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Ceramide and neurodegeneration: Susceptibility of neurons and oligodendrocytes to cell damage and death

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Abstract

Neurodegenerative disorders are marked by extensive neuronal apoptosis and gliosis. Although several apoptosis-inducing agents have been described, understanding of the regulatory mechanisms underlying modes of cell death is incomplete. A major breakthrough in delineation of the mechanism of cell death came from elucidation of the sphingomyelin (SM)-ceramide pathway that has received worldwide attention in recent years. The SM pathway induces apoptosis, differentiation, proliferation, and growth arrest depending upon cell and receptor types, and on downstream targets. Sphingomyelin, a plasma membrane constituent, is abundant in mammalian nervous system, and ceramide, its primary catabolic product released by activation of either neutral or acidic sphingomyelinase, serves as a potential lipid second messenger or mediator molecule modulating diverse cellular signaling pathways. Neutral sphingomyelinase (NSMase) is a key enzyme in the regulated activation of the SM cycle and is particularly sensitive to oxidative stress. In a context of increasing clarification of the mechanisms of neurodegeneration, we thought that it would be useful to review details of recent findings that we and others have made concerning different pro-apoptotic neurotoxins including proinflammatory cytokines, hypoxia-induced SM hydrolysis and ceramide production that induce cell death in human primary neurons and primary oligodendrocytes: redox sensitive events. What has and is emerging is a vista of therapeutically important ceramide regulation affecting a variety of different neurodegenerative and neuroinflammatory disorders.

Keywords

Sphingomyelinases; Ceramide; Signal transduction; Neuronal apoptosis; Oligodendroglial death; Glial activation; Neurodegenerative disorders

Introduction

Neurodegenerative and neuroinflammatory diseases encompass devastating disorders of the central nervous system (CNS) that often strike from age 30 onwards limiting and disabling those in the prime of life. Clinically these diseases are characterized by dementia, disordered movement and gait, ataxia, sleep disorder, and behavioral and psychiatric disturbance [1–3]. Pathologically they are characterized by inflammation, gliosis, axonal degeneration and

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neuronal apoptosis [1–3] and for multiple sclerosis (MS) demyelination. Environmental factors, viruses, genetic mutation, and activation of non-neuronal cells are predisposing influences [1–3]. In some neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and frontotemporal dementia (FTD), aggregated misfolded proteins contribute to the neural pathogenesis, while in MS, autoimmune mechanisms accompany the demyelination and in HIV-associated dementia (HAD), viral products are crucial for neuronal demise [1–4]. In the context of numerous extrinsic and intrinsic factors affecting neurodegeneration and differing among diseases, a shared biochemical cascade of events playing a role in neuronal damage and attrition assumes great importance for understanding of pathogenesis and potential for intervention. Central to this cascade is increased oxidative stress and activation of an evolutionary conserved signaling pathway: the SM-ceramide pathway [5–8]. Here, we review causes of neuronal and oligodendroglial death during neurodegenerative and related/overlapping neuroinflammatory diseases pertaining to ceramide signaling pathways and highlighting neutral sphingomyelinase (N-SMase) as the major player and a potential target to curtail neurodegeneration.

2. Background

2.1. Historical perspectives

Sphingomyelin (SM), a major class of membrane phospholipids, was first isolated from the brain by Thudichum in 1884 though little was known of its metabolism until recently. SM was originally believed to be synthesized enzymatically by transfer of phosphocholine from cytidine 5'-diphosphate choline (CDP-choline) to the free primary hydroxyl group of ceramide [9]. This pathway has not been subsequently established as the major one, but rather the majority of SM is now known to be synthesized by transfer of phosphocholine from phosphatidylcholine to ceramide via sphingomyelin synthase [10]; an enzyme localized in Golgi and plasma membrane. SMs are amphiphiles abundant in mammalian nervous system that were believed to be inert membrane structural components, but were recognized by Hannun and Bell [11] to be involved in diverse intracellular signaling pathways in a variety of and perhaps all cells [12–14]. The SM pathway is triggered by a diverse range of endogenous and exogenous stimulants including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), FAS ligands (FAS L), anticancer drugs, glucocorticoids, antibody cross-linking, heat shock, ionizing and ultraviolet radiation, serum deprivation, and infection by viruses and bacteria [12,13,15]. A transient hydrolysis of sphingomyelin, by specific phospholipase C enzymes, known as sphingomyelinases, with concomitant generation of ceramide has been observed in response to these apoptosis-inducing stimuli. The ceramide formed serves as a second messenger or modulator of diverse signaling pathways in the cell affecting cell behaviors ranging from proliferation and differentiation to cell cycle arrest and apoptosis [16]. The decision to enter these disparate pathways determining diverse neurobiological outcomes depends on levels and molecular species of ceramide formed, cell types and age, expression of specific receptors, and selective targets coupled to specific signaling pathways.

2.2. Role of ceramide in CNS physiology

2.2.1. Lipid rafts (specialized membrane microdomains)—In the outer plasma membrane leaflet of many cell types, including neurons and glia, microdomains called lipid rafts are found that are implicated in a variety of cellular processes including cytoskeletal organization, membrane trafficking and signal transduction [17–19]. Ceramide associates with cholesterol and other sphingolipids and mediates lipid raft organization into larger platforms [20,21]. The distribution of lipid rafts over cell membrane depends upon the cell type. In neurons, lipid rafts accumulate on somal and axonal membranes to a greater extent than on somato-dendritic membranes. Lipid rafts are also found in the post-synaptic sites of neurons [22]. In neurons, rafts play an important role in neuronal adhesion, and in the modulation of

neuronal ion channels and neurotransmitter receptors. Rafts may modulate neurotransmitter release since it has essential components of the vesicular exocytic machinery such as syntaxin, synaptosomal-associated protein-25 (SNAP-25) and synaptobrevin [23]. In oligodendrocytes, rafts mediate the interaction between myelin-associated glycoprotein on myelin, and its receptor on neurons [24].

Raft lipid and protein composition differs in cell types. For example, oligodendrocyte raft composition differs from the neuronal raft in containing galactocerebroside (GalC), sulphatide and proteolipid protein. Raft composition also depends on neuronal developmental stage. Aging neurons contain more raft ceramide in association with members of the Src family protein tyrosine kinases, known to be involved in neuronal differentiation [25]. Aberrant organization of sphingomyelin or cholesterol in rafts has been linked to loss of synapse or changes in nerve conduction [26], and depletion of sphingolipid or cholesterol leads to instability of AMPA receptors and to the gradual loss of synapses (both inhibitory and excitatory) and dendritic spines [26].

2.2.2. Differentiation—Galactolipids such as galactocerebroside (GalC) and sulphatide play functional roles in the regulation of oligodendrocyte (OL) differentiation and myelination [27]. OL differentiation is a critical event during brain development culminating in elaboration of the myelin sheath, and impaired myelination leads to serious neurological disorders; mutation or myelin gene deletion has been extensively used to examine this [28,29]. Another approach has been to reversibly inhibit OL progenitor cell differentiation by a specific monoclonal anti-glycolipid antibody that perturbs myelination: following antibody removal, oligodendrocytes re-differentiate [30].

2.2.3. Neuronal development—Ceramide plays an indispensable role in neuronal development and this action is dependent upon neuronal developmental stage, stimulus type, and ceramide species and concentration. In the first stage of neuronal development, ceramide enhances the formation of minor processes from lamellipodia [31]. Decreasing ceramide content by ISP-1, a specific inhibitor of serine palmitoyltransferase, decreased cell survival and provoked aberrant Purkinje cell dendritic differentiation. Addition of cell-permeable ceramide, sphingosine, or sphingomyelin reversed the effects of ISP-1 treatment in Purkinje cells [32]. And, during axonal development, glycosylated ceramide, glucosylceramide, is essential for normal and for accelerated growth to support intracellular transport [31], while inhibition of ceramide synthesis with fumonisin B1 (FB1), or of glucosylceramide synthesis with 2-decanoylamino-3-morpholino-1-propanol (PDMP) produces a shorter axon plexus and fewer axonal branches of hippocampus neurons in culture. These inhibitors have no effect on axonal morphology during the first 2 days in culture, but cause branch retraction by day 3 [33].

The molecular mechanisms by which ceramide accelerates changes in the cytoskeleton and hence process formation is not known. It may be that ceramide alters cytoskeletal organization by stimulating actin stress fiber formation and focal adhesion assembly as a result of tyrosine phosphorylation of p125 focal adhesion kinase (p125^{FAK}) and paxillin [34]. At both of these stages, ceramide at high concentration induces apoptotic cell death [31] while low ceramide concentrations obtained by pharmacological inhibition of sphingomyelinase or employing the acidic sphingomyelinase knockout were protective. Whether ceramide induces axonal development or apoptosis also depends upon the expression status of neurotrophin receptors. Low concentrations of ceramide generated by p75 neurotrophin receptor (p75^{NTR}) activation in cultured hippocampal pyramidal neurons stimulated their growth [35].

2.3. Role of ceramide in CNS pathophysiology

2.3.1. Apoptosis—Apoptosis is physiologically essential for the elimination of damaged or excess cells during development [4], while pathological triggering of unregulated apoptosis leads to the development of several adult neurological disorders including AD, PD, amyotrophic lateral sclerosis (ALS), cerebral ischemia, and probably demyelinating diseases including MS: all of which are characterized by the loss of specific populations of neurons [6] and for MS of oligodendrocytes. Several studies have implicated ceramide in the apoptosis of neuronal and oligodendroglial cells in the neuroinflammatory and neurodegenerative disorders as shown below.

a) Alzheimer's disease⁴ (AD): AD is a major illness of dementia characterized histologically by the presence of amyloid plaques, neurofibrillary tangles, and extensive neuronal apoptosis. Accumulating evidence indicates elevated levels of ceramide at the very earliest clinical stage of the disease, and levels are elevated more than three-fold when compared with age-matched control [36]. Subsequent studies showed that fibrillar A β injection in mouse increased ceramide levels in hippocampus and cortex after 7 days following injection [37] and that elevated ceramide levels increased the half-life of BACE1 and promoted A β biogenesis. Exogenous C6 ceramide as well as increased levels of endogenous ceramide induced by sphingomyelinase treatment promoted biogenesis of A β . C6 ceramide also restored A β generation in FB1 treated cells [38].

b) HIV-associated dementia (HAD): Human immunodeficiency virus type 1 (HIV-1) infection is known to cause CNS disorders, including HAD. Sphingolipid imbalance plays an important role in neuronal dysfunction and death in HAD. Brain tissues and CSF from patients with HAD evidenced increased oxidative stress with abnormal accumulation of sphingomyelin and ceramide [39]. In a separate study, we have shown that HIV-1 coat protein gp120 (glycoprotein 120) induced neuronal apoptosis in the HAD CNS through the CXCR4-NADPH oxidase-superoxide-NSMase-ceramide pathway.

c) Multiple sclerosis (MS): It is the most common human CNS demyelinating disease, and a disorder in which oxidative stress is proposed to play an important role in oligodendroglial death though molecular mechanisms that couple oxidative stress to the oligodendrocyte losses are poorly understood. Our MS studies have shown that the neutral sphingomyelinase–ceramide pathway is involved in mediating oxidative stress-induced apoptosis and cell death in human primary oligodendrocytes [40]. In a separate study, Lee et al. found that exogenously added bacterial sphingomyelinase exacerbated A β -induced oligodendrocyte death via an oxidative mechanism [8].

d) Amyotrophic lateral sclerosis (ALS) and Stroke: ALS is a progressive neurodegenerative disease characterized by degeneration of motor neurons in the spinal cord and producing progressive paralysis and death. Abnormal buildup of sphingomyelin, ceramide and cholesterol esters has been observed in ALS, and in the mouse model of ALS (Cu/ZnSOD mutant mice) [7], and this abnormal lipid accumulations occurs in transgenic mice prior to any sign of cell death. Pharmacological blockage of sphingolipid synthesis and ceramide accumulation could suppress neuronal death via various inducers of cell death including oxidative stress [7]. In another study, motor neurons over-expressing the ALS-linked SOD1^{G93A} mutation showed greater susceptibility to the p75^{NTR}-activated apoptotic pathway that is associated with decreased antioxidant defenses and increased neutral sphingomyelinase activation. This apoptotic pathway is critically modulated by nuclear factor erythroid 2-related factor 2 (Nrf2) activity [41]. In cerebral ischemia *in vivo*, increased ceramide levels have been attributed to down-regulation of glucosylceramide synthase [42–44]. It has also been reported that transient focal cerebral ischemia induces large increases in A-SMase activity, ceramide levels, and

production of inflammatory cytokines in wild-type mice, but not in mice lacking A-SMase [42]. The production of inflammatory cytokines is largely abolished in wild type mice by administration of A-SMase inhibitor.

e) Batten disease: Batten disease is a rare and fatal inherited disorder of the CNS that begins in childhood. This neurodegenerative disease is characterized by blindness, seizures, cognitive decline, and early death. Puranam et al [45] have reported an increased brain ceramide level in different Batten disease types.

2.3.2. Aging—Aging is accompanied by a progressive increase of brain ceramide content [5]. During aging, alterations in membrane sphingomyelin-cholesterol levels play a critical role in the susceptibility of neuronal cells to oxidative stress including upregulation of N-SMase activity with a significant brain-region-specific alteration in N-SMase activity [46]. Consequently, membrane fractions isolated from the striatum and hippocampus of aging rats were significantly enriched in N-SMase [46]. Additionally, aging induces a switch in the expression of neurotrophin receptors with differential regulation of the processing of APP in AD. The Trk receptor kinase A (TrkA) reduces whereas p75^{NTR} stimulates β -cleavage of APP; an effect that is suppressed in p75^{NTR} knockout mice. In normal mice this signaling pathway downstream of p75^{NTR}, and ceramide generation can be blocked by both caloric restriction and N-SMase inhibitors [47]. Derangement of sphingomyelin metabolism in combination with oxidative stress causes synaptic dysfunction and neurodegeneration [5]. Increased levels of ceramide during aging may also promote inflammation (Cutler et al., 2004). Furthermore, a direct correlation is seen between intracellular accumulation of ceramide and endocytic abnormalities suggesting that the endosomal/lysosomal abnormalities observed in degenerating neurons during pathological aging, and in age-related neurological disorders may be due to aberrant sphingomyelin metabolism [48]. A chronic increase of intracellular ceramide during aging might inhibit axonal elongation and reduce receptor-mediated internalization of nerve growth factor (NGF) [49]. Elevated ceramide levels also reduce receptor-mediated internalization of lipoprotein-associated cholesterol that is involved in regulation of synaptogenesis [49,50]; an effect that may account for the age-dependent reduction in the plasticity of the synaptic circuitry.

2.4. Transduction of apoptotic signaling by ceramide

2.4.1. Breakdown of mitochondria—A growing body of evidence implicates changes at the mitochondria and release of apoptotic inducing factors early in apoptosis. Mitochondria in cells undergoing apoptosis show enhanced production of oxy-radicals, opening of pores and cytochrome *c* release. These events are critical to the cell death process because agents such as manganese superoxide dismutase (MnSOD) and cyclosporine A that suppress mitochondrial oxidative stress state and pore formation prevent neuronal death [51]. Mitochondria contain ceramide, derived either from SM during apoptosis or from endoplasmic reticulum (ER) via intimate membrane contact. The numerous indications of links between ceramide elevation and mitochondrial apoptosis include the following. Firstly, in isolated mitochondria, the ceramide level is increased leading to apoptosis in response to CD95/Fas, TNF α , and radiation [52] and, C2-ceramide induces cytochrome *c* release when added to isolated mitochondria [53] or cultured neural cells [54]. Secondly, bacterial sphingomyelinase that is targeted to mitochondria induces cytochrome *c* release and apoptosis. Exposure of CGC to cell-permeable C2-ceramide markedly alters mitochondrial integrity with marked decrease in mitochondrial membrane potential associated with a massive release of cytochrome *c* [55]. The released cytochrome *c* is then capable of binding to the apoptotic protease activating factor-1 (APAF-1), and this complex activates the caspase cascade for which there is substantial evidence for implication in ceramide-induced apoptosis [54]. In cortical neurons, ceramide treatment leads to Akt dephosphorylation, mitochondrial depolarization and permeabilization, cytochrome *c*

release and activation of caspase-3 [54]. Bongkreik acid, an inhibitor of mitochondrial depolarization, inhibits the hallmark manifestations of apoptosis including chromatin condensation and PARP cleavage, thereby significantly reducing ceramide-induced neuronal apoptosis. Furthermore, co-administration of the selective caspase-3 inhibitor z-DEVD-fmk or caspase-9 inhibitor z-LEHD-fmk significantly reduces C2-ceramide-induced death of cultured rat cortical neuronal cells [56]. In contrast, C2-ceramide does not induce release of cytochrome *c* and caspase cascade in glioma cells, but does activate the Ca^{2+} -activated calpain proteases [57].

Ceramide directly affects the generation of reactive oxygen species (ROS) from mitochondria. Activation of the apoptogenic sphingomyelin-dependent signaling pathway in differentiated neuron-like pheochromocytoma (PC12) cells by cell permeable C2-ceramide produces a transient ROS generation, the site of which has been identified as complex I of the mitochondrial electron transport chain [58]. Further studies of these rat PC12 cells have revealed that C2-ceramide-induced free radical production is accompanied by increased mitochondrial free calcium concentrations producing swelling and rupture (Gueyffier et al., 1998). The ceramide-induced cell death via increase in the mitochondrial free calcium could be prevented by buffering of mitochondrial calcium with the calcium binding protein calbindinD-28K ectopically expressed in mitochondria [59].

Permeabilization of mitochondrial membrane is controlled by pro-apoptotic members of the Bax/Bcl2 family. C2-ceramide increases the Bax level leading to formation of Bax homodimers, mitochondrial permeabilization and neuronal death [55]. Ceramide-derived glycosphingolipids (gangliosides) induce apoptosis in neurons treated with staurosporine [60], and gangliosides either alone or in concert with pro-apoptotic Bcl2 family members such as t-Bid and Bax affect mitochondrial permeability transition pores releasing cytochrome *c*. Suppression of GD3 synthase expression by antisense oligodeoxynucleotides inhibits this neuronal apoptosis.

2.4.2. Activation of death-associated kinase (DAP kinase)—Death-associated protein kinase (DAP-kinase), a novel Ca^{2+} /calmodulin-dependent serine/threonine kinase, acts as a positive mediator of apoptosis for several intra- and extracellular stress signals [61]. The apoptotic function of DAP-kinase is thought to be modulated through its several functional domains, including the death domain and a serine-rich C terminus tail site of auto-phosphorylation [62]. By Northern blot and *in situ* hybridization analysis, DAP-kinase is found to be abundantly expressed in the brain [63]. In developing rat brain, DAP-kinase mRNA is abundant within the proliferative and post-mitotic regions of the cerebral cortex, in the developing hippocampus, and in cerebellar Purkinje cells [63]. The spatial distribution of DAP-kinase expression suggests that it regulates various neuronal functions, including neuron cell death. One example of the possible relationship between DAP-kinase and neuronal cell death via apoptosis and necrosis is indicated by the gradual increase in expression of DAP-kinase mRNA in cerebral cortex at 1–24hr after transient forebrain ischemia. A relationship between ceramide and DAP-kinase activation has been reported in several studies. In PC12 cells, the death-promoting function of DAP-kinase was enhanced in response to ceramide exposure with activity proportional to ceramide concentration [64]. Phosphorylation of serine 308, residing within the calmodulin (CaM) regulatory segment, of DAP-kinase restrained pro-apoptotic functions thereby serving to block this in growing cells [62,64]. Triggering death signals by ceramide removes the restrictions imposed by autophosphorylation of serine 308 suggesting that it becomes a target of regulation when the proapoptotic function of DAPK is active, and ceramide-activated phosphatase may be involved in the dephosphorylation of serine 308. Furthermore, over-expression of wild-type DAP-kinase enhanced the cells' sensitivity to ceramide treatment [64]. In contrast, a catalytically inactive DAP-kinase mutant rescued the cells from ceramide-induced apoptosis [64]. A study of hippocampus neurons derived from

DAP-kinase deficient mice found them significantly less sensitive to ceramide-induced apoptosis and to high concentrations of NGF-generated ceramide [65], while a peptide corresponding to the last 17 amino acids at the C-terminus of DAP-kinase protected the wild type neurons from ceramide-induced death as well as from death induced by addition of exogenous bacterial neutral sphingomyelinase [65]. All the above findings suggest that DAP-kinase is a target of ceramide-induced cell death (Fig. 1). This pathway may not be the only one involved in neuronal death since it has been found that there is not complete resistance to ceramide-induced cell death in DAP kinase $-/-$ neurons [65].

2.4.3. Activation of ceramide-activated protein phosphatase (CAPP)—One proximal target for ceramide is the heterotrimeric cytosolic serine/threonine (class2A) phosphoprotein phosphatase CAPP (Fig. 1) that was found to be activated *in vitro* [66,67]. This effect is also operative *in vivo* [67]. Of the three subunits of protein phosphatase, the 2A (PP2A) catalytic subunit was found to contain the putative lipid binding site, and was found to be essential for its activation [68]. The catalytic subunits of PP2A belong to a gene family that includes the catalytic subunits of phosphatase1 (PP-1) and calcineurin (PP-2B). The regulatory subunits modulate the responses of ceramide *in vivo* either directly by controlling the specificity of the enzyme for different forms of ceramide [66] or controlling the substrate specificity of the enzyme; that is, which substrates are dephosphorylated in response to an increased ceramide level.

The Akt pathway for example is down-regulated in NGF-treated PC12 cells by the cell permeable C2-ceramide as a result of enhanced dephosphorylation of Akt1 at residues T308 and S473 [69]. Interestingly, okadaic acid pretreatment rendered NGF-treated cells insensitive to C2-ceramide mediated down-regulation of Akt1 activity, suggesting that this inhibition was a consequence of the activation of dephosphorylation by CAPP. Inactivation of Akt in cortical neurons is followed by dephosphorylation of proapoptotic regulators such as BAD (proapoptotic Bcl-2 family member), forkhead family transcription factors (FKHR), and glycogen synthase kinase 3-beta (GSK-3 β) [54]. On the other hand, N-myristylated-Akt1 conferred resistance to apoptosis induced by C2-ceramide in NGF-treated PC12 cells. This differential activity of CAPP towards Akt stems from the CAPP cytosolic location [69]. The downstream target of protein kinase B (PKB/Akt), i.e. Bcl-2, is affected by ceramide-induced dephosphorylation by loss of anti-apoptotic potency, and this was further corroborated by the use of the S70E Bcl-2 mutant, in which a serine 70 was mutated to glutamate that mimicked a phosphorylation charge. The mutant efficiently suppresses ceramide-induced apoptosis [70]. And, the *in vitro* treatment of Akt with partially purified PP2A phosphatase reduced its intrinsic kinase activity. Prolonged exposure of cultured hippocampal neurons to sphingosine-1-phosphate (S1P) triggered apoptosis through PP2A activation [71]. S1P, a metabolite of ceramide, may either act as is or be converted to ceramide via the combined actions of S1P-phosphatase and ceramide synthase and execute the apoptotic program. S1P-induced apoptosis releases calcium from inositol triphosphate (IP3)-sensitive internal stores and this (alterations in Ca^{2+} homeostasis) activates calcineurin [71]. Calcineurin in turn dephosphorylates several cellular substrates, including a repressor for PP2A with resultant PP2A activation. One of the targets for PP2A is the c-Fos-containing AP-1 complex. Dephosphorylation of these c-Fos-containing AP-1 complexes increases its binding to, and increased the expression of, death genes containing the TGAGTCA-type enhancer sequences, whose products execute the apoptotic process. Neutralizing antibodies specific for either c-Fos or c-Jun, as well as over-expression of a dominant negative c-Jun mutant, have been reported to be protective against apoptosis whereas enhanced expression of c-Fos leads to apoptosis [72–74]. Another target of CAPP is the retinoblastoma gene (RB) product. Protein phosphatase1 is involved in the ceramide dependent dephosphorylation of the transcriptional regulator RB [75,76], and dephosphorylation of RB and subsequent sequestration by E2F results in cell cycle arrest [76,77].

2.4.4. Activation of stress kinases—Ceramide targets c-Jun N-terminal kinases (JNK) also known as stress-activated protein kinase (SAPK) [78], and this pathway mediates cell death in neurodegenerative diseases [79–82]. There are three JNK genes encoding distinct JNK protein kinases (Davis RJ, 2000). Of these, JNK3 is largely restricted to neurons and JNK1 and JNK2 are ubiquitously expressed [83]. Targeted disruption of the neural-specific *jnk3* gene renders neurons resistant to glutamate excitotoxicity [84].

A correlation of increased ceramide level and JNK activation is reported in serum-deprived neuronally differentiated PC-12 cells [85], and JNK activation and nuclear translocation in neurons have been reported *in vivo* in several different pathological conditions [86]. One potential target of pro-apoptotic signaling by JNK is the transcription factor c-Jun, which is modulated at both transcriptional and post-transcriptional level [87]. An increase in c-Jun phosphorylation was found in neuronal nuclei after ceramide treatment [88], and transient transfections with a dominant negative form of c-Jun partially protected cortical neurons from ceramide-induced apoptosis [88]. Oxidative stress-induced neurodegeneration appears to occur via the JNK pathway and steps of c-jun phosphorylation, mitochondrial cytochrome *c* release, BAX induction, caspase activation and finally neuronal apoptosis [89]. Protection in the dominant negative form of c-Jun is partial suggesting that other pathways and notably p38 MAPK (mitogen-activated protein kinase) cooperate with JNK in inducing neuronal apoptosis. Support for this includes the finding that treatment of dominant-negative c-Jun-expressing cortical neurons with the pharmacological inhibitor of p38 kinase, SB203580 completely blocks ceramide-induced neuronal death [88]. Furthermore, ceramide generated by binding of NGF to p75^{NTR} induced cell death in hippocampal neurons through JNK activation [90]. In addition, in primary cortical neurons, ceramide inactivated the extracellular signal-regulated kinase (ERK) pathway probably through activation of the ceramide-activated protein kinase (CAPK), the protein kinase c ζ (PKC ζ) or the CAPP and simultaneously triggered JNK and p38 MAPK cascades inducing neuronal death through activation of caspase3 and enhanced expression of c-jun, c-fos, and p53 [91]. Ceramide-mediated activation of p38 and oligodendrocyte apoptosis was also seen in oligodendrocytes treated with C2-ceramide, and this apoptosis was attenuated by a p38 inhibitor, SB203580, and by expression of a p38 α dominant negative mutant [92]. Figure 1 illustrates the signaling mechanisms involved in direct killing of neurons once ceramide is generated in response to different neurotoxic agents and in different neurodegenerative disorders.

2.4.5. Production of neurotoxic molecules from glia—Several neurodegenerative diseases, notably AD, HAD, and PD are characterized by neuronal degeneration and simultaneous activation of glial cells with production of toxic factors that aggravate the neuronal damage. A role for neurotoxic molecules in the pathogenesis of neurodegenerative diseases has been supported by several reports demonstrating elevation of these compounds at the lesion site. Subsequent assay by polymerase chain reaction (PCR) and *in situ* hybridization revealed increased mRNA [93] expression of IL-1 β , TNF- α , LT- α , and iNOS respectively in lesions [93]. It should be noted that some studies have failed to show an augmentation of cytokines and this is reported in MS [94–98]. Discrepancies in reports of these findings may be due to sample handling or temporal variability in collection, methodological variability, short cytokine half-lives, or the like. Furthermore, neurotoxic factors might behave differently in proliferating glia. For instance, in astrocytes of AIDS encephalopathy, the HIV-nef gene product triggered TNF- α -induced ceramide generation and NF- κ B activation. On the other hand, blocking AP-1 activation shifted the molecular profile of the cell toward survival and an astrogliosis characteristic of neurodegenerative disorders [99] and consistent with the capability of IL-6 produced from activated astrocytes to induce astrocyte proliferation and activation [100]. Such neurotoxic molecules as inflammatory cytokines, chemokines, excitotoxic molecules, and ROS, elaborated from activated glia increase in the brain and CSF of neurodegenerative disorders, and several of these couple the sphingomyelin-ceramide

pathway with its prominent connection to apoptosis (and to other cellular processes as well). Early on, TNF- α released from astrocytes and macrophages was recognized to activate myelin associated sphingomyelinases to generate ceramide and other downstream products with consequent deleterious effects upon membrane integrity [101,102]. This is fully consistent with our own co-culture studies of genetic knockdown of NSMase in human primary neurons using an antisense strategy; finding that NSMase-impotent neurons resist activated glia-mediated cytotoxicity (unpublished data). A potential bidirectional relationship between cytokine balance and ceramide production has also been well documented. For instance, TNF- α , IL-1 and Fas/FasL that are increased in tissue from neurodegenerative disorders potently induce ceramide production, and ceramide in turn stimulates production of IL-2 and IL-6 [103,104] themselves potent inducers of ceramide and mediating neuronal apoptosis or potentiating other neurotoxins [105,106]. For HAD, this hypothesis is supported by the synergistic neurotoxicity between gp120, Tat, and TNF- α at the ceramide level as shown by negation of the toxic effects by inhibition of sphingomyelinase and/or *de novo* ceramide production [39]. Moreover, the findings that conditioned media from HIV-1 infected monocytes induce neuronal apoptosis by TNF- α and platelet activating factor-mediated induction of ceramide [107] fit this mechanism.

A parallel excitotoxic pathway may well be activated. TNF- α produced by HIV-1-infected macrophages and microglia during HIVD inhibited glutamate uptake by astrocytes leading to an increase in the concentration of extracellular glutamate [108] causing over-activation of glutamate receptors on neurons and neuronal apoptosis by a mechanism involving calcium influx through ionic channels linked to glutamate receptors. Such excitotoxicity may occur in neurodegenerative conditions including stroke, trauma, epileptic seizures, Alzheimer disease and ALS/motor system disorders [109–111]. Perturbations of calcium homeostasis in neurons might induce neuronal death through activation of the redox-sensitive cytosolic calcium dependent PLA2-AA-SMase pathway [112,113]. In addition to cytokines, release of several growth factors including NGF [114] is also associated with gliosis. Although, NGF is critical for differentiation and survival of neurons and for neural plasticity [115,116], it may also promote apoptosis via ceramide depending on the expression levels of neurotrophin receptor p75^{NTR}. Increased NGF levels have been reported in pathological conditions characterized by prominent astrogliosis [117,118]. Further, gliosis is marked by the release of pro-inflammatory mediators such as prostanoids from activated microglia, which is influenced by sphingomyelinase activation and ceramide generation (Akundi et al., 2005).

Lipopolysaccharide (LPS)-induced activation of SMase and ceramide formation is considered essential for cyclooxygenase-2 (COX2) expression and prostaglandin E2 (PGE2) synthesis via activation of the p38 MAPK-dependent pathway, and this suggests that ceramide formation in activated microglia could contribute to the amplification of neuroinflammatory events [119].

2.5. Mechanisms of ceramide production in neurodegeneration

2.5.1. Activation of sphingomyelinases—The catabolic pathway for ceramide formation is located either in lysosomes or at the plasma membrane, and is initiated by the action of SMases, sphingomyelin-specific forms of phospholipase C, which hydrolyze the phosphodiester bond of sphingomyelin yielding ceramide and phosphocholine. There are several isoforms of SMases, distinguished by different pH optima, subcellular localization, and cation dependence. A Mg²⁺-dependent NSMase is found at the plasma membrane [120], and an ASMase is localized in the endosomal-lysosomal compartments [121]. The Mg²⁺-dependent NSMase is very unstable compared to ASMase [122]. The Mg²⁺-independent NSMase localized in the cytosol is found exclusively in the myelin sheath [123], while a bile-salt-dependent alkaline SMase is localized in the gastrointestinal tract [124]. NSMase and ASMase are rapidly and transiently activated by diverse exogenous as well as endogenous stimuli. Neutral and acidic SMases appear responsible for stimulus-induced increases of ceramide within a time frame of seconds and minutes compared to *de novo* ceramide synthesis

that accounts for late and sustained ceramide generation [125]. Therefore, these SMase forms are considered to be major pathways for ceramide generation in early signal transduction. In neurons, both SMase types generate ceramide from SM hydrolysis. Analysis of endogenous sphingomyelinase activities reveal that the NSMase is localized in axons while ASMase is concentrated in the neuron cell body [49].

a) NSMase: the predominant form of sphingomyelinase in the brain: NSMase is the major sphingomyelinase found in the brain [126]. Biochemical characterization of this NSMase revealed that it is sensitive to glutathione [127]. Multiple forms of the membrane-bound NSMases have been identified in bovine brain [128]. This SMase is responsible for ceramide generation in the CNS during normal development and particularly in response to neurotrophins [35]. Neurotrophin such as NGF bind to the p75^{NTR} to stimulate growth and development of cultured hippocampal neurons via the ceramide generation by NSMase [35]. The stimulatory effects of NGF on neurite formation and outgrowth are blocked by scyphostatin, a specific NSMase inhibitor. C2-ceramide and exogenously-added SMase release dopamine via this pathway in dopaminergic neurons of the mesencephalon [129]; a possible explanation for which might be ceramide phosphorylation by a calcium-dependent protein kinase located in synaptic vesicles [130] yielding ceramide 1-phosphate. Ceramide phosphorylation could then foster neurotransmitter release through phosphorylation of such synaptic vesicle proteins as synapsin I [131,132].

Different cellular effects may reflect cellular concentrations. For example, the response of hippocampal neurons to NGF changes in mature neurons that switch from acceleration of axonal outgrowth to apoptotic cell death as levels of p75^{NTR} expression and ceramide generation increase [90]. The NSMase inhibitor, scyphostatin, inhibits NGF-induced ceramide generation and neuronal death, while neurons derived from wild type or ASMase deficient mice are equally susceptible to NGF-induced cell death. Thus, in these neuronal cultures, NSMase rather ASMase is largely responsible for ceramide generation in response to trophic factors. In contrast, an excitotoxic cell death pathway in hippocampal neurons is via ASMase activation suggesting that different pathways generate different ceramide pools in the same neurons in response to different stimuli [42]. The potential players in the regulation of NSMase activation include FAN (factor associated with NSMase activation), arachidonic acid (AA), soluble phospholipase-A2 (PLA2), and intracellular oxidation that is sensitive to glutathione; the prominent negative regulator of NSMase [133–136]. No disease is associated with NSMase deficiency, and therefore signaling pathways associated with NSMase are less well characterized than those involving ASMase.

b) Activation of NSMase by virotoxins: We first reported that NSMase activation plays a major role in neuronal apoptosis and cell death in HAD [137]. Viral proteins such as gp120, induce apoptosis of human primary neurons by coupling with CXCR4 (CXC chemokine receptor 4) receptor in neurons to induce NADPH oxidase-mediated production of superoxide radical in neurons (Fig. 2), NSMase activation and production of ceramide without affect upon ASMase activity [137]. The tight coupling of NADPH oxidase-mediated superoxide production to NSMase activation in gp120-treated neurons was corroborated by antisense knock-down of p22phox, a NADPH oxidase subunit, and by use of the antioxidants diphenyliodonium(DPI) or N-acetylcysteine (NAC). Superoxide radicals exogenously produced in human primary neurons by hypoxanthine and xanthine oxidase also markedly activated NSMase, but not ASMase, in response to HIV-1 gp120 [137]. Similarly, the viral protein Tat induced neuronal apoptosis and cell death via NSMase activation.

c) Activation of NSMase by fibrillar A β : In a separate study [138], we found that fibrillar A β 1–42 peptides induce neuronal apoptosis through the NADPH oxidase-superoxide-hydrogen peroxide-NSMase-ceramide pathway (Fig. 2). Also superoxide radicals exogenously

generated by hypoxanthine and xanthine oxidase induced the activation of NSMase, but not ASMase, activity, through a catalase-sensitive pathway. Moreover, antisense knockdown of p22phox, a subunit of NADPH oxidase, rescued A β 1–42-induced neuronal apoptosis and cell death [138]. Lee et al have found that addition of bacterial sphingomyelinase (mimicking cellular NSMase activity) exacerbated A β -induced oligodendrocyte death, while NSMase inhibition by 3-O-methyl-sphingomyelin or by gene knockdown using antisense oligonucleotides attenuated A β -induced OLG death. Glutathione (GSH) precursors inhibited A β -induced activation of NSMase and prevented oligodendrocyte death, whereas GSH depletors augmented NSMase activity and A β -induced death. These suggest that A β induces oligodendrocyte death by activating the NSMase-ceramide cascade via an oxidative mechanism [8].

d) Activation of NSMase in oligodendrocytes: Cytokines: As in many other cell types, the proinflammatory cytokines TNF α and IL-1 β induced the degradation of SM to ceramide via NSMase in rat primary oligodendrocytes [139]. Interestingly, pretreatment of rat primary oligodendrocytes with N-acetylcysteine, an antioxidant and efficient thiol source for glutathione, prevented cytokine-induced decrease in GSH and degradation of sphingomyelin to ceramide [139].

Reactive oxygen species (ROS): In addition to proinflammatory cytokines, ROS are directly involved in degradation of SM to ceramide via NSMase in human primary oligodendrocytes. Our findings of NSMase activation and ceramide production in human primary oligodendrocytes showed that various ROS producing molecules or ROS itself, such as, superoxide radical produced by hypoxanthine and xanthine oxidase, hydrogen peroxide, aminotriazole that is capable of inhibiting catalase and increasing the intracellular level of H₂O₂, or reduced glutathione-depleting diamide all induced this NSMase activation and ceramide production [40]. Interestingly we found that antisense knockdown of neutral, but not acidic, sphingomyelinase ablated oxidative stress-induced apoptosis and cell death in human primary oligodendrocytes [40]. How NSMase is activated by ROS remains an open question. We speculate that there is either post-translational modification of NSMase by ROS thereby influencing the subcellular localization of NSMase, activation of one of the several protein kinases, inhibition of tyrosine phosphatase or receptor-mediated activation of NSMase. Alternatively, ROS might trigger the generation of second messengers that in turn activate NSMase.

2.5.2. Inhibition of ceramidase—The intracellular level of ceramide can be modulated by regulation of ceramide degradation; i.e., regulation of the hydrolysis of ceramide catalyzed by ceramidases. Three types of ceramidases are distinguished on the basis of pH optima, substrate specificity and subcellular localization [140–143]. Deficiency of lysosomal acid ceramidase leads to a rare form of inborn lipid storage disorder called Farber's disease. The severity of the disease and its rate of progression correlate with the ceramide accumulation, and importantly, ceramidase inhibition by N-oleylethylamine prevents neuronal dysfunction and apoptosis [93].

3. Neurons and oligodendrocytes are more vulnerable than astrocytes and microglia to N-SMase activation!

Both neurons and glia show increased NSMase activation during neurodegenerative diseases but only neurons and oligodendrocytes succumb to apoptosis and cell death. The reasons for this differential fate are summarized below.

3.1. Low levels of antioxidant enzymes

Cells prevent oxidative damage by utilizing several *in situ* antioxidant systems that negate oxidative stress by eliminating ROS. These include such antioxidant enzymes as superoxide dismutase (SOD), glutathione peroxidase, and catalase. The SOD system converts $O_2^{\bullet-}$ to H_2O_2 that in turn is detoxified by catalase and glutathione peroxidase [144,145]. Currently, there are three different known SOD isoforms. One is cytosolic utilizing Cu and Zn as its prosthetic groups and is designated as Cu/Zn-SOD. SOD3 is found in extracellular spaces, and SOD2 uses manganese at its active site, is strictly localized in the mitochondrial matrix, and is designated as MnSOD [146].

Neurons and oligodendrocyte are more prone to oxidative stress-induced damage via NSMase because their levels of catalase and glutathione peroxidase are low [145,147–150]. On the other hand, astrocytes contain higher GSH levels as a means to combat oxidative stress [151,152]. In addition to GSH, intracellular oxidative stress signals for MnSOD gene induction in astroglia [153]. Therefore, we hypothesize that neurons that have limited inflammatory processing capacity are incapable of inducing MnSOD in an oxidative stress milieu and succumb while astrocytes and microglia respond with proliferative and/or inflammatory activation [154].

3.2. Differences in NF- κ B activation

As discussed above, astrocytes have higher constitutive levels of MnSOD than neurons and this level increases during gp120 treatment [155]. The disparity in MnSOD levels stems from the fact that astrocytes have a greater level of NF- κ B p65 subunit than neurons thereby allowing them to generate more MnSOD in response to gp120 treatment. Because activation of NF- κ B is necessary for transcription of the MnSOD gene, over-expression of p65 in neurons increases the MnSOD level and elicited neuronal resistance to gp120 [155] while p65 antisense, p65 siRNA, or a specific inhibitor, NBD peptide, leads to reduced MnSOD and enhanced vulnerability of astrocytes to gp120 treatment [155].

Pahan et al [156] have shown earlier that ceramide induces the expression of MnSOD in rat astrocytes, and this is consistent with ceramide's capability to induce activation of NF- κ B [157]; a step important for the expression of MnSOD [155]. Although this study did not test the neuronal expression of MnSOD, the lower level of NF- κ B p65 in neurons than astrocytes may prevent ceramide induction of MnSOD expression in neurons to save these cells under neurodegenerative conditions. Consistent with this hypothesis, our recent studies demonstrate that gp120 is toxic to neurons, but not astrocytes [155], and that gp120 kills neurons via ceramide production [137].

3.3. Iron content in oligodendrocytes

Iron, an essential compound in brain tissue, is a vital cofactor in many biological pathways including myelin production in oligodendrocytes [158]. Inadequate iron levels impair myelination and lead to mental retardation [159]. Histochemical stains for iron indicate that oligodendrocytes are the predominant brain cell type staining for iron [160–162]. This differential staining arises from the fact that oligodendrocytes have a relatively high metabolic rate and an associated enzyme activity that is iron dependent [150,163]. In addition, ferritin, the major protein involved in iron storage, is abundant in oligodendrocytes [158,164]. This high iron content in oligodendrocytes generates highly toxic hydroxyl radicals by reacting with peroxides through the non-enzymatic Fenton reaction that affects many cellular macromolecules including DNA, proteins and lipids [165]. On the other hand, astrocytes contain little ferritin suggesting that they are en route trafficking of iron to other brain cells [166,167]. They also express ceruloplasmin that functions as a ferroxidase converting ferrous iron to ferric form thereby allowing iron transport out of the cell [166]. In addition, astrocytes express lower levels of transferrin receptors [166]. Fully differentiated microglia resemble

astrocytes in rarely containing histologically detectable levels of iron, transferrin, ferritin or other iron-related protein [168]. From these studies one could speculate that the low levels of iron in astrocytes and microglia bias them to a cellular anti-oxidative machinery.

3.4. High level of unsaturated fatty acids

Myelin sheaths of oligodendrocytes contain polyunsaturated fatty acids (PUFA) that react with peroxides and hydroxyl radicals triggering thereby a cascade of oxidative damage and cell death probably via NSMase activation [40,145]. Furthermore, PUFA enhances iron uptake by modulating iron transporters, and accelerating apoptotic death [169,170]. Similar to oligodendrocytes, neurons have a high content of easily oxidizable polyunsaturated membrane lipids [171]. Docosahexaenoic acid (DHA), a major PUFA in the central nervous system, is used continuously for the biogenesis and maintenance of neuronal membrane and is a target for lipid peroxidation [171].

4. Conclusion and therapeutic perspectives

Neuronal and oligodendrocyte death is a common denominator of many human neurological disorders, and therefore, understanding mechanisms involved in rescuing and sustaining neurons and oligodendrocytes within and near their nervous system lesions is of high priority in developing effective therapies. This is especially important for biochemical ‘final cellular molecular pathways’. Over the past fifteen years, the key role and novel regulatory importance of sphingolipid metabolites has opened new vistas warranting the current vigorous and rigorous investigation of bioactive lyso-sphingolipids for roles in neurodegenerative/inflammatory pathogenesis in diseases as diverse as AD, HAD, ALS, and MS. Our findings and those of other investigators indicate that activation of the neutral sphingomyelinase -ceramide pathway via oxidative stress mechanisms plays a cardinal role in apoptosis of neurons and oligodendrocytes. It follows then that pharmacological inhibitors of neutral sphingomyelinase and/or upstream NADPH oxidase represent a novel way to halt the neurodegeneration that is a prominent feature of several prevalent, different and often devastating neurological diseases.

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Fig. 1. Schematic representation of ceramide-induced various apoptotic pathways

Sphingomyelinases cleave sphingomyelin (represented as small circles around the membrane) to ceramide in response to different extracellular insults. Once ceramide is generated, different downstream targets including death-associated protein kinase (DAPK), kinase suppressor of Ras (KSR), protein kinase C (PKC) ζ , Rac, inducible nitric oxide synthase (iNOS), ceramide-activated protein phosphatase (CAPP), and c-Jun N-terminal kinase (JNK) are activated and the release of cytochrome C is augmented. On the other hand, the anti-apoptotic Akt - Bcl2 pathway is down-regulated. All these events eventually lead to apoptosis.