Published in final edited form as:

J Neurosci Res. 2012 January ; 90(1): 229–242. doi:10.1002/jnr.22748.

Neutral Sphingomyelinase Activation Precedes NADPH Oxidase-Dependent Damage in Neurons Exposed to the Proinflammatory Cytokine TNF α

Brian M. Barth^{1,2}, Sally J. Gustafson², and Thomas B. Kuhn^{2,*}

¹Department of Pharmacology, College of Medicine, Pennsylvania State University, Hershey, PA

²Department of Chemistry and Biochemistry, University of Alaska-Fairbanks, Fairbanks, AK 99775, USA

Abstract

Inflammation accompanied by severe oxidative stress plays a vital role in the orchestration and progression of neurodegeneration prevalent in chronic and acute CNS pathologies as well as in aging. The proinflammatory cytokine tumor necrosis factor alpha (TNF α) elicits the formation of the bioactive ceramide by stimulating the hydrolysis of the membrane lipid sphingomyelin by sphingomyelinase activities. Ceramide stimulates the formation of reactive oxygen species (ROS) and apoptotic mechanisms in both neurons and non-neuronal cells establishing a link between sphingolipid metabolism and oxidative stress. We demonstrated in SH-SY5Y human neuroblastoma cells and primary cortical neurons, that TNF α is a potent stimulator of Mg²⁺-dependent neutral sphingomyelinase (Mg²⁺-nSMase) activity and sphingomyelin hydrolysis rather than de novo synthesis, was the predominant source of ceramide increases. Mg²⁺-nSMase activity preceded an accumulation of ROS by a neuronal NADPH oxidase (NOX). Notably, TNF α provoked a NOX-dependent oxidative damage to sphingosine kinase-1, which generates sphingosine-1-phosphate, a ceramide metabolite associated with neurite outgrowth. Indeed, ceramide and ROS inhibited neurite outgrowth of DRG neurons by disrupting growth cone motility. Blunting ceramide and ROS formation both rescued sphingosine kinase-1 activity and neurite outgrowth. Our studies suggest that TNF α -mediated activation of Mg²⁺-nSMase and NOX in neuronal cells not only produced the neurotoxic intermediates ceramide and ROS but also directly antagonized neuronal survival mechanisms thus accelerating neurodegeneration.

Keywords

sphingomyelinase; ceramide; NADPH oxidase; sphingosine kinase; neuroinflammation

Introduction

A progressively spreading inflammation and oxidative stress are hallmarks of many chronic central nervous system (CNS) diseases, acute CNS injuries, some psychiatric disorders, and aging CNS (Lucas et al., 2006). Inflammatory mediators (cytokines, eicosanoids, A β ₁₋₄₂) and reactive oxygen species (ROS) are largely responsible for the degeneration of neuronal cells (Viviani et al., 2004; Mrak and Griffin, 2005). The proinflammatory cytokine tumor necrosis factor alpha (TNF α) is a potent activator of Mg²⁺-dependent neutral

*Corresponding author: Thomas B. Kuhn, University of Alaska Fairbanks, Department of Chemistry and Biochemistry, 900 Yukon Drive, Reichardt Building Room 194, Fairbanks, AK 99775, USA, Tel: 1 (907) 474-5752; Fax: 1 (907) 474-7827, tbkuhn@alaska.edu.

sphingomyelinase (Mg^{2+} -nSMase), which is coupled to the high affinity TNF receptor type I expressed on the surface of many neuronal cell types through the adaptor protein FAN (factor associated with nSMase) (Adam-Klages et al., 1996). Mg^{2+} -nSMase2 in plasma membranes is predominantly expressed in the CNS and hydrolyzes sphingomyelin to ceramide (Tomiuk et al., 2000; Marchesini and Hannun, 2004). The bioactive lipid ceramide and its metabolites are pleiotropic messenger that stimulate both apoptotic mechanisms as well as cell survival mechanisms (Hannun and Luberto, 2000; Andrieu-Abadie et al., 2001). For example, conversion of ceramide by sphingosine kinases (SphK) to sphingosine-1-phosphate (S1P) generates a messenger with roles in neuronal survival and development (Maceyka et al., 2002; Taha et al., 2006). Toman and co-workers demonstrated that S1P induced neurite outgrowth in both PC12 cells and dorsal root ganglia (DRG) neurons (Toman et al., 2004). SphK1 and SphK2 double knock out mice exhibited neurodevelopment complications and did not develop to term (Mizugishi et al., 2005). In sharp contrast, ceramide and ceramide-1-phosphate are implicated in mediating severe oxidative stress likely via mitochondrial disintegration and activation of NADPH oxidoreductases (Andrieu-Abadie et al., 2001; Lamour and Chalfant, 2005). Members of the NADPH oxidoreductase family with its prototypic isoform NOX2 originally identified in phagocytes have recently been detected in many non-phagocytic cell types including neurons with potential roles in physiological redox signaling mechanisms and oxidative stress (Bedard and Krause, 2007; Kawahara et al., 2007). Functional NOX2 is a multi subunit, superoxide-producing complex composed of the two membrane proteins NOX2, formerly gp91^{phox}, and gp22^{phox} and at least three cytosolic subunits (Groemping and Rittinger, 2005; Sumimoto et al., 2005). NOX2 activation requires a series of complex interactions between cytosolic and membrane subunits as well as anionic phospholipids and posttranslational modification such as phosphorylation of several cytosolic subunits (Cross et al., 1999; Ueyama et al., 2007). It is not clear whether ceramide stimulates NOX activity directly or indirectly via activation of cytosolic phospholipase A2, which produces arachidonic acid, a well document lipid activator of NOX.

Our studies in neuronal cells revealed that TNF α potently stimulated Mg^{2+} -nSMase and the subsequent accumulation of ceramide resulted in the formation of ROS implicating a neuronal NOX2 activity as the primary source. Neuronal stress by ceramide and ROS disrupted growth cone motility and inhibited neurite outgrowth of DRG neurons yet also diminished the survival factor S1P through a NOX2-dependent oxidative damage of SphK1. This shift in ceramide metabolism in conjunction with increased oxidative stress likely exacerbates neuronal degeneration in CNS pathologies exhibiting an inflammatory reaction.

Materials and Methods

Reagents

Unless mentioned otherwise all reagents were purchased from Sigma (St. Louis, MO). Recombinant human TNF α was obtained from Millipore (Temecula, CA). Fetal bovine serum (FBS) was received from Atlanta Biologicals (Atlanta, GA) whereas DMEM (Dulbecco's modified Eagle Medium) and Penicillin/Streptomycin were from Mediatech (Herndon, VA). GlutaMAX-1, Hank's Balanced Salt Solution (HBSS), and trypsin/EDTA solution were purchased from Invitrogen (Carlsbad, CA). For sphingolipid experiments, an Amplex Red Sphingomyelinase assay kit was received from Invitrogen (Carlsbad, CA) whereas sphingolipids were from Avanti Polar Lipids (Alabaster, AL). GW4869 and naphthalene dicarboxylaldehyde (NDA) were from EMD Biosciences (San Diego, CA). A polyclonal rabbit anti-human p67^{phox} antibody and a polyclonal rabbit anti-DNP (dinitrophenyl) antibody were obtained from Millipore (Temecula, CA) and Invitrogen (Carlsbad, CA), respectively. A polyclonal goat anti-mouse SphK1 antibody, a polyclonal goat anti-human phospho-p40^{phox} antibody, a monoclonal mouse anti-human β -actin

antibody, corresponding horseradish peroxidase-conjugated antibodies, and normal goat serum were received from Santa Cruz (Santa Cruz, CA). Streptavidin-agarose beads, protease inhibitor cocktail (PIC), Pico Super Signal chemoluminescent kit, and a BCA protein assay kit were obtained from Pierce (Rockland, IL).

SH-SY5Y neuroblastoma and cortical neuron cultures

Human SH-SY5Y neuroblastoma cells were grown in 100 mm dishes (Falcon) in high glucose DMEM (pH 7.4), containing 10% FBS, 1% GlutaMax-1, and 100 U/ml Penicillin and Streptomycin each (humidified atmosphere, 5% CO₂, 37°C). SH-SY5Y cells were detached with 0.5 mg/ml trypsin/0.2 mg/ml EDTA (5 min, 37°C), collected by centrifugation, and plated at densities of 5×10⁶ cells/well (6 well plates) or 1×10⁵ cells/well (96 well plates), respectively.

Primary cortical neurons were obtained from forebrains of 7 day-old chick embryos (E7), as detailed by Wright and Kuhn (Wright and Kuhn, 2002). Briefly, cortices were removed, stripped of meninges, and remaining tissue dissociated (0.5 mg/ml trypsin, 0.2 mg/ml EDTA, 5 min, 37°C) followed by trituration. Following centrifugation, cortical neurons were resuspended in DMEM (high glucose, pH 7.4), 1% Glutamax, 10% FBS, and 1% N3 supplement (humidified atmosphere, 5% CO₂, 37°C) (Bottenstein and Sato, 1979). Cortical neurons were plated on poly-D-lysine-coated (10 µg/ml) 6-well plates (5×10⁶ cells/well) or 96 well plates (1×10⁵ cells/well).

Neurite outgrowth and growth cone advance

Dorsal root ganglia (DRG) were dissected from 9 day-old chick embryos (E9) and plated in 24 well plates (1 ganglion per well) on laminin (2 µg/cm²) (Kuhn et al., 1998). DRG neurons were maintained in DMEM, 1% GlutaMax-1, 10% FBS, 20 ng/ml 2.5 S NGF (nerve growth factor), and 1% N3 supplement (humidified atmosphere, 5% CO₂, 37°C) (Bottenstein and Sato, 1979). For live-video phase contrast microscopy, DRG ganglia were plated onto glass cover slips (22×22 mm², #1, Carolina Biological Supply Company, Burlington, NC) mounted over a 1.5 cm hole drilled into the bottom of a 35mm culture dish. DRG ganglia were grown until displaying vigorous, radial outgrowth of neurites.

For neurite length measurements, DRG explants were incubated with pharmacological inhibitors (1 h) in the presence of 10% FBS and then acutely exposed to 100 ng/ml TNFα (30 min). Cultures were fixed with 2% glutaraldehyde, and neurite lengths measured in six random segments per ganglion. All measurements were normalized to control conditions. To determine growth cone advance, DRG ganglia were transferred into observation medium (Leibovitz' L15 without phenol red, pH 7.4, 1% GlutaMax-1, 10% FBS, 20 ng/ml 2.5 S NGF, 1% N3). Cultures were placed onto a Nikon TE2000 inverted microscope equipped with a heated stage (38±0.2°C, airstream heater) and allowed to recover for at least 20 min. Images of advancing DRG growth cones were acquired under phase contrast at 20x (Coolsnap_{fx} cooled CCD camera, Roper Scientific, Tucson, AZ) at 3 min time intervals for 10 min prior to incubation with pharmacological inhibitors (pre-images). Image acquisition was continued for an additional 12 min (post images) before exposing DRG ganglia to 100 ng/ml TNFα (post stress). Growth cone advance was measured as the displacement (µm) of the growth cone-neurite border per time interval (Metamorph Software) and plotted as the distance accumulated (µm) over time (min).

Sphingomyelinase assay

Mg²⁺-neutral sphingomyelinase (Mg²⁺-nSMase) activity was quantified in whole cell lysates using an enzyme-coupled Amplex Red fluorescence assay according to the manufacturer (Invitrogen, Carlsbad, CA). Briefly, SH-SY5Y cells or cortical neurons (5

$\times 10^6$ cells/well) were incubated with pharmacological inhibitors (1 h), exposed to 100 ng/ml or 200 ng/ml TNF α for the desired time period, then scraped into cold PBS (phosphate buffered saline), and lysed (sonication). Samples were mixed with reaction buffer (Invitrogen, Carlsbad, CA) and maximum fluorescence of resorufin was quantified using a Beckman Coulter Multimode DTX 880 microplate reader (530 excitation filter, 590 nm emission filter). All data was adjusted to equal amounts of total soluble protein (BCA assay) and normalized to control.

Sphingosine kinase 1 assay

SH-SY5Y cells or primary cortical neurons (5×10^6 cells/well, 6 well plate) were incubated with pharmacological inhibitors (1 h) prior to stimulation with 100 ng/ml TNF α , 10% FBS, or vehicle (1 h). Neuronal cells were scraped into PBS and lysed by sonication. After removing insoluble material by centrifugation, samples (5 μ g total protein) were mixed with 1 mM CaCl₂, 4 mg/ml BSA (bovine serum albumin), 2% Triton X-100, 1% PIC (protease inhibitor cocktail), and 1 mM Na₃VO₄ and incubated (1 h) with 50 μ M D-erythro-sphingosine (150 μ l total reaction volume). After terminating reactions with chloroform (150 μ l), sphingosine-1-phosphate was extracted (chloroform/methanol 50% v/v, basic pH), and derivatized with 1 mM NDA (1 mM NaCN, Na⁺-tetraborate buffer pH 10.3). Samples were subjected to silica thin layer chromatography (chloroform (10)/acetone (4)/methanol (3)/acetic acid (2)/water (1); v/v) and NDA-labeled sphingosine-1-phosphate was quantified (GE Healthcare Typhoon Imager, ImageQuant software, UK).

Quantification of ROS production

SH-SY5Y cells or primary cortical neurons (1×10^5 cells/well, 96-well plates) were loading with 50 μ M 2',7'-dihydrodichlorofluorescein diacetate (H₂DCFDA, 1 h) in the presence or absence of pharmacological inhibitors, 5000 U/ml catalase, or 200 U/ml superoxide dismutase (SOD). Intracellular deacetylation of H₂DCFDA results in 2',7'-dihydrodichlorofluorescein, which increases in fluorescence upon oxidation to 2',7'-dichlorofluorescein (DCF) by peroxide. After recovery in regular growth medium, cultures were exposed to 100 ng/ml TNF α (30 min) or 250 μ M palmitate (4 h), washed with PBS, and lysed (2% SDS, 2 M Tris-HCl (pH 8.0), 10 mM Na₃VO₄). Maximum DCF fluorescence intensity was quantified in cell lysate (100 μ l) using a Beckman Coulter Multimode DTX 880 microplate reader (495 nm excitation filter, 525 emission filter). All data were adjusted per mg total soluble protein and normalized to the average maximum DCF fluorescence intensity under control conditions (relative maximum DCF fluorescence intensities).

Determination of plasma membrane-associated p67^{phox}

Plasma membrane-associated proteins were biotinylated followed by affinity purification on streptavidin according to Li and Shah with minor modifications (Li and Shah, 2002). SH-SY5Y cells (5×10^6 cells/well, 6-well plates) were incubated (1 h) with pharmacological inhibitors or vehicle (equal amount of buffer) prior to stimulation either with 100 ng/ml TNF α (15 min), or with 250 μ M palmitate (4 h). After rinsing with HBSS-CM (Hank's balanced salt solution, pH 7.5, supplemented with 0.1 g/l CaCl₂ and 0.1 g/l MgCl₂), cultures were incubated with 0.5 mg/ml Sulfo-NHS-Biotin in 20 mM HEPES/HBSS-CM (pH 8.0) for 40 min on ice followed by neutralizing excess Sulfo-NHS-Biotin (50 mM glycine, HBSS-CM, on ice, 15 min). Cells were scraped into HBSS-CM, collected by centrifugation ($200 \times g_{\max}$, 2 min), and lysed in 20 mM HEPES (pH 7.5), HBSS, 1% Triton X-100, 0.2 mg/ml saponin, 1% PIC (500 μ l) followed by brief sonication. Biotinylated proteins were adsorbed onto Streptavidin-agarose beads (25 μ l, 2 h, 4°C), collected by centrifugation ($2,500 \times g_{\max}$, 2 min), and resuspended in the same buffer. Bound protein was recovered (5 min boiling water bath and centrifugation $2,500 \times g_{\max}$, 2 min) and total soluble protein determined (BCA protein assay).

Detection of Sphingosine kinase 1 carbonylation

SH-SY5Y cells were incubated with pharmacological inhibitors (1 h) prior to stimulation (1 h) with TNF α (100 ng/ml) or vehicle (buffer) and then lysed (2% SDS, 2 M Tris-Cl pH 8.0, 1 mM Na₃VO₄). Following centrifugation (3,000 $\times$ g_{max}, 4°C, 20 min), supernatants (100 μ l) were cleared using normal goat serum (1 μ g, 45 min, 25°C, agitation) and IgG removed with protein A/G agarose beads (20 μ l). SphingosineKinas-1 (SphK1) was immunoprecipitated from cleared lysates (100 μ l) using a polyclonal goat anti-mouse SphK1 antibody (1 μ g, 2 h, 4°C, agitation) and adsorbed onto protein A/G agarose beads (20 μ l, overnight, 4°C, agitation). Beads were collected (200 $\times$ g_{max}, 2 min), washed three times, and subjected to modification with 2,4-dinitrophenylhydrazine (DNPH) according to Levine et al (Levine et al., 1994). Briefly, immunoprecipitated samples were mixed with 12% SDS (1:1, v/v), incubated with 20 mM DNPH in 2M HCl (1:1, v/v) for 45 min (25°C), and protein recovered by chloroform/methanol precipitation (Wessel and Flugge, 1984).

siRNA-mediated depletion of Mg²⁺-dependent neutral sphingomyelinase

ON-TARGETplus SMARTpool siRNA was from Dharmacon (Lafayette, CO) and Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). SH-SY5Y cells were grown to 50-60% confluency. siRNA targeted against Mg²⁺-nSMase was composed of pool of sequences (accession number NM_018667, gene symbol SMPD3, Dharmacon catalog number: L-006678-03) whereas non-targeted siRNA was pool of scrambled sequences (Dharmacon catalog number: D-001206-14, Pool #2). Both siRNA pools were transfected (100 nM) into SH-SY5Y cells using Lipofectamine 2000 according to the manufacturer's instructions. Knockdown was confirmed by western blotting 24 hours post-transfection. Likewise, ROS assays were performed 24 hours post-transfection.

SDS gel electrophoresis and Western blotting

Total soluble protein (30 μ g per lane) or total immunoprecipitated protein (5 μ g per lane), respectively, were separated on 10% or 15% SDS-polyacrylamide gels (125 volts, 50 watts, 75 mA) and then transferred onto PVDF membranes (2.5 hours, 50 volts, 50 watts, 250 mA) (Laemmli, 1970; Towbin et al., 1979). Membranes were blocked in TBST-BSA (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20, 5 mg/ml BSA) for 1 h and incubated (16 h) with 1 μ g/ μ l primary antibody in TBST. After three washes with TBST, membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (0.2 μ g/ μ l, TBST, 45 min) followed by chemiluminescence detection and quantification (GE Healthcare Typhoon Imager, ImageQuant software, UK).

Statistical analysis

Analysis of variance (ANOVA) was used to determine statistically significant differences between treatments (p<0.05). *Post hoc* comparisons of specific treatments were performed using a Bonferroni test. A Dunette's test was employed for multiple comparisons between treatment means and a single control mean. At least three independent experiments were performed for each condition. All error bars represent standard error of the mean (SEM) unless mentioned otherwise.

Results

TNF α -mediated inhibition of sphingosine kinase 1 activity and attenuation of neurite outgrowth is ceramide and ROS dependent

Various growth factors are potent inducers of SphK1 activity implicated in complex cellular response including cell movement (Olivera and Spiegel, 1993). NGF-dependent neurite outgrowth of DRG neurons is subject to modulation by sphingosine-1-phosphate (S1P), the

product of sphingosine kinase 1 (SphK 1) activity (Toman et al., 2004). Since TNF α interferes with both outgrowth and branching of hippocampal neurons as described by Neumann and co-worker, we explored whether TNF α would interfere with the S1P-dependence of DRG neurite outgrowth (Neumann et al., 2002).

Acute exposure of DRG neurons to 100 ng/ml TNF α in the presence of FBS caused a dramatic reduction of neurite length ($67\% \pm 3\%$, $*p < 0.05$, $n=24$) compared to control ($100\% \pm 5\%$, $n=24$) (Fig. 1A). Inhibiting SphK1 activity with 10 μ M DMS (N',N'-dimethylsphingosine) also reduced neurite length ($60\% \pm 6\%$, $*p < 0.05$, $n=24$), which was not further exacerbated upon a presence of TNF α ($64\% \pm 3\%$, $*p < 0.05$, $n=24$). In contrast, neurite extension in the presence of TNF α was rescued either by inhibiting Mg²⁺-nSMase (10 μ M GW4869, $95\% \pm 3\%$, $n=24$) or NOX (10 μ M DPI, $107\% \pm 3\%$, $n=24$) as well as by ROS scavenging (5 mM NAC, $109\% \pm 2\%$, $n=24$). Notably, pharmacological inhibitors at the indicated concentrations did not interfere with neurite outgrowth regardless of the presence of FBS with the exception of 10 μ M DMS, which attenuated neurite outgrowth in the presence of FBS (Fig. 1B). These findings suggested that TNF α impaired neurite outgrowth of DRG neurons possibly by antagonizing SphK1 function in a ceramide and redox-dependent manner.

Since a reduction of neurite outgrowth could result either from an acute disruption of growth cone motility or indirectly through apoptotic mechanisms originating in the neuronal cell body, we measured growth cone advance using live-video phase contrast microscopy (Fig. 2). Acute exposure of DRG neurons to 100 ng/ml TNF α ceased advance of growth cones accompanied by a degeneration of morphology as opposed to control (Fig. 2, A and B). Similarly, inhibiting SphK 1 activity with 10 μ M DMS also ceased growth cone advance however only in the presence of 10% FBS (Fig. 2C). In contrast, growth cone advance remained intact when blocking de novo ceramide synthesis either with 5 mM L-cycloserine, a serine palmitoyltransferase inhibitor, or 10 μ M fumonisin B1, a dihydroceramide synthase inhibitor. Importantly, inhibiting de novo ceramide synthesis with 10 μ M fumonisin B1 provided no protection for growth cone advance in the presence of TNF α (Fig. 2D). However, incubation of DRG neurons with 10 μ M GW4869 protected growth cone advance in the presence of TNF α with no effect on growth cone advance in the absence of TNF α (Fig. 2E). Taken together, TNF α attenuated neurite outgrowth through a direct, ceramide-dependent disruption of growth cone advance with sphingomyelin hydrolysis as the likely source of ceramide generation rather than de novo synthesis.

To demonstrate SphK 1 inhibition by TNF α , SH-SY5Y neuroblastoma cells or primary cortical neurons (16 h serum-starved) were exposed to 10% FBS (1 h) either in the presence or absence of TNF α and pharmacological inhibitors (Fig. 3, A and B). Acute addition of FBS to serum-starved SH-SY5Y cells or primary cortical neurons significantly increased SphK1 activity (1.81 ± 0.17 , $*p < 0.05$, $n=3$ and 1.89 ± 0.19 , $*p < 0.05$, $n=3$, respectively) compared to basal SphK1 activity in controls (serum-free; 1.00 ± 0.06 , $n=3$). Importantly, 100 ng/ml TNF α abolished FBS-induced SphK1 activation (1.08 ± 0.02 , $n=3$ and 0.95 ± 0.07 , $n=3$, respectively) indistinguishable from the effects of 10 μ M DMS (1.07 ± 0.06 , $n=3$ and 0.80 ± 0.06 , $n=3$, respectively). Addition of 100 ng/ml TNF α to serum-starved neuronal cells had no impact on basal SphK1 activity (SH-SY5Y: 1.07 ± 0.08 , $n=3$ and Cortical: 0.81 ± 0.01 , $n=3$). FBS-induced SphK1 activation was rescued in SH-SY5Y cells and cortical neurons despite a presence of TNF α either by disrupting Mg²⁺-nSMase-dependent ceramide formation (10 μ M GW4869; 1.60 ± 0.07 , $*p < 0.05$, $n=3$ and 1.44 ± 0.02 , $*p < 0.05$, $n=3$, respectively), by preventing NOX-mediated ROS formation (10 μ M DPI; 1.93 ± 0.13 , $*p < 0.05$, $n=3$ and 1.45 ± 0.03 , $*p < 0.05$, $n=3$), or by ROS scavenging (5 mM NAC; 1.72 ± 0.10 , $*p < 0.05$, $n=3$ and 1.48 ± 0.04 , $*p < 0.05$, $n=3$). Taken together, these findings provided

evidence that TNF α antagonized SphK1 activation through ceramide and ROS intermediates.

TNF α stimulates Mg²⁺-nSMase activity in neuronal cells

Activation of a Mg²⁺-dependent neutral sphingomyelinase (Mg²⁺-nSMase) is implicated in cellular stress response evoked by TNF α , A β ₁₋₄₂, virotoxins, and oxidative stress (Jana et al., 2009). We examined whether TNF α stimulates Mg²⁺-nSMase activity in human SH-SY5Y neuroblastoma cells or primary cortical neurons (Fig. 4). Exposure of SH-SY5Y cells to 200 ng/ml TNF α caused a significant and persistent increase in Mg²⁺-nSMase activity (t = 15 min: 1.52 ± 0.09 , *p<0.05, n=4 and t = 30 min: 1.95 ± 0.043 , *p<0.05, n=4) compared to control (1.00 ± 0.13 , n=4) (Fig. 4A). Prior incubation of SH-SY5Y cells with 10 μ M GW4869 (1 h) completely abolished this response. Notably, 10 μ M GW4869 also suppressed Mg²⁺-nSMase activity below basal levels (t=0 min: 0.45 ± 0.03 ; t=15 min: 0.48 ± 0.03 ; t=30 min: 0.45 ± 0.03 , all n=4, respectively). Maximal activation of Mg²⁺-nSMase was already achieved with 100 ng/ml TNF α (1.76 ± 0.13 , *p<0.05, n=4) as compared to 200 ng/ml TNF α (1.52 ± 0.48 , *p<0.05, n=4), and suppressed even below basal levels by 10 μ M GW4869 (100 ng/ml TNF α : 0.91 ± 0.07 , n=4, and 200 ng/ml TNF α : 0.48 ± 0.03 , n=4, respectively) (Fig. 4B). Primary cortical neurons responded correspondingly to 100 ng/ml TNF α with a significant Mg²⁺-nSMase activation (1.26 ± 0.05 , *p<0.05, n=3) within 15 min of exposure as opposed to control (1.00 ± 0.06 , n=3) (Fig. 4C). As expected, TNF α -dependent stimulation of Mg²⁺-nSMase activity was GW4869 sensitive (10 μ M; 0.91 ± 0.03 , n=3). These findings suggested that TNF α potently stimulated Mg²⁺-nSMase activity in neuronal cells as well as a role for Mg²⁺-nSMase in basal neuronal homeostasis.

TNF α elicits a ceramide-dependent ROS formation in neuronal cells

Recently studies demonstrated NOX2 activity, a member of the NOX/DUOX family, in SH-SY5Y human neuroblastoma cells stimulated by TNF α or glutamate in accord with findings in various non-phagocytic cell types including neurons exposed to proinflammatory cytokines, growth factors, or hormones (Nikolova et al., 2005; Barth et al., 2009). We employed the oxidation-sensitive fluorescence indicator 2',7'-dichlorofluorescein (DCF) to quantify intracellular ROS generation and a potential ceramide dependence in SH-SY5Y human neuroblastoma cells and primary cortical neurons.

Serum-starved SH-SY5Y cells responded to 100 ng/ml TNF α (30 min) with a significant increase in maximum DCF fluorescence intensity (1.24 ± 0.05 , *p<0.05, n=5) compared to control (1.00 ± 0.03 , n=12) indicative of ROS formation (Fig. 5A). TNF α -stimulated ROS formation was abolished by exogenously added catalase (5,000 U/ml, 1.02 ± 0.04 , n=3) whereas exogenously added superoxide dismutase (SOD) was ineffective (200 U/ml, 1.39 ± 0.07 , *p<0.05, n=3). Both, 10 μ M DPI (1.02 ± 0.04 , n=8) and 5 mM NAC (0.92 ± 0.01 , n=3) negated ROS accumulation in response to TNF α . Primary cortical neurons also responded with increases in ROS (2.23 ± 0.05 , *p<0.05, n=14) upon exposure to 100 ng/ml TNF α (30 min) compared to control (0.99 ± 0.02 , n=21) (Fig. 5B). This response was suppressed by preincubating cortical neurons with either exogenous catalase (5,000 U/ml, 1.54 ± 0.10 , n=7), 5 mM NAC (0.79 ± 0.05 , n=7), or 10 μ M DPI (1.06 ± 0.12 , n=7). Again, exogenously added SOD (200 U/ml) was ineffective (2.98 ± 0.15 , **p<0.05, n=7). As a standard DCF loading control, application of hydrogen peroxide to neuronal cells dramatically increased DCF fluorescence (data not shown).

Eliminating Mg²⁺-nSMase activity in SH-SY5Y neuroblastoma cells or primary cortical neurons either with siRNA (Fig. 5) or pharmacology (Fig. 6) abolished TNF α -induced ROS formation suggesting a role for the lipid messenger ceramide in agreement with other reports (Andrieu-Abadie et al., 2001; Won and Singh, 2006). As shown in Figure 5C, siRNA

targeted against Mg^{2+} -nSMase (siNSM) substantially decreases Mg^{2+} -nSMase levels in SH-SY5Y cells as opposed to scramble siRNA (SCR), which had no effect on Mg^{2+} -nSMase levels compared to control (Con). Depleting Mg^{2+} -nSMase activity with siRNA successfully negated ROS formation in response to $TNF\alpha$ whereas a scrambled siRNA was ineffective (Fig. 5D). Neither nSMase-targeted siRNA nor scrambled siRNA alter basal ROS levels in SH-SY5Y cells. As expected, exposure of SH-SY5Y cells or cortical neurons with 100 ng/ml $TNF\alpha$ caused a significant formation of ROS (1.24 ± 0.05 , $*p < 0.05$, $n = 4$ and 2.23 ± 0.05 , $*p < 0.05$, $n = 14$, respectively) compared to controls (1.00 ± 0.03 , $n = 12$ and 10.99 ± 0.02 , $n = 21$, respectively), which was abolished by preincubation with 10 μM GW4869 (1.07 ± 0.01 , $n = 4$ and 1.21 ± 0.07 , $n = 7$, respectively) (Fig. 6, A and B). Indeed, the short-chain ceramide analogue C2-ceramide (10 μM Cer, 30 min) elicited a significant increase in ROS formation in SH-SY5Y cells (1.23 ± 0.03 , $*p < 0.05$, $n = 4$) and cortical neurons (1.54 ± 0.04 , $*p < 0.05$, $n = 7$). Increasing intracellular ceramide through a stimulation of *de novo* synthesis in SH-SY5Y cells with palmitate (Pal, 250 μM , 4 h) also caused a significant ROS formation as opposed to control, which was negated by 5 mM L-cycloserine (CS; 1.03 ± 0.03 , $n = 6$), a serine palmitoyltransferase inhibitor, or 10 μM fumonisins B1 (FB1; 0.98 ± 0.03 , $n = 6$), a dihydroceramide synthase inhibitor. Due to the cytotoxicity of palmitate for cortical neurons, we opted to block ceramide degradation with 10 μM of *N*-oleoylethanolamine (NOE), an acid ceramidase inhibitor. Cortical neurons responded with a significant increase in ROS when elevating intracellular ceramide levels with NOE (1.64 ± 0.04 , $*p < 0.05$, $n = 7$). Notably, intracellular ROS levels in neurons were not altered in control conditions when inhibiting sphingomyelin hydrolysis by Mg^{2+} -nSMase (GW4869) or *de novo* ceramide synthesis (L-cycloserine, fumonisins B1) as well as inhibiting NOX with 10 μM DPI (Fig. 6C). However, incubation of neurons with 5 mM NAC resulted in a significant decrease of intracellular ROS formation. Lastly, $TNF\alpha$ -stimulated ROS formation in neuronal cells was not affected when inhibiting *de novo* ceramide synthesis (L-cycloserine, fumonisins B1) (Fig. 6D). Taken together these findings provided evidence that an accumulation of ceramide derived from sphingomyelin hydrolysis by Mg^{2+} -nSMase rather than *de novo* synthesis, mediated the generation of intracellular ROS in neuronal cells exposed to the proinflammatory cytokine $TNF\alpha$.

Ceramide promotes assembly of a neuronal NOX activity

Recent studies demonstrated the expression and function of NOX family members in neuronal cells (Noh and Koh, 2000; Bedard and Krause, 2007). Chick cortical neurons expressed a $TNF\alpha$ -sensitive NOX2-like activity as demonstrated previously in SH-SY5Y neuroblastoma cells (Barth et al., 2009). Immunoreactivity against all NOX2 subunits was present in whole brain homogenates (chick E7, E9, and E11) using human anti-NOX2 subunit-specific antibodies (Fig. 7A). Using native gel electrophoresis in combination with NBT (Nitroblue tetrazolium) staining, NADPH oxidoreductase activities were revealed in E7 whole brain homogenates (Fig. 7B, lane 1) and in plasma membrane preparations obtained from E7 cortical neurons (Fig. 7B, lane 2) with one of these activities corresponding to NOX2 (Fig. 7B, lane 3, western blot). Quantifying the relative increase of the cytosolic NOX2 subunit $p67^{phox}$ in a plasma membrane fraction is a well-documented analysis of NOX activation in combination with ROS measurements (Figures 5 and 6) since NOX2 activation requires an interaction between three cytosolic subunits ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$) with the two membrane subunits NOX2 and $gp22^{phox}$. As shown in Figure 7C, exposure of cortical neurons to 100 ng/ml $TNF\alpha$ indeed increased the relative level of plasma membrane-associated $p67^{phox}$ (1.36 ± 0.03 , $*p < 0.05$, $n = 3$) compared to control (1.00 ± 0.02 , $n = 3$). Importantly, relative $p67^{phox}$ membrane levels were sensitive to preincubation with 10 μM arachidonyl trifluoromethylketone (ATK, 1.13 ± 0.01 , $n = 3$), an inhibitor of cytosolic phospholipase A2 (cPLA2) preventing the formation of the potent NOX2-lipid

activator arachidonic acid, 10 μ M DPI (1.07 ± 0.05 , $n=4$), an NOX inhibitor, or 5 mM NAC (0.9 ± 0.06 , $n=4$) (data not shown).

To corroborate a ceramide-dependent NOX2 activation in neuronal cells exposed to TNF α , we perturbed ceramide accumulation either by inhibiting Mg²⁺-nSMase-dependent sphingomyelin hydrolysis or *de novo* ceramide synthesis and quantified both relative plasma membrane levels of p67^{phox} and phosphorylation of the cytosolic subunit p40^{phox}. Exposure of SH-SY5Y cells to TNF α (100 ng/ml, 15 min) significantly increased the relative levels of both p40^{phox} phosphorylation (1.65 ± 0.12 , $*p < 0.05$, $n=3$) and plasma membrane-associated p67^{phox} (1.48 ± 0.05 , $*p < 0.05$, $n=3$) compared to control conditions (1.02 ± 0.05 , $n=3$ and 1.01 ± 0.01 , $n=3$, respectively) with either response abolished when inhibiting Mg²⁺-nSMase activity with 10 μ M GW4869 (1.19 ± 0.04 , $n=3$ and 1.20 ± 0.02 , $**p < 0.05$, $n=3$) (Fig. 7, D and E). Incubation of SH-SY5Y cells with palmitate (250 μ M, 4 h), which stimulates *de novo* ceramide synthesis, also resulted in a significant increase in the relative level of plasma membrane-associated p67^{phox} (1.34 ± 0.02 , $*p < 0.05$, $n=3$) as opposed to control (1.01 ± 0.01 , $n=3$), a response negated by pretreatment with 5 mM L-cycloserine (1.07 ± 0.01 , $***p < 0.05$, $n=3$) (Fig. 7E). Taken together, these findings implicated a NOX2-like activity as the source of intracellular ROS formation in human SH-SY5Y neuroblastoma cells and cortical neurons in response to TNF α .

TNF α inflicts oxidative damage to SphK1

Our finding suggested that TNF α -mediated inhibition of SphK1 is redox-sensitive likely involving a neuronal NOX2 activity in accordance with a recent study revealing a redox-dependence underlying A β ₁₋₄₂-mediated inhibition of SphK1 in SH-SY5Y cells (Gomez-Brouchet et al., 2007). Since TNF α causes oxidative damage to actin, we explored whether exposure of neuronal cells to TNF α could inflict irreversible oxidative damage (carbonylation) to SphK1. SH-SY5Y cells were exposed to 100 ng/ml TNF α for 1 h after incubation with pharmacological inhibitors or vehicle (1h) followed by immunoprecipitation of SphK1 and modification with dinitrophenyl (DNP) hydrazine. Quantification of DNP immunoreactivity revealed that exposure to TNF α caused a substantial carbonylation of SphK1 (27.91 ± 1.79 , $*p < 0.05$, $n=5$) compared to control (1.00 ± 0.13 , $n=5$) (Fig. 8). TNF α -mediated carbonylation of SphK1 was completely abolished with 10 μ M GW4869 (0.83 ± 0.22 , $n=5$) as well as with 10 μ M DPI (1.00 ± 0.25 , $n=5$) or 5 mM NAC (2.00 ± 1.06 , $n=5$). Conclusively, TNF α blunted SphK1 activity in neuronal cells by inflicting irreversible oxidative damage to SphK1 via NOX2-generated ROS mediated by an accumulation of ceramide through sphingomyelin hydrolysis by Mg²⁺-nSMase.

Discussion

Our investigations revealed that TNF α potently stimulated Mg²⁺-nSMase activity in both SH-SY5Y human neuroblastoma cells and primary cortical neurons causing the subsequent accumulation of ceramide and ROS, two neurotoxic intermediates. Neuronal stress by ceramide and ROS impaired neurite outgrowth and growth cone motility of DRG neurons in part by disabling SphK1 activity due to oxidative damage. Ceramide is a pleiotropic messenger implicated both in cellular stress responses and apoptosis as well as in neuronal survival as a precursor S1P (Hannun and Luberto, 2000; Chalfant and Spiegel, 2005; Jana et al., 2009). Hence neuronal survival is highly sensitive to shifts in ceramide metabolism considering that the two metabolically linked sphingolipids ceramide and S1P exert opposing cellular effects. Our findings suggest that TNF α neurotoxicity is mediated by ceramide and ROS with the consequences of cellular stress activation compounded by direct antagonism of survival pathways (Fig. 9).

The intracellular pool of the bioactive lipid messenger ceramide represents a hub of sphingolipid metabolism (Futerman and Hannun, 2004). Alterations in the ceramide pool could result from sphingomyelin hydrolysis through the action of sphingomyelinases but also from changes in ceramide degradation, conversion to further ceramide metabolites such as glucosylceramide or de novo synthesis. Our results strongly imply a neuronal Mg^{2+} -nSMase activity as the main source of ceramide accumulation in neuronal cells exposed to TNF α rather than de novo ceramide synthesis. First, neuronal responses to TNF α including increased Mg^{2+} -nSMase activity, loss of SphK 1 activity, increased ROS formation, attenuation of neurite outgrowth and cessation of growth cone advance all were highly sensitive to GW4869, a specific Mg^{2+} -nSMase inhibitor, and restored to basal responses as observed in control conditions (Luberto et al., 2002). A contribution of TNF α -mediated increases in ceramide arising from de novo synthesis could be excluded at least for time periods of 30 min or less according to our experimental setup (Meyer and de Groot, 2003; Becker et al., 2005). Inhibition of de novo ceramide synthesis with L-cycloserine or fumonisins B1 did not interfere with neuronal responses (neurite outgrowth, growth cone motility, ROS formation) upon exposure to TNF α . The use of siRNA targeted against Mg^{2+} -nSMase strongly negated TNF α -mediated ROS formation to levels indistinguishable from control thus corroborating pharmacological studies. Moreover, enzyme activity assays were strictly executed at a neutral pH and in the presence of Mg^{2+} . Sphingomyelinase isoforms are distinguished by their respective pH optima, cation dependence, and subcellular localization (Clarke et al., 2006). The predominant isoform in neuronal cells is Mg^{2+} -nSMase, which is associated with plasma membranes and responsible for ceramide generation in the CNS in response to extrinsic stimuli such as TNF α (Hofmann et al., 2000). However, an nSMase activity resides in the Golgi apparatus and acidic sphingomyelinase activity is abundant in the endosomal/lysosomal compartment. Lastly, the antioxidant NAC potentially abolished TNF α -induced Mg^{2+} -nSMase activity in accordance with a reported redox sensitivity linked to glutathione levels (Rutkute et al., 2007). Notably, a significant decrease of Mg^{2+} -nSMase activity in the presence of GW4869 was determined under control conditions suggesting a role for basal Mg^{2+} -nSMase activity in neuronal cells.

Sphingolipid metabolism is intimately linked to cellular redox regulation (Won and Singh, 2006). In our cellular paradigm, exposure of human SH-SY5Y neuroblastoma cells or chick cortical neurons to TNF α resulted in a ceramide-dependent generation of ROS suggested by sensitivity to GW4869 and siRNA-mediated depletion of Mg^{2+} -nSMase. Furthermore, treatment of neuronal cultures with short-chain ceramide analogues, palmitate stimulation of de novo ceramide synthesis, or inhibition of ceramide degradation all increased ROS formation in neuronal cells. However, TNF α stimulated ROS formation despite inhibiting de novo ceramide synthesis implying that this salvage pathway insignificantly contributes to ceramide accumulation. We previously demonstrated in SH-SY5Y cells that exogenous addition of SOD does abolish TNF α -induced ROS generation using simultaneously DCF and dihydroethidium, a superoxide-sensitive fluorescent indicator (Barth et al., 2009). The present study expands these findings into primary cortical neurons suggesting neuronal NOX2-like activity as the primary ROS-generating source stimulated by TNF α . Chick E7 cortical neurons expressed all subunits for from the NOX2 complex including the plasma membrane subunits NOX2 and gp22^{phox} as well as the three cytosolic factors p67^{phox}, p47^{phox}, and p40^{phox}. Various NOX isoforms are expressed in virtually all non-neuronal and neuronal cell types including SH-SY5Y neuroblastoma cells (Bedard et al., 2007). TNF α -induced ROS generation was negated by DPI, and exogenous catalase. In contrast, a presence of exogenous SOD was ineffective likely due the fact that SOD converts NOX-generated superoxide to peroxide and the redox-sensitive fluorescent indicator DCF is predominantly interacting with peroxide. Double labeling cortical neurons with DCF and dihydroethidium was not feasible to cytotoxicity. Proper NOX2 function requires an intricate interaction between cytosolic subunits and plasma membrane subunits. Relative increases in

plasma membrane-associated p67^{phox} or posttranslational modification of p40^{phox} are commonly used biochemical assays. We showed that exposure of neuronal cells to TNF α increased the relative levels of both p40^{phox} phosphorylation as well as plasma membrane-associated p67^{phox} with both biochemical responses highly sensitive to manipulation of ceramide metabolism. Finally, TNF α exposure resulted in an increase in the ratio of relative levels of plasma membrane-associated p67^{phox} and NOX2 indicative of a translocation of p67^{phox} to neuronal plasma membranes (data not shown). Since ceramide and S1P are metabolically linked sphingolipids, we assessed whether a TNF α -induced ceramide accumulation in neuronal cells would affect SphK1 activity. Exploiting the potent activation of SphK1 by serum, serum-starved SH-SY5Y cells or cortical neurons responded with increased SphK1 activity upon serum exposure, which was completely abolished not only by DMS but also by TNF α . In contrast, neither TNF α nor DMS altered basal SphK1 activity in serum-free conditions. Importantly, TNF α -imposed SphK1 inhibition upon serum exposure was largely relieved when inhibiting Mg²⁺-nSMase activity and NOX-mediated ROS formation. Furthermore, TNF α -dependent inhibition of SphK1 resulted from oxidative damage reflected by significant carbonylation, an irreversible oxidative modification, which was rescued either by ROS scavenging, NOX inhibition, or Mg²⁺-nSMase inhibition. In support of this finding, it was recently shown that the antioxidant NAC interferes with A β ₁₋₄₂-dependent impairment of SphK1 activity in SH-SY5Y cells whereas, in contrast, TNF α stimulated SphK1 activity in A549 carcinoma cells (Billich et al., 2005; Gomez-Brouchet et al., 2007).

SphK1 regulates many survival mechanisms in neuronal cells including synaptic plasticity, development of neuronal process, and potentially regeneration (Taha et al., 2006; Alemany et al., 2007). To address biological consequences of SphK1 inhibition in response to TNF α , we examined NGF-dependent neurite outgrowth and growth cone advance of DRG neurons, which is modulated by sphingosine-1-phosphate (Toman et al., 2004). As expected, inhibition of SphK1 with 10 μ M DMS greatly reduced neurite outgrowth but only in serum-containing medium and for the duration of 1h. This would imply that in serum free conditions NGF-dependent neurite outgrowth is SphK1 independent. However, it is feasible that higher doses of DMS for longer time periods would interfere with neurite outgrowth. TNF α also impaired neuronal outgrowth to a similar extent as obtained with DMS. It is noteworthy that conditions equivalent to those that rescued SphK1 activity from TNF α -inflicted damage (inhibition of Mg²⁺-nSMase, inhibition of NOX, scavenging ROS) all rescued neurite outgrowth except for blocking de novo ceramide synthesis. Attenuation of neurite outgrowth could result indirectly from apoptotic mechanisms originating neuronal cell bodies. Although the short time period of TNF α exposure (< 1 h) is unlikely to attribute an apoptotic contribution, we determined the effects of TNF α in conjunction with pharmacology on growth cone advance employing live video phase contrast microscopy. Growth cone responded within minutes to TNF α exposure displaying loss of morphology and motility and ceasing advance while steadily advancing under control conditions. Importantly, inhibiting Mg²⁺-nSMase with GW4869 protected DRG growth cone morphology and advance in the presence of TNF α without effect under control conditions. In contrast, inhibiting de novo ceramide synthesis was unable to rescue growth cones. In accordance with neurite outgrowth, inhibition of SphK1 with DMS halted growth cone advance only in serum-containing medium. These studies implied that TNF α exposure directly impairs DRG growth cone advance and thus neurite outgrowth mediated by ceramide, derived from sphingomyelin hydrolysis, and increased ROS formation. Due to limitations in cell number when working with DRG neurons in contrast to SH-SY5Y cells or cortical neurons, studies relied largely on pharmacology.

A persistent and spreading inflammation in conjunction with severe oxidative stress are hallmarks of neuronal degeneration associated with most chronic CNS disorders, acute CNS

injuries, several psychiatric conditions, as well as aging (Lucas et al., 2006). Dysregulation of sphingolipid metabolism and its interconnection to excessive ROS production are key processes on the molecular level (Won and Singh, 2006). Our findings expand on these concepts and demonstrated in neuronal cells that the proinflammatory cytokine TNF α skews sphingolipid metabolism towards an excessive accumulation of ceramide compounded by a subsequent neuronal NOX-mediated ROS production, and the direct destruction of the S1P survival pathway hence exacerbating neurotoxicity. In this scenario, the neuronal Mg²⁺-nSMase emerges as key to neuronal degeneration and targeting Mg²⁺-nSMase activation could represent a desirable therapeutic strategy to alleviate CNS inflammation.

Acknowledgments

We are grateful to Danielle L. LaVictorie for experimental support. We thank Dr. Larry Duffy, Dr. Marvin Schulte, Dr. Thomas Clausen, and Dr. Thomas Green for helpful insights during the progress of this study and critical review of the manuscript. We are also indebted to Colin McGill and Daniel Kirschner for many discussions. We would like to thank Dr. Mark Kester and Jeremy Haakenson of the Penn State College of Medicine for the use of laboratory facilities and for technical assistance.

This study was supported in part by National Institutes of Health U54 grant NS41069 (NINDS) and United States Department of Agriculture grant 2005-34495-16519.

References

- Adam-Klages S, Adam D, Wiegmann K, Struve S, Kolanus W, Schneider-Mergener J, Kronke M. FAN, a novel WD-repeat protein, couples the p55 TNF-receptor to neutral sphingomyelinase. *Cell*. 1996; 86(6):937–47. [PubMed: 8808629]
- Aleman R, van Koppen CJ, Danneberg K, Ter Braak M, Meyer Zu Heringdorf D. Regulation and functional roles of sphingosine kinases. *Naunyn Schmiedebergs Arch Pharmacol*. 2007; 374(5-6): 413–28. [PubMed: 17242884]
- Andrieu-Abadie N, Gouaze V, Salvayre R, Levade T. Ceramide in apoptosis signaling: relationship with oxidative stress. *Free Radic Biol Med*. 2001; 31(6):717–28. [PubMed: 11557309]
- Barth BM, Stewart-Smeets S, Kuhn TB. Proinflammatory Cytokines Provoke Oxidative Damage to Actin in Neuronal Cells Mediated by Rac1 and NADPH oxidase. *Mol Cell Neurosci*. 2009
- Becker KP, Kitatani K, Idkowiak-Baldys J, Bielawski J, Hannun YA. Selective inhibition of juxtanuclear translocation of protein kinase C β II by a negative feedback mechanism involving ceramide formed from the salvage pathway. *J Biol Chem*. 2005; 280(4):2606–12. [PubMed: 15546881]
- Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*. 2007; 87(1):245–313. [PubMed: 17237347]
- Bedard K, Lardy B, Krause KH. NOX family NADPH oxidases: not just in mammals. *Biochimie*. 2007; 89(9):1107–12. [PubMed: 17400358]
- Billich A, Bornancin F, Mechtcheriakova D, Natt F, Huesken D, Baumruker T. Basal and induced sphingosine kinase 1 activity in A549 carcinoma cells: function in cell survival and IL-1 β and TNF- α induced production of inflammatory mediators. *Cell Signal*. 2005; 17(10):1203–17. [PubMed: 16038795]
- Bottenstein JE, Sato GH. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc Natl Acad Sci U S A*. 1979; 76(1):514–7. [PubMed: 284369]
- Chalfant CE, Spiegel S. Sphingosine 1-phosphate and ceramide 1-phosphate: expanding roles in cell signaling. *J Cell Sci*. 2005; 118(Pt 20):4605–12. [PubMed: 16219683]
- Clarke CJ, Snook CF, Tani M, Matmati N, Marchesini N, Hannun YA. The extended family of neutral sphingomyelinases. *Biochemistry*. 2006; 45(38):11247–56. [PubMed: 16981685]
- Cross AR, Erickson RW, Curnutte JT. Simultaneous presence of p47(phox) and flavocytochrome b-245 are required for the activation of NADPH oxidase by anionic amphiphiles. Evidence for an intermediate state of oxidase activation. *J Biol Chem*. 1999; 274(22):15519–25. [PubMed: 10336445]

- Futerman AH, Hannun YA. The complex life of simple sphingolipids. *EMBO Rep.* 2004; 5(8):777–82. [PubMed: 15289826]
- Gomez-Brouchet A, Pchejetski D, Brizuela L, Garcia V, Altie MF, Maddelein ML, Delisle MB, Cuvillier O. Critical role for sphingosine kinase-1 in regulating survival of neuroblastoma cells exposed to amyloid-beta peptide. *Mol Pharmacol.* 2007; 72(2):341–9. [PubMed: 17522181]
- Groemping Y, Rittinger K. Activation and assembly of the NADPH oxidase: a structural perspective. *Biochem J.* 2005; 386(Pt 3):401–16. [PubMed: 15588255]
- Hannun YA, Luberto C. Ceramide in the eukaryotic stress response. *Trends Cell Biol.* 2000; 10(2):73–80. [PubMed: 10652518]
- Hofmann K, Tomiuk S, Wolff G, Stoffel W. Cloning and characterization of the mammalian brain-specific, Mg²⁺-dependent neutral sphingomyelinase. *Proc Natl Acad Sci U S A.* 2000; 97(11):5895–900. [PubMed: 10823942]
- Jana A, Hogan EL, Pahan K. Ceramide and neurodegeneration: susceptibility of neurons and oligodendrocytes to cell damage and death. *J Neurol Sci.* 2009; 278(1-2):5–15. [PubMed: 19147160]
- Kawahara T, Quinn MT, Lambeth JD. Molecular evolution of the reactive oxygen-generating NADPH oxidase (Nox/Duox) family of enzymes. *BMC Evol Biol.* 2007; 7:109. [PubMed: 17612411]
- Kuhn T, Brown M, Bamburg J. Rac1-dependent actin filament organization in growth cones is necessary for β 1-integrin-mediated advance but not for growth on poly-D-lysine. *Journal of Neurobiology.* 1998; 37:524–40. [PubMed: 9858256]
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227(259):680–5. [PubMed: 5432063]
- Lamour NF, Chalfant CE. Ceramide-1-phosphate: the “missing” link in eicosanoid biosynthesis and inflammation. *Mol Interv.* 2005; 5(6):358–67. [PubMed: 16394251]
- Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* 1994; 233:346–57. [PubMed: 8015469]
- Li JM, Shah AM. Intracellular localization and pre-assembly of the NADPH oxidase complex in cultured endothelial cells. *J Biol Chem.* 2002
- Luberto C, Hassler DF, Signorelli P, Okamoto Y, Sawai H, Boros E, Hazen-Martin DJ, Obeid LM, Hannun YA, Smith GK. Inhibition of tumor necrosis factor-induced cell death in MCF7 by a novel inhibitor of neutral sphingomyelinase. *J Biol Chem.* 2002; 277(43):41128–39. [PubMed: 12154098]
- Lucas SM, Rothwell NJ, Gibson RM. The role of inflammation in CNS injury and disease. *Br J Pharmacol.* 2006; 147(Suppl 1):S232–40. [PubMed: 16402109]
- Maceyka M, Payne SG, Milstien S, Spiegel S. Sphingosine kinase, sphingosine-1-phosphate, and apoptosis. *Biochim Biophys Acta.* 2002; 1585(2-3):193–201. [PubMed: 12531554]
- Marchesini N, Hannun YA. Acid and neutral sphingomyelinases: roles and mechanisms of regulation. *Biochem Cell Biol.* 2004; 82(1):27–44. [PubMed: 15052326]
- Meyer SG, de Groot H. Cycloserine and threo-dihydrosphingosine inhibit TNF- α -induced cytotoxicity: evidence for the importance of de novo ceramide synthesis in TNF- α signaling. *Biochim Biophys Acta.* 2003; 1643(1-3):1–4. [PubMed: 14654222]
- Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL. Essential role for sphingosine kinases in neural and vascular development. *Mol Cell Biol.* 2005; 25(24):11113–21. [PubMed: 16314531]
- Mrak RE, Griffin WS. Glia and their cytokines in progression of neurodegeneration. *Neurobiol Aging.* 2005; 26(3):349–54. [PubMed: 15639313]
- Neumann H, Schweigreiter R, Yamashita T, Rosenkranz K, Wekerle H, Barde YA. Tumor necrosis factor inhibits neurite outgrowth and branching of hippocampal neurons by a rho-dependent mechanism. *J Neurosci.* 2002; 22(3):854–62. [PubMed: 11826115]
- Nikolova S, Lee YS, Lee YS, Kim JA. Rac1-NADPH oxidase-regulated generation of reactive oxygen species mediates glutamate-induced apoptosis in SH-SY5Y human neuroblastoma cells. *Free Radic Res.* 2005; 39(12):1295–304. [PubMed: 16298859]
- Noh K-M, Koh J-Y. Induction and activation by zinc of NADPH oxidase in cultured cortical neurons and astrocytes. *Journal of Neuroscience.* 2000; 20:1–5. [PubMed: 10627575]

- Olivera A, Spiegel S. Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature*. 1993; 365(6446):557–60. [PubMed: 8413613]
- Rutkute K, Asmis RH, Nikolova-Karakashian MN. Regulation of neutral sphingomyelinase-2 by GSH: a new insight to the role of oxidative stress in aging-associated inflammation. *J Lipid Res*. 2007; 48(11):2443–52. [PubMed: 17693623]
- Sumimoto H, Miyano K, Takeya R. Molecular composition and regulation of the Nox family NAD(P)H oxidases. *Biochem Biophys Res Commun*. 2005; 338(1):677–86. [PubMed: 16157295]
- Taha TA, Hannun YA, Obeid LM. Sphingosine kinase: biochemical and cellular regulation and role in disease. *J Biochem Mol Biol*. 2006; 39(2):113–31. [PubMed: 16584625]
- Toman RE, Payne SG, Watterson KR, Maceyka M, Lee NH, Milstien S, Bigbee JW, Spiegel S. Differential transactivation of sphingosine-1-phosphate receptors modulates NGF-induced neurite extension. *J Cell Biol*. 2004; 166(3):381–92. [PubMed: 15289497]
- Tomiuk S, Zumbansen M, Stoffel W. Characterization and subcellular localization of murine and human magnesium-dependent neutral sphingomyelinase. *J Biol Chem*. 2000; 275(8):5710–7. [PubMed: 10681556]
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A*. 1979; 76(9):4350–4. [PubMed: 388439]
- Ueyama T, Tatsuno T, Kawasaki T, Tsujibe S, Shirai Y, Sumimoto H, Leto TL, Saito N. A regulated adaptor function of p40phox: distinct p67phox membrane targeting by p40phox and by p47phox. *Mol Biol Cell*. 2007; 18(2):441–54. [PubMed: 17122360]
- Viviani B, Bartesaghi S, Corsini E, Galli CL, Marinovich M. Cytokines role in neurodegenerative events. *Toxicol Lett*. 2004; 149(1-3):85–9. [PubMed: 15093252]
- Wessel D, Flugge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem*. 1984; 138(1):141–3. [PubMed: 6731838]
- Won JS, Singh I. Sphingolipid signaling and redox regulation. *Free Radic Biol Med*. 2006; 40(11):1875–88. [PubMed: 16716889]
- Wright MV, Kuhn TB. CNS neurons express two distinct plasma membrane electron transport systems implicated in neuronal viability. *Journal of Neurochemistry*. 2002; 83:665–65. [PubMed: 12390528]

Figure 1. TNF α attenuates neurite outgrowth via ceramide and ROS

Dorsal root ganglia (E9) exhibiting radial neurite outgrowth ($2 \mu\text{g}/\text{cm}^2$, laminin, FBS-containing medium) were incubated with pharmacological inhibitors (1 h) or vehicle (equal amount of buffer) prior to acute exposure to TNF α (100 ng/ml, 30 min) or ovalbumin (10 $\mu\text{g}/\text{ml}$, 30 min), our control. Cultures were fixed with 2% glutaraldehyde, and neurite length measured in six random sectors per ganglion (Metamorph Imaging software). All measurements were normalized to control. (A) TNF α significantly attenuated neurite outgrowth (filled bar) compared to control (open bar). Similarly, inhibiting SphK1 activity with 10 μM DMS also abolish neurite outgrowth (hatched bar), which was not further exacerbated upon addition of TNF α (DMS+TNF α). Neurite outgrowth in the presence of TNF α was rescued (grey bars) either by blocking Mg $^{2+}$ -nSMase activity with 10 μM GW4869 thus preventing an accumulation of ceramide, by inhibiting NOX activity (10 μM DPI), or scavenging ROS (2 mM NAC). (B) Neurite outgrowth of DRG neurons is not affected by pharmacological inhibitors either in the presence (filled bars) or absence (graded bars) of FBS with the exception of 10 μM DMS (black shading), which reduced neurite outgrowth only in FBS-containing medium. Neurite outgrowth was insensitive to blocking either sphingomyelin hydrolysis by Mg $^{2+}$ -nSMase (10 μM GW4869) or de novo ceramide synthesis with 5 mM L-cycloserine (CS) or 10 μM fumonisin B1 (FB1) regardless of FBS presence (dark grey bars) Similarly, neurite outgrowth was not affected when preventing ROS accumulation (light grey bars) either by inhibiting NOX (10 μM DPI) or antioxidants (5 mM NAC). All data were obtained in a least three experiments and normalized control. Error bars represent SEM with statistical significance at $*p < 0.05$.

Figure 2. TNF α disrupts growth cone advance of DRG neurons in a ceramide-sensitive manner DRG ganglia (E9) were grown on laminin until displaying vigorous, radial neurite outgrowth and transferred to observation medium for microscopy. Following a recovery period, images of advancing growth cones were acquired (20x, phase contrast, heated stage, 38 $\pm$ 0.2 $^{\circ}$ C, Nikon TE2000) at 3 to 5 min time intervals prior to application of pharmacological inhibitors and/or TNF α . Displacement of the growth cone-neurite border was quantified over time and plotted as the cumulative gain/loss of distance (μ m) as a function of time (min) with slopes indicating growth rates. (A) A DRG growth cone steadily advances upon addition of 10 μ g/ml ovalbumin (control, t = 0min). Note the considerable morphological plasticity displayed. (B) A DRG growth cone exposed to 100 ng/ml TNF α (t = 0min) readily ceases advance and exhibits a degenerating morphology. (C) SphK 1 activity contributes to growth cone motility in the presence of serum. Acute addition of 10 μ M DMS (arrowhead, t = 10min) inhibits growth cone advance in serum-containing medium (filled circles) within 5-10 minutes upon addition whereas in serum-free medium DMS is ineffective (open circles). (D) Growth cone advance is unaffected by an addition (arrowhead, t = 10 min) of L-cycloserine (CS; filled triangles) or 10 μ M fumonisin B1 (FB1; filled circles), two inhibitors of de novo ceramide synthesis. Yet, growth cones remain vulnerable to TNF α exposure (arrow, t = 20min) despite blocking de novo ceramide synthesis with 10 μ M fumonisin B1. This indicates that de novo synthesis does not contribute to a TNF α -stimulated ceramide accumulation. (E) In contrast, blocking Mg $^{2+}$ -nSMase activity with 10 μ M GW4869 (arrowhead, addition at t = 10min) rescues growth

cones advance (open squares) in the presence of TNF α (arrow) without affecting growth cone advance per se (filled squares) suggesting sphingomyelin hydrolysis by Mg²⁺- nSMase as the major ceramide source in response to TNF α . Error bars represent SEM (p* <0.05). Scale bar = 5 μ m.

Figure 3. TNF α inhibits SphK1 activity in neuronal cells in a redox and ceramide-sensitive manner

SH-SY5Y neuroblastoma cells (A) or primary cortical neuron cultures (B) were serum deprived over night prior to addition of 10% FBS (1 h) either in the presence of absence of TNF α and pharmacological inhibitors. SphK1 activity was measured in whole cell lysates using 50 μ M D-erythro-D-sphingosine as a substrate and generation of sphingosine-1-phosphate (fluorescent NDA derivative) was quantified. As expected, exposure to serum (FBS) greatly stimulated SphK1 activity compared to basal levels under control conditions (serum-free). Serum-stimulation of SphK1 activity was potentially negated in the presence of 100 ng/ml TNF α or 10 μ M DMS. However, TNF α did not affect basal levels of SphK1 activity. Serum-mediated stimulation of SphK1 activity in the presence of TNF α was restored when preventing ceramide formation with 10 μ M GW4869 (Mg²⁺-nSMase inhibitor) or ROS accumulation with 10 μ M DPI (NOX inhibition) or 5 mM NAC (ROS scavenging). All data were obtained in at least three independent experiments and error bars represent SEM (statistical significance *p<0.05).

Figure 4. TNF α stimulates Mg^{2+} -dependent neutral sphingomyelinase activity in neuronal cells SH-SY5Y human neuroblastoma cells were exposed to 100 ng/ml or 200 ng/ml TNF α or buffer (equal volume) for increasing time periods following incubation with 10 μ M GW4869 or vehicle (1 h). Activity of Mg^{2+} -dependent neutral sphingomyelinase (Mg²⁺-nSMase) was quantified in whole cell lysates (equal amount of total protein) using an enzyme-coupled Amplex Red fluorescence assay. (A) TNF α significantly increased Mg²⁺-nSMase activity (black bars) within 15 min upon exposure, which was abolished by preincubation with 10 μ M GW4869 (grey bars). Note GW4869-treatment also significantly reduced basal levels of Mg²⁺-nSMase activity in the absence of TNF α . (B) Exposure of SH-SY5Y cells to 100 ng/ml TNF α resulted in maximal Mg²⁺-nSMase activation compared to Mg²⁺-nSMase activity obtained with 200 ng/ml TNF α (black bars), both of which were blocked by GW4869 pretreatment (grey bars). (C) TNF α also stimulated an increase in Mg²⁺-nSMase activity in primary cortical neurons (black bar) compared to control (open bar), which was sensitive to 10 μ M GW4869 (grey bar). Data were obtained from at least three independent experiments with error bars representing SEM ($p < 0.05$).

Figure 5. TNF α elicits ROS generation in neuronal cells

To quantify ROS formation in neuronal cells, SH-SY5Y human neuroblastoma cells or primary chick cortical neurons were loaded (1 h) with the oxidation-sensitive fluorescence indicator DCF (50 μ M) in the presence or absence of pharmacological inhibitors or antioxidant enzymes. Following exposure of neuronal cells to 100 ng/ml TNF α for 30 min, maximum DCF fluorescence per mg total protein was quantified in whole cell lysates, and all data was normalized to the average maximum DCF fluorescence under control conditions (relative DCF fluorescence). (A) In SH-SY5Y cells, TNF α elicited a robust formation of ROS (* p <0.05, black bar) compared to control (open bar). TNF α -mediated ROS formation was negated in the presence of 10 μ M DPI, 5 mM NAC, or 5000 U/ml exogenous catalase (CAT) (grey bars) but not with 200 U/ml superoxide dismutase (SOD, ** p <0.05, checkered bar). (B) Acute addition of TNF α to primary cortical neurons also resulted in ROS formation (* p <0.05, black bar) as opposed to control (open bar). TNF α -mediated ROS formation was abolished by a presence of 10 μ M DPI, 5 mM NAC, or exogenous catalase (5000 U/ml CAT) (grey bar) whereas exogenous superoxide dismutase (200 U/ml SOD, ** p <0.05) was ineffective (checkered bar). (C) Western blotting revealed that siRNA targeted against Mg²⁺-nSMase (siNSM) substantially reduces Mg²⁺-nSMase levels in SH-SY5Y cells as opposed to a scrambled siRNA (SCR). Note, Mg²⁺-nSMase levels in SH-SY5Y cells treated with scrambled siRNA were identical to non-treated cells (Con). Actin was used as a loading control. (D) siRNA targeted against Mg²⁺-nSMase successfully negated TNF α -stimulated ROS formation in SH-SY5Y cells (TNF-siNSM, grey bar) compared to a scrambled siRNA (TNF-SCR, * p <0.01, black bar). Neither targeted siRNA (siNSM) nor scrambled siRNA (siSCR) (checkered bars) affected basal ROS levels under control conditions (open bar). All data represent means obtained in a least three independent experiments and error bars represent SEM.

Figure 6. ROS formation in neuronal cells in response to TNF α is sensitive to ceramide derived from sphingomyelin hydrolysis but not de novo synthesis

Neuronal cells were loaded (1 h) with the oxidation-sensitive fluorescence indicator DCF (50 μ M) in the presence or absence of pharmacological inhibitors or vehicle (equal amount of buffer) for 1 h prior to an acute addition of TNF α (100 ng/ml, 30 min), a short-chain C2-ceramide analogue (10 μ M, 30 min), or palmitate (250 μ M, 4 h) to stimulate *de novo* ceramide synthesis. Maximum DCF fluorescence was quantified in whole cell lysates, adjusted to mg total soluble protein, and then normalized to control (relative DCF fluorescence). (A) ROS formation in SH-SY5Y cells exposed to TNF α was abolished when inhibiting Mg²⁺-nSMase-dependent sphingomyelin hydrolysis with 10 μ M GW4869 (TNF-GW) compared to vehicle (TNF) and indistinguishable from control (Con). Both, C2-ceramide (Cer) and palmitate (Pal) stimulated ROS formation. Inhibiting *de novo* ceramide synthesis with 5 mM L-cycloserine or 10 μ M fumonisins B1 interfered with palmitate-stimulated ROS formation (Pal-CS and Pal-FB1, respectively). (B) Cortical neurons responded with a dramatic ROS production in response to TNF α (TNF) compared to control (Con), which was negated by inhibiting Mg²⁺-nSMase (TNF-GW). Elevating intraneuronal ceramide levels with C2-ceramide or by NOE (inhibiting ceramide degradation) both increased intraneuronal ROS formation (Cer and NOE, respectively). (C) Basal ROS formation in SH-SY5Y cells was not affected by Mg²⁺-nSMase inhibition (GW), *de novo* ceramide synthesis (CS, FB1), or NOX inhibition (DPI). However, a presence of 5 mM NAC resulted in a significant reduction of basal ROS levels (NAC, * p <0,05). (D) *De novo* ceramide synthesis did not contribute to ROS formation in SH-SY5Y cells exposed to TNF α . Neither L-cycloserine (TNF-CS, * p <0,05) nor fumonisins B1 (TNF-FB1, * p <0,05) interfered with TNF α -mediated ROS formation (TNF, * p <0,05) compared to control (Con). Data were obtained in at least three independent experiments and errors represent SEM.

Figure 7. Cortical neurons express a TNF α -sensitive NOX2-like activity

(A) Western blot analysis revealed both membrane-bound subunits (gp91^{phox}, p22^{phox}) and cytosolic subunits (p67^{phox}, p47^{phox}, and p40^{phox}) composing the NOX2 complex in chick E7, E9, and E11 whole brain homogenates (loading control: actin). (B) Using native-gel electrophoresis and subsequent in-gel NBT staining, NADPH oxidoreductase activity was found in chick E7 whole brain homogenates (lane 1) and in plasma membrane preparations from E7 cortical neurons (lane 2). One NADPH oxidoreductase activity corresponded with the presence of Nox2 (lane 3) as demonstrated by immunoreactivity against gp91^{phox} (western blot). (C) In primary cortical neurons, TNF α (100 ng/ml, 30 min) stimulated the formation of a functional NOX2 complex (* $p < 0.05$, filled bar) as indicated by a significant translocation of p67^{phox} from the cytosol to plasma membranes compared to control (open bar) determined by SDS gel electrophoresis of a plasma membrane protein fraction (equal amount of total protein per lane), followed by western blotting and quantification of anti-p67^{phox} immunoreactivity (chemiluminescence). A membrane protein fraction was obtained after biotinylation, cell lysis, and affinity purification on streptavidin. Translocation of p67^{phox} to plasma membranes was negated by 10 μ M ATK (grey bar), an inhibitor of cPLA₂ preventing the formation of arachidonic acid, a crucial lipid activator of NOX. All values were obtained in least three independent experiments and normalized to control. (D and E) Ceramide-dependence of NOX2 activation was analyzed by determining phosphorylation of the cytosolic subunit p40^{phox} and membrane translocation of cytosolic subunit p67^{phox}. Ceramide accumulation via Mg²⁺-nSMase activity was assayed by pretreating (1 h) SH-

SY5Y cells with 10 μ M GW4869 or buffer (equal volume) whereas *de novo* ceramide synthesis was addressed by incubation (4 h) with 250 μ M palmitate (Pal) in the presence or absence of 5 mM L-cycloserine (CS, a serine palmitoyltransferase inhibitor). (D) Exposure of SH-SY5Y cells to TNF α (100 ng/ml, 15 min) caused a substantial phosphorylation of the cytosolic NOX2 subunit p40^{phox} (*p<0.05, filled bar) compared to control condition (open bar), which was abolished by inhibiting Mg²⁺-nSMase activity with GW4869 (grey bar) as detected by westernblotting (equal amounts of total protein per lane) and quantification of phospho-p40^{phox} immunoreactivity (chemiluminescence). (E) Palmitate-induced ceramide accumulation via *de novo* synthesis increased translocation of p67^{phox} to plasma membranes (Pal, *p<0.05) as opposed to control (CON), which was completely negated with 5 mM of the serine palmitoyltransferase inhibitor L-cycloserine (Pal+CS, ***p<0.05). Similarly, TNF α stimulated an increased translocation of p67^{phox} to plasma membranes (TNF α , *p<0.05), which was negated in the presence of GW4869 (TNF α +GW, **p<0.05). Data were obtained in at least three independent experiments and error bars represent SEM. Error bars represent standard deviations (*p<0.05).

Figure 8. TNF α provokes oxidative damage to neuronal SphK1

Oxidative damage to proteins was determined by analyzing carbonylation (irreversible oxidation) via DNP incorporation. SH-SY5Y cultures were incubated (1 h) with the Mg^{2+} -nSMase inhibitor GW4869 (10 μ M), the NOX inhibitor DPI (10 μ M), or the ROS scavenger NAC (5 mM). Following exposure to TNF α (100 ng/ml, 1 h) SphK1 was immunoprecipitated from whole cell lysates and derivatized with 2,4-dinitrophenyl (DNP)-hydrazine. Samples containing equal amounts of protein were subjected to SDS gel electrophoresis followed by western blotting, and quantification of anti-DNP immunoreactivity (chemiluminescence). TNF α caused significant carbonylation of SphK1 (* p <0.05, filled bar), which was abolished (grey bars) either by inhibiting Mg^{2+} -nSMase-dependent ceramide accumulation (TNF α +GW), ROS formation (TNF α +DPI), or ROS scavenging (TNF α +NAC). All data were obtained in at least three experiments and normalized to control. Error bars represent SEM.

Figure 9. Compounding neurotoxicity of $\text{TNF}\alpha$ - ceramide and ROS directly antagonize the formation of the survival factor S1P

The two sphingolipid messengers, ceramide and S1P, regulate fundamental cellular processes although their actions are directly opposing. Whereas ceramide is implicated in degenerative processes, S1P plays a key role in survival pathways. Hence, neuronal health thrives when intracellular levels of S1P are substantial (open diamond) yet levels ceramide and ROS (open circle) are low. A presence of $\text{TNF}\alpha$ (black arrow) shifts sphingolipid metabolism not only by elevating ceramide and ROS but also by directly antagonizing S1P levels through oxidative damage of SphK1. Neuronal stress compromises viability reflected by high levels of ceramide and ROS (closed circle) and low levels of S1P (closed diamond). This compounding effect mediated by $\text{TNF}\alpha$ attributes a pivotal role to both Mg^{2+} -nSMase and NOX in neuronal degeneration in CNS pathologies plagued by inflammation.