cerebral microbleeds and 17.8% having prominent perivascular spaces, the majority (>70%) of which also have FS > 2. **Figure 2A** demonstrates the distribution of Fazekas scores in the total ASPIRE cohort and the smaller stroke mimic cohort. Stroke mimics represented a variety of non-ischemic diagnoses ranging from migraine to syncope (**Table 1**).

Sphingolipid Analysis

Two species, SM 38:1 and Cer 34:1, significantly correlated with FS in a Spearman's correlation model in both the overall ASPIRE cohort and the stroke mimic subgroup (**Tables 3**, **4**). In multivariate logistic regression, SM 38:1 (OR 1.129, 95% CI 1.032, 1.236, $p = 0.008$) and Cer 34:1 (OR 1.118, 95% CI 1.031, 1.212, $p = 0.007$) also accurately differentiated between FS 0–2 vs. 2.5–6. When controlling for age, the two species were still independently predictive of WMH load: SM 38:1 (OR 1.125, 95% CI 1.010, 1.253, $p = 0.032$) and Cer 34:1 (OR 1.096, 95% CI, 1.002, 1.200, $p = 0.046$).

A composite lipid score (LS) was computed from the sum of these two lipid species and demonstrated fair discrimination in ROC analysis (**Figures 2B,C**). Leave-one-out validation

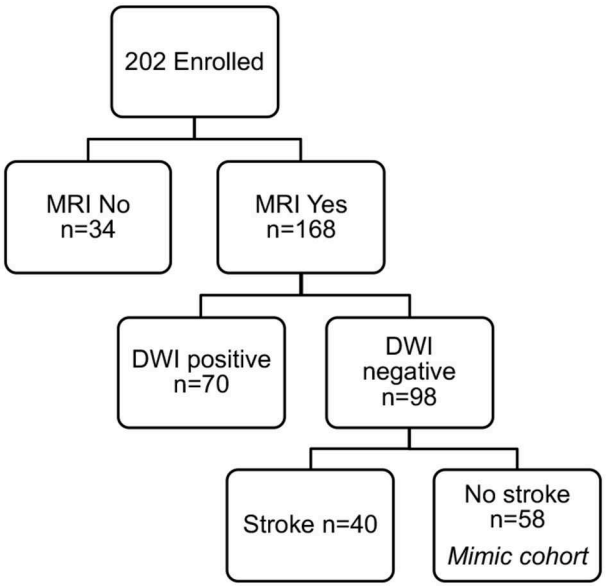

FIGURE 1 | ASPIRE Study subcohorts. The total ASPIRE Study Cohort of enrolled subjects was segmented into those with and without MRI to facilitate accurate measurement of white matter WMH burden. Individual subjects with DWI-positive scan indicating acute ischemia were excluded. Subjects with DWI-negative scans were further subdivided based on clinical diagnosis of stroke. The remaining 58 subjects presented with a neurologic mimic of stroke, did not have neuroimaging evidence of acute pathology, and were included in the analysis for plasma sphingolipid biomarkers.

TABLE 2 | Demographics.

ASPIRE cohort	**Total**	**FS–**	**FS+**	**FS+ vs. –**	
		***n* (%)**		χ^2	***p***
Total	168 (100)	83 (49.4)	85 (50.6)	0.048	0.83
Male	92 (54.8)	45 (54.2)	47 (55.3)	0.02	0.89
Female	76 (45.2)	38 (45.8)	38 (44.7)	0.02	0.89
MetS	42 (25)	21 (25)	21 (25)	0	1
Hx statin	63 (37.5)	17 (20)	43 (51.8)	18	<0.0001**
Hx stroke	63 (39.9)	43 (50.7)	18 (21.7)	15.2	0.0001**
		μ (SD)		***t***	***p***
Age	70.4 (15.2)	62.6 (15.5)	78.4 (9.9)	7.89	<0.0001**
SBP	158 (30.8)	154.4 (27.8)	162 (33.3)	1.6	0.11
DBP	85.5 (17.2)	86.9 (16.5)	84.2 (18.1)	−1.01	0.31
Glucose	133.8 (62.1)	138.5 (76)	129 (44.1)	−0.994	0.32
Stroke mimic cohort	**Total**	**FS–**	**FS+**	**FS+ vs. –**	
		***n* (%)**		χ^2	***p***
Total	58 (100)	31 (53.4)	27 (46.5)	0.548	0.46
Male	28 (48)	19 (61.3)	9 (33.3)	4.45	0.035*
Female	30 (51.7)	12 (38.7)	18 (66.7)	4.45	0.035*
MetS	11 (19)	4 (12.9)	7 (25.9)	1.56	0.21
Hx statin	23 (39.7)	8 (25.8)	15 (55.6)	5.26	0.022*
Hx stroke	25 (43.1)	7 (22.6)	18 (66.7)	11.25	0.0008**
		μ (SD)		***t***	***p***
Age	66.12 (17.5)	59 (18.1)	74 (9)	3.9	0.0003**
SBP	154 (30.7)	144.3 (26)	164.6 (32)	2.67	0.01**
DBP	87.2 (18.7)	86.3 (17.8)	88.1 (19.9)	0.36	0.72
Glucose	123.3 (48.6)	120.6 (55.6)	126.2 (40.7)	0.43	0.67

*n (%), number (percent); p, level of significance; MetS, Metabolic Syndrome; χ^2, chi squared statistic; Hx Statin use, History of statin use; Hx Stroke, History of Stroke; Fazekas –, Fazekas score ≤ 2; Fazekas +, Fazekas score >2; $\bar{x}$ Age (SD), mean age (standard deviation); MSBP (IQR), median systolic blood pressure (interquartile range); M DBP (IQR), median diastolic blood pressure (interquartile range). **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).*

demonstrated a cross-validated R^2 of 12.54%, indicating reproducibility of the LS to predict WMHs resulting from cerebral microvascular disease. To further validate the specificity of the LS, we measured WMH area using FLAIR sequences within the stroke mimic cohort of ASPIRE. Of the 58 stroke mimic subjects, 44 subjects had scans allowing precise measurement of WMH. WMH burden correlated with FS ($r = 0.828$; $p = 4.1 \times 10^{-12}$) and significantly correlated with LS ($r = 0.459$; $p = 0.0017$). **Figure 3** demonstrates the relationship between FS, LS, and WMH in this cohort. In linear regression analysis Cer 34:1 significantly associated with objective white matter measurements ($p = 0.003$), even when controlling for age as a covariate ($p = 0.003$). To evaluate other potential variables to include in the model, mean LS was compared in patients with and without hypertension, diabetes, history of statin use, sex, and low HDL levels. No significant differences in mean LS exist between these groups. Furthermore, these variables were not predictive of FS+ status in univariate binary logistic regression and were therefore excluded from the model.

To demonstrate the specificity of LS for clinically subacute WMH and not the presence of acute ischemic damage, SM 38:1, Cer 34:1, and LS levels were compared between the stroke mimic cohort ($n = 58$) and DWI positive stroke group ($n = 110$). While LS correlated with FS in acute stroke patients ($r = 0.440$, $p < 0.001$), overall SM 38:1 and LS levels were significantly lower in the acute stroke patients compared to stroke mimics (**Table 5**). There was a trend showing lower Cer 34:1 in the acute stroke patients as well, however, this difference did not reach statistical significance.

DISCUSSION

Biomarkers play an important role in timely detection and management of neurological diseases. The availability of a fluid-based biomarker for subacute ischemic white matter injury may aid in early detection of subacute ischemic brain damage and prevention of its progression to symptomatic stroke, global cognitive decline, and functional loss. Currently, no brain-specific biomarkers are in clinical use for the evaluation of WMH. WMH are a neuroimaging finding that may be indicative of ischemic brain damage and are often found incidentally yet convey considerable cerebrovascular risk. The high cost and relative inaccessibility of MRI studies for subacute neurological disease necessitate an alternative to neuroimaging for evaluation of chronic white matter disease.

A number of studies have suggested protein-based circulating biomarkers for ischemic white matter injury but these associations are highly variable among populations (31). Recent work suggests that serum detection of neurofilament light chain is transiently increased within 3 months of a recent small subcortical infarct, adding sensitivity to MRI confirmation of acute stroke but not necessarily reliable in the detection of subacute ischemic white matter injury (32). Here, we demonstrate that serum levels of two brain-specific lipid species, SM 38:1 and Cer 34:1, correlate with WMH load as measured by FS specifically in subjects with imaging confirmation of no recent brain ischemia. Summing the relative intensities in serum of these lipids yields a LS that reliably distinguishes between low and high WMH load with a cut off of FS >2. Quantitative assessments of WMH area further validated the role of LS as a blood-based biomarker for WMH in a clinically relevant study population.

An ideal biomarker for cerebrovascular disease is specific to the brain and can cross the blood-brain barrier (BBB) to be measured in serum or plasma from peripheral circulation. Leakage across the BBB is increasingly recognized as central to the pathogenesis of cerebral microvascular disease with evidence of fibrinogen and other plasma proteins leaking into the brain and white matter in individuals with cerebral microvascular disease (32, 33). While acute stroke can cause adequate disruption to the BBB for lipid species to cross over into systemic circulation, other conditions such as acute infection, migraines, or seizures may cause similar leaks and lead to false positive signals. However, subclinical ischemia is generally confined to the cerebral white matter and using a biomarker specific to myelin components may help tune specificity to white matter damage. Indeed, characterization of specific sphingolipid species indicates that those species increased in acute stroke (Cer 42:1 and SM 36:0) are independent and distinguishable from those relevant to WMHs (Cer 34:1 and SM 38:1) (26).

Brain white matter is enriched for the lipid species investigated in this study. Composition of human brain white matter includes sphingomyelin and ceramide (34). Human myelin within white matter tracts comprises many different sphingomyelin species (26, 35). Both ceramide and sphingomyelin are key components of lipid rafts that play a key role in myelination and the maintenance of myelin (36, 37). CSF measurement of sphingomyelin species has been proposed as a biomarker for demyelination in peripheral neuropathies (38). Given the relative

TABLE 3 | Lipid screening analysis in stroke mimic cohort.

Lipids	M [IQR]		Spearman correlation	
	Total	FS+	*r*	*p*
SM 34:1	3809.5 [3148.2]	3975 [2793.2]	0.132	($p = 0.324$)
SM 36:1	1343.7 [803.7]	458.6 [918.2]	0.182	($p = 0171$)
SM 40:2	1021.3 [439.6]	1015.5 [403.6]	0.032	($p = 0.813$)
SM 34:2	779.6 [609.2]	793.1 [643.4]	0.078	($p = 0.559$)
SM 42:1	873.4 [409.1]	869 [396]	0.039	($p = 0.769$)
SM 38:1	691.6 [402.0]	702.7 [317.5]	−0.035	($p = 0.792$)
SM 36:2	632.0 [316.9]	667 [357.4]	0.173	($p = 0.193$)
SG 19:3	492.6 [307.2]	452.3 [322.7]	−0.121	($p = 0.364$)
SM 38:2	378.7 [174.6]	406.9 [175.7]	0.151	($p = 0.258$)
SG 20:1	237.2 [189.5]	225 [219.7]	−0.112	($p = 0.403$)
SG 18:1	242.7 [183.6]	237.3 [231.6]	−0.107	($p = 0.426$)
SM 39:1	190.1 [134]	181.8 [117.2]	−0.012	($p = 0.93$)
SM 35:1	177.4 [91.4]	193.9 [97.4]	0.291	($p = 0.027$)*
SM 40:3	143.4 [67.9]	156.3 [65.8]	0.194	($p = 0.145$)
SM 37:1	95.4 [45.7]	105.9 [53.2]	0.215	($p = 0.105$)
SM 43:2	86.0 [44]	97.0 [46.4]	0.270	($p = 0.04$)*
SM 43:1	75.6 [46.2]	78.4 [38.8]	0.169	($p = 0.204$)
SM 39:2	42.8 [19]	45.2 [22.7]	0.186	($p = 0.163$)
SM 36:3	35.1 [32.8]	38.6 [37.0]	0.167	($p = 0.209$)
SM 38:3	23.7 [11.8]	26.2 [14.6]	0.235	($p = 0.076$)
SM 38:1	16.9 [9.4]	20.3 [12.3]	0.287	($p = 0.029$)*
CR 34:1	13.3 [11.7]	16.5 [14.5]	0.356	($p = 0.006$)**

*p, level of significance; r, Spearman's rho; Fazekas +, Fazekas score >2; M (IQR), median (interquartile range). **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).*

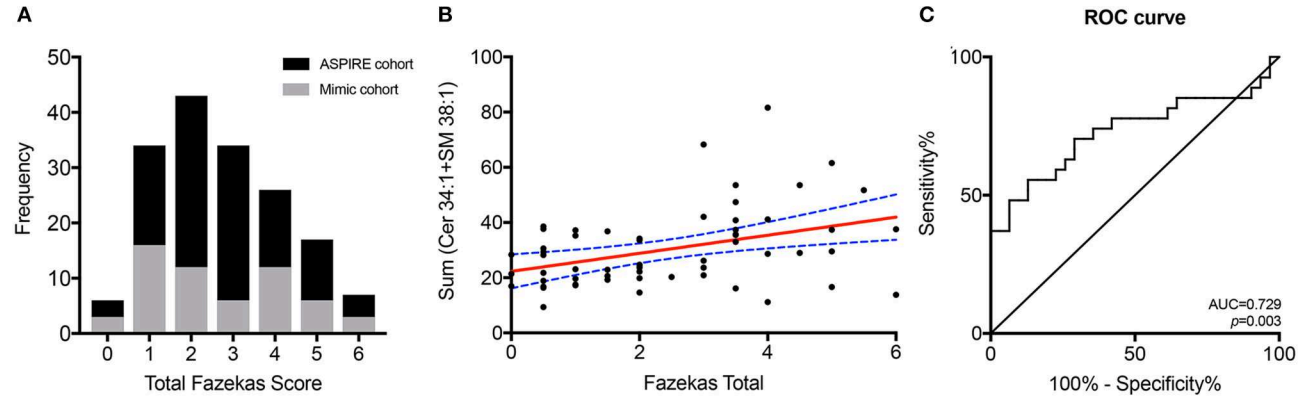

FIGURE 2 | Combined lipid score predicts WMH. The distribution of Fazekas scoring for the total ASPIRE study cohort (black) and the stroke mimic cohort (gray) **(A)**. Correlation of the combined Cer 34:1 + SM 38:1 lipid score with Fazekas scoring in the stroke mimic cohort **(B)**. ROC curve for the combined lipid score as a predictor of WMH **(C)**.

TABLE 4 | Lipid screening analysis in ASPIRE cohort.

Lipids	Spearman correlation		Linear regression		
	r	*p*	β	*p*	95% CI β
SM 34:1	0.032	0.728			
SM 36:1	0.061	0.513			
SM 40:2	−0.091	0.323			
SM 34:2	−0.016	0.860			
SM 42:1	−0.090	0.330			
SM 38:1	−0.094	0.310			
SM 36:2	0.028	−0.143			
SG 19:3	−0.143	0.121			
SM 38:2	−0.051	0.585			
SG 20:1	−0.140	0.880			
SG 18:1	−0.019	0.842			
SM 39:1	−0.137	0.137			
SM 35:1	0.099	0.285			
SM 40:3	0.149	0.105			
SM 37:1	0.051	0.578			
SM 43:2	0.097	0.296			
SM 43:1	−0.055	0.554			
SM 39:2	0.066	0.474			
SM 36:3	0.067	0.468			
SM 38:3	0.152	0.100			
SM 38:1	0.275	0.002**	0. 051	0.010	(1.167, 2.472)
CR 34:1	0.375	0.001**	0.062	<0.001	(0.032, 0.093)

*p, level of significance; r, Spearman's rho; 95% CI, 95% confidence interval. **Correlation is significant at the 0.01 level (2-tailed).*

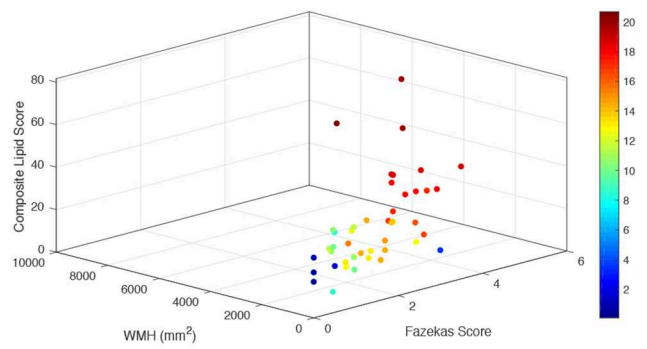

FIGURE 3 | Relationship of combined LS with subjective and objective white matter hyperintensity measurements. For each stroke mimic cohort subject, combined lipid score values are plotted on the vertical z axis (arbitrary units) as a function of measured white matter hyperintensities (mm^2) (left bottom axis) and Fazekas score (right bottom axis). Color scale corresponds to the summated log-normalized composite of each input variable (LS, WMH area, and FS).

abundance of myelin lipids to axonal protein components such as NF-L, leakage of these lipids into the periphery after injury may be a more sensitive indicator of subacute white matter injury. The design of this study enriches for chronic WMH but limits the ability to judge the specificity of LS in other demyelinating injuries.

The original ASPIRE Stroke study cohort of over 200 patients was initially reduced to only 58 to stringently select for those without acute stroke or TIA yet with evidence of WMH

TABLE 5 | Lipid biomarker levels in stroke mimic and tissue-defined stroke cohorts.

	Stroke mimic cohort *n* = 58	Tissue-defined stroke cohort *n* = 110	
	M		*p*
SM 38:1	15.5	12.8	0.0059**
Cer 34:1	11.68	9.67	0.053
LS	28.27	22.46	0.011**

*Medians were compared with a one tailed Mann Whitney U-test. **, significance at the 0.01 level. LS, combined lipid score from sum of SM 38:1 and Cer 34:1; M, median; p, p-value.*

reflective of chronic ischemic damage. Despite excluding patients with acute stroke, this cohort is enriched for systemic disease and advanced age due to the original recruitment criteria of the ASPIRE study. Moreover, our approach of using routine clinical imaging as opposed to research grade MRI sequencing approaches that are often used in evaluation of white matter hyperintensity burden, necessarily reduces the accuracy of white matter hyperintensity load measurements but increases the potential applicability of the LS biomarker in clinical practice. Additional studies using precise volumetric measurements of WMH should be pursued to further validate this biomarker. Details on the microstructural integrity of white matter available using DTI images may also enhance the biological significance of sphingolipids through indirect measurement of myelin sheath breakdown. In ASPIRE, rates of other imaging metrics of cerebral microvascular disease including cerebral microbleeds and perivascular spaces were low or potentially confounded by recent ischemia in the stroke cohort and therefore not included in this analysis. However, since SLs are a major constituent of the myelin sheath, WMH are the logical imaging variable to utilize in determining the value of a fluid-based biomarker for cerebral microvascular disease. Further investigation of this biomarker in healthier and more diverse populations and those with other neurologic and white matter injury phenotypes will help to establish baseline values in both healthy and diseased patients. Furthermore, following patients over months and years will establish the longitudinal value of plasma sphingolipid levels to track longitudinal progression of WMH and clinical outcomes associated with WMH such as stroke risk, cognitive decline, gait changes, and other neuropsychiatric variables.

A fluid-based biomarker for cerebral microvascular disease can serve as a valuable tool for detecting subacute brain ischemia. Exposing subclinical ischemia before its progression to symptomatic stroke or clinically apparent cognitive impairment can reduce disease burden in elderly and frail individuals already at elevated risk for acute stroke. Future studies will likely expand the role of this and other fluid-based biomarkers for WMH and provide a critical role for risk stratification in the study of stroke and vascular cognitive impairment and dementia.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

IA, JH, and SS: conception and design of the study and drafting a significant portion of the manuscript or figures. IA, JH, SS, AI, SL, and VK: acquisition and analysis of data.

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Novel Results and Concepts Emerging From Lipid Cell Biology Relevant to Degenerative Brain Aging and Disease

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While very rare familial forms of proteinopathy can cause Parkinson's disease (PD), Lewy body dementia (LBD) and age-related dementias, recent in-depth studies of lipid disturbances in the majority of the common forms of these diseases instead suggest a primary pathogenesis in lipid pathways. This review synthesizes a perspective from new data that point to an interdependence of lipids and proteinopathy. This article describes disturbed relationships in lipid homeostasis that causes neuropathology to develop over time and with age, which includes altered mechanisms of glia-neuron exchange of lipids and inflammatory signals.

Keywords: neurons, lipids, astrocytes, microglia, inflammation, α-synuclein, apolipoprotein, Parkinson's disease

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LIPID CHANGES IN PARKINSON'S DISEASE AND RELATED NEURODEGENERATION

The relationship between lysosomal storage disorders (LSDs) and Lewy body disorders became apparent through evidence of increased risk for developing Lewy body dementia (LBD) and Parkinson's disease (PD) in carriers of LSD gene mutations, and through glycosphingolipid dysregulation and lysosomal dysfunction implicated in the normal pathophysiology of PD (1, 2) (**Table 1**). Homozygous mutations in the lysosomal hydrolase glucocerebrosidase, *GBA1*, are associated with the LSD, Gaucher disease. Haploinsufficiency of *GBA1*, which causes reduced activity of glucocerebrosidase (GCase), is associated clinically with a significantly increased risk of PD and LBD, and with faster rate of cognitive decline in α-synucleinopathies, including LBD and PD (3, 4). Brain GCase activity is also decreased and corresponding glycosphingolipid substrate levels are elevated in the brain in PD without *GBA1* mutations (1, 5–7) pointing to a much broader age-related decline, and to more complex mechanisms (8). Similar dysfunction of lysosomal hydrolases and disturbances in glycosphingolipid levels to those found clinically in PD are observed in normal aging of both mouse and human brain (1, 9).

Furthermore, PD gene expression and genetic analyses of large cohorts also point to an early involvement of biological processes *upstream* of accumulating alpha-synuclein (αSyn), including involvement of lipids and lipoproteins, oxidative stress, endosomal-lysosomal functioning, endoplasmic reticulum stress, and immune response activation (8, 10). In addition, with age, lysosomal enzyme function, chaperones and transporters present in the endoplasmic reticulum-Golgi complex, may become compromised at an early stage of pathogenesis. Critically, biochemical evidence shows that lysosomal enzyme loss of function and lipid disturbances creates PD-like pathology (see **Table 1**).

TABLE 1 | Biochemical and clinical evidence for lysosomal enzyme loss of function and lipid disturbances creating Parkinson's disease-like pathology.

Relevant lysosomal biochemical pre- and clinical data for parkinson's disease and related disorders	Protein affected	Accumulating substrate(s)
Increased risk for PD and LBD in patients carrying *het*erozygous GBA1 mutations. Glucocerebrosidase activity is reduced in sporadic PD and in normal aging. GluCer and GluSph increased in sporadic PD brain (1).	Glucocerebrosidase	Glucosylceramide and glucosylsphingosine
LIMP-2, which transports GCase to lysosome, is encoded by SCARB2. SCARB2 gene variants are associated with PD and LBD risk (11, 12). LIMP-2 deficiency in mice causes GCase activity reduction, glycolipid accumulation and αSyn aggregates (13).	Glucocerebrosidase	Glucosylceramide
Granulin (GRN) gene variants associated with PD risk (14). Progranulin deficiency in mice leads to reduced GCase activity (15).	Glucocerebrosidase	–
Clinical reports of parkinsonism in Fabry disease patients (16). Reduced activity of α-galactosidase A in leukocytes of PD patients (17).	α-galactosidase A	Globotriasylceramide
Generalized dystonia associated with akinetic-rigid Parkinsonism reported in patients with GM1 gangliosidosis (18) (caused by a β-galactosidase deficiency). Lactosylceramide is utilized in ganglioside biosynthesis.	β-galactosidase	Lactosylceramide
Mutations in SMPD1 lead to Niemann-Pick disease type A or B and accumulation of sphingomyelin, and are also associated with increased risk for PD (19).	Acid sphingomyelinase	Sphingomyelin
Phosphorylated αSyn and Tau in neurons and oligodendrocytes in Niemann-Pick disease type C patient brain (20).	NPC1 and 2	Cholesterol, sphingolipids
α-synucleinopathy reported in the brain of patients with Krabbe's disease (21).	Galactocerebrosidase	Galactosylsphingosine
α-synucleinopathy reported in brain of patients with Sandhoff disease, as well as in Hexb deficient mice (22, 23). Parkinsonism reported in patients with Sandhoff disease (24).	β-hexosaminidase A and B	GM2 ganglioside
Variation in NAGLU associated with risk for PD. Intracellular αSyn accumulation in cortical tissue from Sanfilippo A syndrome cases (25).	N-acetylglucosaminidase, N-sulfoglucosamine sulfohydrolase	Heparin sulfate metabolites

With age, many mechanisms can compromise lysosomal enzyme function, including loss of chaperones and transporters present in the endoplasmic reticulum-Golgi complex. Insufficiency of such lysosomal enzymes puts certain cells and brain regions at risk over the longer time frame associated with a relative increase of longevity in humans, creating risk for age-related neurodegenerative diseases (8).

Over the last decades there has been a large emphasis placed on the idea of "proteinopathy", conceptualized primarily in two versions. First, as a primary mechanism for cell dysfunction and degeneration in PD and other diseases with cellular protein aggregates, which however may only be true in very rare genetic cases with gene copy number variations (CNVs) or rare mutations (26). For example, whilst familial PD cases with CNVs of αSyn (duplication, triplication) cause genetic PD and LBD and protein elevations, such cases are very rare; there are currently ∼80 individuals worldwide carrying these CNVs diagnosed with PD/LBD (26), compared to an estimated 6 million cases of PD worldwide (27). Second, the theory of causative proteinopathy has been extended to encompass extra-neural spread of toxic proteins in order to explain regional patterns of chronic cellular pathology seen in many neurodegenerative disorders. Evidence of physical αSyn spread between cells in human PD and related diseases remains to be established, and so far is demonstrated only in artificial experimental model systems.

Instead, more obvious causes for cell dysfunction and pathologies in PD and other related disorders, are *primary* disturbances from lipids and other metabolic stressors, which in turn can produce protein elevation and aggregation. Lewy body inclusions, widely believed to be predominantly composed of proteinaceous filaments, are in fact more co-labeled with lipids (28, 29). Importantly, recent ultrastructural findings demonstrate that Lewy bodies and neurites in PD post-mortem brain are composed of abundant membranous structures, abnormal vesicles and autophagosome-like structures, in addition to disrupted cytoskeletal elements and dysmorphic mitochondria (30). In summary, the previous almost exclusive focus on aggregating proteins in familial and sporadic cases of PD and LBD, may be replaced by a critical analysis of intracellular lipids and dysfunctional lipid transport as primary mechanisms of disease; in concert with inflammatory processes for PD and LBD. Such analyses may be very useful in the future for selecting candidates, biomarkers and modalities for treatments.

How can perturbation of glycolipid metabolism and lysosomal homeostasis in the aging brain precede or be upstream of protein handling? Glycolipids are abundant in plasma and intracellular membranes and are particularly enriched in the brain where they act as cell surface recognition molecules, as well as having essential roles in the regulation of membrane fluidity and lipid raft formation, modulation of membrane-protein function and signal transduction (31). When GCase in inhibited *in vivo*, there is large accumulation of high molecular weight aggregates of αSyn (32) and *in vivo* genetic models of primary GBA mutations also produce significant α-synucleinopathy over time (33, 34). These α-synucleinopathies can be reversed or prevented by agents that increase GCase, or otherwise reduce the accumulation of glycolipids (33, 35).

In conclusion, the physiological burden of elevated neuronal glycolipid load in aging and in PD/LBD would affect multiple organelles and biological pathways, and may lower the threshold for developing PD and related neurodegenerative disorders, or accelerate pathophysiological processes in vulnerable neurons.

NEURON-GLIA INTERACTIONS DURING LIPID DYSHOMEOSTASIS

Abnormal glycolipid, neutral lipid and protein homeostasis within PD and LBD susceptible neurons are likely to signal to surrounding cells, including microglia and astrocytes, accelerating neurodegeneration (36, 37) **(Figure 1A)**. Understanding how glycolipid changes can drive the inflammatory and neurodegenerative mechanisms will be crucial in enabling the development of novel therapeutics. Elevation of reactive oxygen species, mitochondrial dysfunction and loss of autophagy in neurons leads to elevated lipid particle formation (and peroxidated fatty acids). Such lipid dyshomeostasis in neurons may lead to subsequent accumulation of lipid droplets and eventually larger undigested lipid particles, in neurons, and potentially neighboring microglia (39–41) and Isacson, Brekk, Hallett *unpublished observation*. Appropriate lipid transfer between these cells via lipid transporters such as apolipoproteins, is essential for this process, and for maintaining metabolic integrity of the neuron (40). Peroxidated fatty acids released by neurons are bound to lipoproteins, which are endocytosed by glia (41). Disrupting the transport of lipids from neurons to glia for lipid droplet formation under conditions of neuronal stress, leads to neurodegeneration (39).

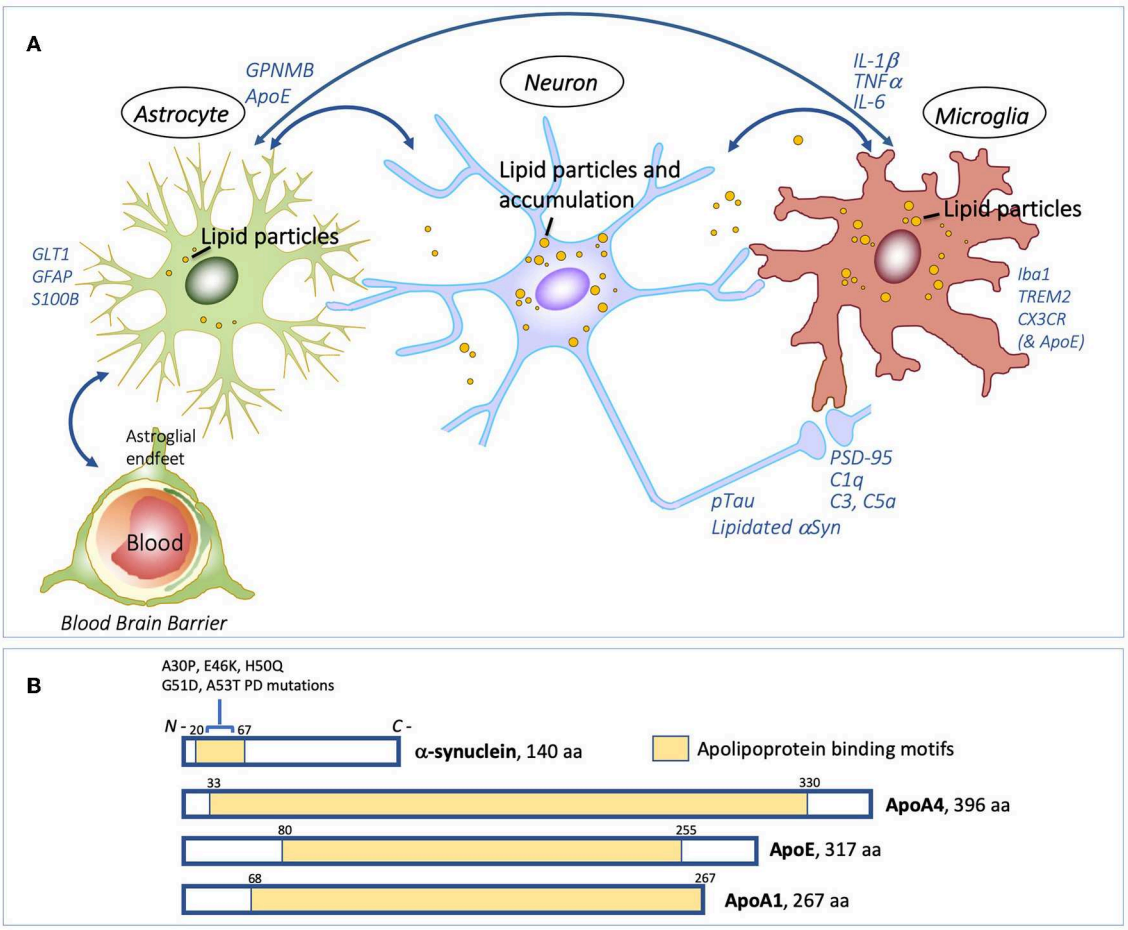

FIGURE 1 | Interdependence of lipid processing and proteinopathy. Lipids, and glia-neuron exchange of lipids and inflammatory signals **(A)** Proposed model for pathogenic lipid perturbations in human PD. Overall accumulation of glycolipids in the substantia nigra with differentially altered lipid droplet deposition in neurons and glial cells could facilitate aberrant protein-lipid interactions (e.g. with αSyn and pTau), in turn perturbing the neuron-glia lipid exchange and activating GPNMB inflammatory protein signals. Cellular dysfunction caused by elevated glycolipids could converge on downstream cytokine-signaling, and other immune responses, in neurons, microglia and astrocytes, causing excessive and aberrant neurite and synaptic damage ultimately leading to neurodegeneration. **(B)** αSyn has a role in lipid binding through its 11-amino acid repeat amphipathic helical region (shaded in yellow). Mutations in αSyn associated with familial PD, A30P, E46K, H50Q, G51D, and A53T, all occur within this region. αSyn shares conserved tandem repeat regions with the apolipoproteins, ApoA4, ApoE, and ApoA1 (yellow shading). Under lipid stress or aging, αSyn can become significantly lipidated, which may also create dysfunction leading to Lewy bodies. Schematic in **(A)** is original by the authors. Schematic in **(B)**, showing apolipoprotein binding motifs, is adapted and modified from Emamzadeh (38), under terms of the Creative Commons CC BY license.

Glycoprotein non-metastatic protein B (*GPNMB*) is a type-I transmembrane glycoprotein that seems to be mechanistically related to altered glycolipid levels (42). In the brain, GPNMB is expressed primarily in glial cells and is thought to play a role as an inflammatory regulator to prevent chronic inflammation (43). Polymorphisms in *GPNMB* are found to associate with idiopathic PD (44), and GPNMB protein levels are selectively increased in the substantia nigra of PD patients (42). In Gaucher disease, increased protein levels of GPNMB correlate with disease severity and progression (45, 46). Upon systemic pharmacological induction of lipidopathy in mice, which also causes α-synucleinopathy, GPNMB is elevated in a brain-region specific manner including the hippocampus, substantia nigra and cerebral cortex (42). This regional specific upregulation of GPNMB may reflect a differential response of brain regions to lysosomal dysfunction and subsequent differential vulnerability of neuronal populations to degeneration (42, 47). In this lipidopathy model, GPNMB elevation is also associated with robust glial activation and GPNMB is localized in astrocytes and microglia (42). Of note, GPNMB is not elevated by increased αSyn alone in transgenic mice overexpressing human αSyn, indicating a selective response to lipid perturbations (42).

αSYN AS A LIPID-CARRYING PROTEIN

αSyn is abundant in neurons and is highly enriched at presynaptic terminals, and it is also associated with some organelles. αSyn has important functional roles in the regulation of vesicles, such as synaptic vesicles, neurotransmitter release, dopamine metabolism, synaptic activity, and plasticity (48). αSyn can interact with various lipid species through its amphipathic N-terminus (amino acid) domain. The N-terminal region of αSyn contains six hexameric repeats of 11 amino acids which are characteristic of the 11 amino acid repeats that mediate lipid interactions of apolipoproteins (38, 49) (**Figure 1B**). Significantly, all mutations in αSyn which are associated with familial PD, are located in this lipid-binding region. In the brain, apolipoprotein E (apoE) is the most abundant lipoprotein, forming lipoprotein particles and binding to ApoE cell surface receptors for the delivery of cholesterol and other lipids to neurons. The *APOE4* isoform allele is associated with increased risk for dementias in Lewy body diseases, including LBD and PD (50). Interestingly, plasma protein levels of another apolipoprotein, apolipoprotein A1, are associated with age of onset and motor severity in early PD (51). αSyn has several apolipoprotein-like characteristics, including regulation of cholesterol efflux in neuronal cells and formation of lipoprotein nanoparticles (52, 53) providing a clear premise for αSyn's function as a lipid-carrying molecule. Statin treatment in a rodent α-synucleinopathy model reduces α-synuclein aggregates and neuronal pathology (54). The role of αSyn as a functional apolipoprotein is also highlighted by the finding that there is a worsening of ApoE4-mediated pathology in mice that carry human apoE4 when mouse αSyn is ablated (55). Levels of neutral lipids are elevated in the brain in mice that lack endogenous αSyn (56). In perspective, given these data it appears that αSyn normally is not participating in lipid transport in roles that involve synaptic transmission and vesicular functions at the synapse, but can become lipidated under cellular stressful conditions that involve glycolipid, sphingolipid, neutral lipid, lipid peroxidation and age-related disturbances (7). In such circumstances, αSyn becomes part of a pathological adaptation to resolve the lipid problems (55), which likely leads to vesicular binding and transport changes that precede Lewy body formation.

Apolipoprotein function is also linked with inflammation, and ApoE is a modulator of immune responses (57) (**Figure 1A**). Mice lacking ApoE show similar immune activation to mice expressing human ApoE4 in response to lipopolysaccharide (58), and expression of complement pathway genes are upregulated in ApoE knockout mice (59). ApoE has also recently been shown to form a complex with C1q within lipid compartments where it is a regulator of the classical complement cascade (59). C1q is implicated as an early mediator of neuronal dysfunction in preclinical models of AD, whereby reduced expression or blockade of C1q rescues synaptic loss and dysfunction upon exposure to toxic amyloid-beta (60); similarly, C1qa knockdown mitigates neurotoxicity in an *in vivo* model of frontotemporal dementia (61). Furthermore, C1qa deficiency delays functional cognitive decline associated with normal aging in mice (62). Activation of the complement system is induced in lysosomal storage disorders, including models of neuronopathic Gaucher disease by inhibiting GCase (using CBE), where protein expression of C1q is robustly elevated in several brain regions (32). Inhibition of the complement pathway, through genetic deficiency of C5R1a completely prevents glycolipid accumulation and inflammation in the brain following similar paradigms of systemic CBE (63).

SUMMARY AND FUTURE PERSPECTIVE

In summary, much evidence points to disruption of lipid cell biology; as glycosphingolipids, gangliosides and possibly several other lipids with metabolic influence can be early initiating factors for age-related neurodegenerative disorders such as PD and LBD (**Table 1** and **Figure 2**). Lipid disturbances in cell types of the brain and/or in specific compartments of such cells, including neurons, astrocytes, microglia and oligodendrocytes, are involved in a large number of neurological diseases. In particular, it is now clear that PD can be triggered by lipid disturbances that are caused by lysosomal genetic or similar age-induced enzymatic loss of function. Relevant to such lipid changes, we find that lipid transport may be compromised by pathological accommodation of αSyn to lipid binding and altered transport roles which are not optimal for normal neuronal function (**Figure 1**). In particular, it is important in future research to identify lipid binding and abnormal lipid droplet or other cellular lipid formations under specific cell

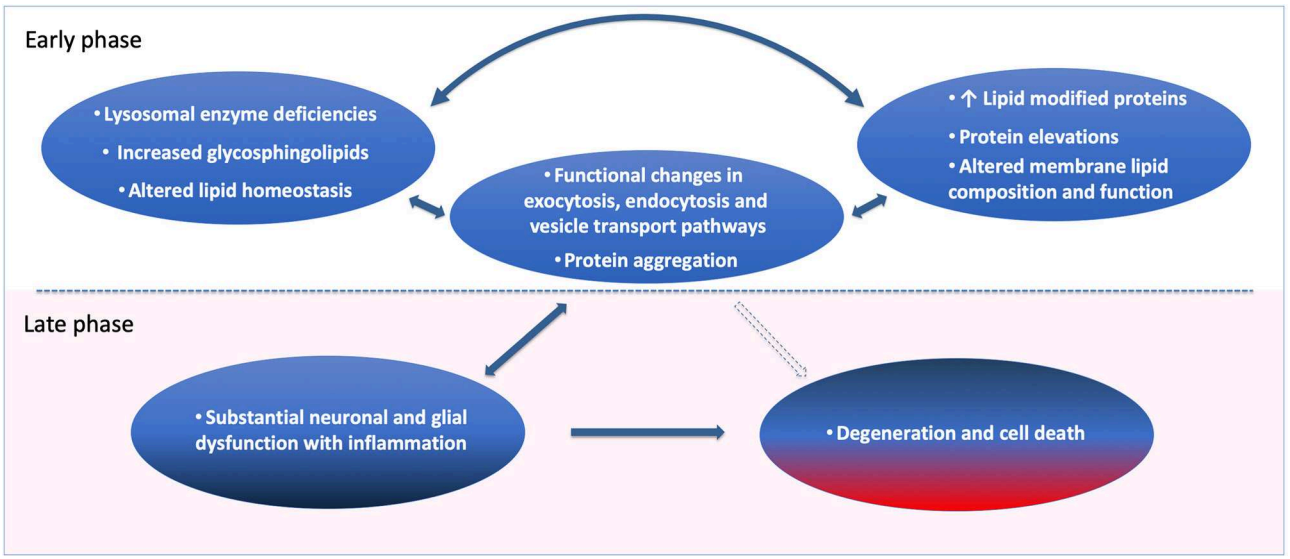

FIGURE 2 | Cell biological pathogenic factors and adaptations leading to neuronal dysfunction, synaptic degeneration, and cell death over time. Loss of lipid homeostasis and lysosomal enzyme deficiencies are the strong genetic and phenotypic drivers of age and Parkinson's disease. Sphingolipid elevations and changes in lipid metabolism can be (Early, see figure) primary pathogenic contributors, leading to protein elevations, lipid modified proteins and sphingolipid-induced membrane composition and loss of function. In the case of CNVs and in mutations of key proteins such as αSyn, the primary event in those extremely rare forms of PD can start with protein elevations followed by altered lipid-modified proteins, and functional changes in exocytosis, endocytosis and vesicle transport, leading to neuronal dysfunction and protein aggregation. It is reasonable to believe that these cellular changes can occur for a long time in an adaptive feedback cycle to keep the cells and neurons functional, as a form of pathological accommodation. Over time with the ongoing disease process (Late, see figure), the cell dysfunction will become severe if no external intervention is obtained, causing substantial neuronal glial dysfunction and inflammation as a result. Such conditions will lead to regional and cell type specific destruction and cell death in the brain tissue.

biological conditions. Lipid storage diseases with excessive lipid handling demand can lead to astrocytic and microglial disturbances. There are several contexts in which such lipid and associated lipid-protein interactions could eventually become pathological. The fact that the lipid-carrying *APOE4* variant is associated with increased risk for AD and dementias may be the most explicit biological situation where apolipoprotein functions are a major driver of brain dementias. Under some conditions, αSyn may even have a cooperative role with apolipoproteins and lipid transport. In addition, basic research demonstrates that several proteins including αSyn (**Figure 1B**) can accommodate pathological lipid disturbances in astrocytic, neuronal and microglial compartments. In such situations, for a time affected cells will handle genetic and age-acquired lipid and metabolic disturbances, and clearly, such cells may even return to a healthier condition when the pathological stimuli are removed, or a treatment is devised that addresses the cause or initiating factor. Regardless, in chronic neurodegenerative diseases when neurons and glia are unable to maintain such cellular component functions, the pathogenic mechanisms will lead to cellular functional failures that are irreversible (see **Figure 2**). Inflammatory responses can be present at any of these pathogenic steps but are potentially most damaging in the later stages of degeneration, as such processes can remove cellular structures, including synapses, permanently at a structural level (**Figure 2**). The continuous expression of elevated amounts or aberrant lipids inside and outside neurons and glia can activate the immune system. In our opinion, novel research needs to focus on the interactions between neurons and glia as an interdependent system that attempts to regulate lipid and protein changes. When such lipid disturbances are significant they can lead to inflammatory reactions and eventually synaptic pathobiology (**Figure 1A**).

Lipid dyshomeostasis, transport and clearance are emerging as central causative factors in neurodegenerative diseases and should help in selecting molecular targets for medical treatments, as well as diagnostic insights to both corrective and anti-inflammatory action to prevent structural degeneration in the brain. This new perspective of pathogenesis relevant, upstream causative mechanisms in several neurodegenerative diseases in PD, LBD and potentially also age-related dementias, provides optimism in developing new therapies for these devastating diseases.

The implication for the new understanding presented here; that lipid and inflammatory mechanisms can precede proteinopathies (**Figure 2**), provides clinical opportunities for identification of relevant and specific lipid and inflammatory biomarkers. It is already possible to measure specific abnormalities from GCase in the blood and brain (1, 64) of

patients with PD. Such patient stratification in other lipid species and specific metabolic disturbances may help to better define effective treatments of neurodegenerative diseases in clinical trials. It is also important that systemic and peripheral biomarkers can be coincident with brain pathology observed in neurodegenerative diseases. There is evidence of such peripheral biomarkers in patients with LSDs, PD, and AD for pathways involved in lipids and inflammation (51, 64–68). In conclusion, determining altered cellular lipid accumulation, transfer and clearance mechanisms in PD and related disorders can be of significant value to helping patients and at-risk individuals.

AUTHOR CONTRIBUTIONS

OI drafted the article with PH. OB contributed to the discussion and analysis of the results mentioned in the article.

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Mitochondrial Cholesterol in Alzheimer's Disease and Niemann–Pick Type C Disease

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Mitochondrial dysfunction has been recognized as a key player in neurodegenerative diseases, including Alzheimer's disease (AD) and Niemann–Pick type C (NPC) disease. While the pathogenesis of both diseases is different, disruption of intracellular cholesterol trafficking has emerged as a common feature of both AD and NPC disease. Nutritional or genetic mitochondrial cholesterol accumulation sensitizes neurons to Aβ-mediated neurotoxicity *in vitro* and promotes cognitive decline in AD models. In addition to the primary accumulation of cholesterol and sphingolipids in lysosomes, NPC disease is also characterized by an increase in mitochondrial cholesterol levels in affected organs, predominantly in brain and liver. In both diseases, mitochondrial cholesterol accumulation disrupts membrane physical properties and restricts the transport of glutathione into mitochondrial matrix, thus impairing the mitochondrial antioxidant defense strategy. The underlying mechanisms leading to mitochondrial cholesterol accumulation in AD and NPC diseases are not fully understood. In the present manuscript, we discuss evidence for the potential role of StARD1 in promoting the trafficking of cholesterol to mitochondria in AD and NPC, whose upregulation involves an endoplasmic reticulum stress and a decrease in acid ceramidase expression, respectively. These findings imply that targeting StARD1 or boosting the mitochondrial antioxidant defense may emerge as a promising approach for both AD and NPC disease.

Keywords: cholesterol, mitochondria, lysosomal disorders, sphingolipids, acid ceramidase

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INTRODUCTION

Neurodegenerative diseases encompass a wide range of neurological disorders caused by different causes, most notably genetic mutations in specific genes. Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases in which the progressive loss of neurons is associated with the upregulation of peptides and activation of proteins, such as amyloid beta (Aβ) or tau phosphorylation, that trigger specific signaling pathways that ultimately contribute to the progression of the disease. In addition, accumulation of other cellular components, such as specific types of lipids, can cause neuronal death and mitochondrial dysfunction in the brain and in peripheral organs. The role of mitochondrial dysfunction in neurodegenerative diseases remains to be fully elucidated. Recent studies have provided evidence that alterations in lipid metabolism can have a deleterious impact on mitochondrial function, which can contribute to the progression

not only of AD but also of the lysosomal storage disorder Niemann–Pick type C (NPC) disease, a neurovisceral disorder primarily characterized by the accumulation of lipids in intracellular organelles, most predominantly in lysosomes. Here, we briefly summarize evidence indicating that increased cholesterol trafficking to mitochondria has emerged as a putative key player in AD and NPC disease through the disruption of mitochondrial routine performance, leading to oxidative stress and cell death.

MITOCHONDRIAL CHOLESTEROL TRAFFICKING IN NEURODEGENERATIVE DISEASES

Cholesterol is an essential component of membrane bilayers, which determines their physico-chemical and functional properties. Cholesterol is particularly enriched in the brain, where it regulates key biological functions, such as signal transduction pathways, myelin formation, and synaptogenesis (1). In the central nervous system (CNS), cholesterol is synthesized *de novo* from acetyl-CoA in the mevalonate pathway in the endoplasmic reticulum (ER) (2). Since cholesterol does not cross the blood–brain barrier (BBB), to ensure steady-state turnover, cholesterol synthesis is matched by its metabolism to 24S-hydroxycholesterol (24-OHC), which crosses the BBB and is delivered to peripheral organs and hence constitutes an intrinsic mechanism to prevent cholesterol accumulation in the brain. During the perinatal period, cholesterol accumulation in the brain is mainly determined by oligodendrocytes due to their key role in myelination, while neurons synthesize their own cholesterol *de novo* needed for neuronal plasticity and function (3). In the adult life, however, the rate of *de novo* cholesterol synthesis declines, forcing neurons to acquire cholesterol from a cross-talk between neurons and astrocytes (1, 4). Although the regulation of cholesterol homeostasis in brain diseases has been reviewed elsewhere (2), here we discuss the specific role of mitochondrial cholesterol in neurodegenerative diseases.

While much of what it is known about the trafficking of cholesterol into mitochondria has been elucidated in the context of steroidogenesis, the relevance of mitochondrial cholesterol in neurodegeneration has been less recognized. Emerging evidence indicates that mitochondrial cholesterol loading can influence mitochondrial function independently of its conversion to pregnenolone or oxysterols, arising as a key factor in the pathology of several neurological diseases associated with mitochondrial dysfunction, such as AD and NPC disease (5). Although the physiological levels of cholesterol in mitochondrial membranes are low compared to other membrane bilayers (e.g., plasma membrane), the limited content of mitochondrial cholesterol is essential for the maintenance of mitochondrial membrane physical properties and synthesis of neurosteroids. The key regulatory enzymes responsible for steroid synthesis in the CNS include the cytochrome P450 side-chain cleavage (P450scc), and the steroidogenic acute regulatory protein StARD1, the founder member of a family of lipid transporting proteins that contain StAR-related lipid transfer (StART) domains. The rate-limiting step in the synthesis of neurosteroids is the availability of mitochondrial cholesterol in the mitochondrial inner membrane (MIM) for metabolism by P450scc. Cholesterol is imported to mitochondrial membranes by the action of lipid transfer multiprotein complex acting at membrane contact sites. StARD1 plays an essential role in the transfer of cholesterol to the MIM, as inferred from the outcome of mice with global StARD1 deletion, which undergo a lethal adrenal lipoid hyperplasia, indicating that other members of the StAR family cannot replace its function in intramitochondrial cholesterol trafficking (6, 7). StARD1 contains a mitochondrial localization sequence and a steroid-binding domain. The exact location of StARD1 in mitochondria has been much discussed, but among StARD1 forms, the 30 kDa phosphorylated form has been described to be localized on the MIM (8). MLN64 (also known as StARD3) provides cholesterol to the mitochondrial outer membrane (MOM) from endosomes (5, 9) and together with StARD1 work in tandem in the net import of cholesterol to the MIM for metabolism.

In the last decade, significant progress has been made on the impact of cholesterol accumulation in mitochondrial function and routine performance in AD and NPC disease using genetic mouse models, such as the APP/PSEN1 transgenic mice overexpressing SREPB-2 (APP/PSEN1/SREBP2) and the $Npc1^{-/-}$ knockout mice (10–14). While much of the deleterious effects of mitochondrial cholesterol accumulation in both diseases is accounted for by the depletion of mitochondrial GSH (mGSH), unfortunately the underlying mechanisms whereby cholesterol accumulates in mitochondria in AD and NPC are not fully understood. Our hypothesis posits that StARD1 is a critical player in mitochondrial cholesterol loading and hence emerges as a putative novel target for intervention in both diseases (**Figure 1**).

INTRACELLULAR CHOLESTEROL HOMEOSTASIS AND MITOCHONDRIAL FUNCTION IN AD

AD is one of the most common neurodegenerative disorders in older adults. The pathological hallmark of AD is the cognitive impairment and associated dementia due to neuronal death caused in part by the accumulation of amyloid plaques in the cortex and hippocampus (15–18). Currently, there is no cure for AD, which reflects our incomplete understanding of AD pathogenesis. AD is a multifactorial disease and several players contribute to its progression, including the disruption of cholesterol homeostasis. In this regard, epidemiological findings showed that hypercholesterolemia is a major risk factor for AD development (19). However, in spite of the association between hypercholesterolemia and AD, the role of cholesterol in AD is controversial and not fully understood. In this regard, a body of literature supports a link between increased cholesterol levels in the brain with the progression of AD. For instance, the specific presence of the enzymes involved in the generation of toxic Aβ peptides in lipid rafts, specific domains of membrane bilayers

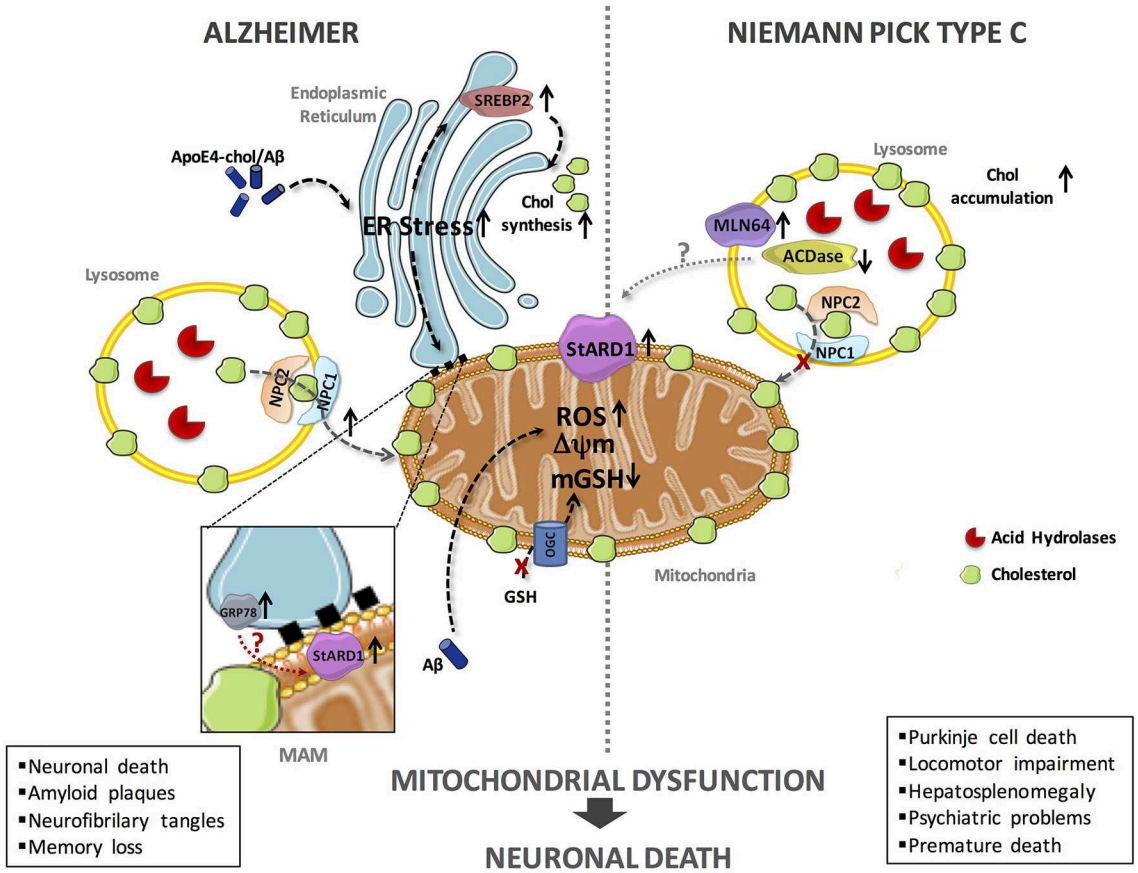

FIGURE 1 | Schematic representation of the putative mechanisms of StARD1 upregulation in AD and NPC. Mitochondrial cholesterol accumulation and subsequent mGSH depletion is a common feature of AD and NPC and its correlates with increased expression of StARD1. Since StARD1 is critical for mitochondrial cholesterol trafficking, it is conceivable that StARD1 is located in the MAM interface between ER and mitochondria. While in AD, ER stress by Aβ may contribute to StARD1 upregulation, this event does not seem to play a role in NPC. Given the repression of StARD1 by ACDase, we propose that ACDase downregulation may contribute to StARD1 induction in NPC disease, which requires further investigation. In both diseases, mitochondrial cholesterol accumulation disrupts membrane physical properties and impairs the transport of GSH into mitochondria, via the 2-oxoglutarate carrier (OGC), disrupting the mitochondrial antioxidant defense and subsequent oxidative stress. Chol, cholesterol; mGSH, mitochondrial glutathione; ROS, reactive oxygen species.

highly enriched in cholesterol, provides a strong association between high cholesterol levels with Aβ generation and AD development (20, 21). Moreover, in the CNS, cholesterol is transported between different cell types by a multicellular trafficking process largely regulated by ApoE (22–24). Consistent with its function in cholesterol trafficking within the brain, ApoE polymorphisms, particularly APOEε4 allele, have emerged as a risk for AD development and correlate with higher levels of Aβ in the serum (25, 26). Moreover, experimental models fed diets enriched in cholesterol have been shown to develop AD-like pathology [(27); reviewed in (28)]. Quite interestingly, besides the association between the increase in cholesterol levels with AD, there is also evidence indicating that low cholesterol levels in the brain can contribute to the AD progression (2). This inverse relationship is of particular significance during aging, as low levels of cholesterol in hippocampus are characteristic of the aged human brain (29). Furthermore, as cholesterol metabolism in the brain to 24-OHC by the action of CYP46A1 represents a unique mechanism to control brain cholesterol homeostasis, CYP46A1 polymorphisms correlate with lower brain cholesterol levels and increased risk of AD (30, 31). In addition, hippocampal cholesterol loss has been shown to contribute to the poor cognition in old rodents, and hence, cholesterol replenishment in aging animals improves hippocampal-dependent learning and memory (32).

In the early stage of AD development, mitochondria undergo significant functional deficits, which correlate with accumulation of neurotoxic Aβ (33–36). Interestingly, it has been shown that Aβ can target mitochondria to stimulate ROS generation, thus contributing to Aβ toxicity in neurons (37, 38). Immunoelectron microscopy analysis indicated the association of APP with mitochondrial protein translocation components, TOM20 and TIM23, which correlated with decreased import of respiratory chain subunits, lower cytochrome oxidase activity, and increased ROS generation (39). Moreover, functional complexes with γ-secretase activity have been found in mitochondria while

insulin-degrading enzyme (IDE), which is known to contribute to Aβ removal, can be targeted to mitochondria (40, 41). Thus, mitochondrial dysfunction is associated with increased Aβ generation and AD progression.

In line with the potential relevance linking intracellular cholesterol to mitochondrial dysfunction in AD, mitochondrial cholesterol enrichment has been shown to sensitize to Aβ-mediated neurotoxicity through depletion of mGSH levels (10). Moreover, APP/PS1/SREBP-2 mice, which exhibit an early mitochondrial cholesterol loading and mGSH depletion, exhibited an accelerated β-secretase activation, Aβ accumulation, and cognitive decline compared to APP/PS1 mice (11), further supporting the correlation between mitochondrial cholesterol accumulation and the subsequent mGSH depletion in AD. Consequently, *in vivo* treatment of APP/PS1/SREBP-2 mice with the cell-permeable GSH ethyl ester, which restored mGSH levels, attenuated synaptic degeneration and improved cognition, suggesting that therapeutic strategy to prevent mitochondrial cholesterol accumulation or the restoration of mGSH levels may represent a relevant approach in the treatment of AD. Further evidence indicated that ER stress acts as a link between Aβ generation and cholesterol upregulation and subsequent mitochondrial cholesterol trafficking due to increased expression of StARD1 (12). Furthermore, administration of chemical chaperones that prevent ER stress ameliorates StARD1 upregulation and cognitive decline in APP/PS1/SREBP-2 mice.

In line with this sequence of events, recent findings provided evidence that StARD1 is an ER stress target gene, as tunicamycin-mediated ER stress induces StARD1 upregulation in primary hepatocytes that was prevented by tauroursodeoxycholic acid (42). Additional evidence linking ER stress and StARD1 upregulation derived from studies on acetaminophen (APAP) hepatotoxicity in which APAP-induced ER stress causally led to StARD1 induction that primed to APAP-induced liver injury (43). Thus, these studies provide strong evidence to support the hypothesis that Aβ-induced ER stress may be a key mechanism for AD pathology by promoting increased brain cholesterol content as a result of enhanced SREBP-2 processing, while stimulating cholesterol trafficking to mitochondria via StARD1 upregulation. Indeed, it has been reported that in the early stage of AD development, Aβ-induced ER stress is indirectly involved as an effector of Aβ-mediated neurotoxicity (44). Furthermore, AD human brains exhibit evidence for increased ER stress markers accompanied by APP accumulation and activation of β-secretase (45). In addition, Aβ peptides are reported to cause alterations in mitochondria-associated membranes (MAMs) (46, 47). In this regard, MAMs act like ER/mitochondria contact sites, transferring stress signals from the ER to mitochondria during the early adaptive phases of ER stress and in the regulation of steroidognesis (**Figure 1**) (48). In addition, different studies have shown a relationship between ER chaperones and StARD1 in MAMs that correlates with an increase in expression of GRP78 in AD patients (45, 49). In line with these findings, elevated STARD1 levels have been reported in the cytoplasm of hippocampal pyramidal neurons from brain samples of AD patients (50), suggesting a mechanistic link between StARD1 and mitochondrial cholesterol loading in human AD. Further work will be needed to critically establish a cause-and-effect relationship between StARD1 upregulation and the mitochondrial cholesterol accumulation and its contribution to AD, which will require the generation of cell-type-specific StARD1 deletion models in brain to examine the sensitivity to AD pathology (Garcia-Ruiz et al., manuscript in preparation).

MITOCHONDRIAL CHOLESTEROL ACCUMULATION IN NPC DISEASE

Lysosomal lipid accumulation is the hallmark of NPC disease, which is characterized by neuronal and visceral symptoms, spleen dysfunction, hepatosplenomegaly, deficits in motor coordination, and premature death (51, 52). NPC disease is caused by mutations in genes encoding NPC1 and NPC2, two lysosomal-resident proteins responsible for the egress of cholesterol from lysosomes to cytosol. Most NPC cases are due to loss of function of NPC1, and consequently, mice with NPC1 deletion ($Npc1^{-/-}$ knockout mice) reproduces many of the deficits seen in NPC patients, including the neurological symptoms, ataxia by 6–7 weeks of age, and reduced maximal life span to about 10–12 weeks (52, 53). Consistent with the crucial role of NPC1/2 in intracellular cholesterol trafficking, the primary biochemical feature of NPC disease is the accumulation of cholesterol in lysosomes. However, due to the mutual regulation of cholesterol and sphingolipids to maintain a constant ratio in membrane bilayers, which is crucial for the maintenance of their physical properties, the increase of lysosomal cholesterol loading in NPC disease is accompanied by accumulation of specific sphingolipids species (54–56). In addition to the accumulation of cholesterol/sphingolipids in lysosomes, cholesterol also has been reported to accumulate in mitochondria in affected organs from $Npc1^{-/-}$ mice, particularly liver and brain and in fibroblasts from NPC patients (57, 58). As recent findings have demonstrated that diet-induced mitochondrial cholesterol enrichment impairs mitochondrial routine performance and disrupts the assembly of respiratory supercomplexes (59), the increase in mitochondrial cholesterol seen in NPC models can contribute to the reported mitochondrial dysfunction and subsequent oxidative stress associated with NPC disease, which is largely due to mGSH depletion and subsequent disruption of mitochondrial antioxidant defense (10, 57, 58, 60–63). In line with these findings, defective ATPase activity has been reported in brain mitochondria from $Npc1^{-/-}$ mice, and this outcome has been causally linked to cholesterol accumulation in mitochondrial membranes as its extraction with methyl-β-cyclodextrine (βCD) restored ATP activity (57). Moreover, mGSH replenishment with GSH ethyl ester (GSHEE) in cerebellum of $Npc1^{-/-}$ mice was able to reverse mitochondrial dysfunction and improve oxidative phosphorylation. GSHEE treatment enhanced neurological performance and motor activity and, more importantly, resulted in increased median survival and maximum life span of NPC1 null mice, similar to treatment with βCD (13). However, combination of GSHEE with βCD had no additive effects, suggesting that both agents act in a common pathway affecting

mitochondrial function. Furthermore, liver samples from 8 week-old NPC null mice exhibited increased protein carbonylation, decreased ATP levels, and activated caspase 3 activity, whereas isolated mitochondria revealed increased MitoSox fluorescence and reduced mitochondrial membrane potential, effects that were reversed by GSHEE therapy. Liver injury, inflammatory foci, and hepatosplenomegaly increased in liver of $Npc1^{-/-}$ mice, and these signs of liver disease were attenuated by GSH-EE treatment. In contrast to the therapeutic effects of GSHEE, N-acetylcysteine (NAC) did not restore mGSH and failed to improve NPC pathology, although NAC was effective in increasing cytosol GSH pool. These findings illustrate the relevance of mGSH depletion in NPC disease and the need to implement specific strategies to bypass the block of GSH transport in mitochondria imposed by the accumulation of cholesterol and subsequent decrease in membrane fluidity.

As mitophagy stands as a specific mechanism to maintain mitochondrial quality control, the increase in lysosomal cholesterol, which has been shown to impair mitophagy, could contribute to the perpetuation of mitochondrial dysfunction in NPC disease due to impaired mitochondrial turnover, as illustrated in drug-induced liver injury (64). Besides the impact in quality control, recent findings have provided evidence for impaired mitochondrial biogenesis in NPC disease by a mechanism involving transcriptional repression of mitochondrial biogenesis (65). This outcome is mediated specifically by the transcription factors KLF2 and ETV1. Both are induced in NPC cells and their silencing restored mitochondrial biogenesis. Increased expression of ETV1 is regulated by KLF2, while the increase in KLF2 levels is caused by impaired signaling downstream of sphingosine-1-phosphate receptor 1, which normally represses KLF2 (65). Quite intriguingly, as mitochondrial respiratory chain deficiency regulates lysosomal homeostasis and hydrolysis (66), it is tempting to speculate that mitochondrial cholesterol-mediated dysfunction and lysosomal cholesterol accumulation engage in a mutual regulatory cycle that is of relevance to NPC disease.

Although mitochondrial cholesterol is recognized to contribute to mitochondrial dysfunction and has emerged as a putative player in NPC pathogenesis, the molecular mechanism involved in the stimulated trafficking of cholesterol to mitochondria remains poorly understood. In this regard, increased expression of StARD3 (MLN64) has been reported in NPC cells, which correlated with enhanced cholesterol accumulation in mitochondria and mitochondrial depolarization (63). In parallel with these observations, StARD1 upregulation has been observed in affected organs of $Npc1^{-/-}$ mice and in fibroblasts from NPC patients (67). Since StARD3 is thought to transfer cholesterol from endosomes to the MOM, the increased expression of StARD1/StARD3 suggests that both proteins work together in the intramitochondrial trafficking of cholesterol, with StARD1 playing a key role in the transfer to MIM. Consistent with this possibility, hepatocyte-specific StARD1 deletion has been shown to prevent cholesterol accumulation in MIM and protect against APAP-mediated liver failure despite unchanged MLN64 expression (43). Thus, in view of the crucial role of StARD1 in mediating mitochondrial cholesterol loading, understanding the molecular mechanisms involved in StARD1 induction may be of relevance for NPC pathogenesis and could emerge as a druggable target for the treatment of NPC disease.

DISCUSSION

Although current evidence indicates that mitochondrial cholesterol emerges as a common event in both AD and NPC, the molecular mechanisms contributing to its accumulation in mitochondria are not well-understood. In both models, we observed increased expression of StARD1 and enhanced MLN64 expression (70–90%) (12, 63, 67). In spite of the shared upregulation of StARD1 in AD and NPC disease, the putative mechanisms underlying its induction appear to be different in both diseases (**Figure 1**). In this regard, although ER stress is known to induce the upregulation of StARD1 and AD is characterized by ER stress, there is no evidence for induction of ER stress markers in NPC (68), dissociating the relationship between ER stress and StARD1 upregulation in NPC and AD. Alternatively to the onset of ER stress, we hypothesized that acid ceramidase (ACDase) may be involved in the upregulation of StARD1, as its expression is decreased in liver and brain of $Npc1^{-/-}$ mice (67). As ACDase has been shown to repress StARD1 expression through binding to the nuclear receptor steroidogenic factor-1 (69), it remains to be established whether decreased ACDase in NPC disease contributes to the upregulation of StARD1 and subsequent mitochondrial cholesterol accumulation. Thus, based on these findings, StARD1 upregulation in AD and NPC disease could account for the increased mitochondrial cholesterol loading. Hence, we propose that ER stress determines StARD1 upregulation in AD, while in NPC, ACDase downregulation may stand as the trigger to induce StARD1 induction (67). In conclusion, we propose that StARD1 may be crucial for the mitochondrial cholesterol accumulation, characteristic of AD and NPC. Further research is required to determine that targeting this process may be of relevance for both AD and NPC disease.

AUTHOR CONTRIBUTIONS

ST, CG-R, and JF-C discussed findings, analyzed literature, wrote the manuscript, and designed the schematic figures.

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REVIEW
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Lipid-Binding Proteins in Brain Health and Disease

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A proper lipid management is paramount for a healthy brain. Lipid homeostasis alterations are known to be causative or risk factors for many neurodegenerative diseases, or key elements in the recovery from nervous system injuries of different etiology. In addition to lipid biogenesis and catabolism, non-enzymatic lipid-binding proteins play an important role in brain function and maintenance through aging. Among these types of lipoproteins, apolipoprotein E has received much attention due to the relationship of particular alleles of its gene with the risk and progression of Alzheimer's disease. However, other lipid-binding proteins whose role in lipid homeostasis and control are less known need to be brought to the attention of both researchers and clinicians. The aim of this review is to cover the knowledge of lipid-managing proteins in the brain, with particular attention to new candidates to be relevant for brain function and health.

Keywords: blood–brain barrier, lipid transport, oxidative stress, neuroinflammation, amyloidogenesis, neurodegeneration, demyelination, lysosomal storage disorder

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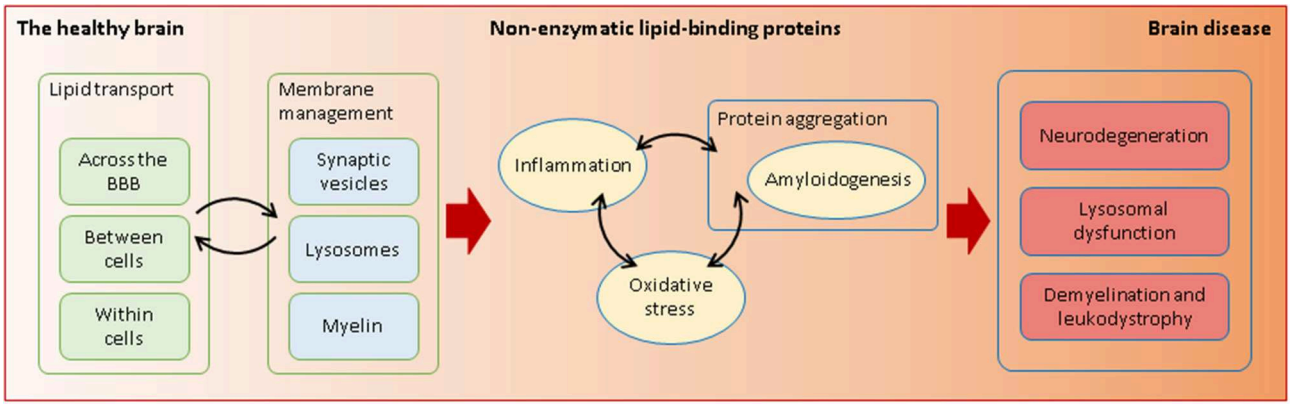
INTRODUCTION

Brain is a lipid-enriched organ. Lipids are on high demand in the brain, since they are required for the long expansions of neurons and, massively, for myelin construction. These structures, characteristic of the nervous system, were initially thought to be static assemblies once development was concluded. Now, neuronal processes (dendrites and axons), their synaptic terminals, and their myelin sheaths are known to be constantly remodeling. Their reorganization is the basis of experience-dependent plasticity and, importantly, of the unexpected endogenous abilities of our nervous system to recover from damages, disease, and aging-related deterioration.

It is estimated that around 5% of the genome codes for proteins dealing with lipids, which are estimated to include 10,000 different species (1–3). Anabolic and catabolic routes generate a plethora of individual lipids with energetic, signaling, or structural functions. To accomplish the lipid-related demands of our nervous system, with its particularly relevant membranous structures, we need not only the set of enzymes that generate, transform, or degrade them. Lipids have properties requiring additional managing within a hydrophilic environment. We devote this review to the cohort of proteins in charge of binding, transporting, or presenting lipids to enzymes, defining this functional group under the umbrella of "non-enzymatic lipid managers."

Taking cholesterol as an example, we can appreciate how lipid metabolism in the central nervous system (CNS) is unique. The brain is the most cholesterol-rich organ in the body (23 mg/g of tissue) (4). Given that there is virtually no exchange of cholesterol with the peripheral circulation, due to the impermeable nature of the blood–brain barrier (BBB), cerebral cholesterol level is dependent on *de novo* synthesis by glial cells (5). However, neurodegenerative diseases as devastating as Niemann-Pick type C (NPC), where the gene affected codes for a lipid transporter in charge of intracellular



GRAPHICAL ABSTRACT | Non-enzymatic lipid-interacting proteins are required in health and disease. Through their transporting and membrane-managing functions, they intervene in processes that are essential for the fragile equilibrium between a healthy and a diseased brain.

cholesterol trafficking, evidence the crucial role of this type of lipid manager in brain function.

Therefore, non-enzymatic lipid-interacting proteins are required to carry out a varied set of functions as lipids need to be transported not only between different tissue compartments (outside vs. inside the brain) or between cells (glia-to-glia, glia-to-neurons), but also within cells, across the cytoplasm toward organelles or the nucleus. Other examples include apolipoprotein E (ApoE) carrying lipids between cells, or fatty acid-binding proteins (FABPs), transporting lipids within the cytoplasm. Some carry lipids to the nucleus (retinoic acid receptors; RXRs). Other proteins bind lipids in membranes and make them accessible to enzymes (saposin B) while some are part of complex lipoprotein particles that transport lipids along separate body compartments (high-density lipoprotein; HDL, low-density lipoprotein; LDL). Finally, other transfer lipids between different classes of lipoprotein particles (phospholipid transfer protein; PLTP).

A literature search using a systematic approach helped us to identify (i) key biological processes essential for nervous system function in which lipid management is required, (ii) a set of non-enzymatic lipid-interacting proteins involved in those processes, and (iii) their relationship to neurodegenerative diseases of diverse etiology. The initial search was then combined with process, protein, or disease-specific searches. **Table 1** summarizes the findings. We have grouped the biological processes in five major categories (sections Lipid Transfer Across the BBB and Between Cells in the Nervous System to Lipoproteins Involved in Myelin Management), and reviewed the knowledge accrued about the lipid-binding proteins involved. Finally, neurodegenerative diseases recovered in our search (through the published work devoted to non-enzymatic lipid-binding proteins) are discussed (sections Alzheimer's Disease and Cerebrovascular Dementias: Dealing With Amyloid Deposition in a Compromised BBB State to Lysosomal Storage Diseases and Their Inseparable Companions: Leukodystrophies). Our aim is to promote new views in the understanding of neurodegeneration that might seed ideas for potential clinical interventions.

LIPIDS AND THEIR PROTEIN MANAGERS ARE INVOLVED IN KEY PROCESSES ESSENTIAL FOR A HEALTHY BRAIN

Lipid Transfer Across the BBB and Between Cells in the Nervous System

Our brain constitutes a separate compartment for lipid management due to the highly controlled BBB. Brain capillary endothelial cells constitute the barrier itself, with tight junctions blocking the passage of substances across the capillary walls, and a set of specific receptors and transporters controlling the traffic across them. Astrocytes and pericytes help in the development and maintenance of the barrier through constant exchange of intercellular signaling that conditions endothelial gene expression and ultimately results in BBB permeability properties. They also modulate the availability of substances crossing the BBB by their effect on activity-dependent blood flow control (6, 7).

In spite of the BBB, metabolic diseases altering systemic lipid profiles clearly affect brain function and homeostasis. A well-documented example is the inverse relationship between levels of cholesterol in plasma high-density lipoprotein (HDL-C) and risk of Alzheimer's disease (AD) and other dementias (8). Lipoprotein particles are emulsions of metabolites, lipids, proteins, and microRNAs (9). They are classified in two major types (HDL and LDL) that differ in their density and lipid content, the main protein serving as scaffold (apolipoprotein A-I, ApoA-I in HDL; apolipoprotein B, ApoB in LDL) and their lipid transport effect (removing excess lipids from cells or supplying lipids to cells, respectively, **Figure 1A**). HDL-like particles are formed in the brain compartment as a separate pool independent of plasma HDL, and they do not cross the BBB under normal conditions (**Figure 1B**). By contrast, LDL biogenesis occurs outside the brain, and LDL receptors (LDLR) mediate its passage from blood to brain by transcytosis (10) (**Figure 1C**). Various members of the LDLR family are expressed by glial cells and neurons as well. However, we must take into account that despite their name, these receptor proteins have a wide array of ligands and functions

TABLE 1 | Non-enzymatic lipid-binding proteins and their involvement in biological processes underlying neurodegenerative diseases.

Lipid-binding protein	Biological process	Lipid binding	Neurodegenerative disease
LIPOPROTEIN PARTICLE SCAFFOLDING PROTEINS AND TRANSFER PROTEINS			
ApoA-I	Blood–brain barrier function, oxidative stress/inflammation, amyloidogenesis/Aβ clearance	Cholesterol, phospholipids, triglycerides, membranes	Alzheimer's, multiple sclerosis, cerebral amyloid angiopathy, Parkinson's
ApoB100	Blood–brain barrier function	Cholesterol, phospholipids, triglycerides, docosahexaenoic acid	Cerebral amyloid angiopathy, multiple sclerosis.
PLTP	Blood–brain barrier function	Cholesterol, phospholipids, triglycerides	
MEMBRANE TRANSPORTERS AND RECEPTORS			
ABCA1	Blood–brain barrier function, HDL biogenesis	Cholesterol, phospholipids, triglycerides	Alzheimer's
ABCG1	Blood–brain barrier function, HDL biogenesis	Cholesterol, phospholipids, triglycerides	
SR-B1	Blood–brain barrier function, HDL biogenesis	Cholesterol, phospholipids, triglycerides	
LDLR	Blood–brain barrier function, amyloidogenesis/Aβ clearance	Lipoprotein particles	Alzheimer's
LRP1	Blood–brain barrier function, Amyloidogenesis/Aβ clearance	Lipoprotein particles	Alzheimer's
Megalin/LRP2	Blood–brain barrier function, amyloidogenesis/Aβ clearance	ApoJ-containing lipoprotein particles	Alzheimer's
S1PR	Blood–brain barrier function, amyloidogenesis	ApoM-containing lipoprotein particles	Cerebral amyloid angiopathy
LSR	Blood–brain barrier function	24(S)-hydroxycholesterol	Alzheimer's
MCT1	Blood–brain barrier function	Ketone bodies	Alzheimer's
LIPOPROTEIN PARTICLE-ASSOCIATED PROTEINS AND OTHER EXTRACELLULAR LIPID TRANSPORTERS			
ApoJ/Clusterin	Blood–brain barrier function, oxidative stress/inflammation, amyloidogenesis	Cholesterol	Alzheimer's
ApoE	Blood–brain barrier function, oxidative stress/inflammation, amyloidogenesis	Cholesterol	Alzheimer's, Parkinson's
ApoM	Blood–brain barrier function	Sphingosine-1-P	Vascular dementia
ApoD	Oxidative stress/inflammation, lysosome, myelin	Fatty acids (arachidonic acid), sphingomyelin, anandamide, lysophosphatidylcholine, membranes	Alzheimer's, Parkinson's, multiple sclrerosis, spinocerebellar ataxia 1, lysosomal storage diseases (Niemann–Pick type C)
Lcn2	Oxidative stress/inflammation, lysosome, myelin	Bacterial siderophores	Fabri's disease, neuronal cerois lipofuscinosis
INTRACELLULAR LIPID-BINDING PROTEINS			
FABP7/B-FABP	Cytoplasmic lipid transport	Fatty acids, retinoids, eicosanoids	Alzheimer's, Parkinson's, Down syndrome
FABP3/H-FABP	Cytoplasmic lipid transport	Fatty acids, retinoids, eicosanoids	Alzheimer's, Parkinson's, Lewy body, vascular dementia, Creutzfeldt–Jakob
FABP5	Cytoplasmic lipid transport	Fatty acids (docosahexaenoic acid)	Alzheimer's
Alfa-synuclein	Synaptic vesicle	Membranes (acidic phospholipids)	Alzheimer's, Parkinson's
NPC1 and NPC2	Lysosome, myelin	Cholesterol	Lysosomal storage diseases (Niemann–Pick type C)
Saposin B	Lysosome, myelin	Membranes (glycosphingolipids)	Lysosomal storage diseases (metachromatic leukodystrophy)
Hsp70	Lysosome	Membranes (phospholipids)	Lysosomal storage diseases (Niemann–Pick type A)
LIPID BINDING TRANSCRIPTION REGULATORS			
RXR	Transcription regulation	Retinoic acid	
LXR	Transcription regulation	24(S)-hydroxycholesterol	

Lipid-binding proteins are grouped by type of lipid management and are assigned one or various of the five general biological processes reviewed: (1) blood–brain barrier function (including transport across and functional maintenance), (2) control of oxidative stress and inflammation, (3) Aβ dynamics (amyloidogenesis or Aβ clearance), (4) organelle membrane-related functions (including lysosomal and synaptic vesicle functions), and (5) myelin management function. Major lipid ligands for each protein are listed. Neurodegenerative diseases that are directly related or modified by the function of the lipid-manager proteins are listed.

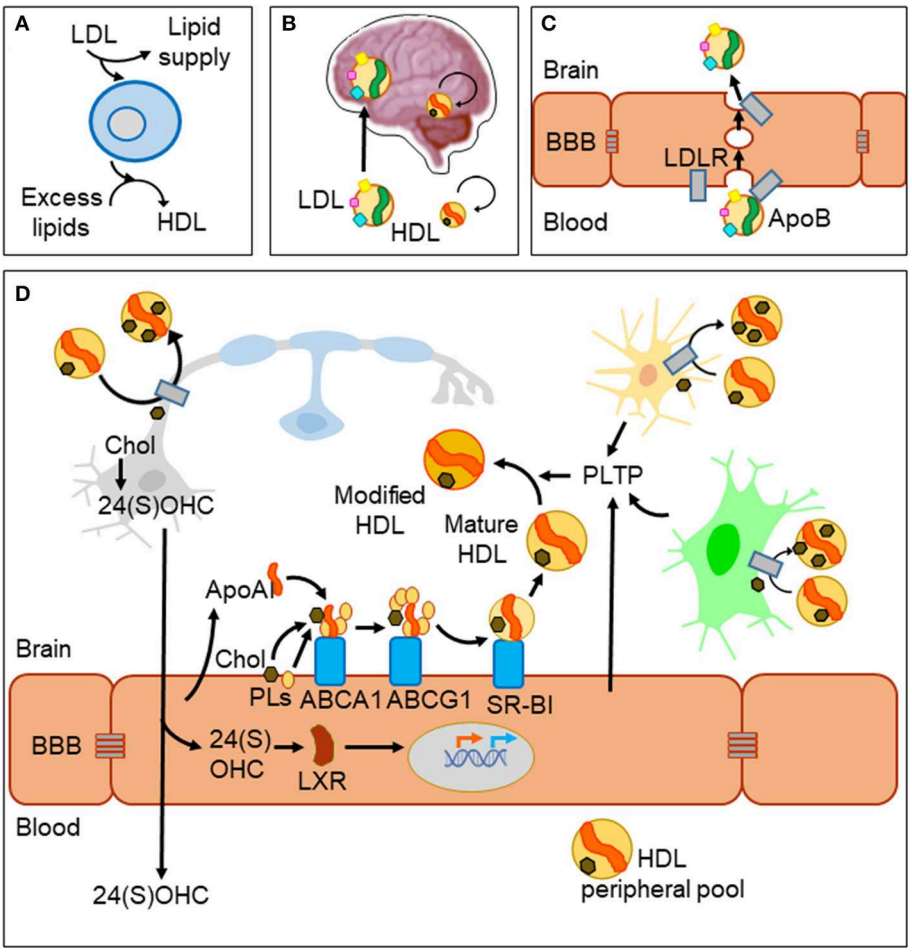


FIGURE 1 | Lipid management in the brain and across the blood–brain barrier. **(A)** Two major classes of lipoprotein particles are in charge of providing lipids to cells (LDL) and removing excess lipids (HDL) to fulfill cellular demands. **(B)** The blood–brain barrier (BBB) imposes limitations to traffic from the circulation to the brain parenchyma. LDL particles originated outside the brain cross the BBB, while biogenesis of HDLs occurs in two separate pools. HDLs cross the barrier only under conditions of damaged BBB. **(C)** LDL uses a receptor-mediated transcytosis mechanism to cross BBB endothelial cells. **(D)** HDL biogenesis and cholesterol recycling within the brain. HDL scaffolding protein ApoA-I and its receptors are expressed by endothelial cells and biogenesis takes place at the brain side of the BBB. Nascent HDLs are then modified by lipid transfer proteins (PLTP) secreted from endothelial and glial cells. HDL particles have receptors in all cell types of the nervous system where they uptake excess lipids (cholesterol and others). Under normal conditions, HDLs exit the brain through bulk flow from the extracellular space to the circulation and lymphatic systems. Part of cholesterol recycling takes place thanks to the production of 24(S)-hydroxycholesterol by the neuronal-specific CYP46A1 enzyme. 24(S)OHC freely crosses the BBB and is also able to bind LXR proteins in the cytoplasm of endothelial cells, which translocate to the nucleus and promote the transcription of ApoA-I and the receptors involved in HDL biogenesis.

and are not limited to the classical ApoB-containing LDL particles (11). As an example, the LDL receptor-related protein 1 (LRP1), a membrane-associated protein highly expressed by BBB endothelial cells, has been described as the major pump responsible for the efflux of amyloid-β (Aβ) out of the brain (12).

Acting in conjunction with HDL particles, a panel of lipid-interacting proteins are expressed by brain endothelial cells, contributing to cholesterol recycling within the brain and trafficking excess cholesterol out of the brain (13, 14) (**Figure 1D**). HDL biogenesis takes place at the basolateral side ("brain-side") of BBB endothelial cells. They express ApoA-I (15, 16), contributing to the CNS pool of this apolipoprotein, one of the most abundant in the cerebrospinal fluid (CSF) (17). BBB endothelial cells also express the set of membrane proteins required to initiate ApoA-I uptake of lipids (ABCA1; ATP-binding cassette transporter A1), and to complete its maturation (ABCG1 and SR-BI; scavenger receptor B1), all of them lipid-interacting proteins transferring cholesterol and phospholipids from the cell to the nascent HDL particle (9). Other lipid-binding proteins are involved in remodeling HDL particles, like PLTP (secreted from endothelial and glial cells), giving rise to pre-β-HDL and HDL_2, HDL forms particularly suitable as acceptors of excess cellular cholesterol (13). As a parallel mechanism, a brain-specific enzyme (CYP46A1) converts excess cholesterol to 24(S)OHC [24(S)-hydroxycholesterol] in neurons with two effects: (1) It can readily cross the BBB and activate nuclear liver-X-receptor

(LXR) transcription factors within endothelial cells. (2) LXRs turn on the expression of proteins involved in HDL biogenesis mentioned above (14) thus resulting in cholesterol turnover. Yet another lipoprotein receptor plays a role in cholesterol exchange: the lipolysis-stimulated lipoprotein receptor (LSR). A reduced LSR expression in aged mice results in higher cortex levels of 24(S)OHC (18). This constitutes an example of an indirect mechanism by which alterations in an LDL receptor impairs efflux of brain cholesterol, affecting the cholesterol turnover rate.

Some lipid-binding proteins associated with HDL-like lipoprotein particles can be conceptualized as chaperones of particular lipids that define particle sub-types. ApoJ/Clusterin, ApoE, or ApoM are good examples of this class of proteins. In spite of their name, they belong to different protein families, and bind lipids in a different way. They contribute to modulate not only the lipid cargo of HDLs but also their docking at specific cell surface receptors. ApoJ binds cholesterol and uses megalin/LRP2 as receptor (17, 19, 20). ApoE binds cholesterol and uses preferentially LRP1 receptor (21). Finally, ApoM binds specifically to sphingosine-1-P (S1P) and uses the receptor S1PR to dock ApoM-HDL particles to the receiving cell surface (22, 23).

Protein carriers transporting single lipid molecules also mediate lipid transport across BBB endothelial cells. An example of this is the monocarboxylic acid transporter (MCT1), expressed by endothelial cells and also by glial cells within the brain. MCT1 transports ketone bodies, originated by liver metabolism, across the BBB (24). This process takes place in conditions of diminished glucose availability, when the brain undergoes a metabolic switch toward the utilization of fatty acids as energetic fuel (25).

Extracellular lipocalins and cytoplasmic fatty acid-binding proteins (FABPs) are single-domain small proteins with a β-barrel structure (of 8 and 10 anti-parallel β-strands, respectively) that holds a ligand-binding pocket. Brain expression is found for various members of the lipocalin family, like apolipoprotein D (ApoD), mainly in myelinating glia and reactive astrocytes, lipocalin-type prostaglandin D synthase (LPGDS), in oligodendrocytes, or lipocalin 2 (Lcn2), in reactive astrocytes (26). On the other hand, cytoplasmic FABPs like FABP3/H-FABP or FABP7/B-FABP (the later uniquely expressed in astrocytes) or FABP5 (expressed by BBB endothelial cells) are also brain-born. They can be described as "chaperones" that facilitate the transport of small hydrophobic ligands [retinoic acid (RA), docosahexaenoic acid (DHA), arachidonic acid (AA), or endocannabinoids like arachidonoylethanolamide (AEA) and 2-arachidonoyl-glycerol (2-AG)] (27). For example, DHA, an essential omega-3 long-chain polyunsaturated fatty acid, is known to have anti-inflammatory properties on glial cells and influences memory and cognitive functions (28). After its transport in blood via the lipocalin retinol-binding protein 4 (RBP4), LDL lipoparticles, or serum albumin (29), it crosses the cell membrane and travels across the brain endothelial cell cytoplasm via FABP5 (30). Lipocalins and FABPs manage lipids not only across the BBB but also between cells in the nervous system (glia-to-glia or glia-to-neuron). This system can be visualized as an extracellular–intracellular relay race, serving intercellular exchange of lipid-mediated signaling, whose disruption contributes to many neurodegenerative conditions.

Lipoproteins Keeping Oxidative Stress and Inflammation Under Control

As depicted above, carrying lipids from one compartment to another or from one cell to another uses diverse systems, with lipids managed either in lipoprotein particles or by single domain small protein carriers. To this complexity, we have to add that lipoprotein particles, lipocalins, and cellular FABPs are not mere lipid transporters, but execute other functions that are especially relevant in the context of nervous system homeostasis: they contribute to control oxidative and inflammatory states.

Oxidative stress (OS) is an imbalance between the production of reactive oxygen species (ROS) due to aerobic metabolism in mitochondria and the antioxidant defenses that counteract them. When ROS are not properly neutralized by antioxidants, they can oxidize DNA, lipids, and proteins, altering their normal function. OS is also implicated in many neurodegenerative diseases, including Parkinson's disease (PD) (31) and AD (32), and is a landmark of physiological aging (33). ApoD is one of the most consistently upregulated proteins in the mammalian aging brain (34), a process highly related to OS production and deficient antioxidant capabilities. While in normal conditions ApoD is expressed at low levels by astrocytes and myelinating glia, its expression is quickly increased upon a neural insult due to trauma, exposure to exogenous toxics, or a wide array of neurodegenerative processes (35). Aging and disease-triggered overexpression of ApoD constitutes an endogenous mechanism of protection, as demonstrated by the mirror effects of the OS-generator Paraquat in loss-of-function and gain-of-function ApoD mutants in *Drosophila* and mouse (36–39). ApoD absence results in a pro-oxidative state in the brain, specifically altering lipid peroxidation (37) and resulting in an accelerated aging of brain functions (40). A mechanism of inhibition of lipid peroxidation has been demonstrated for human ApoD, which is able to reduce lipid hydroperoxides (LOOHs) to inert lipid hydroxides (LOHs) thanks to a particular methionine residue (41). It is interesting to note that one of the mechanisms behind the antioxidant properties of ApoA-I in HDL particles is also based on a methionine-dependent reducing activity on LDL-associated lipids (9). ApoA-I also helps to keep LDL particles resistant to oxidation by removing lipids prone to oxidation. These ApoA-I antioxidative functions counteract the deleterious effect of oxLDL particles on the BBB and contribute to keep HDL lipids in their reduced form. Finally, ApoJ/Clusterin is also thought to be a sensitive biosensor of OS. Its promoter has some OS-responsive binding sites (42), and ApoJ has been postulated as a protective molecule against OS based on gene silencing experiments (43). In addition, ApoJ also contributes to manage inflammation through binding to misfolded proteins and peptides, an interesting aspect shared by various lipid-binding proteins (see below).

In addition to OS, inflammation is a common pathophysiological factor present in the aging brain and neurodegenerative disorders. During aging, the organism suffers an imbalance between inflammatory and anti-inflammatory pathways, rendering a state of low-grade chronic inflammatory status known as "inflammaging." This status is characterized by a higher propensity to inflammation and a lower efficiency of inflammatory responses (44). Lipoproteins are in fact able to modulate polarization of macrophages and microglia in the anti/pro-inflammatory axis. For example, ApoE-LRP1 interaction mediates downregulation of microglial pro-inflammatory activity, by reducing JNK pathway activity (45). Also, ApoE induces polarization of macrophages to the M2 anti-inflammatory phenotype upon binding to LRP1 or ApoER2 receptors (46). These effects are relevant in disease conditions, where macrophages infiltrate the nervous system parenchyma or generate pro-inflammatory mediators (like TNF-α) (47) that cross the BBB and alter basal neuroinflammatory state. In addition, HDLs inhibit cytokine-induced expression of adhesion molecules in endothelial cells (48), which would restrain the macrophage–endothelial cell contacts required for access into the brain, and thus would hold back neuroinflammation. The lipocalin ApoD is also known to have anti-inflammatory functions (49, 50). ApoD controls the extent and duration of the inflammatory response upon peripheral nerve injury, by binding lysophosphatidylcholine (LPC) and arachidonic acid (AA) and dampening the Schwann cell signaling that recruits and activates macrophages.

In summary, lipid-binding proteins not only contribute to keep an adequate redox state in the healthy brain by mechanisms directly related to their lipid management properties but also contribute to the degree and duration of cellular responses to brain injury and disease by influencing pro- or anti-inflammatory signaling cascades.

Lipid-Binding Proteins' Influence on Amyloidogenesis

Misfolded proteins and toxic peptides are a primary cause in many neurodegenerative diseases. Curiously, these toxic peptides are often hydrophobic (hence their tendency to form aggregates) and can be bound by lipid-binding proteins or lipoprotein particles (Aβ being a well-known example). Therefore, because of shared biophysical properties, lipid-binding proteins are also responsible for managing hydrophobic peptides, like Aβ, in the brain. In other instances, the lipid-binding moiety of the protein can become susceptible of aggregation, as in α-synuclein (see below), becoming part of the pathogenic mechanism.

Aβ peptides originate from the proteolytic processing of amyloid precursor protein (APP), a ubiquitously expressed type I transmembrane protein that traffics between the plasma membrane and acidic intracellular compartments (5). **Figure 2A** summarizes the two alternative APP processing pathways. The non-amyloidogenic pathway is predominant in the healthy brain and takes place at the cell surface, where the α-secretase initiates APP processing. The amyloidogenic pathway, which results in the synthesis of Aβ peptides, requires internalization of APP into acidic compartments where β-secretase is at its optimal pH. In both pathways, membrane-anchored C-terminal fragments (CTFs) are subsequently processed by γ-secretase to generate

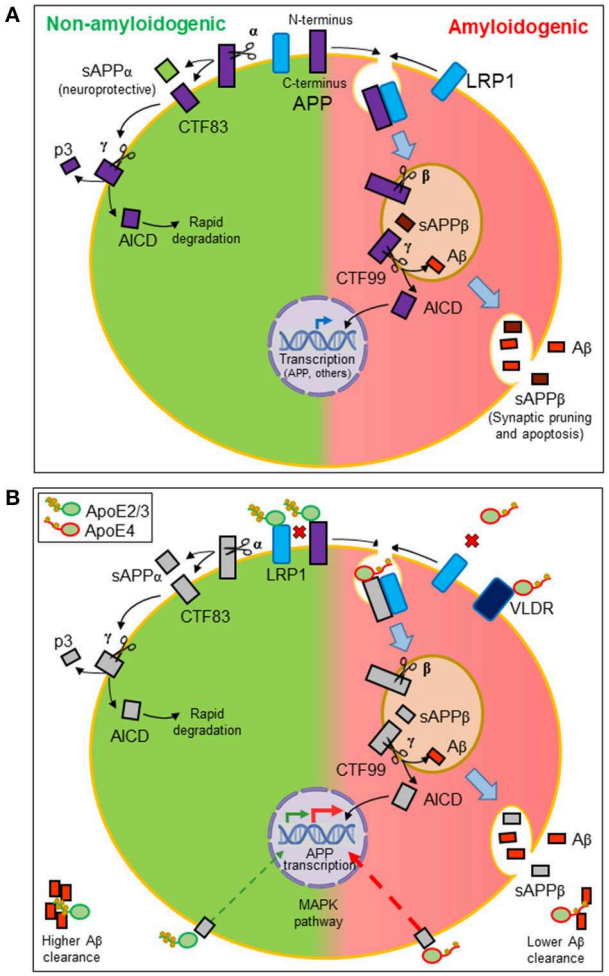


FIGURE 2 | Amyloidogenesis and the influence of lipid managing proteins. **(A)** Alternative proteolytic processing of amyloid precursor protein (APP). Plasma membrane α-secretase initiates the non-amyloidogenic pathway, releasing a soluble ectodomain (sAPPα) and generating a membrane-anchored C-terminal fragment (CTF) of 83 amino acids. In contrast, the amyloidogenic pathway is initiated by β-secretase in intracellular acidic organelles. Its processing results in a 99-amino-acid CTF and a soluble extracellular sAPPβ. CTFs are subsequently processed by γ-secretases generating p3 or Aβ peptides and releasing an APP intracellular domain (AICD). The AICD resulting from the amyloidogenic pathway translocates to the nucleus and regulates amyloidogenic and lipid metabolism gene transcription. The AICD generated by the non-amyloidogenic pathway is rapidly degraded in the cytosol. **(B)** Effects of ApoE on the production and dynamics of Aβ peptides. Direct interaction of ApoE with APP modulates its internalization. Also, LRP1 receptor promotes APP internalization. Because ApoE2/ApoE3 isoforms have more affinity for this receptor than ApoE4, ApoE2, and ApoE3 avoid APP internalization, preventing the amyloidogenic pathway (disfavored interactions depicted as red crosses). Moreover, ApoE triggers a signaling pathway that stimulates APP transcription. ApoE4 is the most potent isoform in triggering this pathway and thus Aβ production. Finally, ApoE also binds hydrophobic Aβ peptides through its lipid-binding domain. The lower lipid load of ApoE4 isoform results in a lower efficiency in Aβ clearance.

p3 or Aβ peptides, concomitantly with the release of an APP intracellular domain (AICD) into the cytosol. APP metabolites play their own roles in brain function: sAPPβ seems to be involved in synaptic pruning and apoptosis (51), while sAPPα is considered neuroprotective (52). The AICD resulting from the amyloidogenic pathway is known to translocate to the nucleus and regulate gene transcription, with APP, β-secretase, neprilisin, and several enzymes involved in lipid metabolism as target genes. In contrast, the AICD generated by the non-amyloidogenic pathway is rapidly degraded in the cytosol (5).

Brain HDL scaffolding proteins like ApoA-I, or associated proteins like ApoJ, have been demonstrated to impact Aβ-related pathogenesis, by influencing its oligomerization, its traffic through the BBB (in or out of the brain), as well as its endocytosis and degradation by cells within the brain or at the vascular endothelium. ApoA-I is able to bind soluble Aβ (53), and this interaction is thought to facilitate Aβ efflux from the brain through bulk flow into the CSF, from which it would be transferred to the circulatory and lymphatic systems. Clearance will avoid Aβ oligomerization and toxicity within the parenchyma. ApoJ is also able to interact with Aβ, apparently altering amyloid aggregation and promoting Aβ clearance (54). However, the specific role that ApoJ plays on Aβ-induced pathology remains unclear. It has been proposed that Aβ-ApoJ interaction is dependent on the ApoJ:Aβ ratio, which could determine whether ApoJ exhibits neuroprotective or neurotoxic effects (55). Endocytosis of Aβ-ApoJ complexes is mediated by interaction with megalin/LRP2 receptor at the BBB endothelial cells (19).

ApoE is highly expressed in the CNS, with no exchange of brain-derived and peripheral ApoE, therefore constituting two independent pools (21, 56, 57). Astrocytes are the primary source of ApoE in the brain (58–60), and it is also expressed at a lesser extent by microglia (61). ApoE plays a major role in cholesterol and phospholipid management within the CNS, and uses preferentially the LRP1 receptor (21). The primary function of ApoE is the maintenance of specific lipoprotein particles structure and docking them to specific cell receptors, carrying out lipid transport not only between cells in the nervous system but also across a defective BBB in pathological conditions (57).

ApoE takes part in HDL-like particles biogenesis, by interaction with ABCA1 receptors, which leads to varied degrees of lipid uptake by ApoE (62, 63). Its C-terminal domain constitutes its lipid-binding moiety, while the N-terminal one binds to receptors. The three most frequent alleles in humans (originating protein isoforms ApoE2, ApoE3, and ApoE4) have variations in amino acids in the receptor-binding domain (21). These ApoE isoforms present differential affinities for lipoprotein receptors located at the BBB and neuronal membranes, with ApoE2 and ApoE3 being the main interactors with LRP1, while ApoE4 binds preferentially to very-low-density lipoprotein receptor (VLDR) (60). The differential interaction with receptors results in differences of lipid-binding capacity for the different ApoE isoforms. ApoE4 is thought to have a molten globule structure, different to the structure of the other two isoforms (64), which makes it less stable, more easily degraded by astrocytes, with a lower level of lipid-loading capacity, and less affinity for LDLR family receptors and for APP (65).

With these properties, ApoE participates in various processes related to APP processing (**Figure 2B**). On the one hand, ApoE direct interaction with APP (66) regulates its internalization and the amyloidogenic pathway. On the other hand, LRP1 accelerates the endocytic traffic of APP, therefore promoting amyloidogenesis (67). We can define these relationships as competitive interactions. When ApoE2 or ApoE3 isoforms are expressed, HDL-ApoE performs its normal lipid transport, docking into the LRP1 receptors in neurons or endothelial cells while preventing APP to be internalized and processed through the amyloidogenic pathway. The lower lipid uptake capacity of ApoE4 and its lower affinity for LRP1 and APP has two consequences: (1) a less efficient intercellular lipid transport, and (2) a higher probability of LRP1-APP interaction, mostly in neuronal membranes, initiating APP internalization and the amyloidogenesis pathway (21, 57). Additionally, ApoE is able to trigger a non-canonical MAP kinase pathway that stimulates APP gene transcription, with ApoE4 being the isoform showing the greatest potency in such stimulation, thus promoting Aβ production further (68).

In addition to the effects on Aβ production, ApoE also binds hydrophobic Aβ peptides through its lipid-binding domain, an interaction that again depends on the lipid load of each isoform. This binding is also pH-sensitive (57), making the interaction to be location-dependent (intracellular acidic organelles vs. neutral extracellular space). Moreover, ApoE4 shows less affinity for Aβ peptides, making it a less efficient apolipoprotein in Aβ clearance from brain to blood (69). In summary, ApoE4 isoform is less suited for its intercellular lipid management function: ApoE4 uptakes a smaller lipid load at origin, and binds poorly to LRP1 at destination. Furthermore, during its traffic between cells, ApoE4 is a poor Aβ acceptor, and at destination, it promotes yet another deleterious effect: amyloidogenesis.

An important aspect of the just discussed effects of lipid-managing proteins on the production and dynamics of Aβ or other misfolded proteins in the extracellular space is that, consequently, inflammatory responses are inhibited. Thus, lipid-binding proteins also contribute to keep neuroinflammation under control not only because of their signaling properties mentioned above but also as a side effect of their ability to manage hydrophobic toxic peptides like Aβ.

Lipid-Binding Proteins Taking Care of Organelle Membranes and Proper Vesicle Trafficking

To the transport of lipids across water-based fluids, we have to add lipid management and interactions within biological membranes as a second major role for non-enzymatic lipid-binding proteins. We will focus next on three biological membranes that have special interest for the healthy and diseased nervous system: synaptic vesicles, lysosomes, and myelin.

Synaptic Vesicle Management

α-Synuclein is located in the cytoplasm of neurons and glial cells, but is known to interact with membranes through acidic

phospholipids (70). α-Synuclein–membrane interactions have gained recent attention, with data accumulating on its function as regulator of membrane lipid composition, with putative roles in fatty acid traffic within the cell (71). Within neurons, α-synuclein is enriched in synaptic terminals, where it serves important functions in synaptic vesicle traffic (72), participating in the vesicle docking system at the presynaptic terminals, as well as in clathrin-dependent vesicle recycling essential for proper neuronal function (73). The fact that mutations in the α-synuclein locus cause familial forms of PD has triggered much research on its biochemical and biophysical properties. In this context, a curious property is shared between some apolipoproteins like ApoA-I and α-synuclein. They contain 11 amino acid repeats that form amphipathic helices that allow these proteins to be inserted into cell membranes. Thanks to this interaction both lipid-binding proteins are able to modulate membrane curvature (74). Thus, a common mechanism is used by ApoA-I/ABCA1 complexes to initiate lipid uptake by ApoA-I and HDL biogenesis (9), and by the α-synuclein for its membrane interaction.

Securing Lysosomal Function

Stability of lysosomal membrane is crucial for cellular homeostasis and becomes a strategic factor for cell survival or death (75). Many proteolytic and lipolytic enzymes can only work properly within a well-controlled lysosome, able to maintain a luminal pH lower than the surrounding cytoplasm. In addition to those with enzymatic activity, various lipid-binding proteins exert functions within the lysosome. Saposin B works as glycosphingolipid presenter for lysosomal enzymes (76). Others regulate lipid-modifying enzymes, like Hsp70, whose binding to endolysosomal phospholipids regulates the activity of acid sphingomyelinase (ASM) (77). Furthermore, a healthy lysosome is required for a proper autophagic flux, which conditions the clearance of aggregation-prone proteins and other deleterious materials invariably produced in cells along their lifetime. Neurons are particularly vulnerable to autophagy disruption, since they are post-mitotic long-living cells. Lysosomes are also involved in the degradation of exogenous material by phagocytosis, a process particularly important after nervous system injury or upon demyelinating conditions, where myelin debris have to be efficiently removed if neuronal regeneration is to take place. Therefore, the composition of lysosomal membranes is crucial for both types of lysosomal function: (1) it has an impact on its permeability properties, and thus the maintenance of an intra-lysosomal milieu optimal for the enzymatic activities; (2) it conditions the association of particular proteins and chaperones to the lysosomal membrane and its fusion to autophagosomes or phagosome membranes.

A somehow surprising finding is that lipid-binding proteins, classically viewed as lipid transporters across extracellular fluids, have also essential roles in managing lysosomal membrane stability. That is the case of the lipocalin ApoD in both glial cells that express it (astrocytes and oligodendrocytes) and neurons (in a paracrine manner) (39, 78). As stated above, ApoD prevents lipid peroxidation, and its presence in lysosomes is required to avoid lysosomal permeabilization in oxidation-promoting situations. In OS conditions ApoD traffics from plasma membrane to lysosomes and guarantees that optimal pH for lysosomal enzymes is kept under control (78). In addition, Glial Lazarillo, a close homolog of ApoD in Drosophila, is required for proper lysosome–autophagosome fusion, which maintains an adequate autophagy flux in a proteinopathic condition (Type I Spinocerebellar Ataxia, SCA1) (79). Other lipocalins like Lcn2 have also been implicated in lysosomal membrane management. Curiously, Lcn2 promotes lysosomal permeabilization, inhibiting autophagic flux in cardiomiocytes (80) and promoting apoptotic cell death (81). This Lcn2 function, discovered outside the nervous system, also takes place in the nervous system cells expressing Lcn2 (astrocytes) or receiving it (neurons, microglia, and oligodendrocytes). Reactive astrocytes induce Lcn2 expression in response to inflammatory conditions and induce their own cell death as well as neuronal death (82). Neurodegenerative conditions of diverse etiology trigger the expression of Lcn2 and ApoD (35, 83). Therefore, these two lipid-binding proteins of the Lipocalin family seem to represent the opposite sides of the important equilibrium between neurodegeneration and neuroprotection, with the control of lysosomal functions as the center stage.

Lipoproteins Involved in Myelin Management

Although not previously anticipated, lysosomal stability and proper lysosomal function are also essential for adequate myelin maturation and for the physiological constant recycling of myelin membranes. Myelin is a very specialized type of plasma membrane assembled by specific glial cells in both CNS and PNS. Extensive membrane growth and recycling through endosomal and lysosomal pathways take place during myelin biogenesis (84), and remodeling continues through adulthood in an activity-dependent manner (85). Myelin properties are essential in determining the conduction velocity and thus neural circuit performance. Altered myelin function clearly leads to behavioral and cognitive deficits (86). Lysosome–myelin relationship is based on the fact that many of the lipidic components of myelin (accounting for 70–80% of its dry weight) are produced or recycled in lysosomes. The lipocalin ApoD was recently shown to be essential for the completion of the myelin compaction process (87). Without ApoD, myelin membrane recycling through the lysosome is altered, leading to lipid composition changes. An excess of glycosylated lipids (gangliosides) prevents the compaction of the extracellular leaflet, resulting in a less hydrophobic myelin. As a result, nerve conduction velocity is reduced (49).

In addition to the lipid management required during myelin construction and remodeling, myelin catabolism represents another process where proper lipid-binding proteins must be necessary, in addition to the involved enzymes. Myelin catabolism in the aging female brain has been proposed as an adaptive response (88). White matter degeneration generates ketone bodies as part of a shift in brain bioenergetics. As a result, levels of ceramides and various fatty acids (DHA, AA,

palmitic, and oleic acids) increase, posing an inflammatory challenge to the aging brain. It is therefore worthwhile to analyze how myelin catabolism-derived lipids are managed during physiological or pathological aging. Upon nervous system injury or in demyelinating diseases, myelin debris are phagocytosed either by resident microglia or Schwann cells or by infiltrating macrophages. Again, in those processes, lipid-binding and membrane-interacting lipoproteins are required. In these situations, ApoD and Lcn2 also play opposite roles, with ApoD favoring myelin phagocytosis and removal (50, 87) and Lcn2 promoting demyelination (89).

NEURODEGENERATION UNDERSTOOD FROM A LIPID MANAGER POINT OF VIEW

Alzheimer's Disease and Cerebrovascular Dementias: Dealing With Amyloid Deposition in a Compromised BBB State

Alzheimer's disease is the most common form of dementia, characterized by deposition of amyloid plaques in brain extracellular space and neurofibrillary tangles inside neurons. Aβ toxic oligomers cause synaptic dysfunction and a series of downstream events, ultimately leading to neuronal cell death. The majority of AD cases are sporadic with a late onset and do not have strong genetic components as the primary cause. Hence, we need to understand all putative risk factors in order to deal with this excruciating health problem. As mentioned above, the best known and objective risk factor for sporadic AD is the ApoE4 allele, which brings lipid-binding proteins up front in the management of the lipophilic Aβ peptides.

Once Aβ is generated by amyloidogenic processing of APP (see above; **Figure 2**), it is released to the interstitial fluid where clearance takes place by different mechanisms: efflux across BBB via ApoE receptors (mainly LRP1 and VLDLR), uptake by cells for lysosomal degradation, and cleavage by Aβ-specific proteases. As reviewed above, members of the LDLR gene family have a broad set of biological functions beyond lipid metabolism. LRP1 interactions with either APP or lipid-loaded ApoE in the cell membrane promote or discourage APP internalization, and therefore amyloidogenesis (67), in an isoform-dependent manner (**Figure 2B**). LRAD3, a newly identified member of the LDLR family, also binds APP and promotes its amyloidogenic processing (90). LRP1B has a slower rate of endocytosis and retains APP at the cell surface (91). Finally, LRP10 binds to APP and promotes its traffic to the Golgi complex, which also prevents amyloidogenesis (11). LRP1, expressed in neurons, influences not only amyloidogenesis but also clearance of extracellular Aβ by promoting its uptake and degradation (92). A similar Aβ clearance mechanism, though taking place in astrocytes and microglia, has been described for ApoJ–Aβ complexes that use the Megalin/LRP2 receptor instead (93, 94). Yet, an additional effect of Aβ is its ability to be inserted in brain cell membranes, altering membrane fluidity and initiating a lipid peroxidation chain reaction. Among the multiple consequences of the OS generated, lipid peroxidation results in the addition of 4HNE adducts to LRP1. The oxidized LRP1 is less active in clearing ApoE–Aβ complexes and, in turn, causes more accumulation of Aβ in the AD brain (12), thus generating a negative synergy between OS and Aβ management.

The majority of AD patients have co-morbid vascular diseases and cerebrovascular pathologies. Most also have Aβ deposition in cerebral arteries, known as cerebral amyloid angiopathy (CAA) (8). Therefore, lipid-binding proteins that influence the cerebrovascular system integrity and control Aβ clearance across the BBB become relevant targets toward treating AD and vascular dementias in general. Leakage of molecules across the BBB could be caused by loss or defective tight junction proteins between endothelial cells and/or enhanced transcytosis across the BBB (95). For example, the CSF of AD patients shows low levels of the Lipocalin ApoM (96). ApoM binds S1P in the blood and carries it to the membrane receptor S1PR in endothelial cells, where S1P promotes their barrier properties, preventing leakage and subsequent neuroinflammation. S1PR activation also decreases the expression of adhesion molecules used by leukocytes to adhere to endothelial cells before infiltration into damaged tissues (23). Therefore, a dysfunction of this ApoM-dependent mechanism might contribute to the BBB breakdown observed in AD patients. ApoA-I is also a relevant player at the cerebrovasculature. ApoA-I originated outside the brain acts from the lumen of blood vessels, precluding deposition of amyloid at the blood side of the BBB, thus preventing amyloid angiopathy and BBB disruption (97). ApoA-I deficiency in APP/PS1 mice increases CAA, total cortical Aβ deposition, and several markers of neuroinflammation (8).

Yet, other factors might influence Aβ clearance at the BBB, early in the disease progression. Multiple studies have shown impaired regional brain uptake of glucose before neurodegeneration, suggesting reduced glucose brain utilization caused by decreased glucose transport across the BBB via endothelial-specific glucose transporter GLUT1 (98). Moreover, diminished GLUT1 expression in brain endothelium leads to transcriptional inhibition of LRP1, accelerating Aβ pathology (99).

In summary, in the brain of AD and related vascular dementias, deleterious effects of amyloid peptides are combined with pro-oxidative and pro-inflammatory conditions in a disrupted BBB situation. Independently of the primary cause of each particular disease, lipid-binding proteins are involved in every step.

Parkinson's Disease: An Oxidative Challenge for Dopaminergic Neurons

PD is an age-related neurodegenerative disorder characterized by dopaminergic neuronal cell death in the brain substantia nigra. The main triggering factor of the disease are Lewy bodies, intraneuronal protein aggregates composed of α-synuclein (100). Various events associated with PD such as OS, endosomal–lysosomal dysfunction, endoplasmic reticulum stress, and inflammatory responses, have been described as processes in which lipids play a key role (3).

Mutations in α-synuclein are causative for familial PD; thus, PD is considered as one of the synucleinopathies, which

include diseases that affect both neurons and oligodendroglia (101). In addition to the functional consequences derived from synaptic malfunction in PD, research aiming at understanding factors triggering α-synuclein aggregation brings its membrane interaction up front. Although some controversy exists about the structure and oligomer conformation of native α-synuclein (102, 103), there is consensus on the concept that α-synuclein interaction with membranes is the key factor conditioning transitions between different structural modes and, therefore, its susceptibility to form pathogenic fibrillar aggregates (70). Factors reducing α-synuclein/membrane interaction promote its aggregation. Furthermore, this membrane interaction is regulated by polyunsaturated phospholipids (PUFAs), the cellular membrane components with the highest susceptibility to oxidative damage. They interact with α-synuclein and stabilize its binding to cell membranes when they are in their reduced state. The OS that develops in dopaminergic neurons of PD patients results in oxidation of PUFAs and oxidative modification of α-synuclein. Both effects reduce the affinity of α-synuclein–lipid interaction and promote the formation of toxic protein oligomers (104).

In vitro studies on toxin-mediated proteasomal impairment in the dopaminergic cell line SH-SY5Y have suggested that ApoJ/Clusterin also prevents α-synuclein aggregation due to its chaperone activity (105), probably involving their hydrophobic lipid-binding moieties. Consistent with this idea, Lewy bodies in PD and other synucleopathies show an inverse correlation between ApoJ and α-synuclein content (106). ApoA-I levels also decrease in PD, especially at early stages of the disease. The increase in oxidation markers produced in PD brains concurs with ApoA-I oxidative damage, resulting in malfunction of cholesterol processing, dysregulation of the inflammatory response, and acceleration of neurodegeneration. Among other effects, oxidized ApoA-I loses its ability to inhibit TNF-α release, which is exacerbated by OS and can cross the BBB, thereby leading to neuronal death (97).

Since ApoD possesses neuroprotective effects against OS, and pro-oxidative stimuli regulate its expression in astrocytes (39), upregulation of ApoD in the brain of PD patients is not unexpected. Increase in ApoD immunoreactivity has been in fact observed in glial cells of the substantia nigra (107) and in brainstem neurons and glia of PD patients (108). This upregulation can be interpreted as a protective mechanism against neurodegeneration, where glia-derived ApoD is internalized by stressed neurons (109). This neuroprotective function is supported by the work on model organisms (36, 37). Lack of ApoD in mouse promotes astrogliosis in the substantia nigra and functional alterations in dopaminergic neurons (39).

Finally, cholesterol deficiency in the brain causes impaired neuronal plasticity and reduced neurotransmission. Since ApoE is mainly responsible for the maintenance of cholesterol homeostasis within the CNS, it is plausible that ApoE might also be involved in PD. In fact, ApoE has been proposed as a risk factor for PD, and the three ApoE isoforms might play different roles in the pathogenesis of PD through their differential interaction with LRP1, as it is the case for AD. An *in vitro* study evaluating the effects of ApoE isoforms on α-synuclein aggregation showed that ApoE4 increases aggregation of α-synuclein more than other isoforms (110) in a mechanism resembling Aβ management in AD.

In summary, various lipid-binding proteins, in addition to α-synuclein, are implicated in lipid interactions and management in the altered conditions present in PD. Interestingly, most of them also influence AD and other common neurodegenerative pathologies. Knowledge of their functional connections can definitely help to broaden the strategies to palliate or counteract disease progression.

Multiple Sclerosis and Other Demyelinating Diseases: BBB and Myelin at Stake

Multiple sclerosis (MS) and other demyelinating diseases are caused by autoimmune attacks toward myelin components in a pro-inflammatory context associated with BBB disruption and infiltration of immune system cells. In these pathologies, the influence of lipid-managing proteins in BBB stability becomes a relevant factor and a process where preventive or repairing interventions would be desirable.

Three ApoM-related processes influence BBB permeability. ApoM and ApoM-positive HDL particles contribute to control neuroinflammation at different levels (23, 111, 112). First, S1P-ApoM complex restrains lymphopoiesis, thus controlling inflammatory state at origin. Second, ApoM brings S1P to brain endothelial cells, influencing their permeability properties through signaling cascades initiated by S1PR binding. Finally, brain endothelial cells express ApoM, with favored secretion toward the BBB brain side, thus contributing to intra-cerebral transport of S1P. The beneficial effects of the S1P analog FTY720 in animal models of MS are coherent with a central role of ApoM in neuroinflammation control. Also, the anti-inflammatory properties of ApoA-I-containing HDLs represent an endogenous protective mechanism against MS (113). ApoA-I levels negatively correlate with disease severity, and MS patients with high levels of serum ApoA-I respond better to IFNβ therapy. The anti-inflammatory effects of this apolipoprotein are thought to be mediated, at least in part, through its prevention of LDL oxidation. ApoA-I avoids the oxLDL-mediated BBB leakage.

Regardless of demyelination being triggered by neuroinflammation, or derived from intrinsic alterations in oligodendrocytes (differentiation, myelin biogenesis, or myelin recycling), we need to keep investigating how demyelination takes place and how the myelin debris generated are dealt with. Myelin recognition and phagocytosis by resident microglia or infiltrated macrophages is a central process whose failure can trigger anomalous myelin destruction, prevent myelin clearance, or halt myelin repair. A recent gene expression analysis of MS lesions, where active demyelinating lesions are compared with inactive ones and with healthy myelinated tissue, revealed genes related to lipid binding and uptake (scavenger receptors) upregulated in the rim of chronic active lesions, where demyelination is taking place (114). Also, myelin phagocytosis control by ApoD is key for proper myelin clearance after injury (50). By regulating the presence at the injury site of lipid mediators of inflammation, and of "eat-me" signals triggering recognition of myelin debris by phagocytic cells, this Lipocalin influences the initiation of myelin phagocytosis. In addition,

ApoD targeting to lysosomes and functional stabilization of lysosomal membranes (78) contributes to optimize the process of myelin degradation after autophagosome-lysosome fusion. Finally, the process of myelin reconstruction after injury is expected to require ApoD at its latest phase: myelin sheath compaction (87). It is therefore not unexpected that ApoD overexpression is detected in MS patients (115), and that its presence in MS lesions (116) is low in sclerosis plaques, particularly in inactive ones, but recovers high levels in the re-myelinating lesions when myelin reconstruction is taking place.

Myelin repair has been recently shown to depend on LRP1 (117). In addition to all signaling cascades triggered by LRP1, its action as a lipoprotein receptor (preferentially for ApoE-HDL, as mentioned above) is required for myelin repair. The cholesterol supply required for myelin sheath growth is impaired in oligodendrocytes lacking LRP1, thus hindering repair after demyelination.

Therefore, a combination of lipid-binding proteins are involved in various phases of demyelinating diseases, with effects at different levels, from BBB stability to inflammatory signaling, myelin destruction, and reconstruction. Knowledge of lipid management at each of these steps is a necessary challenge we need to take up, if we want to control myelin-related ailments.

Lysosomal Storage Diseases and Their Inseparable Companions: Leukodystrophies

One cruel evidence that myelin–lysosome functional link is essential for human health is the existence of lysosomal storage diseases (LSDs), all of them associated to leukodystrophy or myelination problems. LSDs usually show an early onset and are suffered by children that present both systemic and nervous system-related problems and show poor survival chances. LSDs are cataloged as rare diseases, but together they add up to 1 case/8000 (118).

NPC disease has been extensively studied (119). In NPC, mutations in NPC1 and NPC2 genes cause impaired trafficking of cholesterol in neurons, after LDLR-mediated internalization, or in glial cells, where it is endogenously produced. Both proteins are located in endosomes and lysosomes and have a cholesterol-binding domain. In NPC patients, cholesterol and other lipids accumulate in late endosomes and lysosomes and are unable to travel to the plasma membrane. These lipid management alterations result in progressive neurodegeneration, starting early in cerebellar Purkinje cells (120), as well as in myelin defects, revealing the importance of cholesterol mobilization from lysosomes, both in neurons and in oligodendrocytes, to form and maintain a functional myelin sheath (121). Interestingly, concomitant with this cholesterol dyshomeostasis, genomic instability, and trisomy 21 mosaicism have been described in NPC patients (122), an effect that is thought to be a consequence of changes in membrane fluidity. Thus, lipid transport defects can also have consequences in the genetic material of cells and their correct segregation during cell division.

Niemann–Pick type A (NPA) disease is caused by a different genetic alteration, but also alters both lysosomes and myelin. It results from loss-of-function mutations in the ASM gene, leading to accumulation of sphingomyelin in lysosomes (123). NPA is an infantile disease with a rapid progression leading to premature death. It causes early neurodegeneration, particularly in vulnerable Purkinje cells, and also leads to abnormal myelin formation (124). Primarily caused by a lipid-processing enzyme deficit, this disease is also modulated by non-enzymatic lipid managing proteins, like Hsp70. As mentioned above, its lipid presenting role in lysosomes promotes ASM activity (77), and it is able to correct the lysosomal defects in cells of NPA patients (125). Analogous to Hsp70 lipid presenting role, the lysosomal Saposin B binds cerebroside sulfate and other membrane glycosphingolipids (126) to make them available to water-soluble enzymes. Defective Saposin B causes metachromatic leukodystropy, a rare LSD affecting mostly myelinating glia both in CNS and PNS, and resulting in motor and cognitive deterioration (127).

Most lysosomal dysfunctions in LSDs are primarily caused by aberrant or decreased enzymatic activities, and some, like NPC, are directly linked to non-enzymatic lipid manager proteins. It is clear that alterations in the lipid-related lysosomal functions are of particular centrality because of the many downstream consequences, including diverse forms of myelin dystrophy.

CONCLUDING REMARKS

Lipids are essential components of all living cells requiring special management while immersed in a mostly hydrophilic environment. In addition to their energetic or signaling functions, they form structures of special relevance in the lipid-rich nervous system. Lipid-based structures are of high complexity, from apparently simple cell membranes to lipoprotein particles, intracellular lipid droplets, or complex myelin sheaths. Moreover, these structures are dynamic, serving functions as important to the nervous system as synaptic communication, nerve conduction velocity, microglial, or astroglial phagocytosis, or autophagy control of intracellular toxic products.

Lipid processing enzymes as well as lipid carriers with a variety of designs need to act in conjunction to optimize the lipid requirements of each cell type in the nervous system. In this review, we have brought the attention to non-enzymatic lipid managers and their relationship to essential processes that, when disrupted, generate a wide array of nervous system diseases. Moreover, many of the lipid-managing proteins are part of endogenous mechanisms of protection. Knowledge about all steps in each lipid-related process, the proteins involved, and the lipid species affected, should help to design potential therapies for nervous system diseases.

AUTHOR CONTRIBUTIONS

MG, DS, and MC-G reviewed literature and designed the manuscript. MC-G and MG wrote the manuscript. DS reviewed and edited the manuscript. The external Frontiers reviewers helped to improve the manuscript.

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Exploring Sphingolipid Implications in Neurodegeneration

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Over the past decade, it was found that relatively simple sphingolipids, such as ceramide, sphingosine, sphingosine-1-phosphate, and glucosylceramide play important roles in neuronal functions by regulating rates of neuronal growth and differentiation. Homeostasis of membrane sphingolipids in neurons and myelin is essential to prevent the loss of synaptic plasticity, cell death and neurodegeneration. In our review we summarize data about significant brain cell alterations of sphingolipids in different neurodegenerative diseases such as Alzheimer's disease, Parkinson disease, Amyotrophic Lateral Sclerosis, Gaucher's, Farber's diseases, etc. We reported results obtained in brain tissue from both animals in which diseases were induced and humans in autopsy samples. Moreover, attention was paid on sphingolipids in biofluids, liquor and blood, from patients. In Alzheimer's disease sphingolipids are involved in the processing and aggregation of β-amyloid and in the transmission of the cytotoxic signal β-amyloid and TNFα-induced. Recently, the gangliosides metabolism in transgenic animals and the relationship between blood sphingolipids changes and cognitive impairment in Alzheimer's disease patients have been intensively studied. Numerous experiments have highlighted the involvement of ceramide and monohexosylceramide metabolism in the pathophysiology of the sporadic forms of Parkinson's disease. Moreover, gene mutations of the glucocerebrosidase enzyme were considered as responsible for Parkinson's disease via transition of the monomeric form of α-synuclein to an oligomeric, aggregated toxic form. Disturbances in the metabolism of ceramides were also associated with the appearance of Lewy's bodies. Changes in sphingolipid metabolism were found as a manifestation of Amyotrophic Lateral Sclerosis, both sporadic and family forms, and affected the rate of disease development. Currently, fingolimod (FTY720), a sphingosine-1-phosphate receptor modulator, is the only drug undergoing clinical trials of phase II safety for the treatment of Amyotrophic Lateral Sclerosis. The use of sphingolipids as new diagnostic markers and as targets for innovative therapeutic strategies in different neurodegenerative disorders has been included.

Keywords: sphingolipids, neurodegeneration, diagnostic marker, therapeutic target, brain

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THE DARK WORLD OF THE BRAIN AND SPHINGOLIPIDS

In the last 20 years the face of sphingolipids (SphLs) has changed. It took a long time to understand that these lipids are not only structural membrane molecules with a stiffening role but they are functional molecules fundamental for cell fate. Today, SphLs have great interest in brain physiopathology and therefore in the entire body considering that in humans the brain

controls their own physical and inner life and allows relations with the outside world. In fact, specific areas of the brain control movement, sensitivity, vision, listening, thought, word, emotions, music and others, but all areas are rich in interconnections and molecular interactions. Today, mechanisms that underlie the functions of neuronal and glial cells are not completely clarified and many studies highlight that SphLs are some of the main actors (1). In the brain, SphLs play crucial roles by regulating the rate of growth, differentiation, and death of central nervous system (CNS) cells. Violation of the balance of the different classes of SphL leads to changes in the fate and functions of neuronal cells. Now there is a plethora of information on SphL metabolism to which contrasting roles are attributed. The most involved SphLs in neurodegeneration are simple molecules such as sfingosines (sphinganin, Sphn; sphingosine, Sph; sphingosine-1-phosphate, S1P) and ceramide (Cer), derived molecules as glucosylceramide or cerebroside (GCer) and galactosylceramide (GalCer), and sphingomyelin (SM), and finally more complex molecules as gangliosides (GM, glicolipids with sialic acid residues). Moreover, relevant for this topic are enzymes involved in SphL metabolism as neutral and acid sphingomyelinase (nSMase, aSMase), ceramidase (Cerase), sphingosine kinase (SphK), glucocerebrosidases 1 and 2 (GBA1 and GBA2) and galactosylceramidase (GalCerase), and glucosylceramide synthase (GCerS) (**Figure 1**).

SM is hydrolyzed by SMase to produce phosphocholine and Cer. The SM breakdown is carried out by different SMase isoenzymes belonging to three families classified based on their activity pH optima into acid, and neutral subtypes: aSMase and nSMase (2). aSMase that is specifically located in lysosomes and under stress conditions, rapidly translocates from lysosomes to the outer leaflet of the plasma membrane (2). An array of nSMases reside in specific subcellular structures, including cell nucleus (3). aSMase is encoded by *SMPD1* gene and the four nSMase isoforms are encoded by different genes, nSMase1 by *SMPD2*, nSMase2 by *SMPD3*, nSMase3 by *SMPD4*, and MA-nSMase (mitochondrial-associated nSMase by *SMPD5* (4).

In this review, we provide an overview on the changes and roles of SphLs in Alzheimer's disease, Parkinson's disease and Amyotrophic lateral sclerosis, and on the possibility of being interesting molecules as diagnostic markers and therapeutic targets.

Abbreviations: aCerase, acid ceramidase; aSMase, acid SMase; Asah1, acylsphingosine amidohydrolase 1; AD, Alzheimer's disease; ALS, Amyotrophic lateral sclerosis; Cer, ceramides; CHO, cholesterol; FD, Farber's disease; GalCBase, galactocerebrosidase; GalCerase, galactosylceramidase; GalCer, galactosylceramide; GalSph, galactosylsphingosine; GM, ganglioside; GMS, ganglioside synthase; GD, Gaucher disease; GCBase, glucocerebrosidases; GCer, glucosylceramide; GCerS, glucosylceramide synthase; GSph, glucosylsphingosine; GSphLs, glycosphingolipids; HEX, hexosaminidase; hCer, hexosylceramides; iNOS, inducible nitric oxide synthase; KD, Krabbe's disease; LacCer, lactosylceramide; LacCerS, lactosylceramide synthase; NDDs, neurodegenerative disorders; nCerase, neutral ceramidase; NP, Niemann-Pick disease; NO, nitric oxide synthase; PD, Parkinson's Disease; Sphn, sphinganin; SphLs, sphingolipids; SM, sphingomyelin; SMase, sphingomyelinase; Sph, sphingosine; SphK, sphingosine kinase; S1P, sphingosine-1-phosphate; S1PL, sphingosine-1-phosphate lyase.

SPHINGOLIPIDS IN STORAGE DISORDERS AS A CAUSE OF NEURODEGENERATION

Disorders of sphingolipids metabolism in lysosomes induced a family diseases identified as lysosomal storage diseases (LSDs). LSDs include Niemann-Pick disease (NP), Gaucher's disease (GD), Farber's disease (FD) and Krabbe's disease (KD) (**Table 1**).

NP type A and B (NPA-NPB) is characterized by defective activities of aSMase resulting in defect of SM degradation. SM cannot be converted to Cer and consequently Cer-SM ratio is altered. It means that signal cascades with participation of ceramide is not developed (5, 6).

GD is caused by the absence of GBA leading to the accumulation of the glucosylceramide (GCer) and glucosylsphingosine (GSph). GCer and GSph accumulate in mononuclear phagocytes (due to the phagocytic activity of these cells), primarily macrophages, which have a foamy appearance and are referred as Gaucher cells (7–10). In the brain, accumulation of GSph causes neuronal storage of gangliosides leading to loss of neurons and their axons, resulting in cortical atrophy and white matter degeneration (11).

FD is characterized by the deficient activity of acid ceramidase (aCerase) and high levels of Cer (12). The rate of Cer synthesis in brain during FD is normal but Cer resulting from degradation of complex SphLs cannot be hydrolyzed and accumulated into lysosomal compartment. The abnormal Cer level in the brain results in neuronal dysfunction (13, 14).

KD is caused by the deficiency of galactosylceramidase (GalCerase) in lysosomas which remove galactose from galactoceramide derivatives. The disease is due to mutations in the GALC gene (14q31) encoding the lysosomal enzyme GalCerase, that catabolizes the hydrolysis of galactose from galactocerebroside (GalCer) and galactosylsphingosine (GalSph) or psychosine. The accumulation of cytotoxic psychosine leads to apoptosis of oligodendrocytes and demyelination of the CNS and PNS. Rarely, infantile KD is caused by a mutation in the prosaposin (PSAP) gene (10q21-q22), encoding SL activator protein saposin-A, necessary for GalCerase activity (15).

SPHINGOLIPIDS IN NEURODEGENERATIVE DISORDERS

Neurodegenerative disorders (NDDs) include specific diseases characterized by a complex pattern of pathological hallmarks including neuroinflammation with progressive loss of structure and/or function of neuronal cells leading to a set of incurable and debilitating conditions with sometimes different and sometimes overlapping symptoms as functional decline of cognition and/or movement (16). The main neurodegenerative diseases are Alzheimer's disease (AD), Parkinson's disease (PD), and Multiple Sclerosis (MS). Many SphL species, as shown so far in few studies, undergo significant changes during NDDs. The important role of SphLs in neurodegeneration is easily understood by looking at the effects induced by their accumulation. These diseases are characterized by a combination of axonal, neuronal, and myelin defects, in addition to astrogliosis, and neuroinflammation and

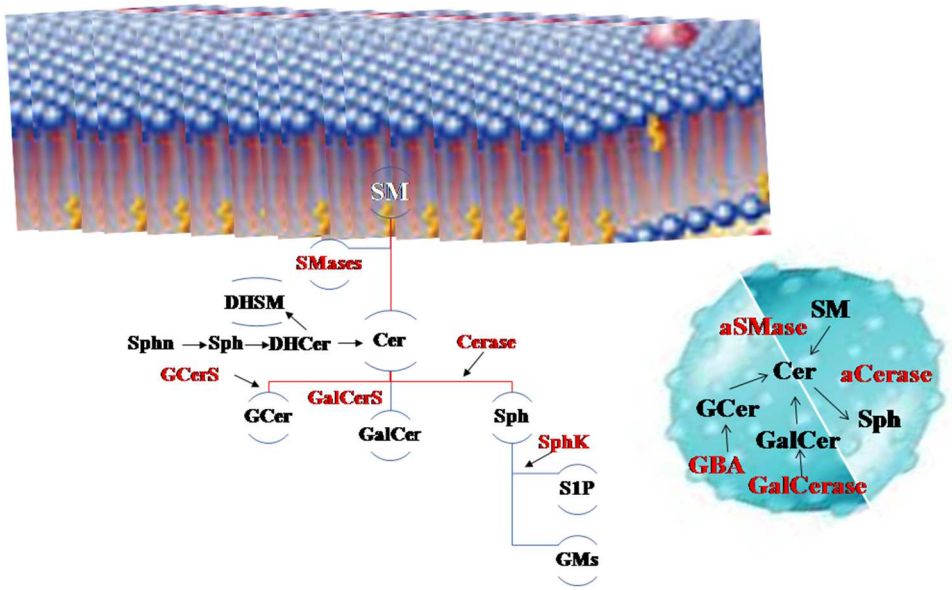

FIGURE 1 | The main sphingolipids (SphLs) and metabolism SphL enzymes in neurodegeneration. Above, the cell membrane, on the right the lysosome. Sphingomyelinase (SMase) degrades sphingomyelin (SM) to ceramide (Cer) and it is catabolized by ceramidase (Cerase) to sphingosine (Sph) that is utilized to form sphingosine-1-phosphate (S1P) by sphingosine kinase (SphK) and gangliosides (GMs). Cer is also used to form glucosylceramide (GCer) by glucosylceramide synthase (GCerS) and galactosylceramide (GalCer). GCer is degraded by glucocerebrosidases (GBA) and GalCer by galactosylceramidase (GalCerase). Also precursors of Cer as sphinganin (Sphn), sphingosine (Sph) and dihydroceramide (DHCer) are present in the brain as well dihydrosphingomyelin (DHSM).

TABLE 1 | Lysosomal storage diseases caused by impairments in sphingolipid metabolism.

Type of disease	Accumulated compound	Deficient enzyme
Krabbe disease, galactosylceramide lipidosis	Galactosylceramide	β-Galactosidase
Niemann-Pick disease, sphingomyelin lipidosis	Sphingomyelin	Sphingomyelinase
Gaucher disease, glucosylceramide lipidosis	Glucosylceramide	β-Glucocerebrosidase
Fabry disease, trihexosylceramidase lipidosis	Trihexosylceramide	α-Galactosidase
Farber disease, ceramidase deficiency	Ceramide	Ceramidase

therefore neurodegeneration (17). It is evident from genetic analysis that mutation affecting lipid manipulating enzymes impact on lipid mediators activities, lipid transport and on important cell functions, such as autophagy and inflammation, thus acting as an independent risk factor for age-related neurodegeneration (18, 19).

Alzheimer's Disease

AD is the most common cause dementia in adults over 65 years old (20). The number of AD patients will increase over 80 million people by 2040 (21). Despite significant efforts undertaken by the international community, precise mechanism of the development of AD still remains unknown. In contrast to other cognitive pathologies causing clinical signs of dementia, the cardinal pathologic features of AD are amyloid beta (Aβ) aggregates, the major components of senile plaques, and neurofibrillary tangles of τ protein (22). It has been shown that amyloidogenesis plays a key role in induction and development of AD (23). Aβ with molecular mass of 4 kDa was originally described in 1985 (24). It is formed from proteolytic cleavage of a constitutive transmembrane protein, Aβ precursor protein (APP), catalyzed by β- and γ-secretases that are at the heart of the AD pathogenesis even if recently also a δ-secretase has been identified (23). Alternative APP cleavage by α-secretase does not result in amyloid peptide formation (25).

Sphingolipids in the Brain

During the last two decades much attention was paid to brain sphingolipids (SphLs) in neurodegeneration (26–28) as molecules involved in the processing and aggregation of Aβ, in the signal transduction of a cytotoxic signal induced by Aβ and of pro-inflammatory signal induced by cytokine TNFα, that are considered the main inducers of AD (29). Neuronal plasma membrane is a primary target for Aβ and its lipid component is directly involved in Aβ neurotoxicity mechanisms. The most important conformational changes of Aβ occur in the presence of SphLs. V3-like domain of Aβ interacts with SM and GalCer in monomolecular films at the air-water interface (30). Such changes in the membrane composition might also influence activity of enzymes involved in APP processing.

In a study of vesicle models to mimic exosomes, GM1 promotes Aβ fibril formation (31). It has been suggested that formation of such structures is a prerequisite for subsequent formation of Aβ plaques (30, 32). Identification of factors resulting in monosialic GM (GM1)/Aβ complex formation would represent a novel mechanism of the pathogenesis of AD and give a possibility for development of a novel strategy for prevention and treatment of this disease.

It has been demonstrated that rafts, special plasma membrane domains enriched in GSphLs, cholesterol (CHO), SphLs, and membrane proteins involved in extracellular signal transduction play a specific role in Aβ aggregation (33, 34). APP is transported and proteolytically cleaved by β-, and γ-secretases located in CHO-SM domains (34). Thus, lipid composition of rafts including GSphLs, CHO, SM and its metabolites, Cers, may strictly control APP processing and Aβ aggregation.

Neutral and acid sphingomyelinase

A soluble Aβ oligomer causes activation of both nSMase and aSMase. Specific inhibition and knockdown of each enzyme provides cell resistance to Aβ- induced apoptosis (35).

In primary neuronal cells the treatment with fibrillar Aβ causes nSMase upregulation and consequently the increase in Cer content (35). Inhibition of nSMase decreases cell death thus suggesting that nSMase is essential for Aβ cytotoxicity (35). The intracerebral administration of Aβ or TNFα to rats results in nSMase activation. This process is more pronounced in hippocampus than in cortex and cerebellum (29). He et al. (27) provided evidence on aSMase role in Aβ cytotoxicity.

Astrocytes, the main representative of glial cells, activated by 1 μM Aβ-142 in combination with 10 ng/ml IL1β were used in studies of mechanisms of their toxic effect on human primary neurons (36). These studies demonstrated a sharp activation of nSMase and Cer accumulation in neurons during their death induced by astroglia activation. Some studies revealed association of the SM cycle signaling system with oxidative stress (29). It was found that reactive oxygen species directly influence SMase or other enzymes involved in regulation of SM metabolism. This may potentiate the toxic effect of cytokines and Aβ on the brain cells during combined action of these compounds. Successful employment of antioxidant therapy in clinical practice confirms effective inhibition of processes related to activation of oxidative systems in the development of AD. The relationship between nSMase and nitric oxide synthase (NO) is controversial. In fact, it has been described that activation of neuronal nSMase is determined by NO generated by activated astroglia. Otherwise, Kumar et al. (37) found that in activated astrocytes nSMase induces mRNA expression of inducible nitric oxide synthase (iNOS) together to an overexpression of pro-inflammatory cytokines (TNFα, IL1β, IL6). Involvement of Aβ in Cer generation in neurons was demonstrated showing that only Aβ142 but not its reverse form (Aβ42–1) induces nSMase activation (38).

In oligodendrocytes, Aβ induces apoptosis accompanied by the increase in the Cer level (39). Addition of exogenous Cer or bacterial nSMase, by increasing the Cer level, to the oligodendrocyte cell culture causes cell death (38, 39). nSMase inhibitor (3methylsphingomyelin) effectively protects oligodendrocytes against Aβ effects (39). In these cells, Aβ influences expression of iNOS induced by TNFα through nSMase/Cer pathway (40). This molecular relationship is fundamental. In fact, neither Cer nor Aβ alone cause iNOS expression that instead is stimulated only by accumulation of TNFα. On the other hand Aβ is only able to activate nSMase. This is relevant for understanding the mechanism of AD development, in which pro-inflammatory components play important roles (40). It should be noted that the natural antioxidant glutathione inhibits nSMase activity. A decrease in the glutathione level causes activation of this enzyme and accumulation of Cer in oligodendrocytes followed by their death (39).

In dendritic cells, the effect of Aβ results in aSMase activation and its inhibition results in the resistance of these cells to Aβ-induced apoptosis (41). A study of postmortem analysis of the AD patient brains revealed activation only of aSMase (38).

Taken together, the above studies suggest the involvement of different SMase forms in the realization of Aβ-induced apoptosis of nerve cells. Analysis of genes encoding aSMase and nSMase2 expression revealed their upregulation in the brain of patients with both AD and other neuro pathologies (42). Since SMase activation was investigated in various cell lines, during actions of various forms of Aβ, in animal experiments and in studies of human postmortem brain preparations, discrepancies in results obtained in different systems is not surprising. There is no reasonable transgenic animal model which would completely mimick the pathological mechanism of AD; still remains unclear which type of Aβ is responsible for cell death and loss of cognitive functions in humans. Although local concentrations of fibrillar Aβ found in the brain of AD patients may differ from Aβ concentrations in primary neurons used in experiments, the data on interrelationship between SMases, Cer accumulation and subsequent death of neurons and oligodendrocytes suggest that SMases may be perspective targets for drugs preventing neurodegenerative impairments in AD. Thus, induction of the SM cascade cycle resulting in accumulation of the proapoptotic agent Cer may be considered as a novel mechanism of the development of AD. This may be considered as a prerequisite for novel approaches to AD therapy by using drugs of a new generation that inhibit SMase activity (43–45).

Ceramides

Cer accumulation is evident in early stages of AD disease while the later stages are characterized by decreased Cer levels in brain structures (46–48). In particular, the authors found in later stages of AD decreased levels of Cer in the white matter of the middle frontal gyrus in AD patients compared with corresponding controls (46). Analysis, performed in early stages of AD and in other brain impairments, of six Cer species, differing on the basis of fatty acid chains, revealed increased levels of Cer16:0, Cer18:0, Cer20:0, and Cer24:0 in comparison with controls (42). High ceramide levels are responsible for the increased susceptibility of neurons and oligodendrocytes to cell death (49). Interesting results were obtained with the gene expression studies for enzymes involved in the control of synthesis and degradation of Cer during the development and course of AD (48). It was

firmly recognized that the early stage of AD is characterized by increased level of Cer *de novo* synthesis, by stimulation of Cer synthases, especially of Cer containing C22:0 and C24:0 long chain fatty acids, while synthesis of GCer decreases (48). These results support above reported studies indicating Cer accumulation in early stages of AD and therefore Cer synthase might by an early target for reducing AD progression.

Sphingosine and sphingosine-1-phosphate

It has been shown an accumulation of Sph, with proapoptotic properties, during a post mortem study of AD patient brains (27). In support, the activity of aCerase converting Cer to Sph is higher in AD patients compared with controls (27, 50, 51).

In contrast to Cer and Sph, S1P, a product of Sph phosphorylation, exhibits antiapoptotic properties and it is involved in the regulation of cell proliferation (27). There are data demonstrating S1P reduction in the cytosolic fraction of the gray matter of the frontotemporal brain region of AD patients compared with controls (27). There is negative correlation between S1P and Aβ and phosphorylated τ protein in the same brain region (27).

Figure 2 summarizes the results of the research work carried out on the brain.

Sphingolipids in Biofluids

Some researchers believe that correlation between changes of cerebrospinal fluid (CSF) and blood SphL species in AD patients imply the applicability of SphLs as AD biomarkers, especially at the early stage of this disease, and as putative targets for novel drug generation (52, 53).

Sphingolipids in the liquor

CSF can most adequately reflect the brain pathological changes in AD patients. Usually, changes in the brain lipids are studied in AD patients at the terminal stage of this disease, while studies in CSF lipids may monitor development of the disease and effectiveness of treatment. Analysis of CSF revealed that SM species increase at the early stage of this disease, while no changes are found in AD patients at the mid and severe stages (53). In AD patients, CSF Cer content is significantly higher than that present in other neurological diseases as cervical spondylosis, lateral amyotrophic sclerosis, metabolic encephalopathy, stroke (54). The highest Cer total content is found in the mid stage of AD compared with early and severe stages (52). Nevertheless, the use of Cer family components as AD markers has been widely discussed in the literature (52–54).

Although the change of Cer species is not specific for AD but it is present also in other neurological pathologies, it may be used as a marker of a particular stage of AD. Unlike the total content of Cer, changes in the Cer species may indicate early impairments in AD, which, possibly, may be responsive to therapeutic correction (52). These results suggest that impairments in SphL metabolism might be useful as a risk marker for the development of AD (53).

Sphingolipids in the blood

At the early stage of AD Cer content decreases in the blood, and increases in the brain and CSF (52, 53). Relationship between

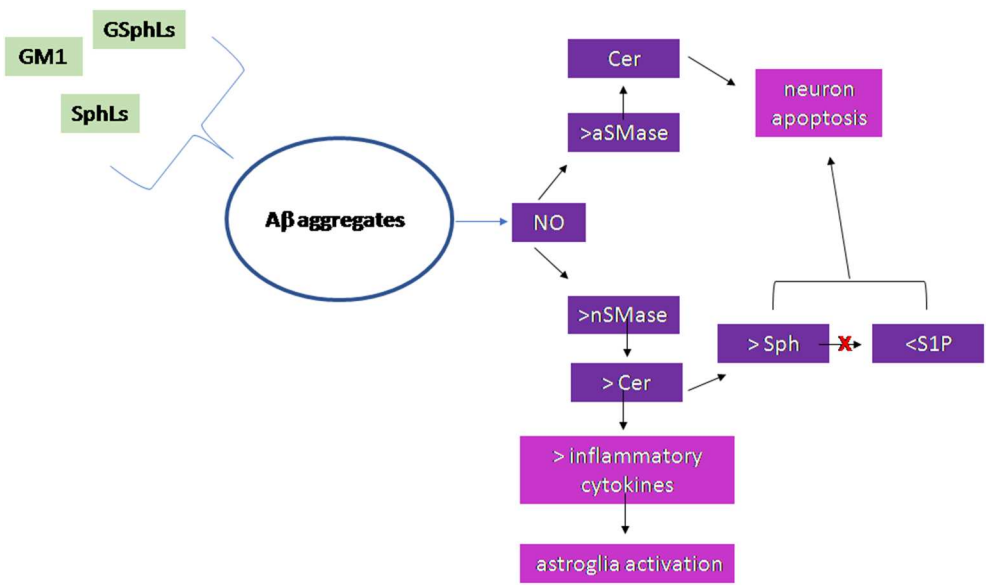

FIGURE 2 | Scheme of the sphingolipid involvement in Alzheimer's Disease. Glycosphingolipids (GSphLs), *ganglioside 1* (GM1), sphingolipids (SphLs) are involved in the formation of Aβ aggregates. Aggregates stimulate nitric oxide that induces activation of both acid sphingomyelinase (aSMase) and neutral sphingomyelinase (nSMase). The increase of aSMase activity is responsible for ceramide (Cer) production in lysosomes that induces neuron apoptosis. The increase of nSMase activity produces cell membrane Cer that either is able to stimulate the synthesis of inflammatory cytokines with consequent astroglia activation or is catabolized to sphingosine (Sph). The sphingosine kinase is inhibited and therefore sphingosine-1-phospahte (S1P) level is very low. Both the accumulation of Sph and the reduction of S1P are responsible for neuron apoptosis.

changes of plasma SphLs and cognitive impairments has been intensively studied in AD patients for predicting of the rate of the development of dementia (52, 53). Mielke et al. (55) studied the content of Cer, DHCer, SM and DHSM in plasma of 120 patients with AD dementia and dementias associated with other neuropathologies. During the period of more than 2 years of observation, authors found an increase in Cer and DHCer associated with rapidly progressive dementia. In patients characterized by increased levels of SM, DHSM, SM/Cer, and DHSM/DHCer ratio, dementia was slowly progressive. The authors also demonstrated that changes in CHO and triglyceride levels were not associated with the rate of dementia. This suggests that the increase in the ratio SM/Cer and DHSM/DHCer in blood of AD patients may be a predictive marker for the rate of the development of this disease. Similar results have been obtained during mass spectrometry analysis of plasma lipids in 26 AD patients and 26 elderly patients with normal cognitive functions (56). Among 33 SM species, the content of eight molecular species containing fatty acid aliphatic chains of 22 and 24 carbon atoms was significantly lower in AD patients compared with control. At the same time, the plasma levels of two Cers, C16:0 and C21:0, were significantly higher in AD patients, while the increase of other 5 Cers was not significant. The ratio of Cers/SMs containing identical fatty acids sharply differed in AD patients compared with normal controls. These changes reflected impairments of cognitive functions in AD patients. Therefore, SphL assay would be useful for monitoring the AD progression. The latter is especially important when patients are treated by novel drugs or novel drugs are under preclinical or early clinical trials.

Parkinson's Disease

PD is the second most common neurodegenerative disease, prevalent in men, without differences for ethnicity, that affects ∼1.5 to 2.0% of people over 60 years old and 4% of people over 80 years old (57). PD is due to the dopamine synthesizing neurons in the substantia nigra degeneration resulting in the decline of neurotransmitter dopamine level in the striatum with consequent movement disorders. The common manifestations are slowness and difficulty with dexterity, tremor, slow walk, unsteadiness and falls, low vocal volume, facial hypomimia (58). Prior to the movement disorders or emerging with motor disease progression may appear non-motor features as cognitive decline, including dementia, constipation, hyposmia/anosmia, depression, anxiety, and other neuropsychiatric features, sexual dysfunction, sleep complaints, urinary frequency, and orthostatic hypotension (58).

The principal cell characteristic of PD is the accumulation in neurons of α-synuclein (α-syn) protein aggregates that may be present in inclusion Lewy bodies (59). α-Syn is an acidic protein of low molecular weight (14 kDa), expressed in normal brain, peripheral nervous system and circulating erythrocytes (60). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin used to reproduce PD in animals, induces a strong expression of e-cadherin, and variation of length and thickness of the heavy neurofilaments (61).

Few observations on simple SphLs and more on complex SphLs as molecules involved in PD pathogenetic mechanisms have been reported.

Sphingolipids in the Brain

Neutral and acid sphingomyelinase

An upregulation of iNO synthase and a downregulation of nSMase protein expression in the hippocampus of mice in which PD was induced by MPTP have been demonstrated (62). Interestingly, the administration of MPTP in Toll-Like Receptor 4 knockout mice causes an increase in nSMase protein expression and enzyme activity in the midbrain with a decrease in SM and an increase in Cer levels, as well as a marked delocalization of the enzyme from the cell membranes, by suggesting a possible role of TLR4 in the change of SM metabolism during MPTP neurotoxicity (63). The authors demonstrate that exposure of Toll-Like Receptor 4-deficient mice to MPTP reduces unsaturated SM species by increasing saturated/unsaturated SM ratio. Since saturated fatty acid make SM a more rigid molecule in the membrane, the change of SM species might contribute to reduce neural plasticity (63). Different role has the aSMase. Interestingly, the well-known antidepressant desipramine, which is used in the clinic to treat depression in patients with Parkinson's Disease (PD), is an inhibitor of aSMase (64). Thus, it is necessary to look for new promising drugs for the treatment of PD among aSMase inhibitors that will block the toxic signals of pro-inflammatory cytokines, protecting dopaminergic neurons from death therefore will reduce cognitive impairment.

Ceramide, sphingosine, and sphingosine-1-phosphate

Disturbances in the Cer metabolism are associated with the appearance of Levy bodies (65). Interestingly, it has been reported by Paciotti et al. (66) that inhibition of aCerase by carmofur in *GBA*-PD derived dopaminergic neurons, resulted in lower levels of α-synuclein, possibly because of its enhanced degradation by Cer activated Cathepsin D. Unlike the proapoptotic SphLs, as Cer and Sph, S1P protects cells from apoptosis. In fact, the addition of exogenous S1P to the culture medium led to an increase in cell survival. Probably, it could perform the same function in PD. Inhibition of SphK causes an increase in synuclein secretion and activation of proapoptotic genes, such as Bcl2 family genes such as Bax (67). Thus, inhibition of the activity of SphK1 responsible for the synthesis of S1P, leads to apoptosis of neuronal cells simulating PD. It is believed that the lipid composition of lipid rafts, including GM, SM, CHO, Cers can strictly control the processing of α-syn and its aggregation (68). More detailed characteristics of rafts, identified at different stages of PD, could serve to diagnose and prevent the disease.

Glucosylceramides and galactosylceramides

Disorders of SphLs in the study of PD mechanisms has been associated with the development of symptoms of this disease in some types of Gaucher disease (GD) (69, 70).

The main trigger of PD is the presence of mutations in the GBA gene encoding the GCase enzyme, which cleaves GC to glucose and ceramide. The two most common mutations were identified in patients of all ethnic groups (71). Although some

representatives have a greater variety of mutations, reaching up to 8. In 2009, an unprecedented international study of mutations in the GBA gene was conducted in 16 centers from 12 countries, including 5,691 patients and 4,898 controls. The obtained data gave grounds to determine a direct relationship between mutations in GD and PD. The presence of mutations in the GBA gene is typical for both familial and idiopathic forms of PD. Since a close relationship between PD and mutations in the GBA gene has been reliably established, studies began in 2010 on the changes in SphLs that are the substrate of this enzyme, primarily GC. A decrease in the GCase activity leads to dysfunction of lysosome with the accumulation of GC inside, that is responsible for the stabilization of oligomeric aggregated toxic form of α-Syn with consequent neuronal cell death (72). Decreased activity of GCerase was found in the substantia nigra and frontal cortex of patients with PD compared to controls (73). Therefore, an increase in GCerase activity is currently being considered as a new therapeutic strategy in the treatment of synucleinopathies, including PD (74). Although most studies focused on the role of GCase in PD, it has been shown that not only GCase is involved in the pathogenesis of disease but also its metabolic products, such as GSph, which has a high toxic effect on neurons (75).

The authors showed that GSph content in GD is even higher than GCer. However, role of this metabolite has not been studied for PD yet. This study gives indications on GSph as potential more striking marker of PD.

From all studies above reported, it is evident that the changes of SphLs in the initial stages of PD are accompanied by an increased risk of developing the disease with consequent dementia complications. The correction of Sph specific specie levels by regulating the activity of enzymes involved in its metabolism can either slow down or prevent the development of pathology. Such studies will certainly make it possible to discover new targets among the enzymes of Sph metabolism and to create new drugs for the prevention and treatment of PD.

Figure 3 summarizes the results of the research work carried out on the brain.

Sphingolipids in Biofluids

Metabolic changes in the sphingolipid system in the initial stages of PD are accompanied by an increased risk of developing this disease with dementia complications, and the correction of the level of certain types of sphingolipids by regulating the activity of enzymes involved in their metabolism can either slow down or prevent the development of this pathology. Such studies will certainly make it possible to discover new targets among the enzymes of sphingolipid metabolism and create new drugs for the prevention and treatment of Parkinson's disease. Fingolimod (FTY720), which is a structural analog of sphingosine and interacts with the S1P1 receptor, can serve as such an example. Treatment with FTY720 attenuates motor deficit and prevents dopaminergic neuronal loss in two mouse models of PD (76).

Sphingolipids in the liquor

β-GCase activity in CSF is reduced in PD patients independent on GBA1 mutation (77).

Sphingolipids in the blood

In plasma Sph changes in PD patients are associated with Lewy body identified in the autopsy sample of the brain (74). Mielke et al. (78) demonstrated that in PD patients the plasma levels of total Cer, monohexosylceramides, and LCer and those of their molecular species, with fatty acid chain from 16 to 26

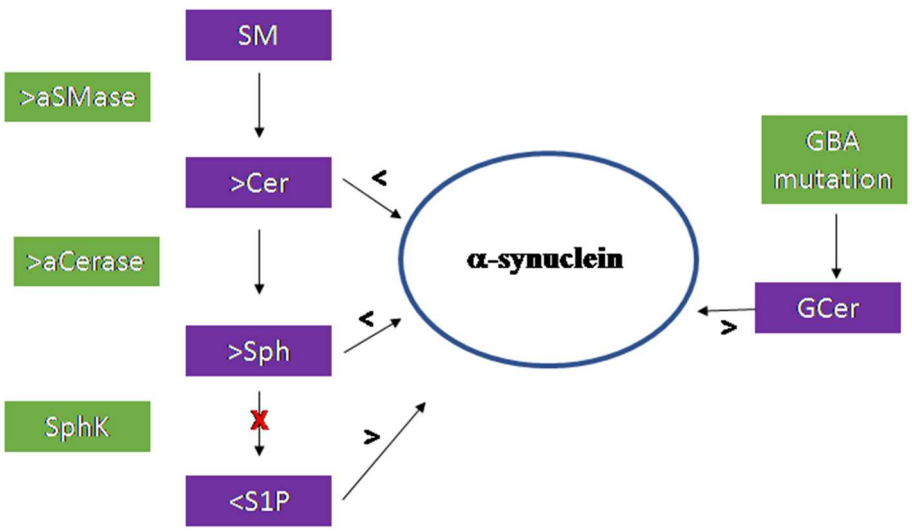

FIGURE 3 | Scheme of the sphingolipid involvement in Parkinson's Disease. Acid sphingomyelinase (aSMase) and acid ceramidase (aCerase), by producing ceramide (Cer) and sphingosine (Sph), respectively in lysosomes, are responsible for the reduction of α-synuclein production and aggregation. The inhibition of sphingosine kinase (SphK) reduces sphingosine-1-phospahte (S1P) level with consequent increase of α-synuclein. Mutation of the GBA gene encoding the glucocerebrosidase enzyme induce accumulation of glucosylceramide (GCer) that induces α-synuclein increase.

carbon atoms, are significantly higher than those of controls. It is important to know that variations of SphLs are specific because CHO and triglycerides in low and high density lipoproteins do not differ respect to the control. Moreover, Cer species change in association with cognitive impairment. In fact, C14:0 and C24:1 Cer levels are significantly higher in PD dementia than in PD with no cognitive impairment and normal controls. In addition, verbal memory is negatively correlated with C14:0 and C24:1, and C22:0, C20:0 and C18:0 are associated with hallucinations, anxiety and sleep behavior disturbances, respectively. In addition, elevated levels of GM3 (79). and ganglioside-NANA-3 (80) are present in the plasma PD patients.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disease characterized by selective degeneration of motor neurons in the spinal cord, motor cortex, and brain stem. Clinically, the disease is manifested as the muscular exhaustion, speech and swallowing impairments, fasciculation, and changes in reflexes and plasticity. The patients die 3 to 5 years after appearance of the first disease symptoms mainly because of the respiratory paralysis. The etiology of ∼90% registered ALS cases is unknown and this form is classified as idiopathic. The remaining 10% are hereditary ALS forms associated mainly with autosomal dominant mutations in particular genes (81). Gene mutations associated with ALS as SOD (82), VCP (83), OPTIN (84), and UBQLN2 (85) for protein responsible for conformational instability and aggregation of specific proteins, genes as C9ORF72 (86), TDP43 (87), and FUS (88, 89) for proteins useful for the impairments in the RNA processing and transport, and genes as PFN1 (1), DCTN1 (90), and TUBA4A (91, 92) for protein responsible for changes in the cytoskeleton dynamics have been described. The outstanding genetic variability of ALS explains its complexity, when different mechanisms result in similar disease pathogenesis. The key features of ALS development are excitotoxicity, oxidative stress, dysfunction of mitochondria (85), neuroinflammatory and immune reactions (86).

Recently, apoptosis has been intensely studied as one of possible mechanisms responsible for the degeneration of motor neurons in ALS (89). In this connection, much attention has been drawn to the studies on the disorders of lipid metabolism in ALS development. Both idiopathic and hereditary forms of ALS are accompanied by lipid metabolism disorders, the most frequent of which is hyperlipidemia (91, 92). One of the characteristic ALS symptoms observed in ∼66% patients is weight loss due to the hypermetabolism with disorder of lipid metabolism (91). Lipids perform the regulatory role by acting as secondary messengers in the inflammation processes during ALS development which are accompanied by the activation of microglia, loss of neuromuscular junctions, and subsequent degeneration of motor neurons. At the same time, the level of neurotoxic molecules synthesized with an active involvement of lipid messengers increases (93).

Sphingolipids in the Brain

Recently, it has been given special attention on the study of the involvement of SphLs in the pathogenesis of ALS in connection with the multiplicity of their functions in the structure and physiology of the brain. Changes in SphL species can be a manifestation of both ALS idiopathic and hereditary forms and affect the rate of disease development (91, 94).

Ceramides

Cutler et al. (95) showed that patients with idiopathic ALS and SOD1G93A transgenic mice that reproduces a model of hereditary ALS have high level of C16:0 and C24:0 Cer, and C16:0 SM in the lumbar spinal cord. Moreover, accumulation of C16:0 Cer in animals is evident even at the pre-asymptomatic stage of ALS. These changes are not present in the cervical spinal cord of transgenic mice, indicating the vulnerability of the lower motor neurons (96). The authors also showed that oxidative stress, which is an early event in the development of ALS can be changed by Cer.

In a subsequent study, Dodge et al. (97) demonstrated a significant increase in the total content of Cer and in its C18:0, C24:1, and C24:0 molecular species in samples of the gray and white matter of the cervical spinal cord of patients with idiopathic ALS. An increase in the Cer content is not associated with a decrease in the activity of enzymes that mediate its degradation which is typical for a group of diseases with impaired lysosomal metabolism (95).

The activity of enzymes responsible for the formation of Cer increases at different pH values. Glucocerebosidase 1 (GCBase1) activity increases at acidic pH values, GCBase2, and GalCerase activity increases at neutral pH values, which indicates the possibility of intensification of SphL hydrolysis both in lysosomes and in the plasma membrane of the cell (97). In the motor neurons of ALS patients, a predominant formation of Cer by catabolic pathways and not as a result of *de novo* synthesis was demonstrated (98). In opposition, it has been shown that apoptosis of motor neurons in SOD1G93A transgenic mice that reproduce ALS is accompanied by the generation of Cer due to the activation of the neutral SMase enzyme (98). The change in the content of Cer and other SphLs in the lumbar spinal cord of transgenic mice essentially depends on the stage of ALS. In the earlier stages of the disease, the level of C24:1 Cer and that of the most complex glycosphingolipids are lowered, and in the terminal stage of disease the level of C24:0 Cer shows an insignificant increase compared to the control (95). However, in the earlier stages of the disease, the level of C24:1 Cer and most complex glycosphingolipids is lowered.

Sphinganine, sphingosine, and sphingosine-1-phoshate

Sphingoid bases—sphinganin (Sphn) and Sph have bright proapoptotic properties. The proapoptotic effect is associated with the ability of these SphLs to interact with DNA, affect the activity of replication and transcription enzymes, the regulation of transcription factors and topoisomerases (47, 99, 100). SphK, that lead to the synthesis of anti-apoptotic S1P, reduces Sph pool thereby saving neuronal cells from death. A pronounced dysregulation in the metabolism of sphingoid bases, including

Sphn, Sph, and S1P, in transgenic FUS mice (1-359) simulating ASL, was demonstrated (101). The study provide evidence that Sphn and Sphincrease sharply mainly in the spinal cord of mice, while their content is low and practically does not change in brain structures during the development of ALS. The ratio of S1P/Sphn-Sph, decreases by indicating a sharp intensification of cell death in the spinal cord.

Significant disturbances were also found in the expression of SphL metabolism genes at different stages of ALS, mainly in the spinal cord. The gene expression of acylsphingosine amidohydrolase 1 (Asah1) and acid Cerase (aCerase), localized in lysosomes, increases while the expression level of Asah2 and neutral Cerase (nCerase), located on the surface of the plasma membrane, significantly decreases during the progression of FUS-mediated proteinopathy (101).

ASAH1 of lysosomes hydrolyzes saturated C10:0 and C14:0 Cer or unsaturated C18:1 and C18:2 Cer. Substrates of ASAH2 are C16:0 and C22:0 or C26:0 and C36:0 Cer. Thus, Sph is generated by lysosomal Cerase from Cer with shorter fatty acids. A change in the activity of lysosomal Cer may indicate the development of lysosomal apoptosis. The sharp increase in the expression of S1P lyase (S1PL) at the early stage of ALS is responsible for a decrease in anti-apoptotic reserves of motor neuron cells and the rapid development of apoptosis at the terminal stage of these disease of ALS, because S1PL degradates S1P that protects cells from apoptosis to the final products ethanolamine phosphate and hexadecenal.

Glucosylceramides and galactosylceramides

An analysis of the cervical homogenates of the post-mortal spinal cord of ALS patients shows an increased content of C18:0 and C24:1 GCer. Analysis of the spinal cord samples of SOD1G93A transegnic mice isolated for different periods of the disease also shows an increase in the content of the C24:1 GCer at the terminal stage of ALS (102). Henriques et al. (103) found significant changes in the composition of GCer already at the pre-symptomatic stage of ALS, and not only in the central nervous system, but also in the muscles of transgenic animals. While the levels of most GCer studied by the authors are reduced in the spinal cord of pre-symptomatic and symptomatic mice, the levels of many of GCer species are increased in muscles at the same stages of ALS.

The content of GCer and, accordingly, the first stage of biosynthesis of complex glycosphingolipids is controlled by glucosylceramide synthase (GCerS), a transmembrane protein of the Golgi complex. The level of expression of GCerS mRNA was significantly increased in muscles at the asymptomatic and symptomatic stage of ALS in SOD1G86R transgenic mice. In the spinal cord, the level of GCerS mRNA does not change, as there are no changes in the GCer content. An increase in GCerS activity, with formation of GluCer, is a known negative regulator of apoptosis Cer-induced (104) by indicating the possibility of a protective role of GluCer in ALS. The content of the C24:1 galactosylceramide (GalCer) form is increased in the white matter of the cervical spinal cord of patients with sporadic ALS.

Lactosylceramides

The formation of C18:0 lactosylceramide (LacCer) due to lactosylceramide (LacCerS) is observed in the spinal cord at the terminal stage of the disease in patients with ALS. This occurs against the background of increased activity of α-galactosidases, enzyme for LacCer synthesis, both at acidic and neutral pH values (105). The formation of LacCer can contribute to the development of the disease, since it is a mediator of inflammation and apoptosis (106) and activates microglia via the NF-κB signaling pathway (107), which is involved in the process of motor neuron death in ALS.

Moreover, LacCer activates microglia along the NF-κB signaling pathway (107), which is involved in the process of motor neuron death in ALS (104).

Gangliosides

Indications for deviations in GM homeostasis in ALS appeared in the literature at the end of the last century (108). Increased levels of single GM (109) due to ganglioside synthase (GMS), GM2 in the motor cortex (110), antibodies against GM2 and GM1 (111), were found in patients with ALS. Yim et al. (112) demonstrated that the total content of the neurotrophic GM3 is significantly increased in SOD1G93A transgenic mice, in relation to the course of disease. In support, a lipidomic study showed an increase of C18:0 and C24:1 molecular species of GCer (79), At the symptomatic stage of ALS in SOD1G86R mice, there is a significant increase in the content of GM1a in the spinal cord, and in the content of GM3 and GM2 in the muscles (113, 114). In the same animals, the amount of hexosaminidase mRNA, an enzyme that metabolizes GM2 to GM3, is increased in the motor neurons of the spinal cord, both at the asymptomatic stage and at the symptomatic stage of the disease (115). Also, in the spinal cord of mice SOD1G93A and patients with idiopathic form of ALS, hexosaminidase activity increases (116).

Figure 4 summarizes the results of the research work carried out on the brain.

CONCLUSIONS

Despite many studies in the field, the exact pathogenetic mechanisms of NDD are poorly understood yet. So far, there are no efficient approaches for the NDD treatment. However, the latest studies have established that degeneration occurs via apoptotic death. Apoptosis is regulated by various interconnected pathways that eventually result in the programmed cell death. In addition to the genetic regulation, apoptosis is controlled by free radicals, death receptors, caspases, proapoptotic proteins of the Bcl2 family, inhibitors of apoptosis proteins, tumor suppressor protein p53, tumor necrosis factor-α, and many other apoptosis associated molecules. Apoptosis can be initiated by the damage to DNA, mitochondria, and lysosomal membranes. Deeper understanding of biological pathways regulating metabolism of proapoptotic and antiapoptotic SphLs in NDD disease can help to clarify pathogenetic mechanisms. Studies of SphL metabolism in the experimental animals in which NDD are induced, in the brain structure from autopsy sampling and in CSF, plasma and serum from patients affected with NDD are of great interest today.

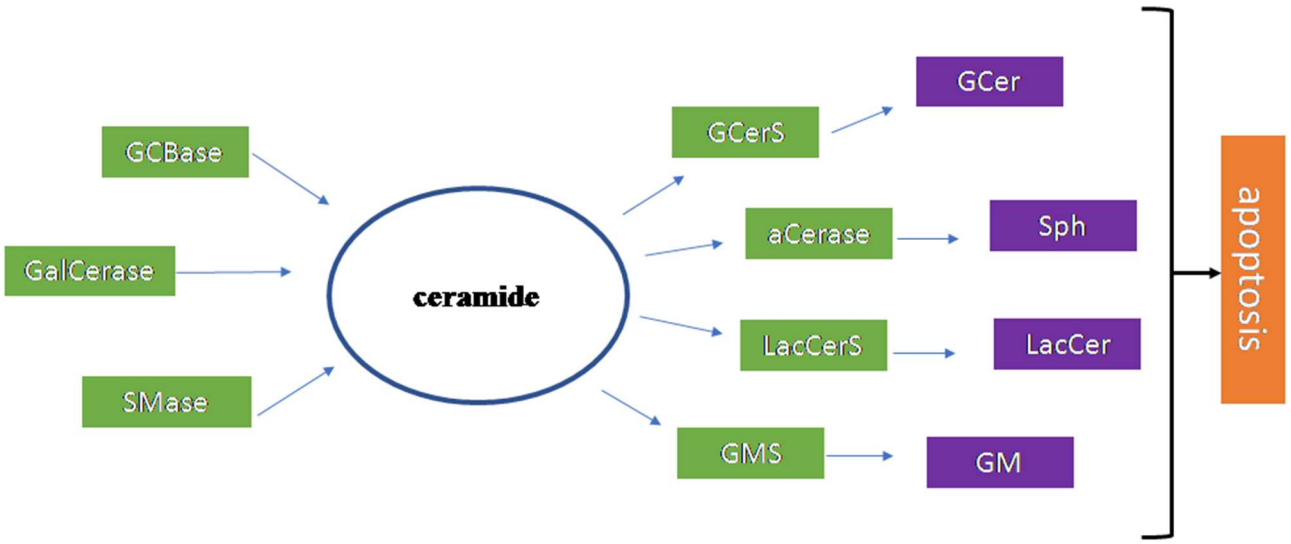

FIGURE 4 | Scheme of the sphingolipid involvement in Amyotrophic Lateral Sclerosis. The ceramide (Cer) is the most important sphingolipid in Amyotrophic Lateral Sclerosis. It is produced by glucocerebrosidase (GCBase), galactosylceramidase (GalCerase), sphingomyelinase (SMase) and it is used by glucosylceramide synthase (GCerS), acid ceramidase (aCerase), lactosylceramide synthase (LacCerS), ganglioside synthase (GMS) to form glucosylceramide (GCer), sphingosine (Sph), lactosylceramide (LacCer), *ganglioside* (GM), respectively. These molecules are all involved in apoptosis that occurs in disease.

Recent studies have shown that SphLs play a decisive role in the neuronal function due to regulation of cell growth, differentiation and death in the CNS. Importantly, aSMase activation, causing accumulation of Cer proapoptotic agent, may be considered as a novel mechanism of the development of NDD as well as accumulation of S1P antiapoptotic agent may be protective for the onset and/or development of diseases. Relevantly, De Wit et al. (117) demonstrated that the in frontotemporal lobar dementia ceramide production in reactive astrocytes is independent of enhanced levels of aSMase but is due to an increase in the expression of CerS. It was observed also in AD (118). In this context it is very important to investigate changes in the SphL profile in the brain of animals with NDD-induced, and in CFS and blood of NDD patients during the course of this disease and its treatment. Analyzing various blood biomarkers in different neuropathologies, a reasonable question arises: does direct correlation between changes in the blood and loss of brain structures exist? It is possible that blood changes may be associated with those of the brain via circulation system and selective permeability of the blood brain barrier? In this connection, an accurate and informative method of analysis of the lipid profile such as mass spectrometry is of special importance. Using this technique, it is possible to analyze rapidly and accurately various lipid components in numerous biological samples using minimal quantities of biological material. Thus, it was found that SphL species might be diagnostic markers of the early stage of NDD and might be very useful to follow the development of disease.

Addictionally, SphL species could be interesting as new targets for innovative therapeutic strategies. An example of such promising pharmaceutical preparation for the ALS treatment is the Fingolimod, an immunomodulatory drug binding the S1P receptors 1, 3, 4, and 5 and playing the antiapoptotic role of the S1P (119).

In conclusion, in this review we described the association between the SL metabolism disorders and the neurodegenerative disease pathogenesis. We reported relevant studies indicating remarkable roles of different SLs in maintaining neuronal health or in inducing cell death. Despite many observations about the possible application of SL pathway molecules in diagnostic and therapy, further studies are still needed to better elucidate the specificity of each SL in the development and/or progression to different stages of neurodegenerative disorders.

AUTHOR CONTRIBUTIONS

AA and EA equally participated in the writing and revision of the review.

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