Galactosylsphingosine (psychosine) -induced expression of cytokine-mediated inducible nitric oxide synthases via AP-1 and C/EBP: implications for Krabbe disease

SHAILENDRA GIRI, MANU JATANA, RAMANDEEP RATTAN, JE-SEONG WON, INDERJIT SINGH, AND AVTAR K. SINGH*,[1]

Department of Pediatrics and *Pathology and Laboratory Medicine, Medical University of South Carolina; and *Department of Pathology and Laboratory Medicine, Ralph Johnson Veterans Affairs Medical Center, Charleston, South Carolina, USA

ABSTRACT **Globoid cell leukodystrophy (Krabbe disease) is characterized by the accumulation of a toxic metabolite, psychosine (galactosylsphingosine), which is a substrate for the deficient enzyme (galactocerebroside β-galactosidase). This study underscores the possible role of psychosine in the effect of inducible nitric oxide synthase (iNOS) -derived NO in the pathophysiology of this demyelinating disease. For the first time, we provide evidence of the expression of iNOS in CNS of Krabbe patient and show that the iNOS-expressing cells in the CNS were astrocytes. Psychosine potentiated the LPS-induced production of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) in primary rat astrocytes and regulated the cytokine-mediated production of NO in C6 glioma and primary rat astrocyte. Psychosine induced cytokine-mediated nuclear translocation of AP-1 and C/EBP by potentiating the expression of Fra-1 and C/EBP-δ proteins. This suggests that psychosine maintained or sustained the cytokine-primed expression of iNOS by further potentiating the nuclear translocation of AP-1 and C/EBP without modulating the cytokine-mediated transcription activity of NF-κB. This study hypothesizes that accumulated psychosine leads to production of cytokines and iNOS expression. The ensuing excessive production of NO and $ONOO^-$ may play a role in pathogenesis of Krabbe disease.—Giri, S., Jatana, M., Rattan, R., Won, J.-S., Singh, I., Singh, A. K. Galactosylsphingosine (psychosine) -induced expression of cytokine-mediated inducible nitric oxide synthases via AP-1 and C/EBP: implications for Krabbe disease. *FASEB J.* 16, 661–672 (2002)**

Key Words: cerebroside · GLD · iNOS · oligodendrocytes · β-galactosidase

GLOBOID CELL LEUKODYSTROPHY (GLD) or Krabbe (1), disease, first described by Krabbe (1), is an autosomal recessive disorder affecting the nervous system (2). It is characterized by PAS-positive material containing globoid cells, gliosis, and loss of oligodendrocytes and axons (2). These characteristic globoid cells show accumulation of galactocerebrosides but with a reduced sulfatide-to-galactocerebroside ratio (2–4). There is limited accumulation of galactocerebrosides, but progressive accumulation of galactosylsphingosine and psychosine (2, 5). Psychosine is essentially undetectable in normal brain tissue whereas in Krabbe brain it may account for as much as 50% of the cerebroside content (6, 7).

Unlike the galactocerebrosides, psychosine is not a substrate for cerebroside:sulfatide transferase. Cerebrosides and sulfatides are the major constituents of myelin lipids (8, 9). Galactocerebrosidase degrades cerebrosides and psychosine to ceramide and galactose and to sphingosine and galactose, respectively (10, 11). The galactocerebrosides are hydrolyzed by GM1 gangliosidase, commonly known as β-galactosidase, but it cannot degrade psychosine (10–14). Therefore, deficient activity of cerebrosidase along with normal activity of GM1 gangliosidase in the tissue of Krabbe patients results in the accumulation of psychosine but not of cerebrosides (6, 7, 15, 16). Cytotoxicity of psychosine in vitro and the fatal effect of intracranially injected psychosine led to the conclusion (known as the 'psychosine hypothesis'; ref 5) that progressive accumulation of psychosine is the critical biochemical pathogenetic mechanism of cell death in the Krabbe brain (17–23). However, the mechanism of action of psychosine in the pathophysiology of Krabbe disease has not been elucidated.

The observation of apoptotic-positive cells (23), expression of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and chemokines (24–26), and correlation of TUNEL-positive cells (23) with the expression of TNF-α (24) in twitcher mice (a murine model of Krabbe) indicates that the inflammatory response may play a role in the pathophysiology of Krabbe disease. Nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) under inflammatory conditions, by itself or as a reaction product of NO with O_2^- ($ONOO^-$), is believed to be a critical factor in the

[1] Correspondence: 316, Clinical Science Bldg., Medical University of South Carolina, Charleston, SC 29425, USA. E-mail: singhi@musc.edu

pathophysiology of neurodegenerative disease (27, 28). Therefore, we examined the possible role of psychosine in the induction of iNOS and production of NO. Studies described here show for the first time the induction of iNOS in Krabbe brain and report that psychosine potentiates the cytokine-mediated induction of iNOS and production of NO in cultured C6 glial cells and rat primary astrocytes. This study indicates a role for MAP kinases and transcription factors AP-1 and C/EBP in psychosine-mediated induction of iNOS.

MATERIALS AND METHODS

Reagents and cell culture

Recombinant rat interferon γ (IFN-γ), DMEM/F-12 medium, fetal bovine serum (FBS), Hank's balanced salt solution, and NF-κB DNA binding protein detection kit were obtained from Life Technologies (Grand Island, NY). Human IL-1β was purchased from Genzyme (Boston, MA). Mouse recombinant TNF-α was obtained from Boehringer Mannheim (Mannheim, Germany). Lipopolysaccharide (LPS) (*Escherichia coli*) was from Sigma (St. Louis, MO). Antibodies against mouse macrophage iNOS, PD98059, and SB compounds were purchased from Calbiochem (San Diego, CA). Immunoassay kit for TNF-α, IL-1β, and IL-6 were obtained from R&D Systems (Minneapolis, MN). Psychosine was from Matreya (Pleasant Gap, PA). Wild-type and mutant oligonucleotides for NF-κB, AP-1, STAT1-, STAT-3, GAS/ISRE, CRE, and SP-1 gel shift and antibodies for C/EBP-α, -β, -δ, -ε, c-fos, fosB, c-Jun, JunB, JunD, Fra I, and Fra II were purchased from Santa Cruz (Santa Cruz, CA). [γ-^{32}P] ATP (3000 Ci/mmol) and the enhanced chemiluminescence (ECL) -detecting reagent were from Amersham Pharmacia Biotech (Arlington Heights, IL), the luciferase assay system from Promega (Madison, WI). NF-κB-SEAP and Great ESCAPE chemiluminescence kits were purchased from Clontech (Palo Alto, CA). C6 glioma, immortalized rat astrocyte, A172, and T98G human astrocytoma cell lines obtained from ATCC (Rockville, MD) were maintained and induced with different stimuli in DMEM/F12 media.

Human autopsy brain specimens

Formaldehyde-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 formalin-fixed tissues were processed for routine histopathology and paraffin-embedded sections were used in subsequent immunohistochemical studies. Sections from normal brains were cut from corresponding anatomical locations.

Immunohistochemistry

Brain sections were deparaffinized, sequentially rehydrated in graded alcohols, and immersed in Tris sodium buffer (0.1M Tris-HCl, pH 7.4, 0.15M NaCl) for 15 min. Slides were then microwaved for 10 min in antigen unmasking fluids (Vector Labs, Burlingame, CA), cooled, and washed 3× for 5 min in Tris-sodium buffer with 0.05% Tween 20 (TNT). Sections were treated with trypsin (0.1% for 10 min) and immersed for 10 min in 3% hydrogen peroxide to eliminate endogenous peroxidase activity. Sections were blocked in Tris sodium buffer with 0.5% blocking reagent (TNB) (supplied with TSA-Direct kit, NEN Life Sciences, Boston, MA) for 30 min to reduce nonspecific staining. Sections were incubated overnight with primary antibody (iNOS diluted in blocking buffer at 1:100) and rinsed 3× for 5 min in TNT. For iNOS detection, slides were incubated for 30 min with anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Sigma) diluted 1:100 in TNB. The signal was enhanced with tyramide signal enhancement technique (Renaissance TSA for Immunocytochemistry, NEN Life Sciences) with cyanine 3 tyramide per manufacturer's instructions. Control rabbit IgG was used instead of iNOS antibody on separate slides and developed in the same manner as the primary antibodies.

Double labeling studies to identify the iNOS-positive cell type

Brain sections were processed for iNOS detection, then incubated with mouse antibodies to GFAP (1:100) for 1 h, followed by anti-mouse IgG conjugated to FITC (1:100) for 1 h. After washing, the slides were air dried and mounted with aqueous mounting media (Vector Labs).

Immunocytochemistry

Cells were grown on Labteck chamber slides. After treatment, cells were briefly washed in Tris sodium buffer (0.1M Tris-HCl, pH 7.4, 0.15M NaCl) and fixed in −20°C methanol for 5 min. Slides were immersed for 5 min in 3% hydrogen peroxide to eliminate endogenous peroxidase activity, then blocked in TNB (supplied with TSA-Direct kit, NEN Life Sciences) for 30 min to reduce nonspecific staining. Vector avidin biotin blocking kit (Vector Laboratories) was used to prevent nonspecific binding of biotin/avidin. Cells were incubated overnight with primary antibody (iNOS diluted in blocking buffer at 1:100) and rinsed 3× for 5 min in Tris sodium buffer. For iNOS detection, slides were incubated for 30 min with biotinylated secondary antibody using Vector Elite ABC kit as per manufacturer's instructions. The purple-colored product was developed by Vector VIP substrate peroxidase substrate kit (Vector Laboratories) according to the manufacturer's protocol.

Induction of NO production in rat astrocyte and C_6 glial cells

Astrocytes were prepared from rat cerebral tissue as described by McCarthy and DeVellis (29). Cells were maintained in DMEM/F-12 medium containing 10% FBS. After 10 days of culture, astrocytes were separated from microglia and oligodendrocytes by shaking for 24 h in an orbital shaker at 240 rpm. To ensure complete removal of the oligodendrocytes and microglia, the shaking was repeated twice after a gap of 1 or 2 days. For induction of NO and cytokines production, cells were plated onto polylysine-coated 24-well and 100 mm plates, then stimulated with LPS in serum-free conditions. C6 glial cells obtained from ATCC were maintained and induced with different stimuli as indicated above.

Assay for NO synthesis

Production of NO was determined by assaying culture supernatants for nitrite, a stable reaction product of NO and molecular oxygen. Briefly, 500 μl of culture supernatant was allowed to react with 500 μl of Griess reagent (30) and incubated at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 570 nm. Fresh culture medium served as the blank in

all experiments. Nitrite concentrations were calculated from a standard curve derived from the reaction of $NaNO_2$ in the assay.

Immunoblot analysis for iNOS

After 24 h of incubation in the presence or absence of different stimuli, the cells were scraped off, washed with Hank's buffer, and sonicated in 50 mM Tris-HCl (pH 7.4) containing protease inhibitors (1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml antipain, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin). Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h in 5% nonfat dry milk TTBS (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20, pH 7.5) and incubated for 1.5 h in primary antisera (anti-mouse iNOS, 1:2000; rabbit anti-p65, 1:1000) containing 1% nonfat dry milk. The blots were then washed four times with TTBS (5 min/wash) and incubated for 45 min at room temperature HRP-conjugated anti-rabbit secondary antibody at a dilution of 1:7000. The blots were washed three times in TTBS and once in 0.1 M PBS (pH 7.4) at room temperature. iNOS and p65 NF-κB proteins were detected by using ECL reagent as per manufacturer specifications (Amersham Pharmacia Biotech).

RNA isolation and Northern blot analysis

Cells were harvested from the culture dish directly by adding Ultraspec-II RNA reagent (Biotecx Laboratories, Houston, TX) and the total RNA was isolated according to the manufacturer's protocol. For Northern blot analyses, 20 μg of total RNA was electrophoresed on 1.2% denaturing formaldehyde-agarose gels, electrotransferred to Hybond-Nylon Membrane (Amersham), and hybridized at 68°C with ^{32}P-labeled cDNA probe using Express Hyb hybridization solution (Clontech). The cDNA probe for iNOS was made by polymerase chain reaction amplification using two primers (forward primer, 5′-CTC CTT CAA AGA GGC AAA AAT A-3′; reverse primer, 5′-CAC TTC CTC CAG GAT GTT GT-3′) (11, 12, 25). After hybridization, the filters were washed two or three times in solution I (2×SSC, 0.05% SDS) for 1 h at room temperature, followed by solution II (0.1×SSC, 0.1% SDS) at 50°C for another hour. Membranes were then dried and exposed to X-ray film (Kodak, Rochester, NY). The same membrane was probed for β-actin to normalized the RNA loading. The relative mRNA content for iNOS (iNOS/β-actin) was measured by scanning the bands with a Bio-Rad (model GS-670) imaging densitometer.

Determination of TNF, IL-1, and IL-6 in culture supernatants

Cells were stimulated with LPS in serum-free media for 24 h in the presence or absence of psychosine and concentrations of TNF, IL-1, and IL-6 were measured in culture supernatants using high-sensitivity enzyme-linked immunosorbent assay (R&D Systems)

Preparation of nuclear extracts and EMSA

Nuclear extracts from stimulated or unstimulated C6 glial cells were prepared using the method of Dignam et al. (31) with slight modification. Cells were harvested, washed twice with ice-cold PBS, and lysed in 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin) containing 0.1% Nonidet P-40 for 15 min on ice, vortexed vigorously for 15 s, and centrifuged at 14,000 rpm for 30 s. The pelleted nuclei were resuspended in 40 μl of buffer B [20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 dithiothreitol,1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin]. After 30 min on ice, lysates were centrifuged at 14,000 rpm for 10 min. Supernatants containing the nuclear proteins were diluted with 20 μl of modified buffer C [20 mM HEPES, pH 7.9, 20% (v/v) glycerol, 0.05 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM PMSF] and stored at −70°C until use. Electrophoretic mobility shift assay (EMSA) was performed as described previously (32) with the NF-κB or AP-1, CREB, C/EBP, OCT-1, SP-1, IRF-1, GAS, and STAT-1 consensus sequence end-labeled with [γ-^{32}P] ATP. Nuclear extracts were normalized for protein (Bio-Rad protein assay) and equal amounts were loaded. DNA–protein complexes were resolved by PAGE on 5% gel in 45 mM Tris, 45 mM boric acid, 1 mM EDTA (0.5×TBE) and run at 11 V/cm. The gels were dried and autoradiographed at −70°C. For competition analysis, nuclear extracts were preincubated with water or competitor DNA for 5 min at room temperature and the samples were processed as for EMSA.

Transfection and decoy experiments

Plasmids were purified using the endotoxin-free plasmid midi prep kit (Qiagen, Santa Clarita, CA). For transient transfections, C6 glioma cells were seeded in 6-well plates and grown to 60–80% confluence in DMEM-F12 + 5% FBS without antibiotics and transfected the next day using Fugene reagent according to the manufacturer's protocol with a total of 2 μg/well of plasmid DNA. For *trans*-activation experiments, 1.5 μg of pNOS-2-luc and 0.5 μg of MLV-C/EBP-δ or insertless expression vector (pBluescript) were used. Twenty-four hours after transfection, cells were maintained another 24 h serum-free, then treated with cytokines with or without psychosine. Sixteen hours later, cell lysates were prepared using lysis buffer (Promega) to measure luciferase activity. Protein content of the lysates was determined using Bradford reagent (Bio-Rad). AP-1 decoy (CGCTTGATGACTCAGCCGGAA) oligonucleotides were annealed to their respective complementary sequence in the buffer containing 20 mM Tris·HCl (pH 8.4), 150 mM NaCl, 50 mM KCl, and 1.5 mM $MgCl_2$. C6 glial cells cultured on 24-well plates were transfected with decoy (0.2 mM) oligonucleotides using Lipotaxi for 4 h. The medium was replaced and cells were treated with cytokine and psychosine/cytokine for 24 h.

Cell viability

Cytotoxic effects of all the inhibitors were determined by measuring the metabolic activity of cells with 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay.

RESULTS

Detection of iNOS in brain of Krabbe patients

Brain sections from patients with Krabbe disease and age-matched controls were immunohistochemically stained for iNOS. The iNOS protein was observed in Krabbe brain but not in normal brain (**Fig. 1**). To identify iNOS-expressing cell types, sections were dou-

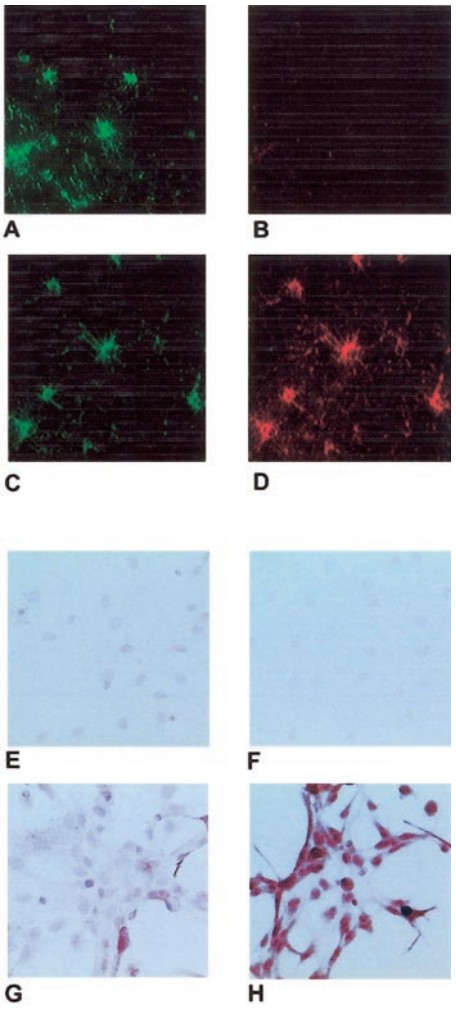

Figure 1. Double immunofluorescence staining for GFAP (green)/iNOS (red) in Krabbe brain autopsy tissue (*A–D*) and immunocytochemical staining for iNOS in primary rat astrocytes (*E–H*). Immunofluorescent microscopy images of brain sections from paraffin-embedded sections of formaldehyde-fixed Krabbe disease brain were stained with antibodies for iNOS (red) and GFAP (green) as described in Materials and Methods. *A, C)* GFAP; *B, D)* iNOS staining. *A, B)* Control age-matched; *C, D)* Krabbe brain. In primary rat astrocytes, cells were grown on Labteck chamber slides and serum starved overnight. Cells were treated with LPS (0.5 μg/ml) with or without psychosine for 24 h and stained for iNOS. *E)* Untreated; *F)* psychosine (15 μM) only; *G)* LPS (0.5 μg/ml); *H)* LPS + psychosine treated. Original magnification = 1000×.

ble-labeled for GFAP, a marker for activated astrocytes. Age-matched control brain sections showed only GFAP whereas in Krabbe brain sections, double-positive cells signaled for iNOS and GFAP (Fig. 1*C, D*), demonstrating the astrocytes as an iNOS-positive cells in Krabbe brain. To study the mechanism of iNOS induction under culture conditions, we performed immunocytochemical staining for iNOS in rat primary astrocytes. As evident in Fig. 1*G*, LPS induced expression of iNOS protein in rat astrocytes, which was significantly potentiated by psychosine (Fig. 1*H*). Expression of iNOS in astrocytes in the brain of Krabbe patients and the up-regulation of LPS-induced expression of iNOS by psychosine in primary rat astrocytes in culture indicate that the induction of iNOS and production of NO may play a role in the pathophysiology of Krabbe disease.

Potentiation of LPS-induced production of proinflammatory cytokines and NO by psychosine in rat primary astrocytes

LPS is known to induce inflammatory cytokine production in primary astrocytes and macrophages (32). To test whether psychosine has any effect on cytokine production, primary astrocytes were treated with different concentrations of psychosine in the absence or presence of LPS (0.5 μg/ml) for 6 h for TNF-α and 24 h for IL-1β, IL-6, and NO production. As expected, LPS induced the expression of cytokines and production of NO. Psychosine up-regulated LPS-mediated induction of cytokines and the production of NO in a dose-dependent manner (**Fig. 2**). Psychosine at the concentration of 20 μM enhanced the LPS-mediated production of NO, TNF-α, IL-1β, and IL-6 by −2.2, −5.3, −20, and −6-fold, respectively. Psychosine by itself had no effect on the production of either cytokines or NO in primary astrocytes.

Psychosine stimulates the cytokine-mediated production of NO

To investigate the possible role of psychosine on cytokine-induced iNOS expression, cells were treated with high (20 ng/ml) or very low concentration (0.1 ng/ml) of cytokines (TNF-α and IL-1β) in the presence/absence of different concentrations of psychosine (1 to 30 μM). C6 glial cells do not produce NO in response to

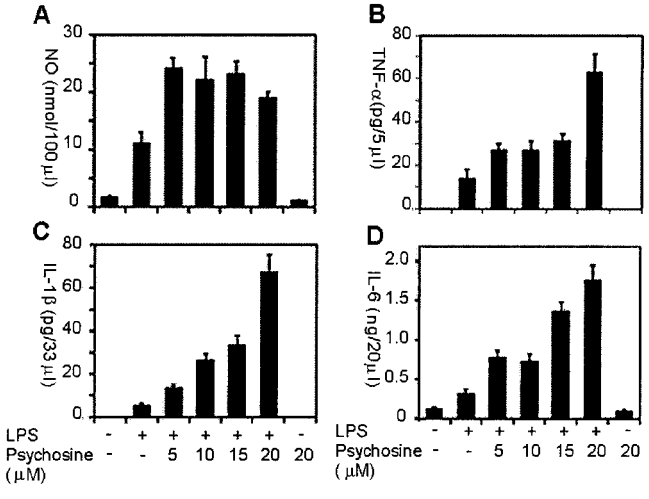

Figure 2. Psychosine potentiated LPS-induced production of NO, TNF-α, IL-1β, and IL-6 in primary rat astrocytes. Under serum-free conditions, cells were treated with 0.5 μg/ml of LPS along with different concentrations of psychosine (5–20 μM). After 6 h of incubation, media was removed to estimate levels of TNF-α; levels of NO, IL-1β, and IL-6 were estimated after 24 h in media supernatant (see Materials and Methods). Data are expressed as mean ± SD of 3 different experiments.

LPS or individual cytokines. A combination of cytokines was required to induce the production of NO in these cells, so a combination of cytokines (TNF-α/IL-1β) was used. After 24 h, nitrite was measured in media. As shown in **Fig. 3*A***, C6 glial cells produced a high amount of NO in response to 20 ng/ml of cytokines and psychosine had little effect on cytokine-induced production of NO (Fig. 3*A–i*). On the other hand, psychosine enhanced the production of NO in a dose-dependent manner when cells were stimulated with lower concentrations of LPS and IFN-γ (0.1 ng/ml and 2 U/ml, respectively) (Fig. 3*A*-ii). These results suggest that high concentrations of cytokines mask the stimulatory effects of psychosine for the production of NO; therefore, low concentrations of cytokines were used in the subsequent studies. As shown in Fig. 3*B, C*, psychosine potentiated the cytokine-induced expression of iNOS in a dose- and time-dependent manner. Similar induction of iNOS was observed in primary rat astrocytes (Fig. 3*D*). Expression of iNOS protein was maximum at 20 μM concentration of psychosine but with a 20% loss of cell viability. Based on MTT and LDH release assay, no cell loss was observed at 15 μM concentration of psychosine; 15 μM concentration of psychosine was used for studies reported here. Psychosine stimulated cytokine-mediated NO production in A172 and T98G, human astrocytoma cell lines (data not shown).

Psychosine sustained cytokine-induced iNOS mRNA synthesis

To understand the mechanism of the stimulatory effect of psychosine on cytokine-mediated induction of iNOS, we examined the effect of psychosine on expression of iNOS mRNA. Cells were treated with cytokine (TNF-α/IL-1β) with or without psychosine (15 μM) and RNA was isolated at different times. Northern blot analysis of iNOS mRNA clearly showed that psychosine does not change the cytokine-mediated induction of iNOS mRNA, but stabilizes in situ levels of iNOS mRNA (**Fig. 4**). The highest levels of iNOS mRNA were observed at 6 h of treatment with cytokines alone, followed by a decline to undetectable levels at 24 h. However, psychosine/cytokine-treated cells showed significant levels of iNOS mRNA at 6 and 24 h of treatment (Fig. 4*A*). A dose-dependent increase in cytokine-induced iNOS mRNA was observed (Fig. 4*B*) when cells were treated with psychosine (1–15 μM) along with TNF-α/IL-1β (0.1 ng/ml each) for 24 h. These studies indicate that the stimulation of NO production derived from iNOS

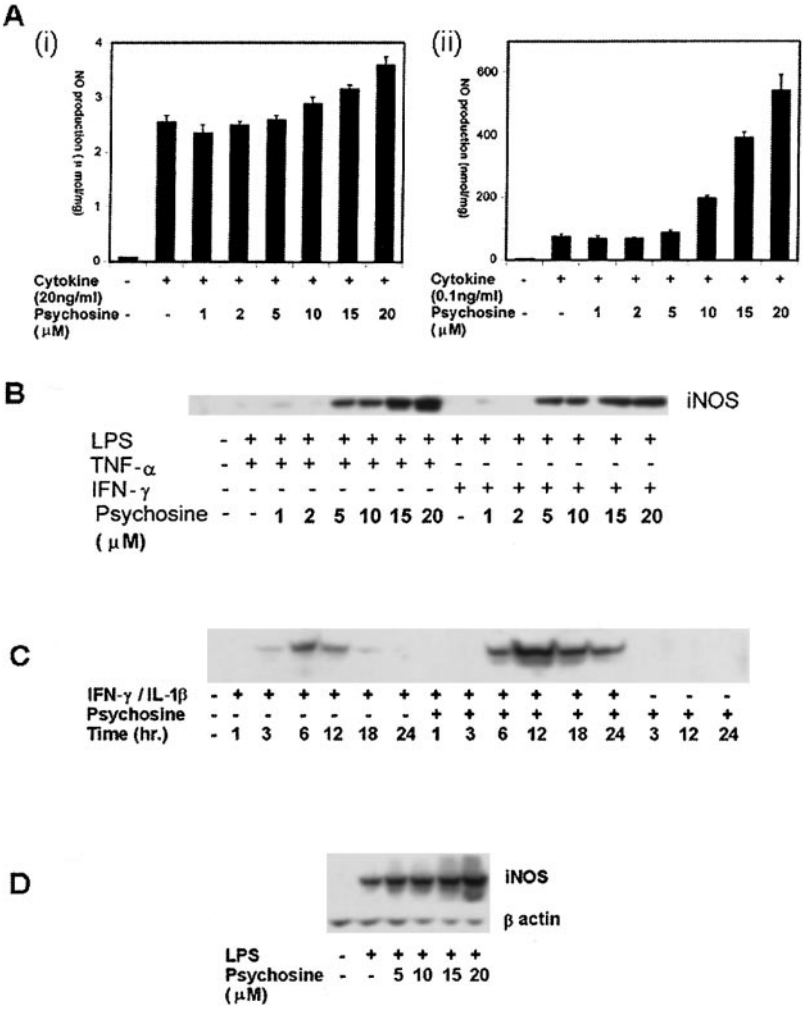

Figure 3. Psychosine potentiates cytokine-induced expression of iNOS in a dose-dependent manner in C_6 glial and primary rat astrocytes. *A)* Cells incubated in serum-free DMEM/F-12 media received high (10 ng/ml) (i) or very low amounts of TNF-α/IL1β (0.1 ng/ml) along with different concentrations of psychosine from 1 to 20 μM. After 24 h, nitrite was estimated in the supernatant as described in text. Data are the means ± SD of 3 different experiments. *B)* C_6 glial cells were treated with LPS/TNF-α (0.1 μg and 0.1 ng/ml) or LPS/IFN-γ (0.1 μg and 2 U/ml) with different concentrations of psychosine. After 24 h, cell homogenates were electrophoresed, transferred to nitrocellulose membrane, and immunoblotted with iNOS antibodies. *C)* C_6 glial cells were treated with IFN-γ/IL-1β (2 U and 0.1 ng/ml) and psychosine (15 μM). Cell homogenates were processed as mentioned above. *D)* Primary rat astrocytes were treated with LPS (0.5 μg/ml) along with different concentrations of psychosine. After 24 h, cell homogenate were processed for iNOS Western blot.

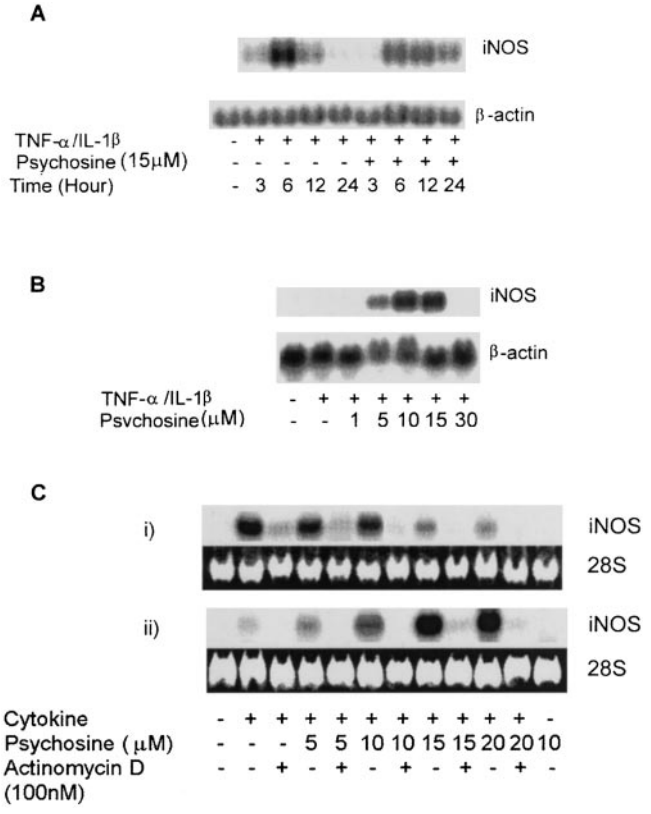

Figure 4. Psychosine stimulates the continuous synthesis of cytokine-induced message of iNOS. *A)* Serum-starved cells were treated with TNF-α/IL-1β (0.1 ng/ml each) with or without 15 μM of psychosine taken out directly by adding Ultraspec-II RNA reagent (Biotecx Laboratories) to the plate for isolation of total RNA; Northern blot analysis for iNOS/β-actin was performed. *B)* C_6 glials cells were treated with different concentration of psychosine (1–30 μM) along with cytokine (TNF-α/IL-1β, 0.1 ng/ml). After 24 h, RNA was extracted and processed for Northern analysis as described above. *C)* (i) Cells were preincubated with actinomycin D (100 nM) for 30 min, then TNF-α/IL-1β (0.1 ng/ml) and psychosine (15 μM) were added into the medium. After 6 h incubation, total RNA was extracted for Northern analysis for iNOS/β-actin. (ii) After 6 h treatment of C_6 glial cells with TNF-α/IL-1β (0.1 ng/ml) and psychosine (15 μM), actinomycin D (100 nM) was added to the media. RNA was extracted after 24 h incubation at 37°C and processed for Northern analysis.

in C6 glial cells by psychosine may be due to either a sustained synthesis of iNOS message or stabilization (down-regulation of its turnover) of cytokine-induced iNOS mRNA. To examine this, cells were treated with actinomycin D (a potent transcription inhibitor). Pretreatment with actinomycin D for 30 min before the addition of cytokine and psychosine completely inhibited the expression of iNOS mRNA in cytokine- and psychosine-treated cells, suggesting that actinomycin D blocks the newly synthesized iNOS mRNA (Fig. 4*C*-i). To examine whether psychosine stabilizes cytokine-induced iNOS mRNA or sustains the synthesis of new iNOS message over longer periods, C6 glial cells were exposed to cytokines with or without psychosine. After 6 h of treatment, 100 nM of actinomycin D was added to the cell culture. After 24 h of treatment, RNA was extracted and processed for iNOS Northern blot. As shown in Fig. 4*C*-ii, psychosine potentiated the iNOS message in a dose-dependent manner, but addition of actinomycin D completely abolished the psychosine-induced, cytokine-mediated iNOS mRNA at 24 h. It suggests that the higher in situ levels of iNOS mRNA in psychosine/cytokine-treated cells is due to continued/sustained expression of mRNA for iNOS rather than stabilization of iNOS message for a longer period.

Involvement of MAPK pathway in psychosine-mediated potentiation of cytokine-induced NO production

MAPKs (ERK1/2 and p38 MAPKs) are known to play a regulatory role in the cytokine-induced iNOS expression (34), therefore, we examined the role of MAPKs in psychosine-induced expression of iNOS. To study the involvement of Ras/ERK1/2 pathway in psychosine-induced, cytokine-mediated induction of iNOS, we examined the effect of PD98059 (a MEK1 inhibitor) and FPT inhibitor II (a selective inhibitor of Ras farnesyl protein transferase) in the cytokine/psychosine and TNF-α/psychosine-treated C6 glial cells and immortalized rat astrocytes, respectively (**Fig. 5*A, B***). In both cell lines, PD98059 and FPT inhibitor II significantly inhibited the psychosine-induced NO production by 50 to 70%. This observation was also confirmed by Western analysis for iNOS (as shown inset of Fig. 5), suggesting that the intracellular signals for the induction of iNOS by psychosine follows the Ras/MAPK signal transduction pathway in these cell lines. To investigate the involvement of p38 MAPK pathway, a specific inhibitor of p38 MAPK (SB203580) was used. As shown in Fig. 5, preincubation of cells with SB203580 almost completely inhibited the psychosine-induced, cytokine-mediated induction of iNOS and production of NO in C6 glial cells and immortalized rat astrocytes. These studies clearly demonstrate that MAPK signal transduction pathways are involved in the psychosine-mediated potentiation of iNOS induction.

Psychosine up-regulates expression of iNOS in cytokine-treated C6 glial cells without attenuating the activation of NF-κB

Since activation of NF-κB is necessary for the expression of iNOS, NF-κB status was examined in response to cytokine/psychosine. The murine iNOS promoter contains two known NF-κB-responsive elements (NF-κB RE); among these, the proximal NF-κB sequence is important in modulating the response to cytokines/LPS (35). In resting cells, the p65/p50 heterodimer is retained in the cytoplasm by its association with IκBα (33). After stimulation with various agents, the cytosolic NF-κB/IκBα complex dissociates as a result of proteolysis of IκBα; free NF-κB (p65/p50) translocates to the nuclei and regulates the transcription of various genes. Activation of NF-κB was monitored by DNA binding

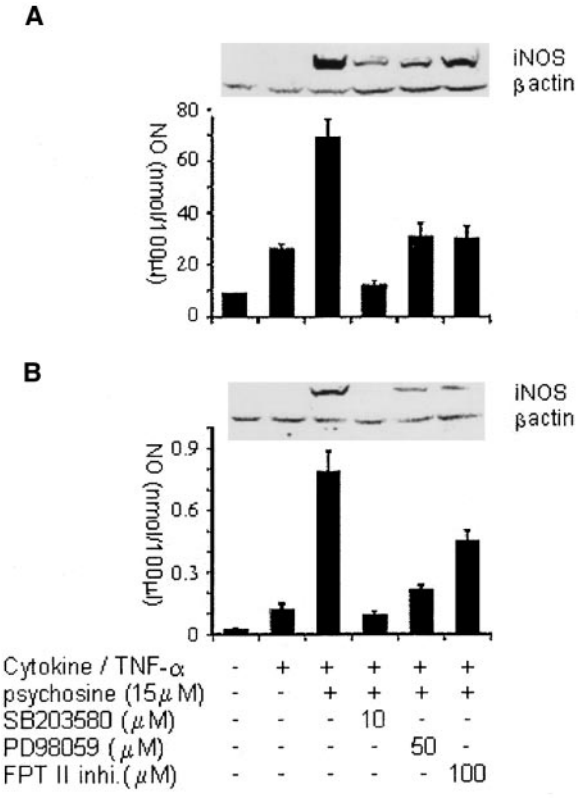

Figure 5. Psychosine follows Ras-ERK and p^{38} MAPK pathways for the induction of cytokine-induced NO production in C_6 glial (*A*) and immortalized astrocytes (*B*). Cells were pretreated with SB 203580 (10 μM), PD98059 (50 μM), and FPT inhibitor II (100 μM) for 30 min and exposed to 15 μM of psychosine and cytokine (C_6 glial cells, *A*) or TNF-α (100 ng/ml, immortalized rat astrocytes; *B*) for 24 h. Supernatant was removed for NO estimation and cell lysates were processed for Western analysis of iNOS and β-actin protein.

and transcriptional activity of NF-κB. The DNA binding activity of NF-κB was evaluated by the formation of a distinct and specific complex in gel shift DNA binding assay (EMSA). Treatment of C6 glial cells with various cytokine combinations induced the DNA binding activity of NF-κB (**Fig. 6*A***). The gel shift assay detected a specific band in response to cytokines that was competed by a 100-fold excess of unlabeled probe. Psychosine alone had no effect on the nuclear translocation of NF-κB and did not modulate the cytokine-mediated DNA binding activity of NF-κB. These results were also supported by Western analysis of p65 (a member of the NF-κB family) from nuclear extract of the cytokine with or without psychosine treatment (Fig. 6*B*). To understand the basis of cytokine-induced synthesis of iNOS mRNA (Fig. 4), the relationship between in situ levels of iNOS mRNA and activation of NF-κB was investigated. NF-κB status was examined at different times (0.5–24 h) in cells treated with only cytokine or cytokine plus psychosine. A time-dependent increase in NF-κB translocation was observed in the cells treated with cytokine alone, but no additional stimulation of NF-κB translocation in response to psychosine/cytokine over that of cytokine treatment alone was seen (Fig. 6*C*).

To further confirm this, we tested the effect of different concentrations of psychosine on NF-κB-dependent transcriptional activity of SEAP in the presence or absence of cytokines. Consistent with the results of DNA binding activity of NF-κB, psychosine alone did not induce the NF-κB-dependent transcription of SEAP and had no effect on the magnitude of TNF-α/IL-1β-induced transcriptional activity of NF-κB (Fig. 6*D*). To examine whether psychosine-induced expression of iNOS in cytokine-treated cells requires activation of NF-κB, the effect of antioxidants (NAC and PDTC, inhibitors of NF-κB activation) was studied on the expression of iNOS and nuclear translocation of NF-κB in cells treated with cytokine and psychosine. NAC and PDTC completely abolished psychosine-induced NO production, iNOS protein expression, and

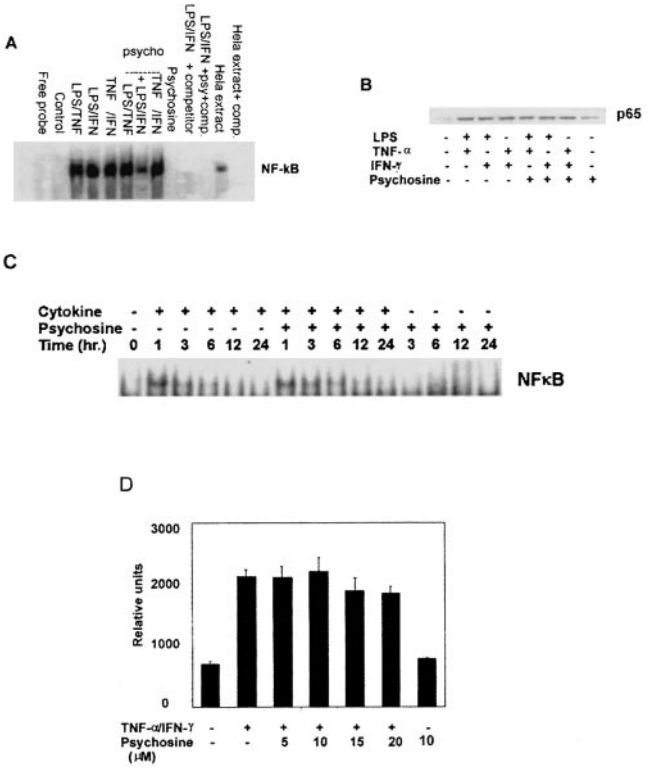

Figure 6. Psychosine does not inhibit nuclear translocation or DNA binding ability of NF-κB. Cells were treated with LPS and different cytokine combinations in the presence and absence of psychosine (15 μM) for 45 min; cytosolic and nuclear extracts were prepared. *A)* NF-κB-specific DNA–protein binding activity in nuclear extracts was determined by EMSA as described in Materials and Methods. *B)* Nuclear level of NF-κB (p65) was immunodetected using p65-specific antibodies. *C)* Cells were treated with TNF-α/IL-1β (0.1 ng/ml) with or without psychosine (15 μM) and nuclear proteins were analyzed for the presence of NF-κB binding activity in a gel shift assay. *D)* Cells were transfected with NF-κB-SEAP plasmid, then stimulated with cytokine and various concentrations of psychosine (5 to 20 μM) for 18 h. Alkaline phosphatase activity was determined in supernatant by a GreatESCAPE chemilumiscence kit according to the manufacturer's instructions. Results are mean ± SE of $n = 5$.

NF-κB DNA binding activity (data not shown). They inhibited NF-κB translocation in cytokine- and cytokine/psychosine-treated cells. These results indicate that NF-κB plays a very crucial role in psychosine-induced, cytokine-mediated production of NO even though psychosine did not have any additive effect on cytokine-mediated translocation of NF-κB.

The promoter region of the iNOS gene contains several binding sites for transcription factors other than NF-κB such as AP-1, C/EBP, ATF/CREB, Stats, GAS/ISRE, OCT-1, Sp-1, and IRF-1, which play a crucial role in optimal iNOS expression (35). It was therefore of interest to examine the effect of psychosine and cytokines on their DNA binding activity. Addition of cytokine leads to the induction of all transcription factors, as evident from **Fig. 7**. STAT1 and STAT3 induction was maximal at 1 h whereas AP-1, CREB, OCT-1, IRF-1, and GAS/ISRE were induced maximally at 3 h in response to cytokines. Addition of psychosine along with cytokine leads to significant induction in DNA binding activity for STAT1, GAS/ISRE, OCT-1, CREB, NF-κB, and STAT3; the maximum effect was observed in AP-1 and C/EBP at 3 and 6 h of incubation. Therefore, we studied the involvement of AP-1 and C/EBP transcription factors in psychosine-induced, cytokine-mediated induction of iNOS.

Psychosine stimulated the cytokine-mediated induction of NO via nuclear translocation of AP-1

As mentioned earlier, AP-1, is known to play an important role in regulating iNOS expression. A specific

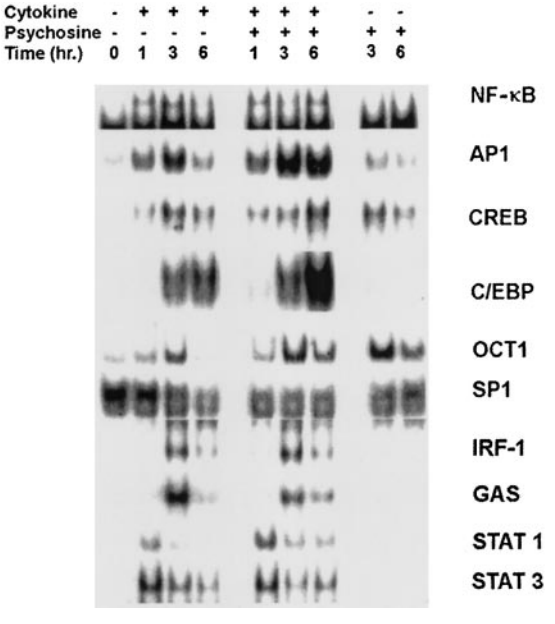

Figure 7. Psychosine modulates the IFN-γ/IL-1β cytokine-induced transcription factors in C_6 glial cells. Under serum-free conditions, C_6 glial cells were treated with IFN-γ/IL-1β (2 U and 0.1 ng/ml) with or without 15 μM psychosine. Cells were removed to prepare nuclear extracts and nuclear proteins were used for EMSA with ^{32}P-labeled double-stranded oligonucleotides for various transcription factors as described in Materials and Methods.

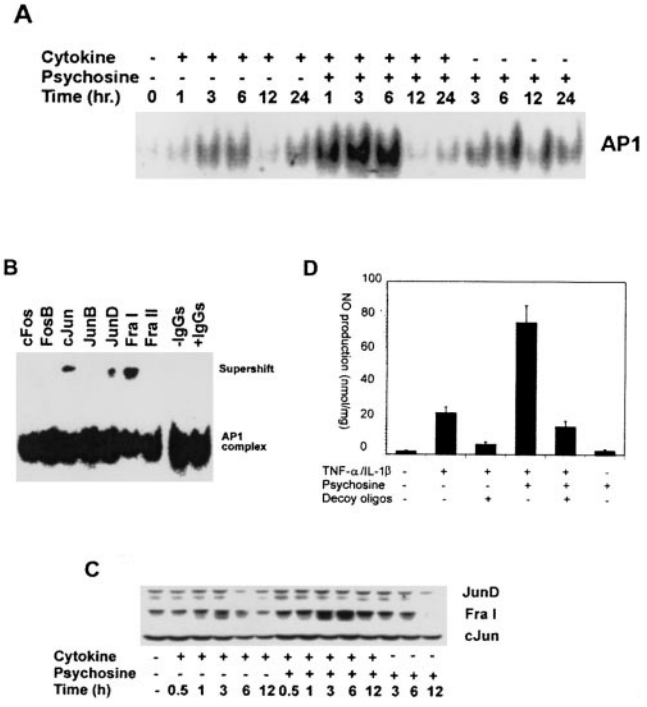

Figure 8. Psychosine potentiated iNOS expression by inducing nuclear translocation of AP-1 transcription factor in C_6 glial cells. *A)* Cells were incubated with cytokines with or without psychosine before preparation of nuclear extracts for gel retardation analysis of AP-1. *B)* Nuclear extracts prepared from cells treated with cytokine and psychosine for 6 h were incubated for 30 min without IgG, with preimmune rabbit serum, or with antibodies against proteins of the Jun and Fos families after they had bound oligonucleotides of AP-1. *C)* Nuclear extracts were treated and subjected to Western analysis with the respective antibodies. *D)* Cells were transfected with decoy oligonucleotides for 6 h, then treated with TNF-α/IL-1β and 15 μM psychosine. After 24 h, nitrite was estimated in supernatant. Results are mean ± SE of $n = 5$.

slow-moving DNA–protein complex that binds to the AP-1 sequence was observed in cytokine-treated cells and the amount of the DNA–protein complex increased with time (**Fig. 8*A***). However, psychosine significantly enhanced the nuclear translocation of AP-1. A 24 h treatment of psychosine/cytokine induced nuclear translocation of AP-1 complex by fivefold vs. cytokine alone. These observations indicate that AP-1 may be one factor responsible for maintaining higher levels of iNOS mRNA, in turn leading to increased levels of iNOS protein and production of NO in response to psychosine treatment. The AP-1 transcription factor is a complex composed of proteins of the Jun and fos proto-oncogene families that need to dimerize in order to promote binding of the complex to the AP-1 recognition site. To identify the proteins responsible for AP-1 complex formation, mobility supershift assays were performed with antibodies raised against proteins of the Jun (c-jun, JunB, and JunD) and Fos (FosB, c-fos, Fra I, and Fra II) families. With oligonucleotide-bearing sequences of AP-1 elements (Fig. 8*B*), antibodies specific for c-Jun, Jun D, and fra I produced marked supershifts, which suggests these

proteins are major components of the AP-1 DNA binding complex. On the other hand, control serum and the other antibodies (JunB, FosB, c-fos, and Fra II) did not supershift the complex formed with nuclear extracts.

In view of the different patterns seen in protein binding of AP-1 oligonucleotides in EMSA experiments, cytokine- and cytokine/psychosine-induced expression of protein constituents of the AP-1 nucleoprotein complex were examined in nuclear extract of C6 glial cells. c-Jun and JunD proteins were constitutively expressed in untreated cells and induction was not observed in their protein levels in nuclear extracts after cytokine and psychosine treatment (Fig. 8*C*). In control cells, very little Fra I was detected in nuclear extracts. Stimulation with cytokines markedly enhanced the Fra I protein level in nuclear extracts, which was dramatically induced by psychosine (see Fig. 8*C*). Immunoblot analysis of c-Jun, JunD, and Fra1 in nuclear extract further supports the increased DNA binding activity of AP-1 in response to cytokine and psychosine treatments.

For additional proof that AP-1 is required for psychosine-induced, cytokine-mediated production of NO, we developed a double-stranded decoy oligonucleotide that corresponds to the AP-1 response element. The rationale for this approach was that the decoy should compete for the AP-1 binding site of the iNOS promoter and thus block AP-1-dependent transcriptional responses. Introduction of the AP-1 double-stranded decoy oligonucleotide inhibited the psychosine-induced, cytokine-mediated NO production (Fig. 8*D*). These observations indicate that AP-1-dependent transcriptional responses are critical for iNOS induction by psychosine.

Psychosine promotes cytokine-induced C/EBP binding activity

Psychosine alone modulated the nuclear translocation of AP-1, OCT1, and CREB, but without cytokines was unable to induce expression of iNOS in these cells, indicating that the critical factors might be STATs, GAS/ISRE, NF-κB, and C/EBP. Of the transcription factors, AP-1 and C/EBP showed the maximum change in response to psychosine (Fig. 7). AP-1 can be induced by psychosine alone, it therefore seems that C/EBP might be a key factor in psychosine-mediated upregulation of the expression of iNOS. To identify the C/EBP protein(s) responsible for the iNOS induction, supershift assays with antibodies specific for C/EBP-α, -β, -δ, and -ε were performed. Nuclear extracts from cytokine- and cytokine/psychosine-treated C6 glial cells were incubated with IgGs specific for each of these C/EBP proteins before reaction with the ^{32}P-labeled C/EBP oligos. Only the IgGs specific for C/EBP-β and -δ significantly supershifted the complex (**Fig. 9*B***) in cytokine- and cytokine/psychosine-treated cells. Expression of C/EBP-β and -δ was examined in nuclear extracts of cytokine- and cytokine/psychosine-treated cells at various times. C/EBP-β was constitutively expressed in untreated cells and its induction was not observed with any treatment; C/EBP-δ was found to be

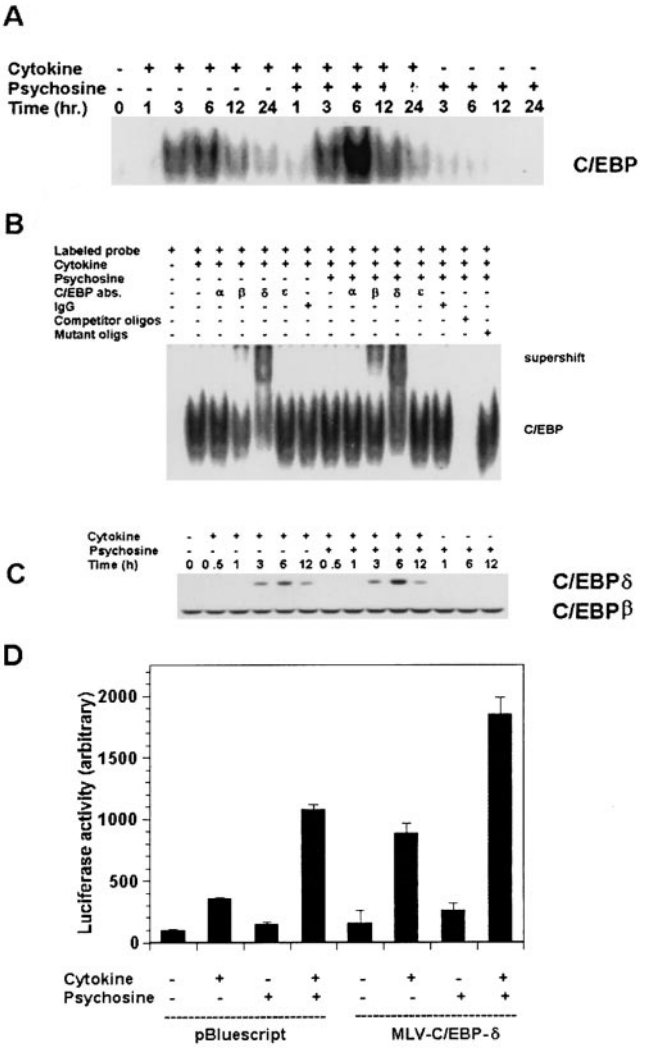

Figure 9. Psychosine induced the expression of C/EBP-δ isoform, thereby increasing the cytokine-induced DNA binding ability of C/EBP in C_6 glial cells. *A)* Cells were incubated with various treatments before preparation of nuclear extracts for EMSA of C/EBP. *B)* Polyclonal IgGs specific for C/EBP α, β, δ, and ε were used in supershift experiments with nuclear extracts from cytokine- and cytokine/psychosine-treated (6 h) C_6 glial cells and the ^{32}P-labeled C/EBP oligomer. Autoradiograms are representative of 2 independent experiments performed on separate preparations of nuclear extracts. *C)* Nuclear extracts prepared from various treatments as in panel *A* were subjected to Western analysis for C/EBP-β and -δ proteins. *D)* C6 glioma cells were transfected with the pNOS2-luc reporter construct in the presence of expression vector for C/EBP-δ (MLV-C/EBP-δ) or an insertless vector (pBluescript). 24 h after transfection, cells were stimulated with IFN-γ 2 U/ml) + IL-1β(0.1 ng/ml) for 16 h and cell lysates were prepared. Luciferase activity in cell lysates was assayed and normalized to cell protein content. Data are the mean ± SD of 3 different values.

inducible in response to cytokine/psychosine treatment as it could not be detected in untreated cells (Fig. 9*C*). To determine whether overexpression of C/EBP could alter cytokine or psychosine-induced iNOS promoter activity, transient transfection was performed with the wild-type iNOS promoter construct (piNOS-luc) together with either empty expression vector

(pBluescript) or expression plasmids for C/EBP-δ (MLV-C/EBP-δ). As evident in Fig. 9*D*, cytokine-induced iNOS promoter activity was further enhanced 2.6-fold when cells were treated with a 15 μM concentration of psychosine along with cytokines. Cotransfection with MLV C/EBP-δ exhibited significantly higher iNOS promoter activity with cytokines and cytokine/psychosine treatments, clearly demonstrating the regulatory role of C/EBP-δ in iNOS regulation. Psychosine potentiates the expression of cytokine-induced C/EBP-δ significantly, which in turn may be responsible for the formation of homodimers and heterodimers with C/EBP-β for increased DNA binding activity of C/EBP in cytokine/psychosine-treated C6 glial cells in a time-dependent manner (Fig. 9*C*).

DISCUSSION

Evidence is provided here for the first time that induction of iNOS in the CNS of patients with Krabbe disease and psychosine (whose level increased significantly in this disease) regulates production of NO via AP-1 and C/EBP transcription factors in rat primary astrocytes and C6 glial cells. These conclusions are based on the following. *1)* Immunohistochemical studies using iNOS-specific antibodies identified iNOS protein in brain tissue of Krabbe patients. Double labeling experiments with cell-specific markers confirmed that iNOS-expressing cells in the Krabbe brain are astrocytes. *2)* Psychosine potentiated the LPS-induced expression of iNOS and the production of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) production in a dose-dependent manner in rat primary astrocytes. *3)* Up-regulation of cytokine/LPS-mediated induction of iNOS protein and production of NO by psychosine may be due to sustained synthesis of iNOS mRNA. These observations indicate that psychosine, which accumulates in Krabbe disease due to the mutation in galactocerebrosidase enzyme, under inflammatory conditions leads to the production of NO, which in turn may play a role in the pathogenesis of the disease. Inducible NOS usually is not present in the normal brain, but is detected after neuroinflammatory diseases such as infection, multiple sclerosis, stroke, Alzheimer disease, experimental allergic encephalomyelitis, injury, as well as during aging of the normal brain (36–43).

Several studies of brain tissue from twitcher mice, an animal model for Krabbe disease, and from patients with Krabbe disease clearly documented this disease to be a demyelinating one due to loss of oligodendrocytes (44–49). Psychosine, a potent neurotoxin, has been postulated to play a major role in the degeneration and/or dysfunction of myelin-forming cells (44, 50) as the number of oligodendrocytes decrease with progression of the disease in demyelinated areas of the brain (51–53). The observed psychosine-induced production of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) in primary rat astrocytes in culture (Fig. 2) and the expression of IL-6, TNF-α, and chemokines (MCP-1, IP-10, MIP-1α, MIP-1β, and RANTES) in twitcher mice (24–26) indicate the involvement of an inflammatory process in the pathogenesis of Krabbe disease. However, the mechanism of psychosine/cytokine-mediated toxicity in the degeneration/dysfunction of oligodendrocyte is not understood at this time. The study reported here hypothesizes that the accumulation of psychosine up-regulates cytokine production, which in turn leads to the induction of iNOS and production of NO in Krabbe brain. Induction of NO by psychosine under inflammatory conditions may be the basis of the oligodendrocyte loss. Since ONOO generated by the reaction of NO and O_2^- is known to modify cellular components (protein, lipids, and DNA), which may lead to inactivation of cellular functions and, finally, to cell death (53, 54).

To understand the mechanism of psychosine-mediated induction of iNOS, we examined the effect of psychosine on the nuclear translocation of various transcription factors—i.e., NF-κB, AP-1, C/EBP, OCT-1, CREB, STATs, SP-1, and IRF-1–that have potential binding sites in the promoter of the iNOS gene (35). Of all the transcription factors studied, psychosine caused the maximum induction of nuclear translocation of AP-1 and C/EBP. Psychosine by itself significantly induced translocation of transcription factors such as AP-1 and CREB and had a further additive effect on cytokine-mediated activation of these transcription factors. Activation of AP-1 is a critical event in psychosine-mediated induction of iNOS. First, psychosine and cytokine together significantly potentiated the nuclear translocation of AP-1 compared with that by cytokine alone. Second, psychosine potentiated expression of Fra I (a component of AP-1) significantly. Third, the double-stranded AP-1 decoy inhibited the stimulatory effect of psychosine on the cytokine-mediated production of NO. These data strongly suggest that psychosine-mediated AP-1 activation is a crucial factor responsible for higher expression of iNOS and production of NO under cytokine/psychosine treatment (Fig. 7). Deletion of the AP-1 site in the promoter of iNOS is known to lead to the complete inhibition of luciferase iNOS promoter activity, indicating its importance for the expression of this gene (55).

Other than AP-1, psychosine showed the most profound effect on cytokine-induced nuclear translocation of C/EBP (Fig. 9). On the other hand, psychosine alone had no effect on the nuclear translocation of C/EBP. C/EBP belongs to the superfamily of basic region/leucine zipper DNA binding proteins. The C/EBP protein α, β, δ, γ, and ε isoforms function as activators of transcription whereas C/EBP homology protein, the liver-enriched transcriptional inhibitory protein, and C/EBP-30 serve as repressors (56). Expression of various C/EBP isoforms is differentially induced during macrophage and/or granulocyte differentiation. C/EBP binding motifs have been identified in the functional regulatory regions of various genes expressed by cells of myelomonocytic lineage, including those encoding the inflammatory (IL-6, IL-1β, and

TNF-α), as well as other cytokines such as IL-8 and IL-12, genes encoding proteins important for macrophagic or granulocytic functions such as iNOS, lysozyme, myeloperoxidase, and neutrophil elastase, the gene encoding the granulocyte colony stimulatory factor, and the macrophage, granulocyte, and granulocyte-macrophage receptor genes (57). It can therefore be speculated that psychosine mediated the induction of proinflammatory cytokines (IL-6, IL-1β, and TNF-α) and NO production through C/EBP.

Most recently, the C/EBP response element present in the promoter region of the iNOS gene has been shown to be involved in the regulation of its expression in various cell lines (58). Supershift studies provided evidence that the C/EBP complex formed in C6 glial cells is composed of C/EBP-β and -δ isoforms (Fig. 9). Our results agree with previous reports of various experimental systems (59, 60) that C/EBP-β isoform is constitutive whereas expression of -δ isoform increased in response to cytokines and psychosine. Different transcription factors have been reported to be able to physically interact with members of the NF-κB family (61, 62). Because induction of C/EBP mRNA occurs relatively late after the inflammatory stimulus, it is likely that transcription factors like NF-κB and STAT3 whose activation is more rapid but transient are responsible for the first burst of induction that is partly replaced after a few hours by C/EBP members. In agreement, it has recently been demonstrated that Stat3 is involved in the IL-6-induced up-regulation of C/EBP-β and C/EBP-δ (63). This hypothesizes a mechanism for psychosine signaling under mild inflammatory conditions in which induction of inflammatory cytokines would first trigger the activation of preexisting inactive forms of Stat3 and NF-κB. These factors would then initiate the activation of iNOS genes; at the same time, psychosine would induce new synthesis C/EBP-δ through STAT3. The preexisting population of C/EBP molecules would participate in this initial induction of acute-phase genes by interacting with NF-κB after phosphorylation. However, the main role played by C/EBP factors in the induction of acute-phase genes is linked to the synthesis of new C/EBP, which will substitute NF-κB and Stat3, allowing the activated status of iNOS genes expression to be sustained in the case of psychosine. Deleted promoter analysis of iNOS is under way that will provide additional insight into the psychosine-mediated signaling in iNOS gene expression.

In summary, we have demonstrated for the first time the presence of iNOS in the CNS of Krabbe patients and shown that psychosine potentiates cytokine-mediated induction of iNOS by involving various transcription factors, mainly AP-1 and C/EBP. The excessively produced NO derived from iNOS and/or ONOO, a reaction product of NO and O_2^-, may play a role in the pathogenesis of Krabbe disease. FJ

We gratefully acknowledge Drs. S. L. McKnight and H. Zhang for providing the C/EBP cDNA and 3.2 iNOS promoter luciferase constructs, respectively. We thank Amy Amerman for secretarial assistance and Joyce Bryan for technical assistance. This work was supported by grants (NS-22576, NS-34741, NS 37766, and NS-40810).

REFERENCES

1. Krabbe, K. (1916) A new familial, infantile form of diffuse brain sclerosis. *Brain* **30,** 74–114
2. Suzuki, K., Suzuki, Y., and Suzuki, K. (1995) Galactosylceramide lipidosis; globoid cell leukodystrophy (Krabbe disease) In The Metabolic and Molecular Bases of Inherited Disease (Scrire, C. R., Beaudet, A. L., Sly, W. S., Valle, D., eds) Vol. 2, pp. 2671–2692. McGraw-Hill, Inc., New York
3. Austin, J. H. (1963) Recent studies in the metachromatic and globoid body forms of diffuse sclerosis. In *Brain Lipids and Lipoproteins, and the Leucodystrophies* (Folch-Pi, J., and Bauer, H., eds) pp. 120–123, Elsevier, Amsterdam
4. Svennerholm, L. (1963) Some aspects of biochemical changes in leukodystrophy. In *Brain Lipids and Lipoproteins, and the Leucodystrophies* (Folch-Pi, J., and Bauer, H., eds) pp. 104–119, Elsevier, Amsterdam
5. Suzuki, K. (1998) Twenty-five years of the 'psychosine hypothesis': a personal perspective of its history and present status. *Neurochem. Res.* **23,** 251–259
6. Igisu, H., and Suzuki, K. (1984) Progressive accumulation of toxic metabolite in a genetic leukodystrophy *Science* **224**, 753–755
7. Svennerholm, L., Vanier, M.-T., and Mansson, J. E. (1980) Krabbe disease: a galactosylsphingosine (psychosine) lipidosis. *J. lipid. Res.* **21,** 53–64
8. Morell, P., and Radin, N. S. (1969) Synthesis of cerebroside by brain from uridine diphosphate galactose and ceramide containing hydroxy fatty acid. *Biochemistry* **8,** 506–512
9. Costantino-Ceccarini, W., and Morell, P. (1972) Biosynthesis of brain sphingolipids and myelin accumulation in the mouse. *Lipids* **7,** 656–659
10. Kobayashti, T., Shinnoh, N., Goto, I., and Kuroiwa, Y. (1985) Hydrolysis of galactosylceramide is catalyzed by two genetically distinct acid β-galactosidases. *J. Biol. Chem.* **260,** 14982–14989
11. Austin, J., Suzuki, K., Armstrong, D., Brady, R., Bachhawat, B. K., Schlenker, J., and Stumpf, D. (1970) Hydrolysis of galactosylceramide is catalyzed by two genetically distinct acid-â-galactosidases. *Arch. Neurol.* **23,** 502–511
12. Miyatake, T., and Suzuki, Y. (1972) Globoid cell leukodystrophy: additional deficiency of psychosine galactosidase. *Biochem. Biophys. Res. Commun.* **48,** 538–543
13. Suzuki, K., and Suzuki, Y. (1970) Globoid cell leukodystrophy (Krabbe's disease); deficiency of galactocerebrocyte-β-galactosidase. *Proc. Natl. Acad. Sci. USA* **66,** 302–309
14. Zlotogora, J., Chakraborty, S., Knowleton, R. G., and Wenger, D. A. (1990) Krabbe disease locus mapped to chromosome 14 by genetic linkage. *J. Hum. Genet.* **47,** 37–44
15. Suzuki, K., Tanaka, H., and Suzuki, K. (1976) Studies on the pathogenesis of Krabbe's leukodystrophy: cellular reaction of the brain to exogenous galactosylsphingosine, monogalactosyldiglyceride and lactosylceramide. In *Current Trends in Sphingolipidoses and Allied Disorders* (Volk, B. W., and Schneck, L., eds) Vol. 50, pp. 99–113, Plenum Press, New York
16. Taniiki, M., Mohri, I., Eguchi, N., Irikura, D., Urabe, Y., Okada, S., and Suzuki, K. (1999) An apoptotic depletion of oligodendrocytes in the twitcher, a murine model of globoid cell leukodystrophy. *J. Neuropathol. Exp. Neurol.* **58,** 644–653
17. Sweet, H. (1986) Twitcher is on Ch 12. *Mouse Newslett.* **75,** 30
18. Luzi, P., Rafi, M. A., and Wenger, D. A. (1996) Multiple mutations in the GALC gene in a patient with adult onset Krabbe disease. *Ann. Neurol.* **40,** 116–119
19. Sugama, K., Kim, S.-W., Ida, H., and Eto, Y. (1990) Psychosine cytoxicity in rat neural cell cultures and protection by phorbol ester and dimethyl sulfoxide. *Pediatr. Res.* **28,** 473–476
20. Igisu, H., and Nakamura, M. (1986) Inhibition of cytochrome C oxidase by psychosine (galactosylsphingosine). *Biochem. Biophys. Res. Commun.* **137,** 323–327
21. Ida, H., and Eto, Y. (1990) Biochemical and morphological studies of dorsal root ganglion and its cultured cells from

twitcher mouse (murine globoid cell leukodystrophy). *Brain Dev.* **12,** 412–416
22. Tanaka, K., and Webater, H., De, F. (1993) Effect of psychosine (galactosylsphingosine) on the survival and the fine structure of cultured Schwann cell. *J. Neuropath. Exp. Neurol.* **52,** 490–498
23. Andrew, J. M., and Menekes, J. H. (1970) Ultrastructure of experimentally produced globoid cells in the rat. *Exp. Neurol.* **29,** 483–493
24. Levine, S. M., and Brown, D. C. (1997) IL-6 and TNF-α expression in brains of twitcher, quaking and normal mice. *J. Neuroimmunol.* **73,** 47–56
25. Pedchenko, T. V., and Levine, S. M. (1999) IL-6 deficiency causes enhanced pathology in Twitcher (globoid cell leukodystrophy) mice. *Exp. Neurol.* **158,** 459–468
26. Wu, Y. P., McMahon, E. J., Matsuda, J., Suzuki, K., and Matsushima, G. K. (2001) Expression of immune-related molecules is downregulated in twitcher mice following bone marrow transplantation *J Neuropathol. Exp. Neurol.* **60,** 1062–1074
27. Kenneth, J. S., Kapoor, R., and Felts, P. A. (1999) Demyelination: the role of reactive oxygen and nitrogen species. *Brain Pathol.* **9,** 69–92
28. Torreilles, F., Salman-Tabcheh, S., Guerin, M., and Torreilles, J. (1999) Neurodegenerative disorders: the role of peroxynitrite. *Brain Res. Rev.* **30,** 153–163
29. McCarthy, K., and DeVellis, J. (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Biol.* **85,** 890–902
30. Feinstein, D. L., Galea, E., Roberts, S., Berquist, H., Wang, H., and Reis, D. J. (1994) Induction of nitric oxide synthase in rat C6 glioma cells. *J. Neurochem.* **62,** 315–321
31. Dignam, D., Lebovitz, R. M., and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11,** 1475–1489
32. Pahan, K., Sheikh, F. G., Namboodiri, A. M. S., and Singh, I. (1997) *N*-Acetyl cysteine inhibits induction of NO production by endotoxin or cytokine stimulated rat peritoneal macrophages, C6 glial cells and astrocytes. *Free Radic. Biol. Med.* **24,** 39–48
33. Pahan, K., Sheikh, F. G., Namboodiri, A. M. S., and Singh, I. (1998) Inhibitors of Protein Phosphatase 1 and 2A Differentially Regulate the Expression of Inducible nitric-oxide synthase in rat astrocytes and macrophages. *J. Biol. Chem.* **273,** 12219–12226
34. Guan, Z., Buckman, S. Y., Springer, L. D., and Morrison, A. R. (1999) Both $p38^{MAPK}$ and JNK/SAPK pathways are important for induction of nitric-oxide synthase by interleukin-1 in rat glomerular mesangial cells. *J. Biol. Chem.* **274,** 36200–36206
35. Eberhardt, W., Kunj, D., Hummel, R., and Pfeilschifter, J. (1996) Molecular cloning of the rat inducible nitric oxide synthase gene promoter. *Biochem. Biophys. Res. Commun.* **223,** 752–756
36. Merrill, J. E., and Murphy, S. P. (1997) Regulation of gene expression in the nervous system by reactive oxygen and nitrogen species. *Metab. Brain Dis.* **12,** 97–112
37. Merrill, J. E., and Scolding, N. J. (1999) Mechanisms of damage to myelin and oligodendrocytes and their relevance to disease. *Neuropathol. Appl. Neurobiol.* **25,** 435–58
38. Merrill, J. E., Ignarro, L. J., Sherman, M. P., Melinek, J., and Lane, T. E. (1993) Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *J. Immunol.* **151,** 2132–2141
39. Hu, J., Akama, K. T., Krafft, G. A., Chromy, B. A., and Van Eldik, L. J. (1998) Amyloid-β peptide activates cultured astrocytes: morphological alterations, cytokine production and nitric oxide release. *Brain Res.* **785,** 195–206
40. Vodovotz, Y., Lucia, M. S., Flanders, K. C., Chesler, L., Xie, Q. W., Smith, T. W., Weidner, J., Mumford, R., Webber, R., Nathan, C., Roberts, A. B., Lippa, C., F., and Sporn, M. B. (1996) Inducible nitric oxide synthase in tangle-bearing neurons of patients with Alzheimer's disease. *J. Exp. Med.* **184,** 1425–1433
41. Koprowski, H., Zheng, Y. M., Heber-Katz, E., Fraser, N., Rorke, L., Fu, Z. F., Hanlon, C., and Dietzschold, B. (1993) In vivo expression of inducible nitric oxide synthase in experimentally induced neurologic diseases. *Proc. Natl. Acad. Sci. USA* **90,** 3024–3027
42. Cross, A. H., Keeling, R. M., Goorha, S., San, M., Rodi, C., Wyatt, P. S., Manning, P. T., and Misko, T. P. (1996) Inducible nitric oxide synthase gene expression and enzyme activity correlate with disease activity in murine experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **71,** 145–153
43. Okuda, Y., Nakatsuji, Y., Fujimura, H., Esumi, H., Ogura, T., Yanagihara, T., and Sakoda, S. (1995) Expression of the inducible nitric oxide synthase in the central nervous system of mice correlates with the severity of actively induced experimental allergic encephalomyelitis. *J. Neuroimmunol.* **62,** 103–112
44. Taniike, M., and Suzuki, K. (1995) Proliferative capacity of oligodendrocytes in the demyelinating twitcher spinal cord. *J. Neurosci. Res.* **40,** 325–32
45. Kobayashi, T., Slinoda, H., Gota, I., Yamanaka, T., and Suzuki, Y. (1987) Globoid cell leukodystrophy is a generalized galactosylsphingosine (psychosine) storage disease. *Biochem. Biophys. Res. Commun.* **144,** 41–46
46. Jacobs, J. M., Scaravilli, F., and De Aranda, F. T. (1982) The pathogenesis of globoid cell leukodystrophy in peripheral nerve of the mouse mutant twitcher. *J. Neurol. Sci.* **55,** 285–304
47. Takahashi, H., and Suzuki, K. (1984) Demyelination in the spinal cord of murine globoid cell leukodystrophy (the twitcher mouse). *Acta Neuropathol. (Berlin)* **62,** 298–308
48. Tanaka, K., Nagara, H., Kobayashi, T., and Goto, I. (1988) The twitcher mouse: accumulation of galactosylsphingosine and pathology of the sciatic nerve. *Brain Res.* **454,** 340–346
49. Takahashi, H., Igisu, H., Suzuki, K., and Suzuki, K. (1983) The twitcher mouse: an ultrastructural study on the oligodendroglia. *Acta Neuropathol. (Berlin)* **59,** 159–66
50. Kobayashi, T., Shinoda, H., Goto, I., Yamanaka, T., and Suzuki, Y. (1987) Globoid cell leukodystrophy is a generalized galactosylsphingosine (psychosine) storage disease. *Biochem. Biophys. Res. Commun.* **144,** 41–6
51. Shinoda, H., Kobayashi, T., Katayama, M., Goto, I., and Nagara, H. (1987) Accumulation of galactosylsphingosine (psychosine) in the twitcher mouse: determination by HPLC. *J. Neurochem.* **49,** 92–99
52. Tanaka, K., Nagara, H., Kobayashi, T., and Gota, I. (1989) The twitcher mouse: accumulation of galactosylsphingosine and pathology of the central nervous system. *Brain Res.* **482,** 347–350
53. Chao, C. C., Hu, S., Sheng, W. S., Bu, D., Burkinsky, M. I., and Peterson, P. K. (1996) Cytokine-stimulated astrocytes damage human neurons via a nitric oxide mechanism. *Glia* **16,** 276–284
54. Mitrovic, B., Ignarro, L. J., Vinters, H. V., Akers, M. A., Schmid, I., Vittenbogaart, C., and Merrill, J. E. (1995) Nitric oxide induces necrotic but not apoptotic cell death in oligodendrocytes. *Neuroscience* **65,** 537–539
55. Marka-Konejalik, J., Chu, C., S., and Moss, J. (1998) Cytokine-mediated transcriptional induction of the human inducible nitric oxide synthase gene requires both activator protein 1 and nuclear factor B-binding sites. *J. Biol. Chem.* **273,** 22201–22208
56. Lekstrom-Himes, J., and Xanthoponlos, K. G. (1998) Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J. Biol. Chem.* **273,** 28545–28548
57. Poli, V. (1998) The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *J. Biol. Chem.* **273,** 29279–29282
58. Gupta, A. K., and Kone, B. C. (1999) CCAAT/enhancer binding protein *trans*-activates murine nitric oxide synthase 2 gene in an MTAL cell line. *Am. J. Physiol.* **276,** F599–F605
59. Cardinaux, Jean-Rene., Allaman, I., and Magistretti, P. J. (2000) Pro-inflammatory cytokines induce the transcription factors C/EBPbeta and C/EBPdelta in astrocytes. *Glia* **29,** 91–97
60. Akira, S., and Kishimoto, T. (1997) NF-IL6 and NF-kappa B in cytokine gene regulation. *Adv. Immunol.* **65,** 1–46
61. Stein, B., Cogswell, P. C., and Baldwin, A. S., Jr. (1993) Functional and physical associations between NF-kappa B and C/EBP family members: a Rel domain-bZIP interaction. *Mol. Cell. Biol.* **13,** 3964–3974
62. LeClair, K. P., Blanar, M. A., and Sharp, P. A. (1992) The p50 subunit of NF-kappa B associates with the NF-IL6 transcription factor. *Proc. Natl. Acad. Sci. USA* **89,** 8145–8149
63. Cantwell, C. A., Sterneck, E., and Johnson, P. F. (1998) Interleukin-6-specific activation of the C/EBPdelta gene in hepatocytes is mediated by Stat3 and Sp1. *Mol. Cell. Biol.* **18,** 2108–2117

Received for publication October 31, 2001.
Revised for publication January 18, 2002.