

Krabbe disease: psychosine-mediated activation of phospholipase A2 in oligodendrocyte cell death

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Abstract Globoid cell leukodystrophy (Krabbe disease) is an inherited neurological disorder caused by the pathogenic accumulation of psychosine (galactosylsphingosine), a substrate for the deficient enzyme galactocerebroside β -galactosidase. This study underscores the mechanism of action of psychosine in the regulation of oligodendrocyte cell death via the generation of lysophosphatidylcholine (LPC) and arachidonic acid (AA) by the activation of secretory phospholipase A2 (sPLA2). There was a significant increase in the level of LPC, indicating a phospholipase A2 (PLA2)-dependent pathobiology, in the brains of Krabbe disease patients and those of twitcher mice, an animal model of Krabbe disease. In vitro studies of the treatment of primary oligodendrocytes and the oligodendrocyte MO3.13 cell line with psychosine also showed the generation of LPC and the release of AA in a dose- and time-dependent manner, indicating psychosine-induced activation of PLA2. Studies with various pharmacological inhibitors of cytosolic phospholipase A2 and sPLA2 and psychosine-mediated induction of sPLA2 enzymatic activity in media supernatant suggest that psychosine-induced release of AA and generation of LPC is mainly contributed by sPLA2. An inhibitor of sPLA2, 7,7-dimethyl eicosadienoic acid, completely attenuated the psychosine-mediated accumulation of LPC levels, release of AA, and generation of reactive oxygen species, and blocked oligodendrocyte cell death, as evident from cell survival, DNA fragmentation, and caspase 3 activity assays. This study documents for the first time that psychosine-induced cell death is mediated via the sPLA2 signaling pathway and that inhibitors of sPLA2 may hold a therapeutic potential for protection against oligodendrocyte cell death and resulting demyelination in Krabbe disease.—Giri, S., M. Khan, R. Rattan, I. Singh, and A. K. Singh. **Krabbe disease: psychosine-mediated activation of phospholipase A2 in oligodendrocyte cell death.** *J. Lipid Res.* 2006. 47: 1478–1492.

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Krabbe disease, also known as globoid cell leukodystrophy, is an inherited demyelinating disease caused by a deficiency of galactosylceramidase. Cytotoxicity of psychosine in vitro and the fatal effects of intracranial-injected psychosine led to the conclusion (known as the psychosine hypothesis) that progressive accumulation of psychosine is the critical biochemical pathogenetic mechanism of cell death in Krabbe brain (1). There is accumulation of two substances, β -galactocerebroside, which accumulates within macrophages to form the characteristic globoid cells, and galactosylsphingosine (psychosine) (2). Galactocerebroside degrades β -galactocerebroside and psychosine into cerebroside and galactose and sphingosine and galactose, respectively. The galactocerebroside is also hydrolyzed by GM1 gangliosidase, but it cannot degrade psychosine; therefore, there is enhanced accumulation of psychosine relative to galactocerebroside (3). Loss of oligodendrocytes, the cells responsible for myelin formation, results in demyelination (2). Psychosine has been shown to induce several responses characteristic of Krabbe disease; for example, psychosine causes oligodendrocyte cell death (4–6), astrocyte activation (7), and the formation of multinuclear globoid-like cells in U937 monocytic cells (8) and NK cells (9). Apoptotic cells and expression of apoptotic-related molecules such as tumor necrosis factor- α (TNF α) have been observed in brains of the twitcher mouse, an animal model of Krabbe disease, and in brains of patients with Krabbe disease, leading to the conclusion that progressive accumulation of psychosine is the critical biochemical pathogenetic mechanism of cell death in the Krabbe brain (5, 6, 10). We have recently reported that psychosine

Abbreviations: AA, arachidonic acid; CAT, chloramphenicol acetyltransferase; CNS, central nervous system; cPLA₂, cytosolic phospholipase A₂; DCFDA, 6-carboxy 2', 7'-dichlorodihydrofluorescein diacetate; DEDA, 7,7-dimethyl eicosadienoic acid; iPLA₂, calcium-independent phospholipase A₂; LPC, lysophosphatidylcholine; NAC, N-acetylcysteine; PLA₂, phospholipase A₂; ROS, reactive oxygen species; sPLA₂, secretory phospholipase A₂; TNF α , tumor necrosis factor- α .

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mediates oligodendrocyte cell death via upregulation of reactive oxygen species (ROS)-JNK-AP-1, a pro-apoptotic pathway, and downregulation of the NF- κ B pathway, an antiapoptotic pathway (4). However, the mechanism of action of psychosine in the pathophysiology of Krabbe disease is not completely understood.

The observed expression of inflammatory mediators such as TNF α , IL-6, and iNOS in the central nervous systems (CNSs) of Krabbe disease patients and in twitcher mice indicates that the inflammatory response may play a role in the pathobiology of Krabbe disease (7, 10). Inflammatory cytokines are known to induce the phospholipase A₂ (PLA₂) enzyme system. PLA₂s hydrolyze phospholipids at the *sn*-2 position and generate lysolipids and free fatty acids, including arachidonic acid (AA). These mediators are critically involved in the regulation of several physiological events, including cell death (11, 12). The PLA₂s have been divided into three major groups based on size, ability to be secreted, and calcium dependency (11). The three groups consist of low-molecular-mass sPLA₂, (13.5–16.8 kDa), calcium-independent phospholipase A₂ (iPLA₂, 80 kDa), and high-molecular-mass cytosolic phospholipase A₂ (cPLA₂, 85 kDa) (11). The cPLA₂, when activated by phosphorylation via an increase in the cytosolic concentration of calcium, translocates from the cytosol to either the nuclear membrane, endoplasmic reticulum, Golgi, or plasma membrane, depending on cell type and stimulus (12–15), to catalyze the hydrolysis of membrane glycerophospholipids at the *sn*-2 position to liberate AA (11). Several mammalian intracellular and sPLA₂s have been described previously (16). Among sPLA₂s, type IIA sPLA₂, also referred to as synovial PLA₂, is a pro-inflammatory enzyme found to be highly elevated, both in the circulation and locally in the tissue, in a number of pathological diseases, such as atherosclerosis, asthma, and acute lung injury (17–19). Additionally, the identity and role of several sPLA₂s is yet to be revealed.

The products of PLA₂, i.e., lysophosphatidylcholine (LPC) and AA are bioactive lipids and are known to regulate a number of physiological and pathological events (11, 12). LPCs have been shown to induce demyelination (20, 21) by activating cell death in mature oligodendrocytes and in oligodendrocyte progenitors (22, 23). On the other hand, metabolism of AA gives rise to ROS, which induce oxidative stress, leading to either cell proliferation or apoptosis, depending on the cell type (24–27) and also regulate redox-sensitive transcription factors AP-1 and NF- κ B by activating p38 mitogen-activated protein kinase (MAPK) (28–30). Released free AA either acts as a second messenger or is further metabolized by three different enzyme systems, i.e., cyclooxygenase, lipoxygenase, and cytochrome P450 epoxygenase, to generate eicosanoid signaling molecules, including prostaglandins, leukotrienes, and thromboxanes (31). These, in turn, may set the stage for augmented synthesis of lipid mediators, free radicals, and ROS, and hence peroxidative and oxidative damage to cellular membranes (32, 33).

The present study was undertaken to gain a better understanding of the underlying mechanisms of psycho-

sine-induced apoptosis of oligodendrocytes. Studies reported here document excessive accumulation of LPC in brains of twitcher mice and of Krabbe disease patients, indicating a possible role of PLA₂ in the pathobiology of Krabbe disease. Activation of psychosine-mediated sPLA₂ activity and inhibition of psychosine-induced cell death by the inhibitor of sPLA₂ demonstrate that the sPLA₂-mediated signal transduction pathway plays a critical role in the psychosine-induced cell death of oligodendrocytes and that these effects of psychosine are independent of TDAG8, a presumed receptor for psychosine. The inhibition of psychosine-induced cell death by the inhibitor of sPLA₂ indicates that psychosine mediates its proapoptotic signaling through the PLA₂ pathway rather than as the originally suggested consequence of its lytic activity (psychosine hypothesis) (1).

MATERIALS AND METHODS

Reagents

DMEM/4.5 gm glucose/L medium, fetal bovine serum, and Hank's balanced salt solution were obtained from Life Technologies (Grand Island, NY). N-(2S,4R)-4-(biphenyl-2-ylmethylisobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]pyrrolidin-2-ylmethyl]-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide (BDDPAA), arachidonyltrifluoromethyl ketone (AACOF3), c(2NapA)LS(2NapA)R, TFA (NNTFA), 7,7-dimethyl eicosadienoic acid (DEDA), were purchased from EMD Biosciences, Inc. (San Diego, CA) and antibodies against p-cPLA₂, p-p42/p44, p-p38, and p-JNK were purchased from Cell Signaling Technology (Beverly, MA). 6-Carboxy 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA) was from Molecular Probes (Eugene, OR). Lipofectamine 2000 was from Invitrogen. Antibodies against TDAG8 were from Santa Cruz Biotechnology (Santa Cruz, CA). Psychosine, gluco-psychosine, ceramide, and sphingosine were from Matreya (Pleasant Gap, PA). MO3.13 oligodendroglial cells were a kind gift from Dr. Catherine Waters (Division of Neuroscience, Biological Sciences, University of Manchester, UK). The expression vector for TDAG8 was a kind gift from Dr. Kevin R. Lynch (University of Virginia School of Medicine, Charlottesville, Virginia).

Cell culture

Rat primary oligodendrocytes were prepared in our laboratory as described previously (34). The cells were maintained in DMEM (4.5 gm glucose/l) containing 10% FBS and 10 μ g/ml gentamicin. All cultured cells were maintained at 37°C in 5% CO₂/95% air. At 80% confluency, the cells were incubated with serum-free DMEM medium for 24 h prior to incubation with various treatments. MO3.13 oligodendroglial cells were maintained in DMEM (4.5 gm glucose/l) containing 10% FBS and 10 μ g/ml gentamicin. This oligodendrocyte cell line was maintained under serum-free conditions for 24 h before treatment. It was observed that such serum-free conditions led to the expression of the oligodendrocyte marker GST- π in these cells. Psychosine was first dissolved in DMSO and then mixed in the medium at a DMSO concentration 0.1% of the medium.

Human autopsy brain specimens

Frozen-fixed tissues of two Krabbe-diseased brains and two normal age-matched controls were from the Brain and Tissue

Bank (University of Maryland at Baltimore, MD). The frozen-fixed tissues were processed for LPC analysis as described below. Sections from normal brains were cut from corresponding anatomical locations.

Animals

All animal work was approved and performed according to the Guidelines for the Care and Use of Laboratory Animals according to the recommendations of the Institutional Animal Care and Use Committee of the Medical University of South Carolina (MUSC). Twitcher heterozygote breeding pairs (C57BL/6J *twi*⁺) were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained at MUSC's animal facility. The genotypes of all newborns were determined according to the PCR-based method described by Sakai et al. (36) with modifications (suggested by N. Sakai, personal communication) using DNA isolated from tails of 7- to 8-day-old pups (35). We used 5'-CAGTCATTCAGAAAG-TCTTCC-3' as forward primer, and 5'-GCCCACTGTCTTCAGGT-GATA-3' as reverse primer. PCR consisted of a 10 min heating step at 95°C, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min. The PCR products were digested with EcoRV and then subjected to 3:1 NuSeive GTG agarose:agarose gel electrophoresis. Twitcher (30 days old) and age-matched control mice were euthanized by decapitation after an overdose of pentobarbital. Brains were removed immediately and kept in liquid nitrogen and later stored at -80°C until used for lipid analysis.

AA release assay

Primary oligodendrocyte or MO3.13 cells were prelabeled with 0.5 μ Ci/ml [3H]AA in DMEM medium containing 10% FBS for 12 h at 37°C in a humidified incubator supplied with 95% air and 5% CO₂. The labeled cells were then washed twice with serum-free media and treated with the indicated treatments in serum-free DMEM medium at 37°C. At various time periods, radioactivity in the medium (100 μ l) was measured using a liquid scintillation counter.

Quantification of LPC levels in mouse brain and in cultured cells

Total lipids were extracted from twitcher mouse brain homogenates (homogenized in 0.25 M sucrose buffer in the presence of protease inhibitor and butylated hydroxytoluene) as described previously (36). Quantification of LPC was performed by one-dimensional high-performance thin-layer chromatography (HPTLC; LHPK from Whatman, Inc.; Florham Park, NJ) using the method described by Weerheim et al. (37) with modification. Briefly, plates were developed in methyl acetate-1-propanol-chloroform-methyl alcohol-0.25% KCl-acetic acid (100:100:100:40:36.5:2; v/v/v/v/v/v) and visualized by heating at 200°C for 6 min after spraying with 10% CuSO₄ in 8% phosphoric acid. Different concentrations (0.2 to 5.0 μ g) of LPC (1-palmitoyl LPC) were resolved on the same plate as standard for quantification. LPC was quantified by densitometric scanning using the Imaging Densitometer (model GS-670; Bio-Rad). Cell monolayers were incubated with 0.1 μ Ci [¹⁴C]choline chloride (55 mCi/mmol) overnight at 37°C. Cells were washed with PBS and incubated in serum-free medium for 3 h before treatment. Total lipids were extracted by the addition to cell pellets of CHCl₃-CH₃OH-HCl (100:100:1; v/v/v) as described previously (38). Total radioactivity in lipid extracts were 1,255, 904 \pm 155, and 104 dpm. Phospholipids were separated on HPTLC as described above for brain samples. Chromatographs were autoradiographed, and LPC was quantified either by densitometric analysis (represented as arbitrary units) or by scraping the spot

and counting the radioactivity in a Beckman Coulter LS 6500 multi-purpose scintillation counter.

sPLA₂ activity

To assess sPLA₂ activity in supernatant, MO3.13 cells were treated with different concentrations of psychosine (5–20 mM) for various time periods (1–8 h) in serum-free media. Cell supernatant (800 μ l) was taken for sPLA₂ enzymatic activity and incubated in 200 μ l of 5 \times incubation buffer finally comprising 100 mM Tris-HCl (pH 9.0), 4 mM CaCl₂, and 2 μ M [¹⁴C]arachidonyl-*sn*-glycero-3-phosphocholine (PerkinElmer Life Sciences) as the substrate at 37°C for 1 h. The [¹⁴C]AA released was extracted and separated on HPTLC. Labeled AA was quantified by scraping the spot and counting the radioactivity in a Beckman Coulter LS 6500 multi-purpose scintillation counter.

Measurement of ROS

ROS were determined using the membrane-permeable fluorescent dye 6-carboxy 2', 7'-dichlorodihydrofluorescein (DCFDA) diacetate in serum-free medium as described previously (39). The cultured cells, with or without treatment, were incubated with 5 μ M DCF dye in PBS for 2 h at 37°C. The change in fluorescence was determined at excitation 485 nm and emission 530 nm using a Soft Max Pro spectrofluorometer (Molecular Devices, Sunnyvale, CA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell growth and viability

The viability of cells was evaluated using the MTT assay. This assay is based on the cleavage of tetrazolium salt MTT to a dark blue formazan product by mitochondrial dehydrogenase in viable cells. The absorbance of viable cells was measured with a test wavelength of 570 nm and a reference wavelength of 630 nm.

DNA fragmentation

After treatment, cells were harvested and then lysed with lysis buffer containing 20 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA, 1% sodium dodecyl sulfate, and 1 mg/ml proteinase K for 24 h in a 55°C water bath. The standard phenol-chloroform-isoamyl alcohol method (25:24:1) was used to remove protein and extract nucleic acid. RNA was digested with RNase A (100 μ g/ml) for 12 h at 37°C, and DNA concentrations were determined. DNA extracts were electrophoresed on a 2% agarose gel at 50 V for 45 min and visualized with ethidium bromide staining under ultraviolet illumination (40).

Transfection studies

Plasmids were purified using the endotoxin-free plasmid midi prep kit (Qiagen). For transient transfections, HEK293 cells were seeded in 6-well plates and grown to 60–80% confluence in DMEM/F-12 plus 5% FBS without antibiotics and transfected using Lipofectamine 2000 reagent. TDAG8 expression vector (1–3 μ g), along with insertless expression vector (pcDNA3.1), was used for transfecting. Cells were treated with psychosine and/or DEDA for 24 h and processed for further experiments. For PathDetect[®] in Vivo Signal Transduction Pathway *trans*-Reporting Systems (Stratagene; La Jolla, CA), MO3.13 cells were transiently transfected with 0.5 μ g pFA2-cJun-Gal4, pFA2-Elk1-Gal4, or pFA2-CHOP-Gal4 DNA binding domain, along with 0.5 μ g pFR-chloramphenicol acetyltransferase (pFR-CAT) reporter, using Lipofectamine 2000 reagent. After 24 h of transfection, cells were treated with DEDA and psychosine for 24 h and processed for CAT by ELISA (Roche). pFC-MEKK (for JNK), pFC-MEK1 (for p42/44), and pFC-MEK3 (for p38) were

cotransfected, followed by phorbol 12-myristate 13-acetate (PMA) (0.1 nM) treatment as a positive control. pCMV-GAL4 binding domain (without insert) and pFR-CAT were transfected as a control to detect the basal levels of CAT activity.

Short interfering RNA experiments

To decrease the levels of endogenous TDAG8, MO3.13 cells were transfected for 48 h with 25 nM of short interfering RNA (SiRNA) of TDAG8 or control (Santa Cruz Biotechnology). SiRNAs were transfected with Lipofectamine 2000 reagent according to the manufacturer's instructions. The cells were then treated with psychosine for 8 h or 24 h for AA release or for caspase 3 activity assay, respectively.

Activation of caspase 3

Caspase 3 activation was determined using substrates [acetyl-Asp-Glu-Val-Asp (Ac-DEVD)-7-amino-4-methyl-coumarin (AMC) for caspase 3 procured from Biomol (Plymouth Meeting, PA)], which are cleaved into fluorescent reaction products by the respective caspase action (40). For analysis of caspase 3 activity, cells were collected and lysed in 100 μ l of lysis buffer containing 10 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1% (w/v) Triton X-100, and 10 mM sodium pyrophosphate. The lysate was incubated with 20 μ M substrate in reaction buffer [20 mM HEPES-10% (v/v) glycerol-2 mM dithiothreitol] at 37°C for 15 min. The fluorescence intensity (expressed as arbitrary U/ μ g protein) of liberated AMC was measured using spectrofluorometry (excitation wavelength, 380 nm; emission wavelength, 460 nm).

Western blot analysis

Western blot analysis for phospho-MAPKs, cPLA₂, and TDAG8 was performed using standard procedures. Cells were harvested and lysed with lysis buffer [50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, and 0.5% Nonidet P-40] containing a protease inhibitor cocktail (Sigma). The lysate was clarified by centrifugation at 10,000 *g* for 15 min at 4°C. Equal amounts of total protein (50 μ g) were subjected to 4–20% SDS-PAGE and electrophoretically transferred to a High-Bond nitrocellulose membrane (Amersham Life Science; Arlington Heights, IL). After blocking with Tween 20-Tris-buffered saline [TTBS; 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.05% Tween 20] containing 5% nonfat milk for 1 h at room temperature, the membranes were incubated overnight at 4°C with the primary antibodies at 1:1,000 dilution in blocking buffer (TTBS with 5% nonfat milk). The membranes were then washed three times for 10 min each in TTBS and incubated with an appropriately diluted horseradish peroxidase-labeled secondary antibody (1:5,000) in blotting buffer for 1 h at room temperature. The membranes were washed three times, reacted with ECL reagent (Amersham Life Science), and subjected to autoradiography.

Statistical analysis

Data are expressed as mean \pm SD for *n* experiments. Statistical comparisons were made using one-way ANOVA followed by Student's *t*-test and Student Newman-Keuls test as required. A *P* value <0.05 was considered statistically significant.

RESULTS

Accumulation of LPC in Krabbe and twitcher brain

The lipid composition of Krabbe and twitcher brains was analyzed, and a significant increase in the levels of LPC

in the Krabbe and twitcher brains was observed (Fig. 1A, B). To examine the possibility that elevated levels of psychosine in Krabbe and twitcher brain are responsible for LPC generation, primary oligodendrocytes were treated with different concentrations of psychosine (5–20 μ M). It was observed that psychosine treatment significantly increased the LPC levels in a dose-dependent manner in primary oligodendrocytes. A similar pattern was also observed in the oligodendrocyte cell line MO3.13 (Fig. 1C, D). These observations indicate that psychosine accumulation due to a genetic defect causes excessive accumulation of LPC in both Krabbe patient and twitcher mice brains as well as in cells treated with psychosine.

Psychosine-induced AA release in primary oligodendrocytes and the MO3.13 cell line

To further evaluate the psychosine-mediated generation of LPC, the effect of psychosine on the hydrolysis of phospholipids was investigated. For this, primary oligodendrocytes were labeled with radioactive [¹⁴C]AA. After 12 h of incubation, cells were washed with media three times, followed by treatment with different concentrations of psychosine (5–40 μ M). As depicted in Fig. 2A, in primary oligodendrocyte cells treated with psychosine, there was a significant release of labeled AA. A similar trend was observed when the oligodendrocyte cell line MO3.13 was used (Fig. 2B). Psychosine treatment induced sPLA₂ activity in media supernatant in a dose- and time-dependent manner (Fig. 2C). Like psychosine (galactopsychosine), glucopsychosine was equally potent in inducing AA release, whereas ceramide and sphingosine had no effect on MO3.13 cells (Fig. 2D), documenting the specificity of the AA-releasing activity of psychosine and glucopsychosine in oligodendrocytes.

Psychosine-induced AA release is dependent on cPLA₂ and sPLA₂

To investigate the enzymatic activity responsible for the psychosine-induced AA release that is dependent on cPLA₂ and sPLA₂, we employed specific pharmacological inhibitors of cPLA₂ and sPLA₂. Treatment with BDDPAA and AACOF3 (cPLA₂ inhibitor) at different concentrations (5–20 μ M) significantly inhibited psychosine-induced AA release in MO3.13 cells, suggesting the possible role of cPLA₂ in psychosine-mediated AA release (Fig. 3A). Because we did not observe complete inhibition of AA release mediated by psychosine, the role of sPLA₂ was also examined using its specific inhibitors, NNTFA and DEDA. For this, MO3.13 cells were treated with NNTFA and DEDA for 30 min prior to the addition of psychosine. After 8 h of treatment, release of labeled AA in supernatant was examined. Inhibitors of sPLA₂ significantly inhibited the psychosine-induced AA release, indicating the role of sPLA₂ (Fig. 3B). We also used a combination of suboptimal doses of the cPLA₂ inhibitor (BDDPAA, 10 μ M) and the sPLA₂ inhibitor (DEDA, 5 μ M) and examined the effect on psychosine-mediated AA release. The combination of suboptimal doses of BDDPAA (10 μ M) and DEDA (5 μ M) inhibitors further significantly inhibited the psychosine-induced AA release (Fig. 3C), indicating that both cPLA₂

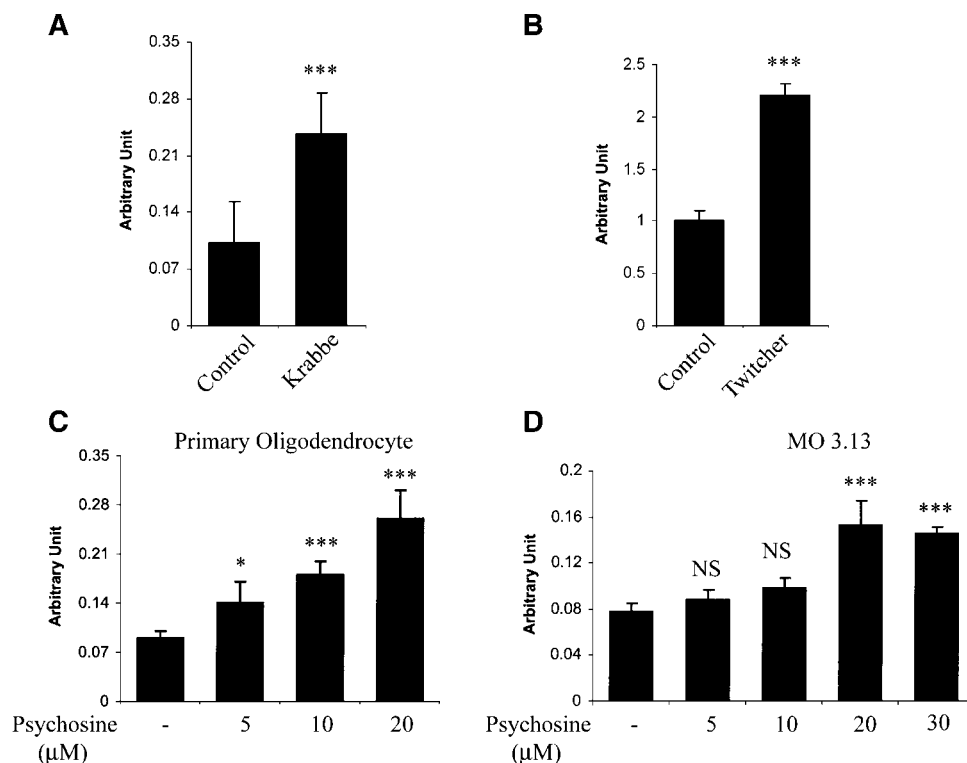


Fig. 1. Accumulation of lysophosphatidylcholine (LPC) in Krabbe patient, in twitcher mice brains, and in psychosine-treated oligodendrocyte cells. LPC analysis in Krabbe patient and normal age-matched control (A), and twitcher mice and control age-matched brains (B) was carried out as described in Materials and Methods. Data are presented as mean \pm SD for triplicate determination. *** $P < 0.01$ compared with normal control human brain/animal. The primary oligodendrocytes (C) and MO3.13 cells (D) were treated with different concentrations of psychosine, as indicated, for 8 h, followed by LPC detection as described in Materials and Methods. Results are mean \pm SD of three determinations. * $P < 0.05$; *** $P < 0.001$; and NS, not significant as compared with untreated cells.

and sPLA2 are involved in the psychosine-mediated release of AA in oligodendrocyte cells.

DEDA inhibits psychosine-induced LPC generation in the oligodendrocyte cell line

To investigate the relationship of AA release and generation of LPC, the generation of LPC was examined in MO3.13 cells treated with DEDA followed by psychosine. After 8 h of treatment with psychosine in the presence or absence of DEDA, cells were processed for LPC detection as described in Materials and Methods. It was observed that the sPLA2 inhibitor (DEDA) completely blocked LPC generation (Fig. 4). However, the cPLA2 inhibitor (BDDPAA) had no effect on LPC generation induced by psychosine (data not shown). The observed complete attenuation of psychosine-induced generation of AA and LPC by the sPLA2 inhibitor as compared with the cPLA2 inhibitor indicates that sPLA2 activity plays a prominent role in this process.

DEDA blocked psychosine-mediated induction of MAPKs in oligodendrocytes

MAPKs such as ERK p42/p44, JNK/SAPK, and p38 of the MAPK pathway have been implicated in the regulation of the signaling pathways leading to apoptosis in response

to oxidative stress. To examine the effect of DEDA on the psychosine-mediated induction of MAPKs (p42/44, JNK, and p38), MO3.13 cells were treated with DEDA (10 μ M) in the presence or absence of psychosine (20 μ M). After 30 min of incubation, cells were processed for the phosphorylation status of these MAPKs using immunoblot analysis. As shown in Fig. 5A, psychosine induced the phosphorylation of p42/44, JNK, and p38 and also induced the phosphorylation of cPLA2. Treatment with DEDA blocked the psychosine-induced phosphorylation of these MAPKs and cPLA2. This observation was further confirmed using PathDetect[®] in Vivo Signal Transduction Pathway *trans*-Reporting Systems (Stratagene; La Jolla, CA). For this, cells were cotransfected with the fusion transactivator plasmid [c-Jun (for JNK), Elk1 (for p42/44), or CHOP (for p38) transcriptional activator fused with the yeast GAL4 DNA binding domain residues 1–147] and reporter plasmid (CAT) and then treated with DEDA in the presence or absence of psychosine. After 24 h incubation, cells were processed to examine the expression of CAT as a reporter using a CAT ELISA kit (Roche Applied Science; Indianapolis, IN). CAT expression from the reporter plasmid in response to psychosine treatment indicated the activation of the fusion transactivator protein and, therefore, the activation of the endogenous protein kinase

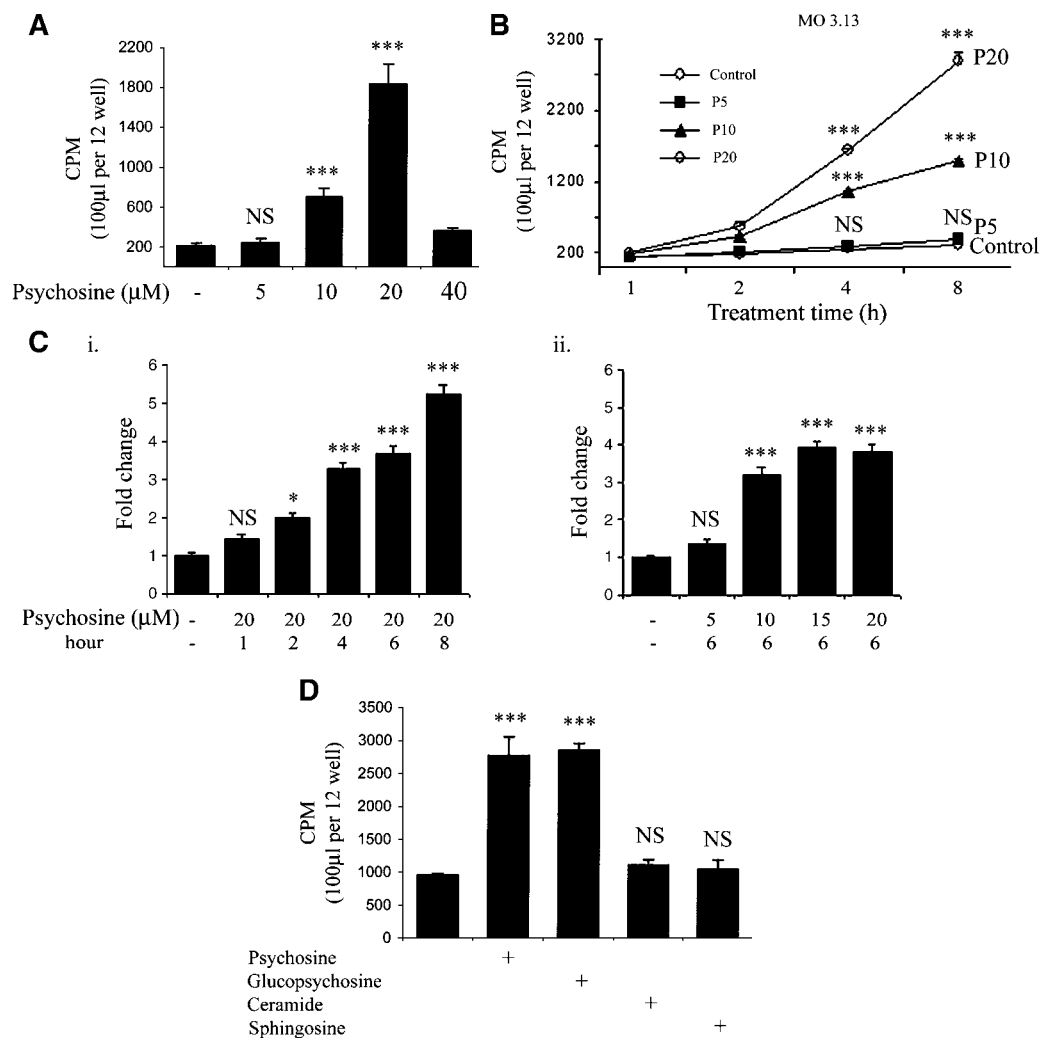


Fig. 2. Effect of psychosine on arachidonic acid (AA) release in oligodendrocytes. Primary oligodendrocytes were labeled with 0.5 µCi/ml [^3H]AA. **A:** After 12 h incubation, cells were washed twice with serum-free media and treated with different concentrations of psychosine (5–40 µM). After 24 h, radioactivity in the medium (100 µl) was measured using a liquid scintillation counter. Results are mean \pm SD of three determinations. *** $P < 0.001$ as compared with untreated cells. **B:** MO3.13 cells were labeled with [^3H]AA as described in Materials and Methods. Cells were treated with different concentrations of psychosine as indicated (5–20 µM). At various time periods (1–8 h), radioactivity in the medium (100 µl) was measured using a liquid scintillation counter. *** $P < 0.001$ as compared with untreated cells. **C:** MO3.13 cells were treated with psychosine (20 µM) at different time periods (1–8 h) (i) and different concentrations of psychosine (5–20 µM) (ii), and supernatant was taken for secretory phospholipase A2 (sPLA2) activity, as described in Materials and Methods. Total AA released in sPLA2 activity from untreated cell supernatant was 1,255 \pm 155 dpm. Results are mean \pm SD of three determinations. * $P < 0.05$; *** $P < 0.001$ as compared with untreated cells. **D:** MO3.13 cells were labeled with [^3H]AA as described in Materials and Methods. Cells were treated with 20 µM concentrations of psychosine, glucopsychosine, ceramide, and sphingosine for 8 h, followed by counting of radioactivity released in medium. Results are mean \pm SD of three determinations. *** $P < 0.001$ as compared with untreated cells. NS, not significant.

(ERK p42/p44, JNK/SAPK, and p38) (Fig. 5B). Treatment with DEDA in these transfected cells significantly blocked the in vivo activation of these MAPKs.

Psychosine-induced AA release by ROS generation

Psychosine has been shown to induce oxidative stress and to regulate peroxisomal functions and apoptosis in primary oligodendrocytes and the MO3.13 cell line (4, 39). As reported earlier, psychosine treatment induced ROS generation in a dose- and time-dependent manner (Fig. 6A, B).

Glucopsychosine was observed to be more potent in inducing ROS generation in MO3.13 cells as compared with psychosine, whereas, ceramide and sphingosine had no effect (Fig. 6C). To investigate whether ROS plays any role in AA release, we examined the effect of antioxidant [*N*-acetylcysteine, (NAC)] on AA release. Treatment with NAC completely blocked psychosine-induced ROS generation (Fig. 6D) and also inhibited AA release and LPC generation (Fig. 6E, F), documenting that oxidative stress plays a role in the observed psychosine-mediated effects. We have

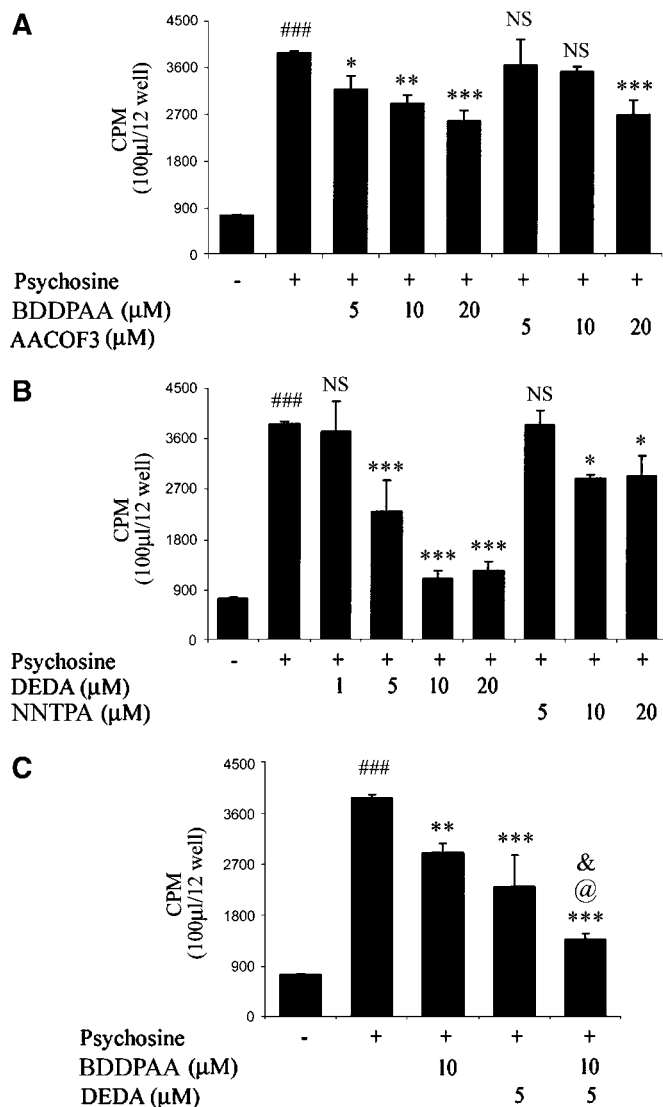


Fig. 3. Effect of cytosolic phospholipase A2 (cPLA2) and sPLA2 inhibitors on psychosine-mediated release of AA. **A:** MO3.13 cells were labeled with [3 H]AA as described in Materials and Methods. Cells were treated with different concentrations (5–20 μ M) of cPLA2 inhibitors (BDDPAA and AACOF3) for 30 min, followed by psychosine treatment. After 8 h, radioactivity released in the medium (100 μ l) was measured. ### $P < 0.001$ as compared with untreated cells; * $P < 0.05$ and *** $P < 0.001$ as compared with psychosine-treated cells. NS, not significant. **B:** Cells were treated with different concentrations (5–20 μ M) of sPLA2 inhibitors (DEDNA and NNTPA) for 30 min after labeling with labeled AA, followed by psychosine treatment for 8 h and counting of radioactivity. ### $P < 0.001$ as compared with untreated cells; * $P < 0.05$ and *** $P < 0.001$ as compared with psychosine-treated cells. NS, not significant. **C:** Labeled MO3.13 cells were treated with BDDPAA (10 μ M) and DEDNA (5 μ M), followed by psychosine treatment for 8 h; this was followed by radioactivity counting in media. ### $P < 0.001$ as compared with untreated cells; ** $P < 0.01$ and *** $P < 0.001$ as compared with psychosine-treated cells; @ $P < 0.001$ and & $P < 0.01$ as compared with psychosine/BDDPAA- and psychosine/DEDNA-treated cells, respectively.

previously reported that treatment with NAC attenuated psychosine-induced cell death in oligodendrocytes (4). Because DEDNA inhibited psychosine-mediated generation of AA and LPC, it was of interest to examine the effect of

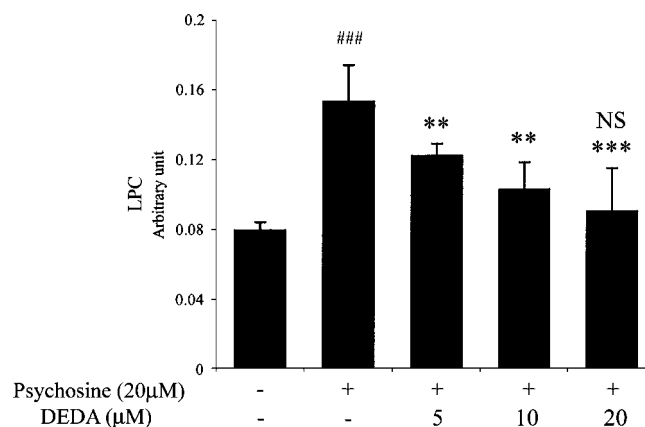


Fig. 4. DEDNA inhibits the psychosine-mediated accumulation of LPC. MO3.13 cells were treated with different concentrations of DEDNA for 30 min, followed by psychosine treatment. After 8 h of incubation, cells were processed for LPC measurement as described in Materials and Methods. Results are mean \pm SD of three determinations. ### $P < 0.001$ as compared with untreated cells. ** $P < 0.01$ and *** $P < 0.001$ as compared with psychosine-treated cells. NS, not significant as compared with untreated cells.

DEDNA on psychosine-mediated induction of ROS. For this, cells were treated with different concentrations of DEDNA (5–20 μ M), and ROS was measured in psychosine- and DEDNA-treated cells. As depicted in Fig. 6G, the sPLA2 inhibitor (DEDNA), but not the cPLA2 inhibitor (BDDPAA), inhibited the psychosine-induced ROS generation.

DEDNA attenuated psychosine-mediated oligodendrocyte cell death

Loss of oligodendrocytes is a hallmark in demyelinating Krabbe disease, and progressive accumulation of psychosine has been implicated in oligodendrocyte cell death in vivo and in vitro. Because the sPLA2 inhibitor attenuated AA release and LPC generation, we further examined its effect on psychosine-induced oligodendrocyte cell death. For this, MO3.13 cells were treated with DEDNA and the cPLA2 inhibitor BDDPAA for 30 min, followed by psychosine (20 μ M) treatment. After 48 h, MTT was performed to detect cell survival. As reported previously, psychosine at a concentration of 20 μ M induced cell death significantly (4). The sPLA2 inhibitor blocked the psychosine-mediated cell death, whereas the cPLA2 inhibitor BDDPAA had no effect (Fig. 7A, B), documenting the role of sPLA2 in psychosine-induced cell death. This observation was further supported by morphologic analysis, DNA fragmentation, and caspase 3 activity assays. As evident from morphologic analysis, MO3.13 cells treated with psychosine detached from the plate. However, their detachment was completely blocked when they were treated with DEDNA (Fig. 7C). To examine the effect of DEDNA on psychosine-mediated DNA fragmentation, cytoplasmic DNA isolated from cells treated in a similar manner was subjected to agarose gel electrophoresis (Fig. 7C). A DNA ladder was observed in cells treated with psychosine but not in control cells, and this ladder was reduced by the treatment with DEDNA at 5 μ M and 10 μ M concentrations at 48 h of treatment. DEDNA treat-

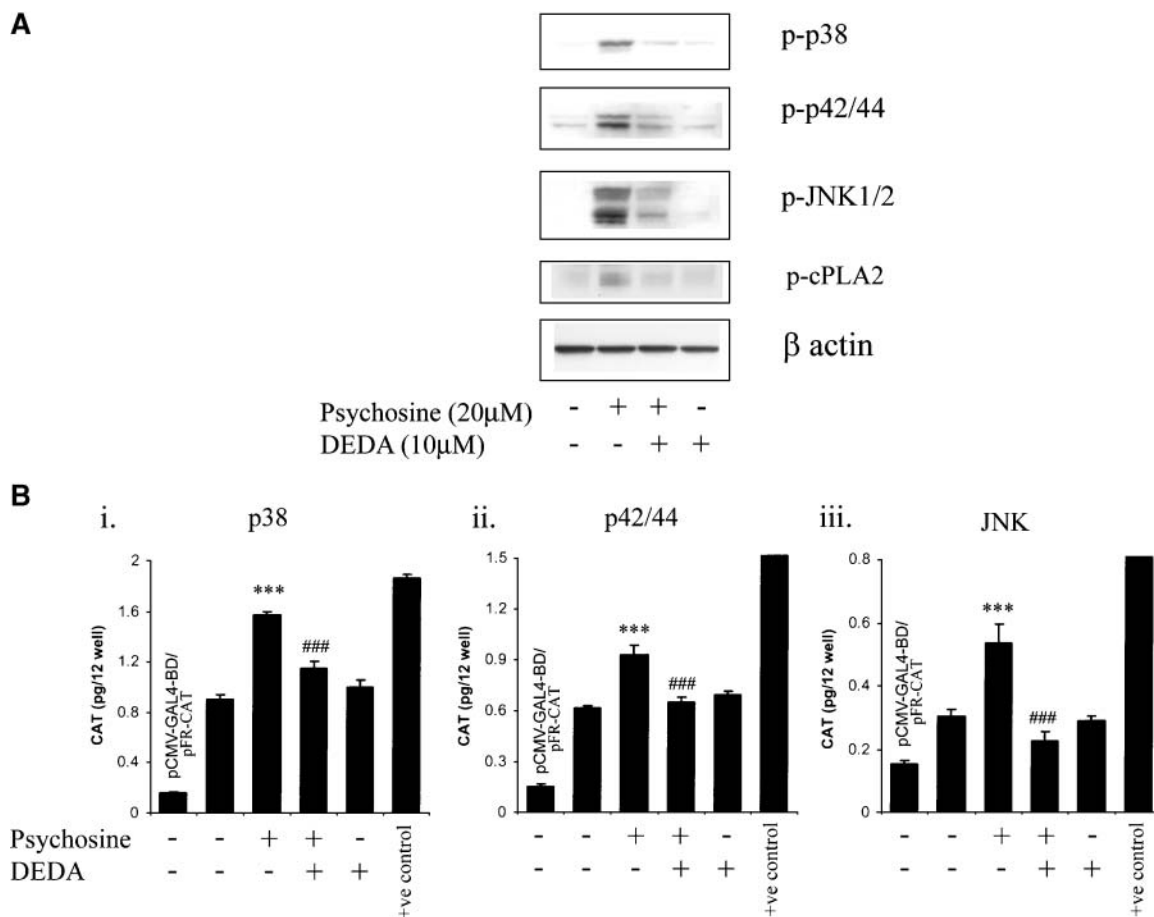


Fig. 5. DEDA inhibits psychosine-induced mitogen-activated protein kinases ERK1/2, p38, and JNK1/2 in the oligodendrocyte cell line. **A:** MO3.13 cells were incubated with DEDA (10 μ M) for 30 min, followed by psychosine treatment (20 μ M) for 30 min. Cells were washed with chilled phosphate buffer saline (PBS) and scraped in lysis buffer as discussed in Materials and Methods. Fifty milligrams of total protein was loaded on SDS-PAGE, followed by immunoblot analysis with phospho-specific antibodies against p42/44, JNK1/2, p38, and cPLA2. The anti- β actin antibody was used as an internal control for equal protein loading. **B:** To examine the effect of DEDA on in vivo mitogen-activated protein kinase pathways using PathDetect[®] in Vivo Signal Transduction Pathway *trans*-Reporting Systems, MO3.13 cells were transiently transfected with fusion transactivator plasmid [CHOP for p38 (i), Elk1 for p42/44 (ii), or c-Jun for JNK (iii)] transcriptional activator fused with the yeast GAL4 DNA binding domain residues 1–147 and pFR-chloramphenicol acetyltransferase (pFR-CAT) as a reporter using Lipofectamin 2000 according to the manufacturer's instructions. After 48 h, cells were treated with DEDA (10 μ M) and psychosine (20 μ M) for 24 h, followed by CAT assay by ELISA (Roche). The pCMV-GAL4 binding domain (without insert) and pFR-CAT were transfected as a control to detect the basal levels of CAT activity (first lane). pFC-MEKK (for JNK), pFC-MEK1 (for p42/44) and pFC-MEK3 (for p38) were cotransfected, followed by PMA (0.1 nM) treatment as a positive control (last lane). Data are the mean \pm SD of three values. *** $P < 0.001$ as compared with untreated cell; ### $P < 0.001$ as compared with psychosine-treated cell.

ment also attenuated psychosine-induced caspase 3 activity in the oligodendrocyte cell line (Fig. 7D). Taken together, these studies document that the sPLA2 inhibitor DEDA inhibits psychosine-induced oligodendrocyte cell death.

Psychosine-mediated effect is independent of TDAG8 receptor

TDAG8, a G protein-coupled receptor, has been proposed to be the receptor for the psychosine effect (41). To examine whether the psychosine-mediated effects are dependent on its receptor, the effect of SiRNA for TDAG8 was investigated on psychosine-induced AA release and caspase 3 activity. As depicted in Fig. 8A, psychosine treatment induced AA release; however, transfection with SiRNA of TDAG8 did not alter the psychosine-mediated AA or caspase 3 activity (Fig. 8B). However, TDAG8 SiRNA

transfection was able to reduce the approximately 50% levels of endogenous TDAG8 in MO3.13 cells (Fig. 8A, inset). To confirm further the TDAG8-independent effects of psychosine, we employed the HEK293 cell line, which is deficient for TDAG8, to study psychosine effects (41). For this, HEK293 cells labeled with [¹⁴C]AA were treated with different concentrations of psychosine (5–30 μ M). In agreement with studies on primary oligodendrocytes and MO3.13 cells, psychosine treatment induced the release of labeled AA in media in a dose-dependent manner (Fig. 8C), suggesting that the psychosine-mediated effect on AA release is independent of the TDAG8 receptor. Moreover, psychosine also induced LPC generation in HEK293 cells (Fig. 8D). To further support these observations, we transfected HEK293 cells with a mammalian expression vector of TDAG8, followed by the

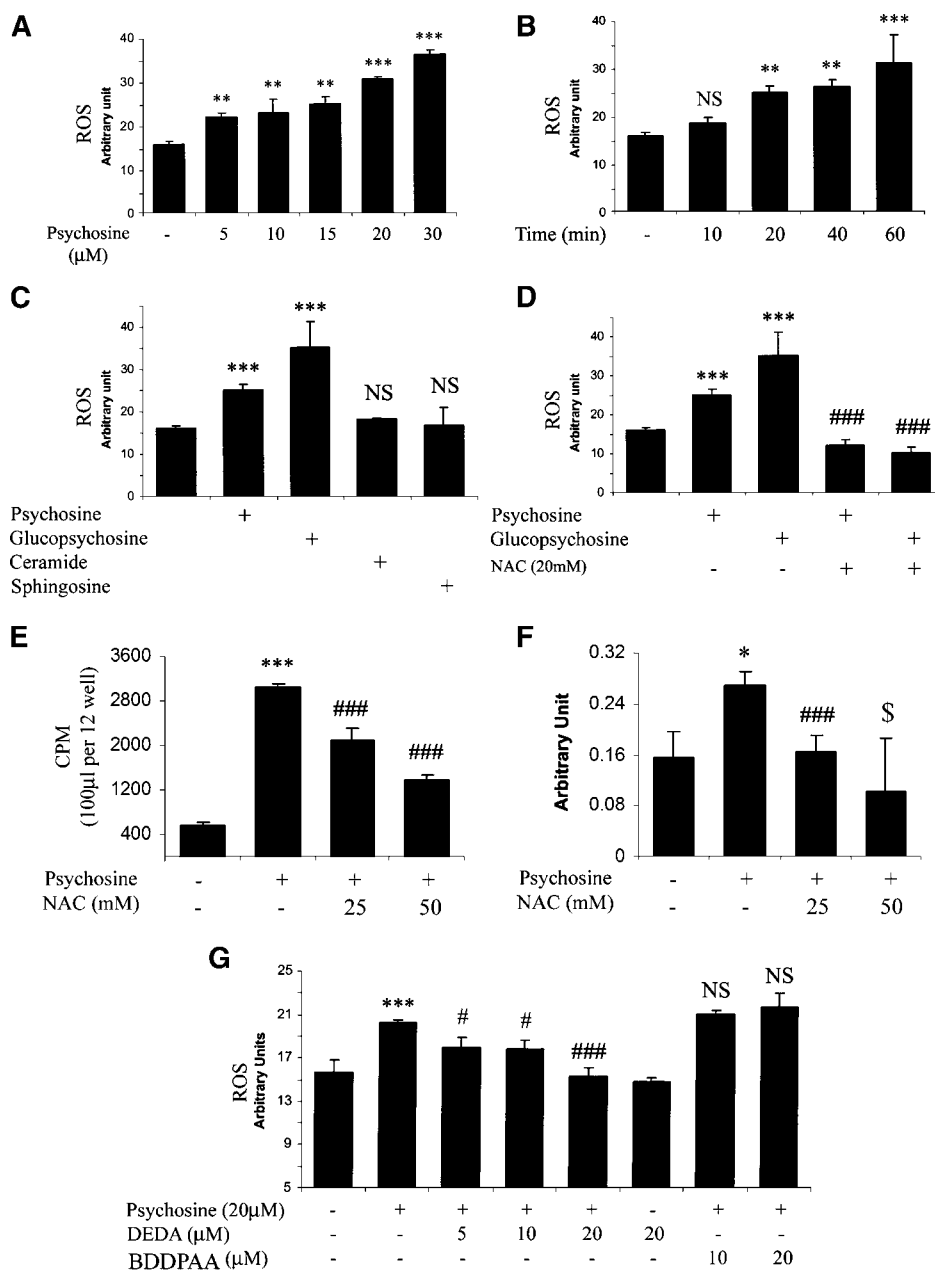


Fig. 6. Oxidative stress plays an important role in psychosine-mediated effects in the oligodendrocyte cell line. **A:** MO3.13 cells were treated for 60 min with different concentrations of psychosine (5–30 μM) in the presence of 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (DCFDA) dye, and fluorescence was recorded as described in Material and Methods. Data are the mean of three values \pm SD. ** $P < 0.01$ and *** $P < 0.001$ as compared with untreated cells. **B:** MO3.13 cells were treated with psychosine (20 μM) for various time periods (10–60 min) in the presence of DCFDA dye followed by fluorescence measurement. Data are the mean of three values \pm SD. NS, not significant; ** $P < 0.01$ and *** $P < 0.001$ as compared with untreated cells. **C:** MO3.13 cells were treated with 20 μM concentrations of psychosine, glucopsychosine, ceramide, and sphingosine for 60 min in the presence of DCFDA dye, followed by fluorescence measurement. Results are mean \pm SD of three determinations. NS, not significant; *** $P < 0.001$ as compared with untreated cells. **D:** MO3.13 cells were pretreated with *N*-acetylcysteine [NAC (20 mM)] followed by psychosine and glucopsychosine (20 μM) treatment for 60 min in the presence of DCFDA dye, followed by fluorescence measurement. Results are the mean \pm SD of three determinations. *** $P < 0.001$ as compared with untreated cells; ### $P < 0.001$ as compared with psychosine- or glucopsychosine-treated cells, respectively. **E:** To examine the effect of NAC on psychosine-induced AA release, MO3.13 cells were labeled with [^3H]AA as described in Materials and Methods. After 12 h, cells were washed and treated with NAC for 2 h prior to the addition of psychosine for 8 h. Release of labeled AA in medium was counted as described in Materials and Methods. Results are the mean \pm SD of three determinations. *** $P < 0.001$ as compared with untreated cells; ### $P < 0.001$ as compared with psychosine-treated cells. **F:** To examine the effect of NAC on LPC accumulation, cells were treated with NAC as described in Materials and Methods. After 8 h treatment with psychosine, cells were processed for LPC accumulation. Results are the mean \pm SD of three determinations. * $P < 0.05$ as compared with untreated cells; ### $P < 0.05$ and \$ $P < 0.01$ as compared with psychosine-treated cells. **G:** To examine the effect of DEDA on reactive oxygen species, MO3.13 cells were treated with DEDA and BDDPAA for 30 min prior to the addition of psychosine (20 μM) for 60 min in the presence of DCFDA dye, and fluorescence was recorded as described in Materials and Methods. Data are the mean of three values \pm SD. *** $P < 0.001$ as compared with untreated cells; # $P < 0.01$, ### $P < 0.001$, and NS, not significant as compared with psychosine-treated cells.

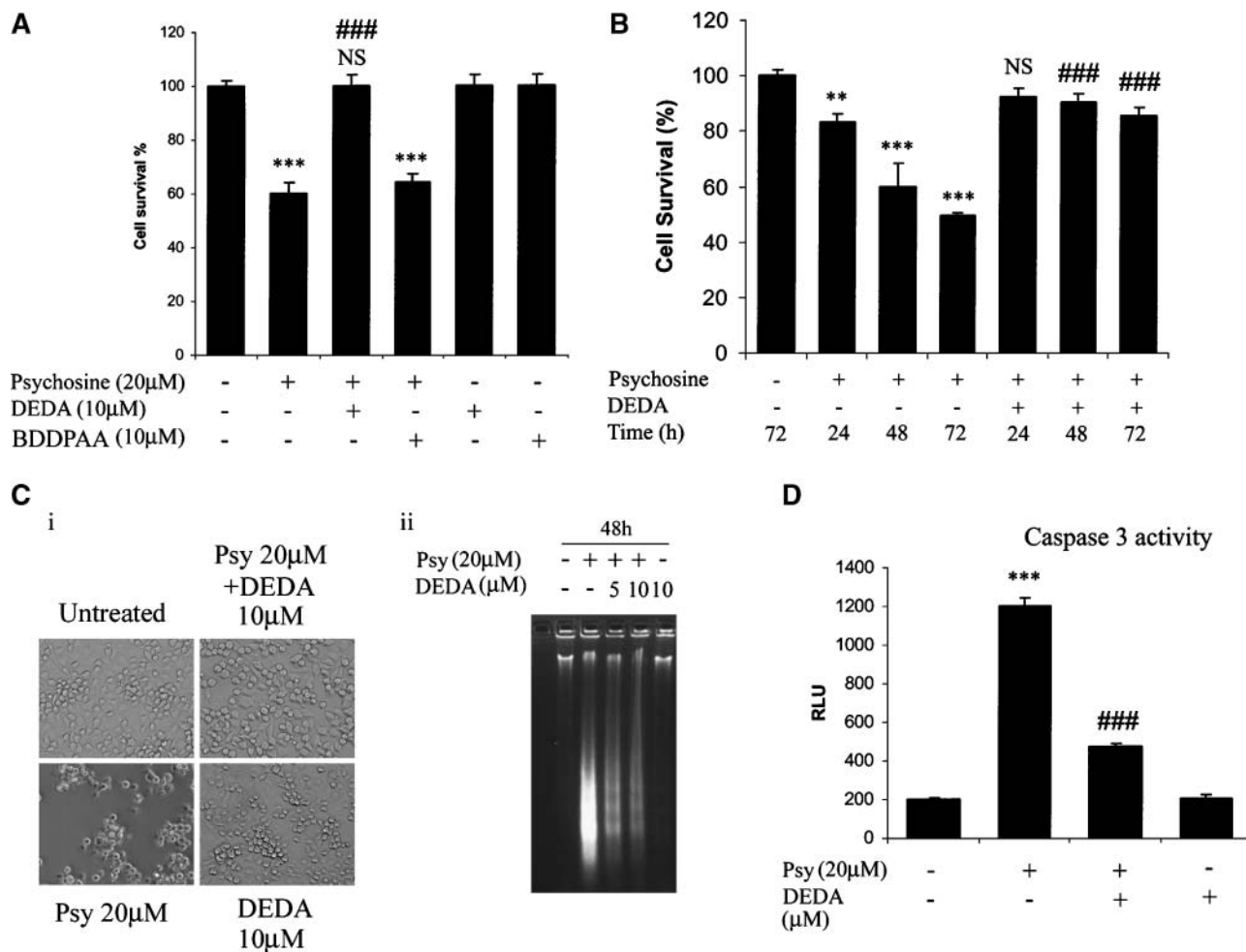


Fig. 7. DEDA blocked psychosine-mediated oligodendrocyte cell death. **A:** To examine cell viability, MO3.13 cells were plated and treated with DEDA (10 μ M) and BDDPAA (10 μ M), followed by psychosine treatment (20 μ M) for 48 h. Viable cells were determined by MTT assay as described in Material and Methods. Data are the mean of six values \pm SD. *** $P < 0.001$ and NS, not significant as compared with untreated cells; ### $P < 0.001$ as compared with psychosine-treated cells. **B:** MO3.13 cells were plated and treated with DEDA (10 μ M), followed by psychosine treatment (20 μ M) for 24, 48, and 72 h. Viable cells were determined by MTT assay as described in Materials and Methods. Data are the mean of six values \pm SD. ** $P < 0.01$ and *** $P < 0.001$ as compared with untreated cells; NS, not significant; ### $P < 0.001$ as compared with psychosine-treated cells at 24, 48, and 72 h, respectively. **C:** MO3.13 cells were plated in 2-well chambers, followed by DEDA and psychosine treatment as described in Materials and Methods. After 72 h, cells were analyzed under the microscope for morphological analysis (i). Data are representative of three different observations. MO3.13 cells were treated as in Materials and Methods and at 48 h were lysed in DNA lysis buffer; the extracted DNA obtained was electrophoresed on a 2% agarose gel (ii). The gel is representative of two individual experiments. **D:** Cells were treated as in Materials and Methods; cell lysates were prepared from treated cells and subjected to caspase 3 activity assay. Caspase 3 activation was determined by measuring the fluorescence intensity (expressed as arbitrary U/ μ g protein) of liberated amino-4-methyl-coumarin using spectrofluorometry as described as in Materials and Methods (40). Data are the mean of three values \pm SD. *** $P < 0.001$ as compared with untreated cells; ### $P < 0.001$ as compared with psychosine-treated cells.

AA-release assay. Transient transfection of the TDAG8 expression vector did not alter the psychosine-mediated release of AA (Fig. 8E). However, its transfection significantly induced the caspase 3 activity in these cells in both the presence and the absence of psychosine (Fig. 8F), which is in agreement with previous studies (42–44). Altogether, these sets of experiments clearly demonstrate that psychosine-mediated AA release and LPC generation is independent of TDAG8. These conclusions are in agreement with a very recent report using TDAG8 knock-out mice, which established that TDAG8 is not a receptor for psychosine (45). In summary, the results described in this study clearly document that psychosine induces cell death

via the sPLA2-mediated signal transduction pathway, independent of TDAG8.

DISCUSSION

Progressive accumulation of psychosine, loss of oligodendrocytes, and demyelination are a hallmark of Krabbe disease; however, the mechanism of psychosine-induced loss of oligodendrocytes resulting in demyelination is not well understood (1, 2). In the present study, we have shown that the mechanism of action of psychosine-mediated oligodendrocyte cell death is via activation of sPLA2

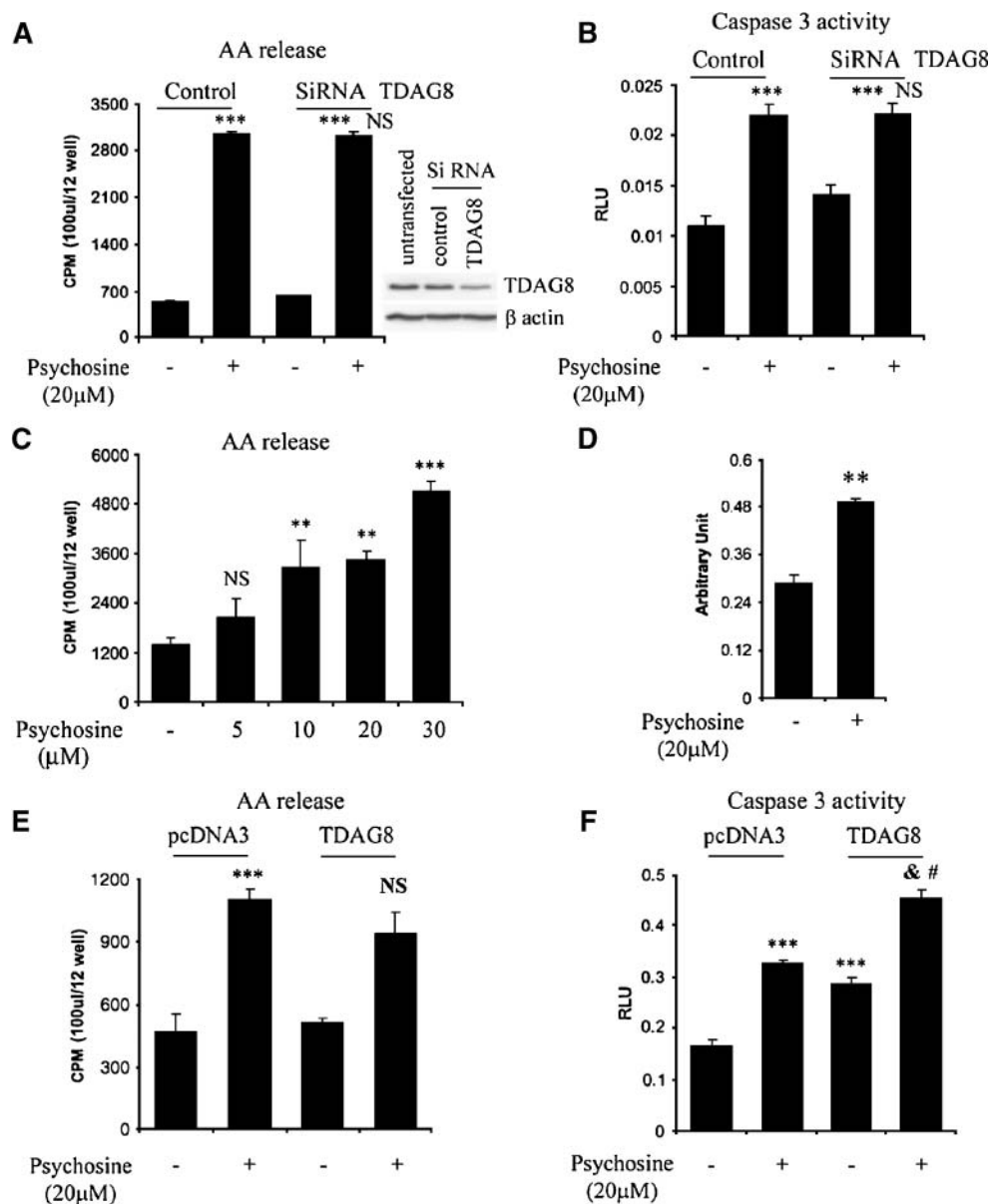


Fig. 8. The psychosine-mediated effect is independent of the TDAG8 receptor. **A:** To examine the role of TDAG8 in psychosine-mediated effects, MO3.13 cells were transfected with TDAG8 short interfering RNA (SiRNA) according to the manufacturer's instructions. After 48 h, cells were labeled with [³H]AA as described previously. After 12 h, cells were washed and treated with psychosine for 8 h. Release of labeled AA in medium was counted as described in Materials and Methods. Results are the mean \pm SD of three determinations. *** $P < 0.001$ as compared with control and TDAG8 SiRNA-untreated cells; NS, not significant as compared with psychosine-treated control SiRNA cells. To examine the effect of SiRNA on TDAG8 protein levels, cells were transfected with control and TDAG8 SiRNA, and cell lysate (50 μ g) was used for immunoblot analysis for the detection of TDAG8 levels as described in Materials and Methods (inset). **B:** To examine the caspase 3 activity, cells were transfected with TDAG8 SiRNA as described previously, followed by psychosine treatment for 48 h and caspase 3 activity assay. Results are the mean \pm SD of three determinations. *** $P < 0.001$ as compared with control and TDAG8 SiRNA-untreated cells; NS, not significant as compared with psychosine-treated control SiRNA cells. **C:** HEK293 cells were labeled with [³H]AA as described in Materials and Methods. After 12 h, cells were washed and treated with psychosine (5–30 μ M) for 24 h. Release of labeled AA in the medium was counted as described in Materials and Methods. Data are the mean of six values \pm SD. ** $P < 0.01$, *** $P < 0.001$; NS, not significant as compared with untreated cells. **D:** To examine the LPC accumulation, HEK293 cells were treated with psychosine (20 μ M) for 24 h, followed by LPC detection as described in Materials and Methods. Data are the mean of three values \pm SD. ** $P < 0.01$ as compared with untreated cells. **E:** HEK293 cells were transiently transfected with the expression vector of TDAG8 with Lipofectamine 2000 as described in Materials and Methods. After 24 h, cells were labeled with [³H]AA and treated with psychosine (20 μ M). After 24 h, media was taken for radioactive counting. Results are the mean \pm SD of three determinations. *** $P < 0.001$ as compared with pcDNA3- or TDAG8-transfected untreated cells; NS, not significant as compared with psychosine-treated pcDNA3-transfected cells. **F:** For caspase 3 activity, HEK293 cells were transfected with the expression vector of TDAG8 as described in Materials and Methods. After 48 h treatment with psychosine, the cell lysate was processed for caspase 3 activity assay. Results are the mean \pm SD of three determinations. *** $P < 0.001$ as compared with pcDNA3-transfected untreated cells; and & $P < 0.001$ and # $P < 0.001$ as compared with psychosine-treated pcDNA3-transfected cells and TDAG8-transfected untreated cells, respectively.

and generation of LPC and AA. This conclusion is based on the following observations: *i*) Brains of Krabbe patients and twitcher mice exhibited significant accumulation of LPC. *ii*) Psychosine treatment significantly induced the generation of LPC and AA release in primary oligodendrocytes and the MO3.13 cell line. *iii*) Studies using various pharmacological inhibitors of cPLA2 and sPLA2 and enzymatic activation of sPLA2 activity document that psychosine-induced release of AA is contributed mainly by sPLA2. *iv*) The sPLA2 inhibitor DEDA completely attenuated psychosine-induced generation of LPC and AA. *v*) The sPLA2 inhibitor DEDA also inhibited the generation of ROS and oligodendrocyte cell death, as evident from cell survival, DNA fragmentation, and caspase 3 activity, and these effects of psychosine are independent of the presumed receptor of psychosine, TDAG8. Taken together, these results indicate that the sPLA2 inhibitor may have therapeutic potential, either alone or in combination with anti-inflammatory drugs for twitcher/Krabbe disease.

TDAG8 (T-cell death-associated gene 8) was initially cloned as an orphan G protein-coupled receptor, which was upregulated during the programmed cell death of T-lymphocytes (42–44). Im et al. (41) reported that TDAG8 is a putative receptor for psychosine and that its overexpression results in the formation of multinuclear cells by psychosine. Our results are in contrast with these observations. We observed that psychosine-mediated oligodendrocyte cell death is independent of TDAG8 in MO3.13 oligodendrocyte cells. In support of our finding, recently a study using TDAG8 knockout mice has also reported that psychosine-mediated effects are independent of TDAG8 (45). Moreover, psychosine-mediated effects were also observed in the HEK293 cell line, where TDAG8 expression is deficient (41), further suggesting that psychosine-mediated effects such as the AA release and LPC and ROS generation observed in oligodendrocytes and in the HEK293 cell line are independent of TDAG8. Overexpression of TDAG8 induces caspase 3 activity in the HEK293 cell line, which is consistent with a previous report in which its expression was upregulated during the programmed cell death of T-lymphocytes (42–44).

Several studies have reported a role for psychosine in regulating apoptotic cell death in Krabbe disease (4, 5, 46–49), yet its molecular mechanism(s) remain an enigma. While investigating the lipid composition of brains from Krabbe patients and from twitcher mice, we observed increased levels of LPC. In the present study, we have documented the possible role of LPC and AA generated by activation of sPLA2 in psychosine-mediated oligodendrocyte cell death. PLA2-mediated alterations of phospholipid metabolism have been suggested to be responsible for neurodegeneration in ischemia, spinal cord trauma, head injury, and Alzheimer's disease (50). Under these pathological conditions, PLA2 may be involved in the massive release of AA and accumulation of eicosanoids. ROS generated during phospholipid degradation produce oxidative stress and are closely associated with the apoptotic as well as necrotic cell death that occurs in neurological disorders (50). We have documented recently that psy-

chosine induces ROS generation and depletes GSH levels in twitcher mice and oligodendrocyte cells (4).

Inhibitors of cPLA2 (BDDPAA and AACOF3) partially inhibited the release of AA, whereas the sPLA2 inhibitor DEDA completely inhibited psychosine-induced oxidative stress, generation of AA and LPC, caspase 3 activity, and apoptotic cell death. DEDA is considered to be a specific inhibitor of sPLA2 over cPLA2 (51), but it is also a weak inhibitor of 5-lipoxygenase with a higher IC_{50} (56 μ M), suggesting the possible involvement of 5-lipoxygenase, in addition to cPLA2 and sPLA2, in psychosine-mediated effects. We also observed that DEDA attenuated psychosine-mediated activation of p42/44 MAPK, stress MAPKs (p38 and JNKs), and phosphorylation of cPLA2. Therefore, the complete inhibition of AA release by DEDA may be due not only to direct inhibition of sPLA2 but also to the inhibition of cPLA2 phosphorylation via downregulation of p42/44 and p38, a critical for cPLA2 phosphorylation, membrane translocation, and its enzymatic activity (11, 12). Accordingly, DEDA treatment inhibited MAPK phosphorylation as well as phosphorylation of cPLA2. Our efforts to identify the sPLA2 involved in psychosine-mediated cell death have not been successful thus far. Moreover, the involvement of iPLA2 can also be ruled out, because a specific iPLA2 inhibitor, bromoenol lactone, did not affect the psychosine-mediated AA release in MO3.13 cells (data not shown). Studies are in progress to identify the sPLA2 specific for psychosine-induced signaling pathways.

A direct correlation between psychosine treatment and dose-dependent elevated levels of LPC generation and AA release in primary oligodendrocytes and MO3.13 cells suggests that increased levels of psychosine may be responsible for the higher levels of LPC found in Krabbe and twitcher brains. LPC is a well-known lysolipid, responsible for inducing demyelination in an animal model (20, 21) and cell death of mature oligodendrocytes and their progenitors *in vitro* (22, 23). The demyelinating effects of LPC are known to appear very rapidly *in vitro* as well as *in vivo*, and result from an increase in the hydrophilic nature of the molecular components of membranes, leading, in the case of myelin, to disruption of the intraperiod line, and finally to solubilization of the sheath (20). Elevated levels of psychosine elicit unique cellular reactions, such as formation of multinucleated cells (globoid cells) from resident microglia/macrophages, and reactive astrogliosis (52, 53). Activated glial cells produce various inflammatory cytokines and related mediators (7, 10, 54) and chemokines, including macrophage chemoattractant protein-1, which are likely to play an important role in recruiting peripheral macrophages into the brain (54). These recruited macrophages further upregulate the inflammatory disease and participate in the progressive demyelinating disease process in Krabbe disease and in twitcher mice. LPC is known to act as a chemoattractant for monocytes (55) and T lymphocytes (56, 57) and also enhances the production of interferon- γ by activated T cells, thus promoting an inflammatory reaction (58, 59). Because elevated levels of LPC are observed in Krabbe and twitcher brain, it can be postulated that LPC may, at least

in part, be responsible for the inflammatory response observed in Krabbe disease and in twitcher mice. Moreover, LPC is also a precursor for platelet-activating factor, which is a well-known inflammatory mediator in the regulation of several inflammatory diseases (60–63). Therefore, inhibition of LPC generation should play an important part in therapeutics against the disease process in Krabbe disease and twitcher mice brains.

On the other hand, AA and its metabolites are important second messengers involved in the regulation of several biological processes, such as tissue inflammation and cell growth, differentiation, and apoptosis, through the activation of different signal transduction pathways (64–68), leading to the activation of transcription factors such as redox-sensitive transcription factors AP-1 and NF- κ B by the activation of p38 MAPK (28–30). The metabolism of AA gives rise to ROS, which may induce oxidative stress leading to either cell proliferation or apoptosis, depending on the cell type (24–27). The inhibition of psychosine-induced cell death by the inhibitor of sPLA2 in oligodendrocytes indicates that LPC- and/or AA-mediated activation of signaling pathways is responsible for the loss of oligodendrocytes in Krabbe disease.

Due to variable forms of onset of clinical signs in patients with Krabbe disease and the lack of correlation between disease progression and enzyme levels or mutation status, other factors, such as environmental and/or other genetic factors, have been suggested to influence the late onset of Krabbe disease. Environmental factors include the possibility that trauma, i.e., a blow to the head (69) or viral infections (70, 71), may trigger the onset of symptoms in humans with Krabbe disease, perhaps by induction of proinflammatory mediators. Genetic factors include the presence of modifier genes that interact with the disease, causing gene or influence the disease process and, thus, impact disease progression. The saposin A and acid β -galactosidase genes are perfect examples of genetic factors,

inasmuch as mutations in these genes can lead to a slowly progressive form of Krabbe disease in twitcher mice (72, 73). Similarly, a change in the genetic background of twitcher mice, from C57BL/6 to C57BL/6 \times CAST/Ei background, significantly increases the life span (61.4 ± 2.5 vs. 37.0 ± 0.6 days), with fewer lectin-positive globose cells, less gliosis, and a greater preservation of myelin, compared with C57BL/6 twitcher mice under moribund conditions, without any changes in the levels of psychosine, suggesting the involvement of a factor critical for the progression of Krabbe disease other than the accumulation of psychosine in the CNS (74). These observations raise a question as to whether the use of inhibitors of sPLA2, along with anti-inflammatory drugs (e.g., NAC or statins), will protect against the loss of oligodendrocytes and thus increase the life span of twitcher mice or patients with Krabbe disease. At present, there is no treatment for Krabbe disease except hematopoietic stem cell transplantation, which is not a cure, but does slow down the course of the disease in humans as well as in twitcher mice (75–79). Recently, anti-inflammatory therapy with the phosphodiesterase inhibitor ibudilast has been reported to downregulate the inflammation and cell death in twitcher mice (80).

Our findings are summarized in Fig. 9, where we have documented that accumulated levels of psychosine induce the generation of LPC and the release of AA via activation of sPLA2 activity. The initial event may be Ca^{2+} -induced activation of PLA2, with subsequent generation of ROS, and eventually, it may result in a vicious cycle. Using a pharmacological approach, it was confirmed that the psychosine-mediated effects could be nullified by the sPLA2 inhibitor DEDA, which blocks the activation of MAPKs and the generation of ROS by psychosine in oligodendrocyte cells. Antioxidant treatment (NAC) also attenuated LPC accumulation, AA release, ROS generation, and cell death induced by psychosine (4), suggesting the partial involvement of oxidative stress in psychosine-me-

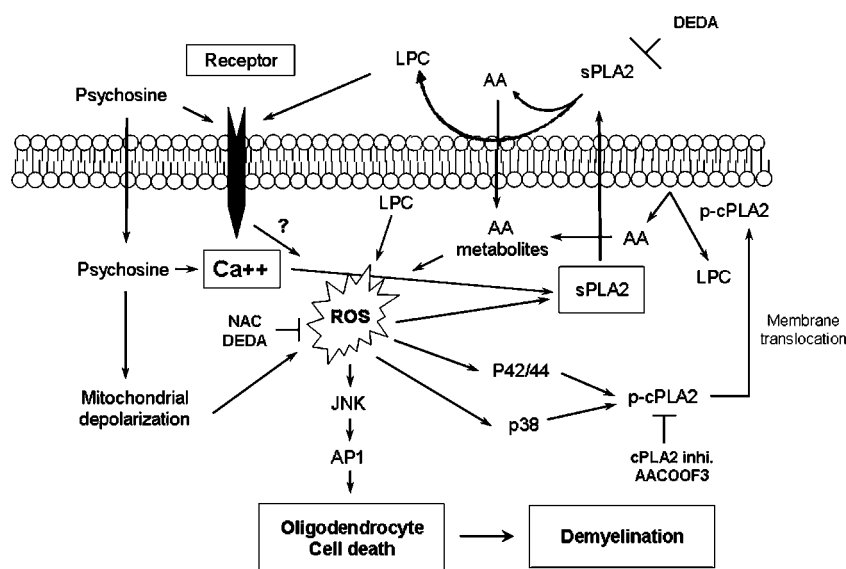


Fig. 9. A schematic representation of the effect of psychosine on the generation of LPC and AA in oligodendrocyte cells.

diated effects in oligodendrocytes. Moreover, generation of LPC has been implicated in demyelination (20, 21) by the activation of cell death in mature oligodendrocytes and their progenitors (22, 23). On the other hand, metabolites of AA give rise to oxidative stress, regulating redox-sensitive transcription factors (28–30) and cell death (25, 27). Psychosine-mediated effects are independent of the links to its putative receptor TDAG8 supported by others (45). Altogether, this study documents that psychosine-mediated oligodendrocyte cell death is mediated via the sPLA2 signaling pathway and that inhibitors of sPLA2 may have therapeutic potential for protection against oligodendrocyte cell death and the resulting demyelination in Krabbe disease. ■

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