Amyloid-β peptide induces oligodendrocyte death by activating the neutral sphingomyelinase–ceramide pathway

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Amyloid-β peptide accumulation in senile plaques, a pathological hallmark of Alzheimer’s disease (AD), has been implicated in neuronal degeneration. We have recently demonstrated that Aβ-induced oligodendrocyte (OLG) apoptosis, suggesting a role in white matter pathology in AD. Here, we explore the molecular mechanisms involved in Aβ-induced OLG death, examining the potential role of ceramide, a known apoptogenic mediator. Both Aβ and ceramide induce OLG death. In addition, Aβ activated neutral sphingomyelinase (nSMase), but not acidic sphingomyelinase, resulting in increased ceramide generation. Blocking ceramide degradation with N-oleoyl-ethanolamine exacerbated Aβ cytotoxicity; and addition of bacterial sphingomyelinase (mimicking cellular nSMase activity) induced OLG death. Furthermore, nSMase inhibition by 3-O-methyl-sphingomyelin or by gene knockdown using antisense oligonucleotides attenuated Aβ-induced OLG death. Glutathione (GSH) precursors inhibited Aβ activation of nSMase and prevented OLG death, whereas GSH depletors increased nSMase activity and Aβ-induced death. These results suggest that Aβ induces OLG death by activating the nSMase–ceramide cascade via an oxidative mechanism.

Introduction

The amyloid-β peptide (Aβ), a 39–43-amino acid cleavage product of amyloid precursor protein (Estus et al., 1992; Haass et al., 1992), has been implicated as the primary neurotoxic factor in Alzheimer’s disease (AD) pathology (Yankner et al., 1989). In vitro, Aβ is toxic to neurons (Yankner et al., 1989; Behl et al., 1994; Yu et al., 1998), endothelial cells (Thomas et al., 1996; Huang et al., 1998), astrocytes (Brera et al., 2000), vascular smooth muscle cells (Kawai et al., 1993; Davis-Salinas et al., 1995), and oligodendrocytes (OLGs; Xu et al., 2001); and Aβ depositions in senile plaques are postulated to cause neuronal and vascular degeneration in AD brains (Masters et al., 1985; Yankner et al., 1989; Thomas et al., 1996). Although Aβ-mediated cell death demonstrates morphological, biochemical, and molecular features of apoptosis, the molecular mechanism underlying Aβ cytotoxicity remains largely undefined but may involve oxidative stress (Behl et al., 1994; Schapira, 1996). NF-kB and AP-1, redox-sensitive transcription factors, are activated in Aβ-treated OLGs, and N-acetylcysteine (NAC), an antioxidant, prevents Aβ-mediated OLG apoptosis (Xu et al., 2001). OLGs are susceptible to oxidative stress because they have low levels of reduced glutathione (GSH) and high concentrations of iron, resulting in a compromised ability to scavenge peroxides (Thorburne and Juurlink, 1996; Back et al., 1998; Juurlink et al., 1998).

Abbreviations used in this paper: 3-OMe-SM, 3-O-methyl-sphingomyelin; Aβ, amyloid-β peptide; AD, Alzheimer’s disease; aSMase, acidic sphingomyelinase; bSMase, bacterial sphingomyelinase; BSO, buthionine sulfoximine; DEM, diethyl maleate; ESI/MS, electrospray ionization/mass spectrometry; GSH, glutathione; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetylcysteine; nSMase, neutral sphingomyelinase; NOE, N-oleoyl-ethanolamine; OLG, oligodendrocyte; PLP, protocollid protein.

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Key words: Alzheimer’s disease; apoptosis; cell death; oxidative stress; white matter

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The Journal of Cell Biology, Volume 164, Number 1, January 5, 2004 123–131
http://www.jcb.org/cgi/doi/10.1083/jcb.200307017

Published Online: 5 January, 2004 | Supp Info: http://doi.org/10.1083/jcb.200307017
Downloaded from jcb.rupress.org on July 24, 2018
Ceramide, a lipid second messenger that increases the cellular oxidative state, has been implicated in several apoptosis paradigms including trophic factor withdrawal and exposure to proinflammatory molecules (Coroneos et al., 1995; Kyriakis and Avruch, 1996; Kolesnick and Kronke, 1998). Cellular ceramide synthesis increases in response to stress or death signals (Haimovitz-Friedman et al., 1994; Tepper et al., 1995; Verheij et al., 1996). One pathway of ceramide formation involves sphingomyelin hydrolysis by either neutral sphingomyelinase (nSMase) or acidic sphingomyelinase (aSMase; Testi, 1996); both enzymes are involved in several cell death paradigms (Kolesnick and Kronke, 1998; Levade and Jaffrezou, 1999). Another pathway involves ceramide synthase–catalyzed de novo ceramide synthesis (Bose et al., 1995; Spiegel and Merrill, 1996; Xu et al., 1998).

Aβ and ceramide share cell death signaling characteristics. Aβ-induced apoptosis involves TNF-α, p75 neurotrophin receptor, and Fas ligand (Blasko et al., 1997; de la Monte et al., 1997; Yaar et al., 1997), which are cell surface receptors that relay death signals through the sphingomyelin–ceramide pathway (Dobrowsky et al., 1995; Hannun, 1996). Moreover, both Aβ (Kaneko et al., 1995; Askanas et al., 1996; Bruce-Keller et al., 1998; Xu et al., 2001) and ceramide (Garcia-Ruiz et al., 1997; Singh et al., 1998) cause mitochondrial dysfunction and induce oxidative stress. In vitro, OLG death induced by Aβ (Xu et al., 2001) or ceramide (Larocca et al., 1997; Singh et al., 1998; Scurlock and Dawson, 1999) share similar apoptotic characteristics. Lower sphingomyelin levels and higher ceramide levels in AD brains have been reported (Soderberg et al., 1992), thereby implying that increased sphingomyelin degradation and ceramide accumulation contribute to AD pathogenesis. We have previously shown that Aβ-induced OLG death with characteristic features of apoptosis (Xu et al., 2001). In this paper, we demonstrate that ceramide mediates Aβ-induced OLG death by activating the nSMase–ceramide cascade.

**Results**

**Aβ and C2-ceramide are cytotoxic to OLGs**

We have previously shown that Aβ induced apoptosis in primary OLG cultures derived from neonatal rat brains, characterized by nuclear and cytoskeletal disintegration, DNA fragmentation, and mitochondrial dysfunction (Xu et al., 2001). In this paper, we used neurosphere-derived differentiated OLGs because of their ease of preparation compared with primary OLG isolation. The neurosphere-derived differentiated OLGs exhibited characteristic OLG morphology, which is composed of large cell bodies with multiple branching processes (Fig. 1, A–H). Immunocytochemical analysis revealed that virtually all cells in culture immunostained for the OLG-specific antigens, CNP (A), Rip (C), GalC (E), and PLP (G). Bar, 30 μm.

![Figure 1. Characterization of OLG cultures.](image1)

**Figure 2.** Aβ 25-35 and C2-ceramide induced OLG death. (A) OLGs treated with 10–100 μM Aβ 25-35 for 48 h. (B) OLGs exposed to 10–100 μM C2-ceramide for 24 h. The assessment of cell survival based on MTT assay or cell death based on LDH assay showed a dose-dependent cytotoxic effect of Aβ 25-35 and C2-ceramide. Data shown are representative of three separate experiments of triplicate samples with similar results; error bars represent SD.
Ceramide is derived from sphingomyelin hydrolysis catalyzed by nSMase or aSMase (Hannun, 1996). Aβ 25-35 treatment increased nSMase activity in OLGs as early as 2.5 min after exposure and reached maximal levels at ~16 h (Fig. 5 A). Aβ 1-40 treatment also increased OLG nSMase activity in a similar manner (unpublished data). In contrast, Aβ 25-35 treatment did not alter aSMase activity (Fig. 5 A). The addition of recombinant bacterial sphingomyelinase (bSMAse), an exogenous source of sphingomylinase that mimics nSMase action by degrading membrane sphingomyelin to increase cellular ceramide levels (Okazaki et al., 1989; Jarvis et al., 1994; Zhang et al., 1997; Tonnetti et al., 1999), caused OLG death (Fig. 5 B). Furthermore, the nSMase inhibitors, 3-O-methyl-sphingomyelin (3-OMeSM; Lister et al., 1995) and NAC (Liu et al., 1998a; Yoshimura et al., 1999), were effective in protecting OLGs against Aβ cytotoxicity (Fig. 5, C and D). Confirming the effects of the nSMase inhibitors, a marked reduction in nSMase enzymatic activity was observed in OLGs treated with 3-OMeSM or NAC (Fig. 6 A). Moreover, significant decreases in endogenous ceramide content and increases in sphingomyelin levels were detected in OLGs treated with the inhibitors (Fig. 6, B and C). To further confirm the contribution of nSMase in Aβ-mediated cell death, sense and antisense oligonucleotides specific for nSMase were generated. Antisense oligonucleotides reduced nSMase activity (Fig. 7 A), reduced ceramide content in cell lysates (Fig. 7 B), and attenuated Aβ-induced cell death (Fig. 7 C), but sense oligonucleotides had no effect on nSMase activity, ceramide content, or cell survival.
It has been shown that Aβ decreases GSH in cortical neurons in vitro (Muller et al., 1997), which correlates well with the ability of Aβ to heighten cellular oxidative stress (Cafe et al., 1996). GSH depletion has been shown to activate nSMase activity (Liu and Hannun, 1997; Liu et al., 1998a,b). The fact that NAC, a GSH precursor, inhibited Aβ-mediated Aβ25-35 activation of nSMase and prevented Aβ-mediated OLG death suggests that Aβ25-35 depletion of cellular GSH is involved in the Aβ–nSMase–ceramide OLG death signaling cascade. Buthionine sulfoximine (BSO) and diethyl maleate (DEM) have been shown to deplete GSH in various cell types (Anderson and Meister, 1983; Masukawa et al., 1983; Szekely and Lobreau, 1987). Both BSO and DEM selectively increased nSMase activity (Fig. 8 A), increased ceramide levels (Fig. 8 B), decreased cellular GSH levels (Fig. 8 C), and were cytotoxic to OLGs (Fig. 8 D).

Because Aβ25-35 did not activate aSMase, it is unlikely that aSMase is involved in Aβ25-35-mediated OLG death. Selective aSMase inhibitors such as desipramine and chlorpromazine (Albouz et al., 1986) were ineffective in protecting OLGs from Aβ25-35-induced death (unpublished data). Furthermore, fumonisin B2, a ceramide synthase inhibitor, did not block Aβ25-35 cytotoxicity in this cell death paradigm (unpublished data). Together, these results suggest that ceramide generation catalyzed by nSMase, but not aSMase or ceramide synthase, mediates the Aβ25-35 death pathway in OLGs.

Results from this study also support the contention that ceramide mediates, at least in part, Aβ-mediated OLG death. Both Aβ and C2-ceramide (but not the biologically inactive dihydroceramide) caused OLG death in a time-dependent manner. Aβ treatment increased ceramide formation in OLGs. In addition, increasing cellular ceramide release from sphingomyelin by exogenous hSMase, which mimics cellular nSMase action, also induced OLG death. Inhibition of ceramide degradation by NOE, a ceramidase inhibitor, enhanced Aβ cytotoxicity in OLGs.

Discussion

Several lines of evidence support the contention that ceramide mediates, at least in part, Aβ-mediated OLG death. Both Aβ and C2-ceramide (but not the biologically inactive dihydroceramide) caused OLG death in a time-dependent manner. Aβ treatment increased ceramide formation in OLGs. In addition, increasing cellular ceramide release from sphingomyelin by exogenous hSMase, which mimics cellular nSMase action, also induced OLG death. Inhibition of ceramide degradation by NOE, a ceramidase inhibitor, enhanced Aβ cytotoxicity in OLGs.

Results from this study also support the contention that Aβ cytotoxicity is mediated via activation of nSMase leading to increased cellular ceramide generation. Aβ25-35 and Aβ1-40 activated nSMase, but not aSMase, in OLGs. Addition-
nSMase inhibitors such as 3-OMe-SM and NAC (also an antioxidant) prevented Aβ 25-35–induced nSMase activity, which resulted in decreased ceramide synthesis from sphingomyelin and protected OLGs from Aβ 25-35 cytotoxicity. Antisense oligonucleotides specific for nSMase also attenuated Aβ-induced OLG death, further implicating nSMase as a mediator. Chemical agents such as BSO and DEM that deplete cellular GSH content also activated nSMase in OLGs and caused cell death. The specific role of nSMase in Aβ-mediated OLG death is supported by the finding that pharmacological inhibition of nSMase, but not αSMase or ceramide synthase, prevented Aβ 25-35–induced OLG death.

The exact mechanism underlying Aβ-mediated nSMase activation remains to be elucidated but may involve changes in the cellular redox state and/or GSH metabolism (Sawai and Hannun, 1999). GSH is the most abundant thiol-containing compound in living cells. nSMase enzymatic activity is directly regulated by cellular GSH content (Liu and Hannun, 1997; Liu et al., 1998a,b). Aβ has been shown to deplete GSH in cultured cortical neurons (Muller et al., 1997), and depletion of cellular GSH stores by oxidative stress has been proposed as a prime mechanism underlying the Aβ cytotoxic action (Muller et al., 1997; Pereira et al., 1999). Thus, it is plausible that a decrease in GSH level subsequent to Aβ exposure may activate nSMase in OLGs. NAC, a GSH precursor, inhibited Aβ 25-35 activation of nSMase and protected OLGs against Aβ-induced death, whereas depletion of cellular GSH stores by BSO or DEM resulted in selective activation of nSMase and OLG death. Although the agents used to manipulate GSH levels may be relatively nonspecific, these findings raise the possibility that the activation of nSMase by Aβ may involve the depletion of cellular GSH content.

Oxidative stress plays a prominent role in Aβ-mediated neuronal and OLG death (Behl et al., 1994; Behl, 1999; Markesbery, 1999; Xu et al., 2001). Brain tissue is especially sensitive to oxidative injury because of its higher metabolic rate driven by glucose, lower concentrations of protective antioxidants, and higher levels of polyunsaturated fatty acids that are susceptible to lipid peroxidation (Behl and Sagara, 1997; Behl, 1999; Markesbery, 1999). Although Aβ-mediated oxidative stress induces mtDNA damage (Bozner et al., 1997; Xu et al., 2001) and activates selected transcription factors including NF-κB and AP-1 (Abate et al., 1990; Schreck et al., 1991; Pinkus et al., 1996; Xu et al., 2001), the mechanism by which Aβ induces oxidative stress in the AD brain remains unknown. Ceramide has emerged as a potent second messenger in oxidative stress-induced apoptosis (Hannun and Luberto, 2000). Hydrogen peroxide (Goldkorn et al., 1998), 1-β-D-arabinofuranosylcytosine (Bradshaw et al., 1996), daunorubicin (Jaffrezou et al., 1996), TNF-α (Bezombes et al., 1998), γ-rays (Bruno et al., 1998), hypoxia (Yoshimura et al., 1998), CD40 activation (Segui et al., 1999), and sindbis virus infection (Jan et al., 2000) are among the agents that can mediate cell death via nSMase activation, thus emphasizing the central role of the nSMase–ceramide signaling cascade. Results shown here provide a unique perspective on the nSMase–cell signaling pathway from cell surface Aβ engagement, induction
of oxidative stress, and activation of the nSMase–ceramide cascade culminating in OLG death.

In summary, this work demonstrates a novel mechanism for Aβ-induced OLG death. These results reveal a causal relationship between Aβ exposure and the activation of the nSMase–ceramide pathway, which is likely to involve heightened oxidative stress after depletion of cellular GSH stores. In addition, we have evidence that activation of the nSMase–ceramide cascade may also contribute to Aβ-induced death of cortical neurons and cerebral endothelial cells (unpublished data), thereby suggesting that this cascade may be operating in many cell types other than OLGs. Aβ→nSMase→ceramide cascade represents a novel signaling pathway that contributes at least in part to Aβ cytotoxicity to various types of brain cells. Identification of this pathway may lead to the development of more effective therapeutic strategies aimed at preventing Aβ-induced cell death. For instance, blockade of the Aβ-activated death signaling process can be achieved by pharmacological modulation of nSMase activity as demonstrated in this work.

Materials and methods

Reagents and cell culture

All chemicals were purchased from Sigma-Aldrich, and all cell culture reagents were purchased from Invitrogen unless otherwise specified. B104 cells (a gift from David Schubert, Salk Institute for Biological Studies, La Jolla, CA) were cultured in DME supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

OLG culture

Neurospheres were cultured using the methods of Zhang et al. (1999) with modifications. In brief, embryonic rat brains (E14–16 d) were dissected, homogenized gently in DME/Ham's F12, and centrifuged at 350 g for 10 min. The pellet was digested with 0.05% trypsin in 1.5 ml of 0.53 M EDTA for 30 min at 37°C, followed by the addition of 1.5 ml DME/Ham's F12 with 20% FBS, and filtered through 10-µm nylon mesh. The filtrate was centrifuged at 350 g for 5 min, and the pellet was washed twice with DME/Ham's F12. Dissociated cells were layered on a preequilibrated Percoll gradient (formed by centrifuging 50% Percoll and 50% DME/Ham's F12 at 23,500 g for 1 h at 4°C) and centrifuged at 3,500 g for 15 min. The fraction containing glial progenitors banding between myelin and blood cell layers was recovered and washed twice with DME/Ham’s F12 followed by another wash with neurosphere culture medium (DME/Ham’s F12/2%FBS, 10% supplement, 25 µg/ml insulin, 130 ng/ml progesterone, 20 ng/ml of basic FGF, and 20 ng/ml of EGF). The cell pellet was resuspended in 20 ml of neurosphere culture medium and seeded in 75-mm culture flasks. After 24 h, when neurospheres had formed, 5 ml of fresh medium was added to each culture every other day for 7 d, and then the neurosphere cultures were split (1:2). The neurospheres were dissociated gently 10 times with a syringe and a 25-gauge needle and centrifuged at 350 g. The resulting cell pellets were treated with 0.05% trypsin/0.53 M EDTA and centrifuged at 350 g for 10 min. The cells were resuspended in progenitor medium (69% DME/Ham’s F12/2%FBS containing N1 supplement, 10 µg/ml insulin, 20 nM progesterone, 30% conditioned medium from B104 cells, and 1% FBS) and plated on 100-mm culture dishes precoated with polyl-ornithine. For differentiated OLG cultures, progenitor cells were detached with trypsin/EDTA and cultured on poly-l-ornithine–coated plates or coverslips in mature OLG medium (DME/Ham’s F12; N1 supplement, 20 µg/ml biontin; 20 µg/ml triiodo-l-thyronine, T3, and 1% FBS).

Immunocytochemistry

Differentiated OLG cultures were fixed in 4% PFA, washed in PBS, and blocked with 5% goat serum. Fixed cells were incubated with the primary antibody overnight at 4°C. The following primary antibodies were used: anti-mouse CNP, anti-mouse Rip, anti-mouse PLP (Chemicon), and anti-mouse GaLC (Cedarlane Laboratories) at a concentration of 1:100. The secondary antibody, anti-mouse IgG conjugated to FITC (Vector Laboratories), was added for 1 h at RT. The cells were washed in PBS and visualized using a confocal microscope (model LSM 5 Pascal; Carl Zeiss Microimaging, Inc.) equipped with a CCD camera (model AxioCam HR; Carl Zeiss Microimaging, Inc.). Images were collected and processed using Adobe Photoshop software.

Sphingomyelinase assay

The cells were washed twice with PBS, pH 7.4, and lysed in 0.2% Triton X-100 for 10 min at 4°C. The lysates were sonicated for 30 s in ice-cold bath, and protein concentrations were determined by Lowry assay (Lowry et al., 1951). A phosphorimine/luciferin–luciferase assay [methyl-3H]sphingomyelin (0.23 nmol) in nSMase or aSMase assay buffer was incubated for 2 h at 37°C. The reaction was terminated with 200 µl CHCl3/methanol (1:1) and 90 µl H2O followed by vigorous agitation. The samples were centrifuged at 6,000 g for 5 min. [3H]Phosphocholine in the aqueous phase (120 µl) was collected for liquid scintillation counting. Phosphocholine is the degraded moiety of sphingomyelin after ceramide is released by nSMase or aSMase. The aSMase or nSMase activity was calculated as picomoles of sphingomyelin hydrolyzed by 1 mg of total proteins per hour and expressed as a percentage of control values.

TLC

OLGs with or without Aβ 25-35 treatment were cultured with 10 µCi [3H]palmitate (1 mCi/ml; Amersham Biosciences; Kaneko et al., 1995). The labeled cells were collected and washed twice with PBS, pH 7.4, to remove free isotope before lipid extraction (Xu et al., 1998). The cell pellet was resuspended in 400 µl methanol/1 N HCl (100:6, vol/vol) followed by 800 µl chloroform and 240 µl H2O. The sample was mixed and centrifuged at 6,000 g for 5 min. The lipid fraction was reextracted with 1 ml chloroform/methanol (2:1, vol/vol) and applied to a TLC plate. The solvent was chloroform/methanol/acetic acid/H2O (85:4.5:5:0.5, vol/vol) for ceramide and chloroform/methanol/acetic acid/water (65:23.8:8.4:5 vol/vol) for sphingomyelin. Plates were air dried and sprayed with 1 M sodium selenocytate for autoradiography. Standard lipids were stained by rhodamine 6G (Sigma-Aldrich) and visualized by UV light.

Quantitative ceramide analysis by ESI/MS

After three washes with PBS, pH 7.4, OLGs harvested from 100-mm culture plates with or without Aβ 25-35 treatment were homogenized in 0.5 ml PBS, pH 7.4, with a glass tissue grinder. A bicinchoninic protein assay kit was used to determine the protein concentration before lipid extraction (Pierce Chemical Co.). Lipids from the homogenates were extracted as described previously with modifications (Bligh and Dyer, 1959) using 50 mM LiOH in the aqueous layer and C17:0 ceramide (2 nmol/mg protein) as an internal standard for quantitation of ceramide content. These molecular species represent <1% of the endogenous cellular lipid mass. The lipid extracts were dried under nitrogen stream, dissolved in chloroform, desalted with Sep-Pak columns, and filtered with 0.2 µm PTFE syringe filters (Fisher Scientific). Lipids were reextracted with 20 mM LiOH in the aqueous layer, dried under a nitrogen stream, and resuspended in 0.5 ml of chloroform/methanol (1:1, vol/vol) for ESI/MS analysis. ESI/MS analysis was performed using a spectrometer (model TSPQ-7000; Finnigan) equipped with an electrospray ion source as described previously (Han et al., 1996, 2001). A 5-min period of signal averaging in the profile mode was used for each spectrum of a lipid extract. All extracts were infused into the ESI emitter using a syringe pump at 1 µl/min flow rate. Ceramide in the lipid extracts was quantitated directly as deprotonated ions (M+H)− in comparison with an internal standard (C17:0 ceramide) after correction for 13C isotopic effects in the negative-ion mode. Ion peaks were identified using tandem mass spectroscopic analyses as described previously (Han and Gross, 1995).

Cell death assays

OLG viability was quantitated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and trypan blue exclusion method. Cell death was also assessed by the amount of lactate dehydrogenase (LDH) release into the culture medium after Aβ or C2-ceramide treatment (Koh and Choi, 1987; Shaikh et al., 1997; Xu et al., 1998). The amount of LDH released by cells killed with Triton X-100 was considered maximal cell death or “full kill” (Xu et al., 1998).
Assay for cellular GSH content

Cellular GSH levels were determined using a GSH-400 colorimetric assay kit (Calbiochem-Novabiochem). Triplicate samples (3 x 10^6 cells) were collected by centrifugation and washed twice with PBS, pH 7.4. The cell pellets were treated with 5% metaphosphoric acid (Sigma-Aldrich). A Teflon pestle was used to homogenize the cells. Protein concentrations were determined by Lowry assay (Lowry et al., 1951). The homogenates were centrifuged at 3,000 g for 10 min at 4°C. Supernatants were assayed for GSH according to the instructions provided with the kit. A standard curve was generated with graded concentrations of GSH (5–40 μM). GSH concentration was measured by absorbance at 400 nm with a spectrophotometer.

nSmase antisense oligonucleotides

Morpholino sense (GCCCGCAAGAAAGTGTGGCTCAT) and antisense (CCTTTCACCTGCTACATTTATA) oligonucleotides were generated for nSmase (Gene Tools, LLC). OLGs in serum-free medium were treated with 1.4 μM oligonucleotides in EPEI delivery solution (per manufacturer’s instructions; Gene Tools) for 4 h. The medium was exchanged and Aβ was added for 24 h, after which cells were harvested for nSmase activity or cell death determination.

Statistical analysis

Results are expressed as mean ± SD. Differences among groups were analyzed by one-way ANOVA followed by Bonferroni’s post-hoc t test to determine statistical significance. Comparison between two experimental groups was based on two-tailed t test. P < 0.05 was considered statistically significant.

We would like to thank Dr. Yannan Ouyang for his technical expertise in cortical microscopy.

This work was supported by National Institutes of Health grants NS37230 and NS40525.

Submitted: 2 July 2003
Accepted: 19 November 2003

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