Neurobiology of Disease

Fibrillar Amyloid-β-Activated Human Astroglia Kill Primary Human Neurons via Neutral Sphingomyelinase: Implications for Alzheimer's Disease

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Glial activation plays an important role in the pathogenesis of various neurodegenerative disorders including Alzheimer's disease. However, molecular mechanisms by which activated glia could kill neurons are poorly understood. The present study underlines the importance of neutral sphingomyelinase (N-SMase) in mediating the damaging effect of fibrillar amyloid-β 1-42 ($A\beta_{1-42}$) peptide-activated astroglia on neurons. In transwell experiments, soluble products released from activated primary human astroglia induced the activation of neutral sphingomyelinase (N-SMase), production of ceramide, and cell death in primary human neurons. Protection of neurons from cytotoxic effects of activated astroglia by antisense knockdown of N-SMase, but not acidic sphingomyelinase (A-SMase), suggests that soluble products released from activated astroglia kill neurons via N-SMase but not A-SMase. Next we examined the role of N-SMase in the activation of human astroglia. Interestingly, knockdown of N-SMase, but not A-SMase, by either antisense oligonucleotides or chemical inhibitor, prevented the induction of proinflammatory molecules [tumor necrosis factor-α, inducible nitric oxide synthase, interleukin-1β (IL-1β), and IL-6] and the activation of nuclear factor-κB in $A\beta_{1-42}$-activated astroglia. Subsequently, fibrillar Aβ peptides also induced the activation of N-SMase and ceramide *in vivo* in mouse cortex. Most importantly, antisense knockdown of N-SMase, but not A-SMase, decreased the activation of astroglia and protected neurons from fibrillar Aβ toxicity *in vivo* in the cortex. Together, it is apparent that both the activation of astroglia by Aβ and that the cytotoxicity of activated astroglia on neurons depend on N-SMase.

Introduction

Alzheimer's disease (AD) (Martin, 1999) is a neurodegenerative disorder resulting in progressive neuronal death and memory loss. Neuropathologically, the disease is characterized by neurofibrillary tangles and neuritic plaques. Neurofibrillary tangles are composed of the hyperphosphorylated tau protein (Braak et al., 1994). On the other hand, neuritic plaques are mainly composed of aggregates of amyloid-β (Aβ) protein, a 40–43 amino acid proteolytic fragment derived from the amyloid precursor protein that is overexpressed in AD (Martin, 1999). Histopathologically, this disease is characterized by infiltration of inflammatory cells into the CNS, gliosis, and neuronal apoptosis (Stalder et al., 2005; Simard et al., 2006; Town et al., 2008).

Although deposition of Aβ peptides is one of the primary causes of neuronal loss in AD (Hsiao et al., 1996), mechanisms by which Aβ causes neuronal loss are mostly unknown. Several lines of evidence suggest that neurons are killed through neurotoxic molecules elaborated from glial activation. Consequently, activated astrocytes and microglia are characteristically found in abundance near amyloid plaques in AD and in mouse models of AD, and amyloid peptides have been reported as glial activators in the presence of an immunological cofactor (Meda et al., 1995; Tan et al., 1999, 2002). Activated glia are also found to increase substantially in the hippocampus and neocortex of the aging brain (Nichols, 1999). Consistently, microinjection of pre-aggregated $A\beta_{1-42}$ peptides into the nucleus basalis of the rat produces a congophilic deposit followed by microglial and astrocyte activation and a strong inflammatory reaction surrounding the dystrophic neuritis (Giovannini et al., 2002; Jantaratnotai et al., 2003).

Therefore, controlling glial activation and, hence, inflammation is of great therapeutic interest in mitigating neuronal cell death in different neurological disorders including AD. Because ceramide, the lipid second messenger molecule, produced from the degradation of sphingomyelin by sphingomyelinases (neutral and acidic) induces apoptosis and cell death in various cell types, including glial and neuronal cells (Brugg et al., 1996; Hannun, 1996; Wiesner and Dawson, 1996; Keane et al., 1997; Singh et al., 1998; Pettus et al., 2002), we decided to investigate the neutral sphingomyelinase (N-SMase)–ceramide pathway in Aβ-activated glia-mediated neuronal death. Here, we present the evidence that Aβ plus interleukin-1β (Aβ+IL-1β)-activated astrocytes induce the activation of sphingomyelinases and the production of ceramide in human primary neurons. We also show that the activation of neutral sphingomyelinase, but not acidic
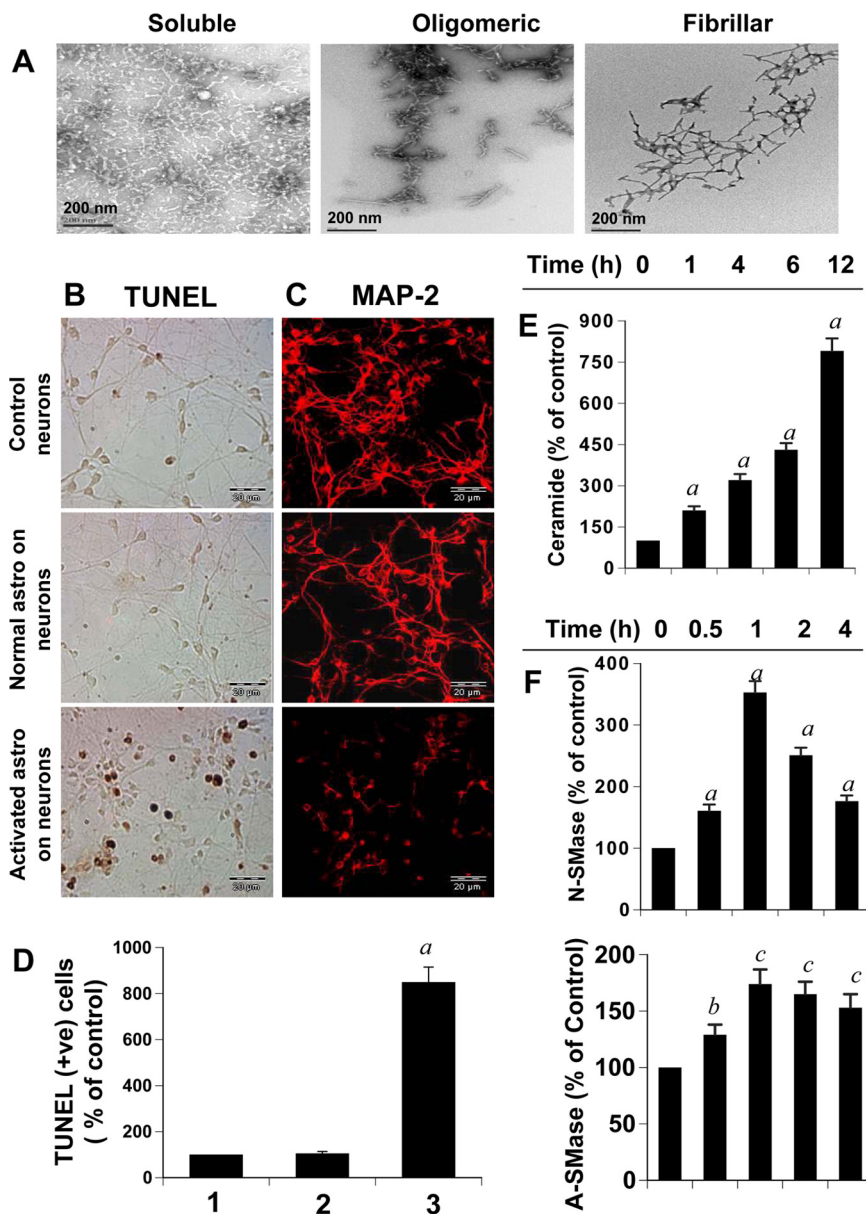
Figure 1. Activated primary human astrocytes induce apoptosis and the activation of the N-SMase– ceramide pathway in primary human neurons in neuron–astrocyte transwell cultures. **A**, Morphology of oligomeric and fibrillar form of Aβ_{1-42} peptides was examined by transmission electron microscopy. **B**, Primary human astrocytes seeded in inserts were stimulated with a combination of 1 μM fibrillar Aβ and 10 ng/ml IL-1β in serum-free media. After 24 h, media were removed and inserts with activated astrocytes were placed on coverslips containing primary neurons for 6 h followed by TUNEL. **C**, After 18 h of treatment of neurons with activated astrocytes, cells were immunostained with MAP-2. **D**, TUNEL-positive cells were counted manually in four different images of each of three coverslips by three individuals blinded to the experiment. Values obtained from the control group (1) served as 100%, and data obtained from other two groups, i.e., the normal astrocyte–neuron group (2) and the activated astrocytes–neuron group (3) were calculated as a percentage of control accordingly. Results are the mean ± SD of three different experiments. $^a p < 0.001$ versus control. Activated astrocytes were placed on human neurons and further incubated for different time intervals. **E**, Lipids were extracted from neurons at respective time points, and ceramide levels were determined. $^a p < 0.001$ versus 0 h. **F**, Activities of N-SMase and A-SMase were assayed in total cell extract of neurons. Control group served as 100%, and data from other groups were expressed as a percentage of control. Results are the mean ± SD of three different experiments. $^a p < 0.001$ versus 0 h; $^b p < 0.05$ versus 0 h; $^c p < 0.001$ versus 0 h.

sphingomyelinase (A-SMase), plays a key role in neuronal apoptosis in response to neurotoxins released from activated astrocytes. Furthermore, blocking of astroglial activation by N-SMase inhibition prevented the release of neurotoxic substances. Subsequently, inhibition of astroglial N-SMase suppressed nuclear factor-κB (NF-κB) activation in activated astroglia. The *in vitro* data were further substantiated by *in vivo* findings where we found that antisense oligo-

nucleotides (ASOs) against N-SMase prevented astrogliosis and microgliosis and protected neurons in the cortex of Aβ-injected C57BL/6 mice.

Materials and Methods
Reagents
Neurobasal medium and B27 supplement were purchased from Invitrogen. Fetal bovine serum and DMEM/F-12 were obtained from Mediatech. Human Aβ peptides (1-42) and (42-1) were obtained from Bachem Bioscience. Glial fibrillary acidic protein (GFAP) was purchased from Santa Cruz Biotechnology. Phosphorothioate-labeled antisense and scrambled oligodeoxynucleotides were synthesized in the DNA-synthesizing facility of Invitrogen.

Isolation of primary human neurons
Human primary neurons were prepared as described previously (Jana and Pahan, 2004a,b; Jana et al., 2007). All of the experimental protocols were reviewed and approved by the Institutional Review Board of the Rush University Medical Center. Briefly, 11- to 17-week-old fetal brains obtained from the Human Embryology Laboratory (University of Washington, Seattle, WA) were dissociated by trituration and trypsinization (0.25% trypsin in PBS at 37°C for 15 min). The trypsin was inactivated with 10% heat-inactivated fetal bovine serum (Mediatech). The dissociated cells were filtered through 380 and 140 μm meshes (Sigma) and pelleted by centrifugation. The cell pellet was washed once with PBS and once with Neurobasal medium containing 2% B27 and 1% antibiotic-antimycotic mixture (Sigma). In the first step, neurons were enriched by allowing the cells (3 × 10^6/ml) to adhere to poly-D-lysine-coated plates or coverslips for 5 min. Nonadherent cells were removed, and adherent cells (mostly neurons) were further treated with 10 μM arabinosylcytosine to prevent the proliferation of dividing cells. After 10 d of Ara-C treatment, the cells were used for this study. More than 98% of this preparation was positive for microtubule-associated protein-2 (MAP-2), a marker for neurons.

Preparation of primary human astrocytes
Primary human astrocytes were prepared as described previously (Jana et al., 2005, 2007; Saha et al., 2007). Briefly, human CNS tissue (Human Embryology Laboratory, University of Washington, Seattle, WA) from each specimen was processed separately and independently, as were subsequent cell cultures. There was no pooling of CNS tissue from distinct specimens. All of the experimental protocols were reviewed and approved by the Institutional Review Board of the Rush University Medical Center. By immunofluorescence assay, these cultures homogeneously expressed GFAP (Liu et al., 2002).

Astrocyte-neuron transwell study
Primary human astrocytes were grown to confluency on inserts. After 24 h of stimulation by the combination of Aβ (1 μM) and IL-1β (10

ng/ml), inserts were washed thrice with HBSS and then placed onto the wells containing primary human neurons. Therefore, in this transwell model, although neurons and astrocytes face each other, they are separable, and the effect of soluble factors released from activated astrocytes on neurons can be studied, allowing analysis of neuronal and glial populations separately.

Preparation of fibrillar Aβ

Fibrillar $A\beta_{1-42}$ and control reverse peptide $A\beta_{42-1}$ (Bachem Bioscience) were prepared by incubating freshly solubilized peptides at 50 μM in sterile distilled water at 37°C for 5 d (Pike et al., 1993). On the other hand, oligomeric $A\beta_{1-42}$ was prepared after incubation at 37°C for 2 d (for morphology of oligomeric and fibrillar forms of $A\beta_{1-42}$, see Fig. 1A).
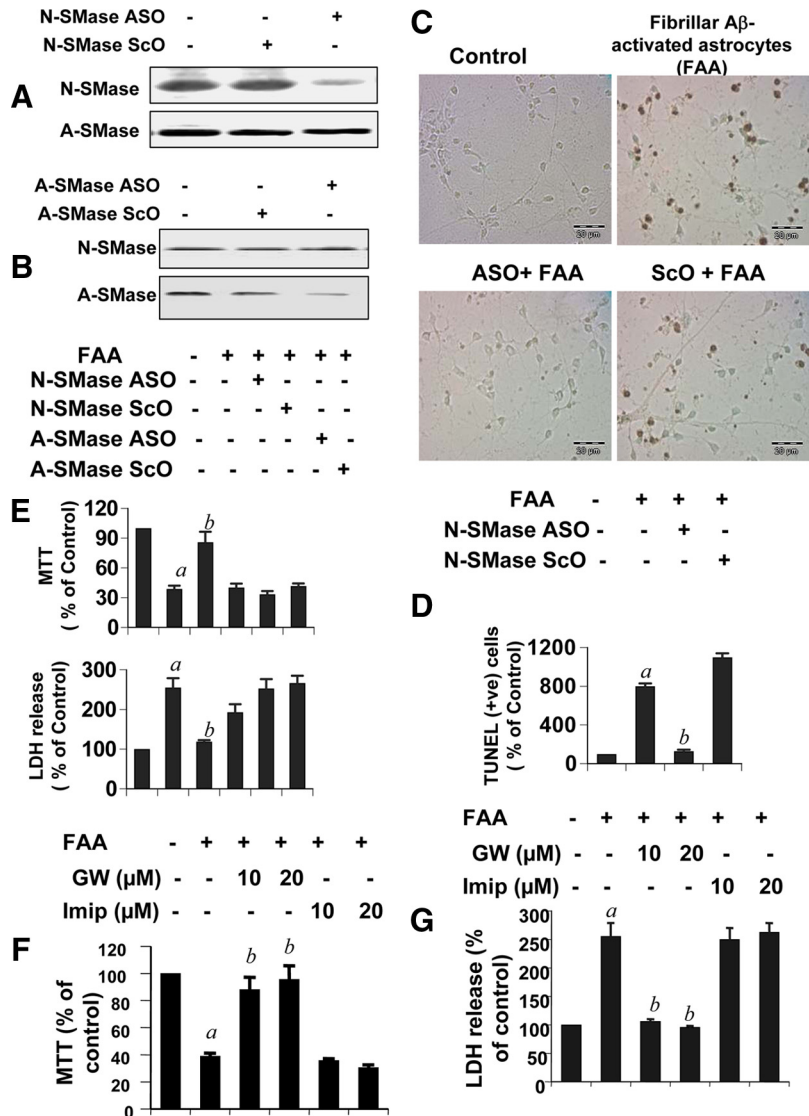
Treatment of primary neurons

During treatment with fibrillar Aβ peptides, cells were incubated in Neurobasal medium containing 2% B27 supplement without antioxidant (Invitrogen).

Assay of neutral and acidic sphingomyelinases

Activities of SMase(s) were assayed as described previously by Jana and Pahan (2004a,b, 2007). Briefly, after treatment, the cells were washed with PBS, harvested in PBS, divided into two halves, and centrifuged. The fraction for N-SMase was resuspended in buffer A (100 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 1 mM EDTA, and protease inhibitors), and the cell suspension was sonicated and centrifuged at 500 × g at 4°C for 5 min. The supernatant was used as the enzyme source for N-SMase. The reaction mixture contained enzyme preparation in buffer A containing 5 nmol of [^{14}C] sphingomyelin, 5 nmol of phosphatidylserine, 5 mM dithiothreitol, and 5 mM MgCl$_2$ in a final volume of 100 μl. Similarly, the fraction for A-SMase was resuspended in buffer B (100 mM sodium acetate, pH 5.0, 0.1% Triton X-100, and protease inhibitors). The cell suspension was sonicated and centrifuged. The supernatant was used as the source of A-SMase. The activity of A-SMase was measured in 100 μl of reaction mixture consisting of the enzyme preparation in buffer B and 5 nmol of [^{14}C] sphingomyelin. The enzyme reaction was initiated by the addition of 50 μl of substrate and stopped by the addition of 1.5 ml of chloroform: methanol (2:1, v/v) and 0.2 ml of water. After vortexing and phase separation, the aqueous phase was removed for counting.

Quantification of ceramide levels by diacylglycerol kinase assay

After treatment, lipids were extracted from cells as described previously (Pahan et al., 1998, 2000a). Ceramide content was quantified using diacylglycerol (DAG) kinase and [γ- ^{32}P] ATP as described previously (Pahan et al., 1998, 2000). Briefly, dried lipids were solubilized in 20 μl of an octyl β-D-glucoside/cardiolipin solution (7.5% octyl β-D-glucoside and 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid) by sonication in a sonicator bath. The reaction was then performed in a final volume of 100 μl containing the 20 μl of sample solution, 50 mM imidazole HCl, pH

6.6, 50 mM NaCl, 12.5 mM MgCl$_2$, 1 mM EGTA, 2 mM dithiothreitol, 6.6 μg of DAG kinase, and 1 mM [γ- ^{32}P] ATP (specific activity of 1–5 × 10^5 cpm/nmol) for 30 min at room temperature. The labeled ceramide-1-phosphate was resolved with a solvent system consisting of methyl acetate: n-propyl alcohol/chloroform/methanol/0.25% KCl in water/acetic acid (100:100:100:40:36:2). A standard sample of ceramide was phosphorylated under identical conditions and developed in parallel. Both standard and experimental samples had an identical R_F value (0.46). Quantification of ceramide-1-phosphate was performed by autoradiography and densitometric scanning using a Fluor Chem 8800 imaging system (Alpha Innotech). Values are expressed either as arbitrary units (absorbance) or as a percentage of control, considering

Figure 2. Antisense or chemical knockdown of N-SMase, but not A-SMase, protects neurons from Aβ-activated primary human astrocytes in astrocyte–neuron transwell cultures. **A**, **B**, Primary human neurons were incubated with ASOs and ScOs against N-SMase (**A**) and A-SMase (**B**). After 48 h, neurons were analyzed for protein expression of N-SMase and A-SMase. **C**, Primary human astrocytes seeded in inserts were stimulated by the combination of Aβ and IL-1β as described above. After 24 h, media were removed, and inserts were washed and placed on neurons that were already pretreated for 40 h with 1 μM ASO or ScO against N-SMase and A-SMase. After 6 h of treatment with activated astrocytes, apoptotic events in neurons were detected by TUNEL. **D**, TUNEL-positive cells were counted manually in four different images of each of three coverslips by three individuals blinded to the experiment. **E**, After 18 h of stimulation, cell viability was examined by the metabolism of MTT and the release of LDH. Activated astrocytes were placed on neurons that were pretreated with different concentrations of GW4869 (GW) and imipramine. **F**, **G**, Cell viability was checked by MTT (**F**) and LDH (**G**). Values obtained from the control group served as 100%, and data obtained in other groups were calculated as a percentage of control accordingly. Results are the mean ± SD of three different experiments. $^a p <$ 0.001 versus control; $^b p <$ 0.001 versus FAA. FAA, Fibrillar Aβ+IL-1β-activated astrocytes.

control as 100%. Statistical comparisons were made using one-way ANOVA followed by Student's *t* test.

Assay for NO synthesis

Synthesis of NO was determined by assay of culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen, using Griess reagent as described previously (Jana et al., 2001; Liu et al., 2002).

Immunostaining

Coverslips containing 200–300 cells/mm^2 were fixed with 4% paraformaldehyde for 20 min followed by treatment with cold ethanol ($-20°C$) for 5 min and 2 rinses in PBS. The samples were blocked with 3% bovine serum albumin in PBS containing Tween 20 (PBST) for 30 min and incubated in PBST containing 1% bovine serum albumin and goat anti-MAP-2 (1:50), as described previously (Dasgupta et al., 2003b, 2004). After three washes in PBST (15 min each), the slides were further incubated with Cy5 (Jackson ImmunoResearch Laboratories). For negative controls, a set of culture slides were incubated under similar conditions without the primary antibodies. The samples were mounted and observed under a Bio-Rad MRC1024ES confocal laser-scanning microscope.

GFAP and NeuN immunostaining

Two days after microinjection, mice were perfused with 4% paraformaldehyde and their brains were processed for immunohistochemical studies. For double labeling of GFAP with inducible nitric oxide synthase (iNOS), sections (6 μm) were incubated with polyclonal anti-GFAP (GFAP, 1000 dilution; Calbiochem) and anti-iNOS (iNOS, 1000 dilution; Calbiochem) for 24 h at 4°C. After three washes in PBST (15 min each), slides were further incubated with Cy5 and Cy2 (Jackson ImmunoResearch Laboratories) secondary antibodies for 2 h at room temperature. For negative controls, a set of tissue sections was incubated under similar conditions without the primary antibodies. The samples were mounted and observed under a Bio-Rad MRC1024ES confocal laser-scanning microscope.

Fragment end labeling of DNA

Fragmented DNA was detected *in situ* by the terminal deoxynucleotidyl transferase (TdT)-mediated binding of 3′-OH ends of DNA fragments generated in response to fibrillar Aβ_{1-42}, using a commercially available kit (TdT FragEL) from Calbiochem. Briefly, coverslips were treated with 20 μg/ml proteinase K for 15 min at room temperature and washed before terminal deoxynucleotidyl transferase staining.

Cell viability measurement

MTT assay. Mitochondrial activity was measured with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Sigma). The cells were grown on 24-well culture plates with 500 μl of medium and treated with various reagents according to the experimental design. At the end of the treatment period, 300 μl of culture medium were removed from each well, and 20 μl of MTT solution (5 mg/ml) were added and incubated for 1 h.

Lactate dehydrogenase measurement. The activity of lactate dehydrogenase (LDH) was measured using the direct spectrophotometric assay using an assay kit from Sigma.

Microinjection of Aβ into the frontal cortex of C57BL/6 mice. C57BL/6 mice (10–12 weeks old) were anesthetized with ketamine and xylazine and underwent cortical operations in a Kopf small animal stereotaxic instrument. Briefly, the animal was mounted in a stereotaxic frame on a heating blanket. Body temperature was maintained at 37 $\pm$ 0.5°C during the time of surgery. A midsagittal incision was made to expose the cranium and a hole <0.5 mm in diameter was drilled with a dental drill over the frontal cortex according to the following coordinates: 1.5 mm anterior to bregma, 1.5 mm lateral to bregma, and 1 mm ventral (see Fig. 9A). One microgram of either Aβ_{1-42} or Aβ_{42-1} in 2 μl of saline was injected using a 5 μl syringe (Hamilton) over a period of 3 min, and the needle was held in place for another 2 min before withdrawing it from the skull to prevent reflux up the needle tract. Similarly, control mice received 2 μl of saline. During antisense treatment, 1 μg of either ASO or scrambled oligonucleotide (ScO) against
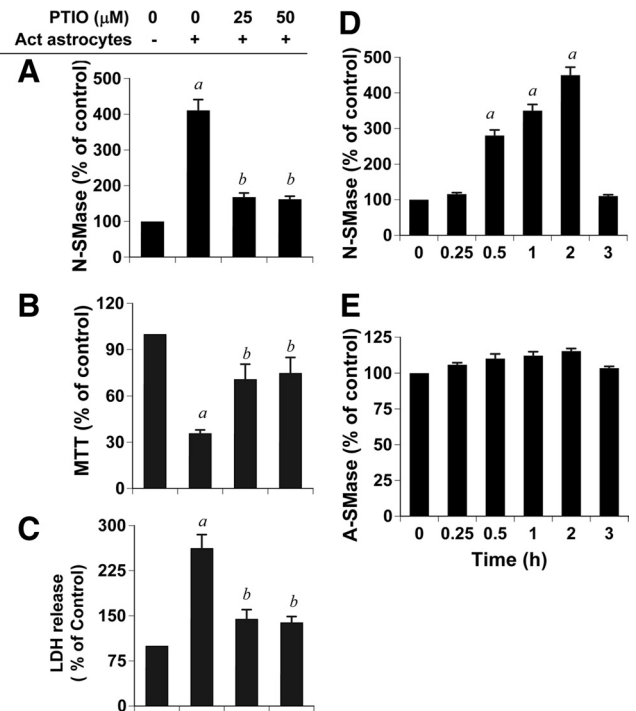
Figure 3. Role of nitric oxide in activated astrocyte-induced cell death in neurons. Primary human astrocytes seeded in inserts were stimulated by the combination of Aβ and IL-1β as described above. After 24 h, media were removed and inserts were washed and placed on neurons that were pretreated with PTIO for 30 min. *A*, After 1 h, activity of N-SMase was measured in total cell extracts of neurons as described above. *B*, *C*, After 18 h, neuronal viability was examined by the metabolism of MTT (*B*) and the release of LDH (*C*). Values obtained from the control group served as 100%, and data obtained in other groups were calculated as a percentage of control accordingly. Results are the mean $\pm$ SD of three different experiments. $^a p < 0.001$ versus control; $^b p < 0.001$ versus activated astrocytes. *D*, *E*, Primary human neurons were treated with 25 μM DETA-NONOate (an NO donor), and at different time points of treatment, activities of N-SMase (*D*) and A-SMase (*E*) were monitored. $^a p < 0.001$ versus control (0 h).

N-SMase dissolved in 2 μl saline was stereotaxically injected into the frontal cortex of C57BL/6 mice. After 24 h of ASO/ScO microinjection, 1 μg of fibrillar Aβ_{1-42} in 2 μl volume was again microinjected at the same site as described above. The incision was closed with surgical staples and covered with a mixture of Bacitracin and Hurricane (20% benzocaine).

Semiquantitative reverse transcriptase-coupled PCR. Total RNA was isolated from human primary astrocytes using RNA-Easy Qiagen kit following the manufacturer's protocol. To remove any contaminating genomic DNA, total RNA was digested with DNase. Semiquantitative reverse transcriptase-coupled (RT)-PCR was performed as described previously (Jana and Pahan, 2005) using oligo (dT)$_{12-18}$ as primer and moloney murine leukemia virus reverse transcriptase (Clontech) in a 20 μl reaction mixture. The resulting cDNA was appropriately diluted, and diluted cDNA was amplified using Titanium Taq polymerase and the following primers for human proinflammatory genes: tumor necrosis factor-α (TNF-α; 378 bp), sense, 5′-CTG AGT CGG TCA CCC TTC TCC AGC T-3′; antisense, 5′-CCC GAG TGA CAA GCC TGT AGC CCA T-3′; IL-1β (263 bp), sense, 5′-GGA TAT GGA GCA AC A AGT GG-3′; antisense, 5′-ATG TAC CAG TTG GGG AAC T-3′; IL-6 (236 bp), sense, 5′-TTT TGG AGT TTG AGG TAT ACC TAG-3′; antisense, 5′-GCT GCG CAG AAT GAG ATG AGT TGT-3′; iNOS (368 bp), sense, 5′-CTG CAG ACA CGT GCG TTA CTC CAC C-3′; antisense, 5′-GCA GGG CGT ACC ACT TTA GCT CCA G-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 300 bp), sense, 5′-GGT GAA GGT CGG AGT CAA CG-3′; antisense, 5′-GTG AAG ACG CCA GTG GAC TC-3′.

Amplified products were electrophoresed on a 1.8% agarose gels and visualized by ethidium bromide staining. Message for the GAPDH gene

was used to ascertain that an equivalent amount of cDNA was synthesized from different samples.

Quantitative real-time PCR analysis. Quantitative real-time PCR analysis was performed using the ABI-Prism7700 sequence detection system (Applied Biosystems) using TaqMan Universal Master Mix and optimized concentrations of FAM-labeled probe, forward and reverse primers (Applied Biosystems) as described previously (Jana and Pahan, 2005). The mRNA expression of iNOS was normalized to the label of GAPDH mRNA. Data were processed by the ABI Sequence Detection System 1.6 software and analyzed by ANOVA.

Electrophoretic mobility shift assay. Nuclear extracts were prepared, and electrophoretic mobility shift assay (EMSA) was performed as described previously (Pahan et al., 2001; Dasgupta et al., 2003a; Auch et al., 2004) with some modifications. Briefly, IRDye infrared dye end-labeled oligonucleotides containing the consensus binding sequence for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) were purchased from Licor Biosciences. Six micrograms of nuclear extract was incubated with binding buffer and with infrared-labeled probe for 20 min. Subsequently, samples were separated on a 6% polyacrylamide gel in 0.25× TBE buffer (Tris borate-EDTA) and analyzed by the Odyssey Infrared Imaging System (LI-COR Biosciences).

Transmission electron microscopy sample preparation. Aliquots of sample (5 μl) were pipetted on to the surface of carbon-coated electron microscope grids and adsorbed for 2 min at room temperature. After rinsing with 20 μl of sterile deionized water, 5 μl of 1% (w/v) uranyl acetate was added for 15–20 s. Grids were then blotted dry and examined under a JEOL JEM-1220 transmission microscope.

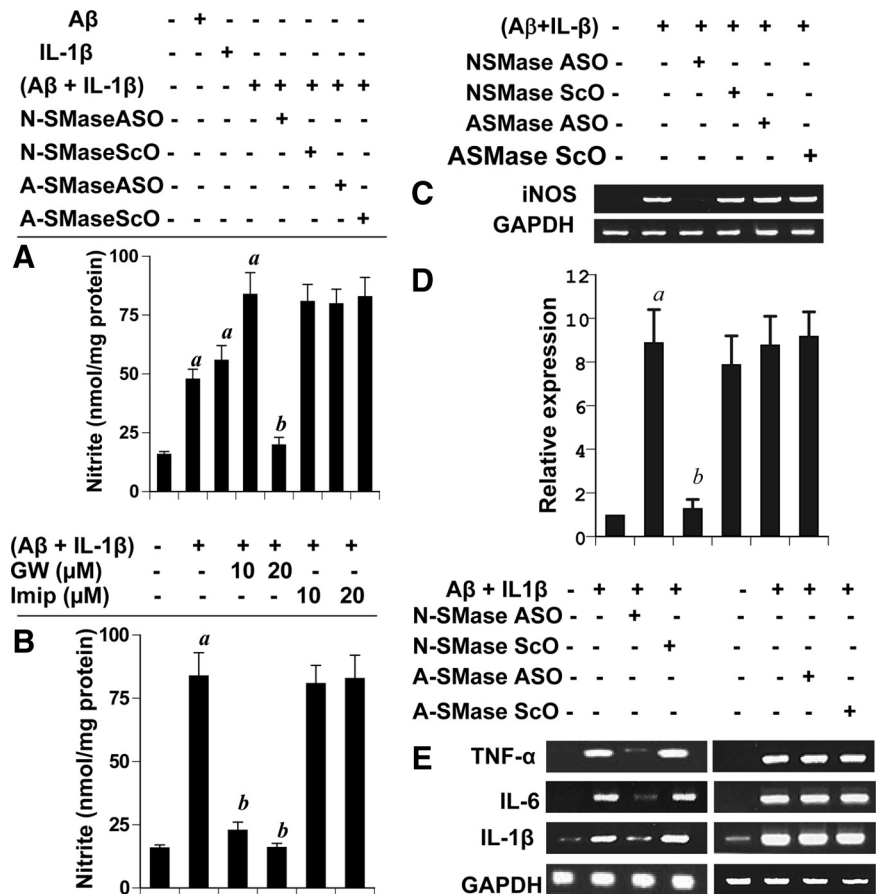*Statistical analysis.* All values are expressed as the mean ± SD of three independent experiments. Statistical differences between means were calculated by Student's *t* test. A *p* value of <0.05 (*p* < 0.05) was considered statistically significant.

Figure 4. Antisense and chemical knockdown of N-SMase, but not A-SMase, blocks the expression of iNOS and proinflammatory cytokines in Aβ+IL-1β-activated primary human astrocytes. Cells pretreated with ASO and ScO against N-SMase and A-SMase for 40 h were stimulated with Aβ+IL-1β under the serum-free condition. *A*, After 24 h of stimulation, the concentration of nitrite was measured in supernatants by Griess reagent. *B*, Cells preincubated with different concentrations of GW4869 and imipramine for 30 min were stimulated as above and nitrite level was measured after 24 h of stimulation. Cells were treated with ASOs/ScOs for 40 h followed by stimulation with the combination of fibrillar Aβ and IL-1β as above. *C*, *D*, After 6 h, the expression of iNOS mRNA was analyzed by both semiquantitative RT-PCR (*C*) and quantitative real-time PCR (*D*). *E*, The expression of TNF-α, IL-6, and IL-1β mRNAs were analyzed by semiquantitative RT-PCR. Results are the mean ± SD of three different experiments. *$^a p < 0.001$ versus control; $^b p < 0.001$ versus Aβ+IL-1β.*

Results

Activated primary human astroglia induce apoptosis and cell death in primary human neurons

Recently, glial activation has been attributed to be one of the major causes of neuronal loss in several neurodegenerative disorders, including AD. However, mechanisms by which activated glia facilitate neuronal loss are poorly understood. Because fibrillar Aβ$_{1-42}$ peptide is an important pathogenic molecule for AD and astrocytes are major glial cells in the CNS, we examined the effect of Aβ$_{1-42}$-activated primary human astrocytes on the survival of primary human neurons. To potentiate the neurotoxic effect of Aβ peptides and to simulate the *in vivo* situation, astrocytes were stimulated with a combination of fibrillar Aβ$_{1-42}$ and IL-1β. Furthermore, reports from our lab and elsewhere have reported a critical role of IL-1β in the expression of iNOS and the production of NO in primary human astrocytes either alone or in combination with other neurotoxins (Hua et al., 2002; Jana et al., 2005).

Primary human astrocytes were stimulated with 1 μM fibrillar Aβ, 10 ng/ml IL-1β, or a combination of the two for 24 h under

serum-free conditions followed by the addition of activated astrocytes on neurons in a transwell setting, where activated astroglia present on inserts were not in direct contact with neurons cultured on 12-well plates. Under this experimental setting, cytotoxic mediators released from activated astrocytes were able to reach human neurons. After 6 h of challenge of neurons by activated astrocytes, apoptosis and cell death were analyzed in neurons by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL). TdT-mediated labeling of DNA fragments was not observed in control unchallenged neurons (Fig. 1*A*, top, *C*). Unstimulated human astrocytes were also unable to induce any apoptosis in human neurons (Fig. 1*A*, middle, *C*). Although either fibrillar Aβ- or IL-1β-activated astrocytes induced some apoptosis in neurons, astrocytes stimulated by the combination of Aβ and IL-1β were much more potent than either Aβ- or IL-1β- stimulated astrocytes in inducing neuronal apoptosis (Fig. 1*B*, bottom, *D*; supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In another parallel set of experiments, the effect of unstimulated and Aβ+IL-1β-stimulated astrocytes on neuronal structure was examined after 18 h of challenge by MAP-2 immunofluorescence.

Control, unchallenged neurons stained MAP-2 uniformly and exhibited neuronal processes (Fig. 1C, top). Unstimulated astrocytes were not able to alter MAP-2 immunoreactivity and neuronal morphology (Fig. 1C, middle). On the other hand, we observed loss of neuronal processing and marked decrease in MAP-2 immunoreactivity after challenge by Aβ-stimulated astrocytes (Fig. 1C, bottom).

Activated primary human astroglia induce the activation of N-SMase and the production of ceramide in primary human neurons
Next, to gain insight into molecular mechanisms involved in activated astroglia-induced apoptosis and death of primary human neurons, we explored the role of ceramide, a well-known inducer of apoptosis. Neurons were challenged with Aβ+IL-1β-activated astrocytes, and the level of ceramide was measured in neurons at different time periods using the DAG kinase assay. Within 30 min of challenge, activated astroglia were able to induce the level of ceramide by approximately twofold, and with additional increase in duration of insult, the level of ceramide increased markedly (Fig. 1E). After 12 h of insult, an approximately eightfold increase in ceramide production was recorded in human neurons (Fig. 1E). Because DAG kinase phosphorylates both DAG and ceramide using $[\gamma\text{-}^{32}P]$ATP as a substrate, both lipids can be quantified in the same assay. Although the DAG content was much higher than the ceramide content, in contrast to a time-dependent increase in the production of ceramide, the level of DAG in insulted neurons remained almost same at different time points of astroglial insult (data not shown).

Next, to determine whether Aβ-stimulated astroglia induced the production of ceramide in neurons through the activation of SMases, we measured activities of N-SMase and A-SMase in neurons before and after challenge with activated astroglia. As shown in Figure 1F, a marked induction of N-SMase activity was observed in primary human neurons in response to activated astrocytes in a transwell setting. Significant increase in N-SMase activity in human neurons was detected as early as 30 min of challenge with activated astrocytes, and the maximum induction (∼3.5-fold) was observed at 1 h of challenge (Fig. 1F). In contrast, Aβ-activated astrocytes were able to increase the activity of A-SMase by ∼70% in neurons (Fig. 1F) under a similar experimental setting. These results suggest that Aβ-activated human astroglia increase the level of ceramide in human neurons through SMase-mediated catabolism of sphingomyelin.

Activated primary human astroglia induce apoptosis and cell death in primary human neurons via N-SMase
Next we examined the role of N-SMase and A-SMase in activated astroglia-mediated neuronal apoptosis. Because ASOs are powerful tools to inhibit target gene expression in nondividing cells in a sequence specific manner, we used ASOs to knock down N-SMase and A-SMase in primary human neurons. As reported in our previous studies (Jana and Pahan, 2004b, a), the following ASOs and ScOs were used to knock down these molecules: N-SMase, ASO, 5′-CAGCGAGCCCGTCCACCAGCC-3′; ScO, 5′-CACGCGTCCGACGCCGCACGA-3′; A-SMase, ASO, 5′-GACATCTCGGAGCCGGGGCA-3′; ScO, 5′-GGAAACCCG-GTTAGGCCCGG-3′.

Because these ASOs have become a very critical part of our study, we checked the efficacy of N-SMase and A-SMase ASOs in inhibiting the protein expression of N-SMase and A-SMase by Western blot. As evident from Figure 2A, ASOs against N-SMase
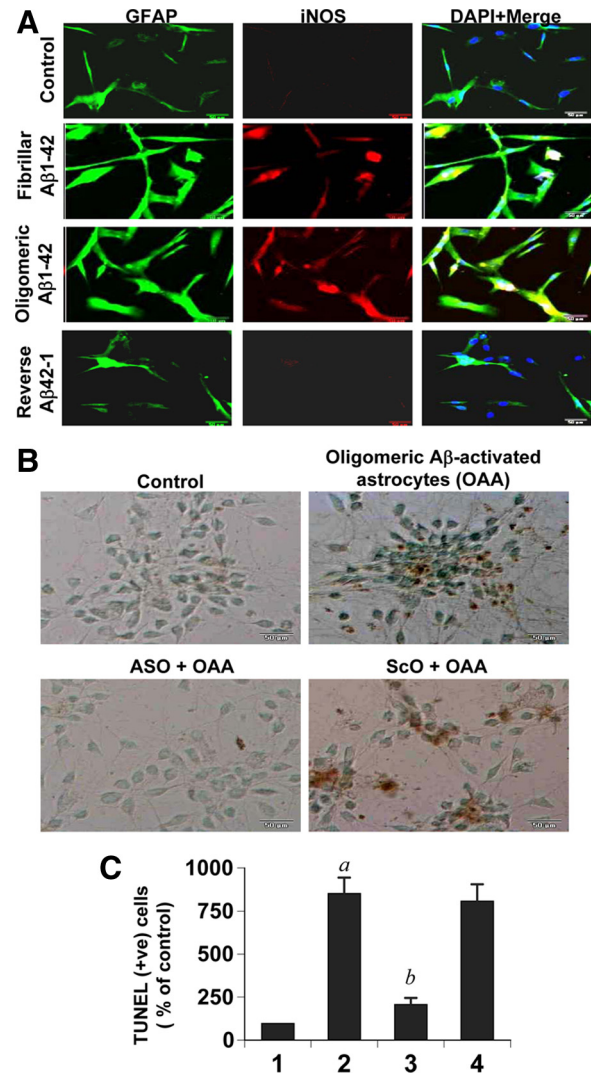
Figure 5. Oligomeric Aβ$_{1-42}$-activated astrocytes induce apoptosis in neurons via N-SMase in neuron-astrocyte transwell cultures. ***A***, Primary astrocytes were stimulated with fibrillar Aβ$_{1-42}$, oligomeric Aβ$_{1-42}$, and reverse peptide Aβ$_{42-1}$ for 24 h followed by double labeling of GFAP and iNOS. ***B***, Primary human astrocytes seeded in inserts were stimulated with a combination of 1 μM oligomeric Aβ$_{1-42}$ and 10 ng/ml IL-1β in serum-free media. After 24 h, media were removed and inserts with activated astrocytes were placed on coverslips containing primary neurons that were already pretreated for 40 h with 1 μM ASO or ScO against N-SMase. After 6 h, neuronal apoptosis was monitored by TUNEL. ***C***, TUNEL-positive cells were counted manually in four different images of each of three coverslips by three individuals blinded to the experiment. Values obtained from the control group (1) served as 100%, and data obtained from other three groups, i.e., the oligomeric Aβ-activated astrocytes (OAA) group (2), the ASO-OAA group (3), and the ScO-OAA group (4), were calculated as a percentage of control accordingly. Results are the mean ± SD of three different experiments. $^{a}p < 0.001$ versus control; $^{b}p < 0.001$ versus OAA.

effectively inhibited the level of N-SMase, but not A-SMase, protein. Similarly, ASOs against A-SMase suppressed the level of A-SMase, but not N-SMase, protein (Fig. 2B). On the other hand, ScOs had no effect on the protein level of either N-SMase or A-SMase (Fig. 2A,B), suggesting the specificity of the effect. Next, we investigated the effect of antisense knockdown of neuronal N-SMase and/or A-SMase on activated glia-induced neuronal apoptosis by TUNEL. As evident from Figure 2C, control neurons showed a very few apoptotic bodies after 6 h of incubation with unstimulated astrocytes in neuron–astrocyte transwell culture, but exhibited a marked increase in apoptosis in response

to activated astrocytes (Fig. 2*C,D*). However, ASOs, but not ScOs, against neuronal N-SMase markedly protected neurons from the insult of activated astrocytes (Fig. 2*C,D*). However, under similar experimental conditions, ASOs against A-SMase failed to rescue neurons (data not shown). To further strengthen these results, we also monitored neuronal death by MTT and LDH. As evidenced by a decrease in MTT metabolism, activated astroglia reduced neuronal viability (Fig. 2*E*). Consistent with TUNEL data, ASOs against N-SMase, but not A-SMase, effectively prevented activated astroglia-mediated loss of MTT metabolism in human neurons (Fig. 2*E*). Similarly, treatment of human neurons with ASOs against N-SMase, but not A-SMase, resulted in the significant reduction in LDH release from neurons (Fig. 2*E*) in response to insult from activated astrocytes. On the other hand, ScOs against N-SMase showed no effect on cell viability under similar experimental settings (Fig. 2*E*).

To confirm these results further, we used specific pharmacological inhibitors of N-SMase and A-SMase. Whereas GW4869 is a specific synthetic inhibitor of N-SMase (Luberto et al., 2002), imipramine has been shown to block the activity of A-SMase, but not N-SMase (Hauck et al., 2000). At first, we checked the specificity of these two inhibitors in our experimental setting. Activated human astrocytes induced the activation of both N-SMase and A-SMase in human neurons (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Whereas GW4869 strongly suppressed the activity of N-SMase, but not A-SMase, imipramine inhibited the activity of A-SMase, but not N-SMase, in activated astroglia-insulted human neurons (supplemental Fig. 2, available at www. jneurosci.org as supplemental material), suggesting the specificity of these compounds. As evident from MTT metabolism in Figure 2*F* and LDH release in Figure 2*G*, pretreatment of neurons with GW4869, but not imipramine, was able to protect human neurons from activated astroglia-induced cell death. These findings suggest that Aβ-activated human astrocytes induce apoptosis and cell death in human neurons via N-SMase.

Identification of an astroglial soluble mediator, which is responsible for neuronal death

In AD, an inflammatory response characterized by glial activation is seen surrounding the plaque region. Results described above clearly suggest that once astroglia become activated, soluble gliotoxins released from these glial cells destroy human neurons via N-SMase. In an effort to identify such an astrogliotoxin that could be responsible for neuronal death, at first we considered the possibility that ceramide was released from activated astrocytes to act intercellularly on neurons as a proapoptotic signal. Primary astrocytes were activated by the combination of Aβ and IL-1β for different time periods followed by assay of ceramide in conditioned media. As evident from supplemental Figure 3*A* (available at www.jneurosci.org as supplemental material), we did not notice any increase in extracellular cer-

(Aβ+IL-1β)	-	+	+	+	+		-	+	+	+		-	+	+	+
NSMase-ASO	-	-	+	-	-		-	-	-	-		-	-	-	-
NSMase-ScO	-	-	-	+	-		-	-	-	-		-	-	-	-
ASMase-ASO	-	-	-	-	+		-	-	-	-		-	-	-	-
GW (μM)	-	-	-	-	-		-	-	10	20		-	-	-	-
Imip (μM)	-	-	-	-	-		-	-	-	-		-	-	10	20
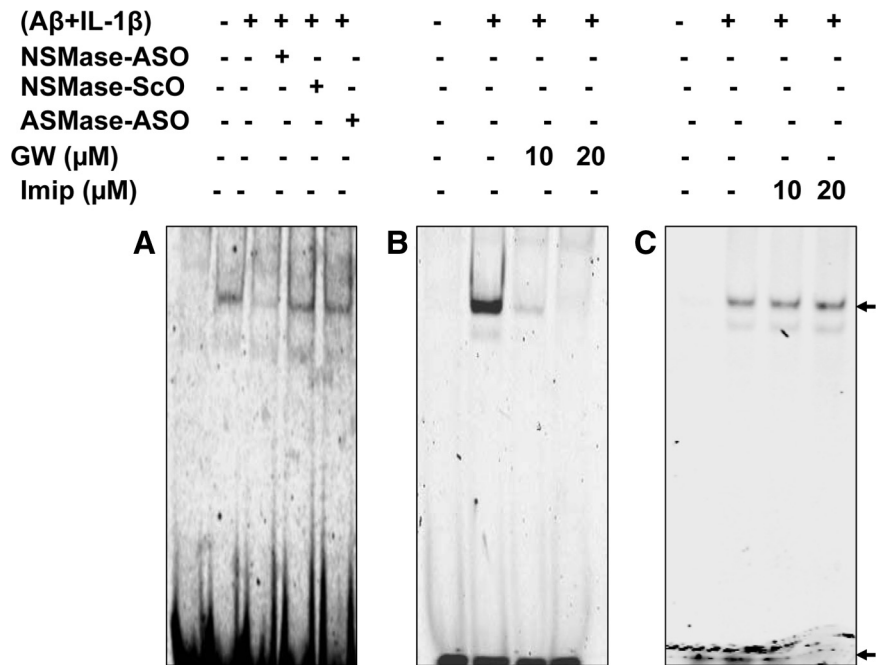
Figure 6. Inhibition of N-SMase, but not A-SMase, suppresses the activation of NF-κB in activated primary human astrocytes. *A*, Cells treated with 1 μM ASO or ScO against N-SMase and A-SMase for 40 h in complete media were stimulated with Aβ+IL-1β under the serum-free condition. After 1 h of stimulation nuclear extracts were prepared and subjected to EMSA for the detection of NF-κB. *B*, *C*, In the other set of experiments, cells preincubated with different concentrations of GW4869 (*B*) and imipramine (*C*) for 30' were stimulated with Aβ+IL-1β for 1 h followed by EMSA. The top and bottom arrows indicate the induced NF-κB band and the unbound probe, respectively. Results represent three independent experiments.

amide. However, as expected, the level of intracellular ceramide increased significantly under the same experimental condition (supplemental Fig. 3*B*, available at www.jneurosci.org as supplemental material). These results rule out the involvement of extracellular ceramide as a soluble mediator for neuronal death in our transwell culture model. Because activated astroglia also release nitric oxide (NO), a multifunctional molecule, next we investigated any possible role of NO in neuronal death. Interestingly, pretreatment of neurons with 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide (carboxy-PTIO) (a scavenger of NO) reduced the ability of activated astrocytes to induce the activation of N-SMase (Fig. 3*A*), decrease MTT metabolism (Fig. 3*B*), and increase the release of LDH (Fig. 3*C*), identifying NO as a possible astroglial soluble mediator for neuronal death. Although reactive oxygen species are known to activate N-SMase, it was not known whether NO could do the same. We examined whether NO alone was sufficient for the activation of N-SMase in neurons. As evident from Figure 3, diethylenetriamine (DETA)-NONOate, an NO donor, alone markedly induced the activation of N-SMase (Fig. 3*D*), but not A-SMase (Fig. 3*E*), in neurons.

Is the activation of human astrocytes dependent on N-SMase?

Although soluble gliotoxins released from activated human astrocytes destroy human neurons via N-SMase, it is not known whether N-SMase is also involved during the activation of glial cells. After activation, glial cells release various proinflammatory molecules. Previously we (Jana et al., 2005) found that among various stimuli (lipopolysaccharide, TNF-α, IL-1β, and interferon-γ) tested, only IL-1β was capable of inducing the production of NO in human fetal astroglia. It is evident from Figure 4*A* that fibrillar Aβ_{1-42} alone was also capable of inducing the

production of NO in primary human astroglia at a comparable level to that by IL-1β. However, the combination of IL-1β and Aβ induced the production of NO at a level higher than that found by these stimuli alone (Fig. 4A). Therefore, we used this combination to stimulate primary human astroglia. Next we investigated the effect of antisense knockdown of N-SMase on the production of NO. It is clear from Figure 4A that antisense knockdown of N-SMase, but not A-SMase, markedly abrogated Aβ+IL-1β-induced production of NO in human astroglia. On the other hand, ScOs had no effect on NO production suggesting the specificity of the effect. Next we examined the effect of chemical knockdown of N-SMase on astroglial production of NO. While GW4869, a specific inhibitor of N-SMase, markedly suppressed Aβ+IL-1β-induced production of NO, A-SMase inhibitor imipramine showed no effect on NO production (Fig. 4B). These results suggest that N-SMase, but not A-SMase, is involved in the production of NO from activated human astroglia.

Next, to confirm these results from another angle; we analyzed the mRNA expression of iNOS in activated astrocytes. As expected, the combination of Aβ and IL-1β induced the expression of iNOS mRNA (Fig. 4C). Consistent with the effect of N-SMase ASOs on the production of NO, antisense knockdown of N-SMase, but not A-SMase, strongly inhibited the expression of iNOS mRNA, whereas ScOs had no impact on Aβ+IL-1β-induced expression of iNOS (Fig. 4C). Real-time PCR data (Fig. 4D) also corroborated these findings. In addition to expressing iNOS, activated astrocytes are also known to express various proinflammatory cytokines. Consistently, Aβ+IL-1β induced the expression of TNF-α, IL-1β, and IL-6 mRNAs in human astroglia (Fig. 4E). However, similar to the effect of antisense knockdown of N-SMase on the expression of iNOS mRNA, ASOs against N-SMase, but not A-SMase, abrogated the ability of Aβ plus IL-1β to induce the expression of TNF-α, IL-1β, and IL-6 mRNAs (Fig. 4E). Together, these results suggest that the expression of proinflammatory molecules in activated human astroglia depends on N-SMase but not A-SMase.

Do oligomeric Aβ_{1-42} peptides also activate primary human astrocytes?
Although we are using fibrillar Aβ peptides to mimic activated astroglia-induced neuronal death, recent studies have shown that in addition to fibrillar Aβ, oligomeric Aβ is also capable of inducing profound inflammatory response in glial cells (White et al., 2005). Therefore, we wondered whether oligomeric Aβ_{1-42} induced the activation of human astroglia and whether oligomeric Aβ-activated astroglia also kill neurons via N-SMase. As expected, fibrillar Aβ induced the expression of iNOS protein and increased the level of GFAP in human astrocytes (Fig. 5A). Similarly, oligomeric Aβ also induced iNOS and upregulated GFAP in astrocytes (Fig. 5A). These results are specific, as reverse Aβ_{42-1} peptides did not have any effect on astroglial GFAP and iNOS (Fig. 5A). Therefore, we next examined the effect of oligomeric Aβ-activated astrocytes on the apoptosis of neurons. Similar to fibrillar Aβ-activated astrocytes, oligomeric Aβ-activated astrocytes also markedly induced apoptosis of human neurons (Fig. 5B,C), and this effect was blocked by antisense knockdown of N-SMase (Fig. 5B,C), suggesting that oligomeric Aβ-activated astrocytes also kill neurons via N-SMase.

*Does the activation of NF-κB in primary human astrocytes also depend on N-SMase?*
Among various transcription factors that are required for the transcription of proinflammatory molecules, NF-κB is a key
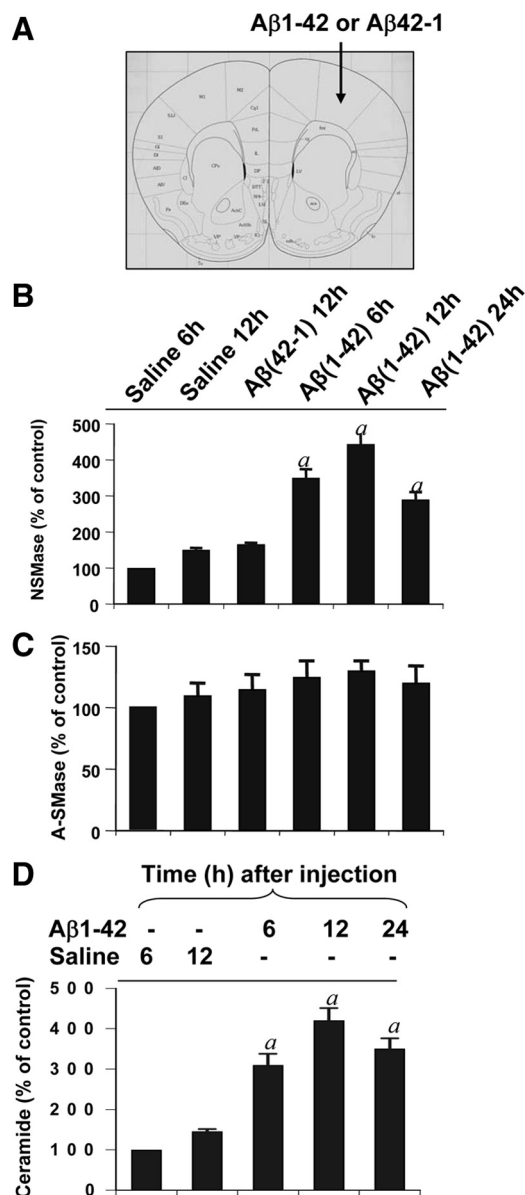
Figure 7. Fibrillar Aβ_{1-42} peptides induce the activation of N-SMase and the production of ceramide *in vivo* in the cortex of C57BL/6 mice. *A*, One microgram of either fibrillar Aβ_{1-42} or reverse Aβ_{42-1} peptides dissolved in saline was stereotaxically injected into the frontal cortex of C57BL/6 mice. Control mice received saline injection. *B–D*, At indicated time points, cortex was dissected out and divided into two halves: one half for measuring activities of N-SMase (*B*) and A-SMase (*C*) and the other for assaying ceramide (*D*). The control (saline, 6 h) group served as 100%, and data from other groups were expressed as a percentage of control. Results are the mean ± SD of five different mice ($n = 5$). $^a p < 0.05$ versus saline (6 h).

transcription factor involved in the transcription of almost all proinflammatory molecules (Blackwell and Christman, 1997). Therefore, to understand the basis of suppression of proinflammatory molecules by inhibition of N-SMase, we examined whether the activation of NF-κB in Aβ-stimulated astroglia was also regulated by N-SMase. Activation of NF-κB was monitored by DNA binding activity of NF-κB (Pahan et al., 2000b; Brahmachari et al., 2006). DNA binding activity of NF-κB was evaluated by the formation of a distinct and specific complex in a gel shift DNA binding assay. Treatment of primary human astroglia with Aβ+IL-1β resulted in the induction of DNA binding activity of NF-κB (Fig. 6A). This gel shift assay detected a specific band in
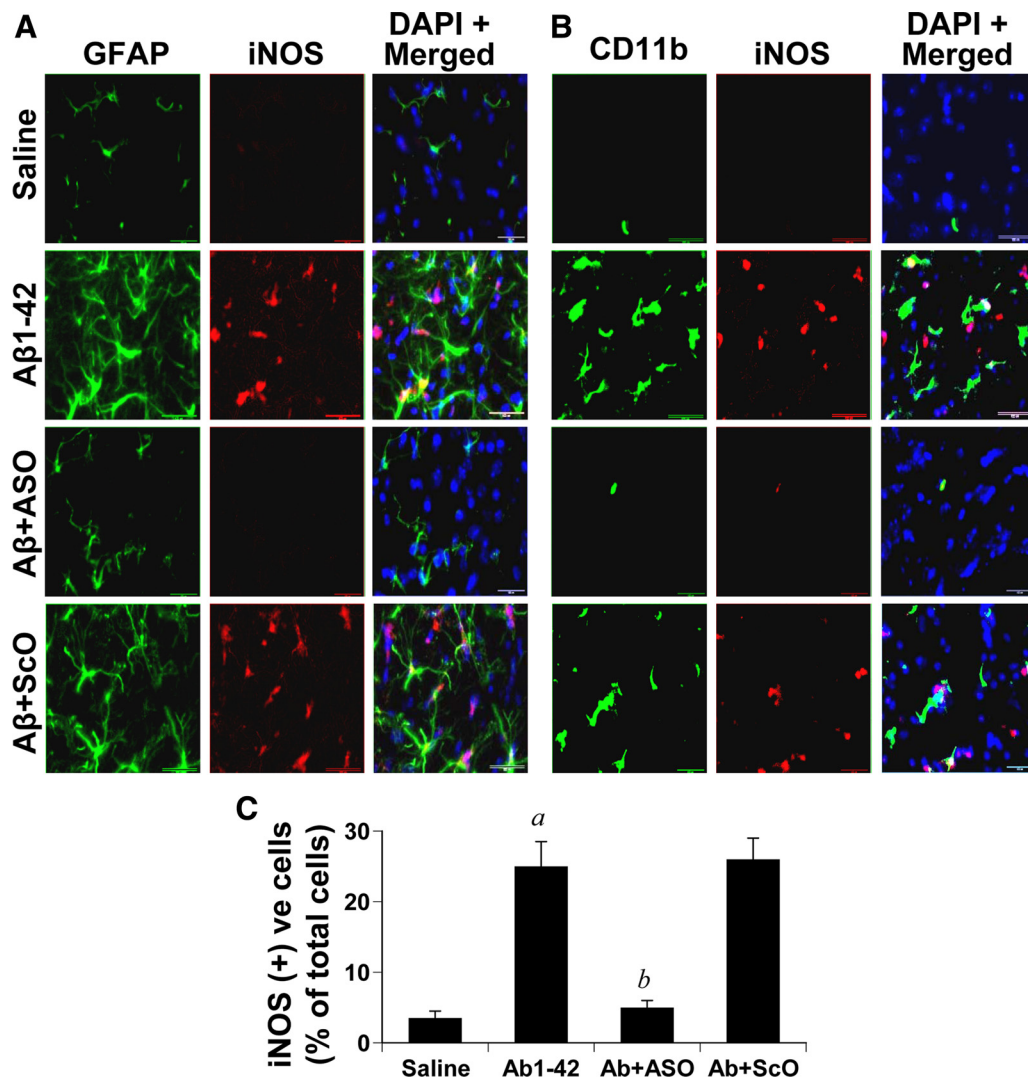
Figure 8. Antisense knockdown of N-SMase attenuates the activation of astroglia and microglia and reduces the expression of iNOS *in vivo* in the cortex of C57BL/6 mice. One microgram of either ASO or ScO against N-SMase dissolved in 2 μl of saline was stereotaxically injected into the frontal cortex of C57BL/6 mice. After 24 h of microinjection, 1 μg of fibrillar Aβ_{1-42} in a 2 μl volume was again microinjected at the same site. **A**, **B**, After 6 h of Aβ microinjection, mice were perfused, and double immunofluorescence for GFAP (green) and iNOS (red) (**A**) and CD11b (green) and iNOS (red) (**B**) was performed. Five mice ($n = 5$) were used in each group. **C**, Cells positive for iNOS as well as total 4′,6′-diamidino-2-phenylindole-positive cells were counted in three cortical sections (two images per slide) of each of five different mice with an Olympus IX81 fluorescence microscope using the MicroSuite imaging software. Results are expressed as the number of iNOS-positive cells per 100 cells. [a]$p < 0.001$ versus saline; [b]$p < 0.001$ versus Aβ.

response to Aβ+IL-1β (Fig. 6) that was competed off by an unlabeled probe (data not shown), suggesting that Aβ+IL-1β induces the DNA-binding activity of NF-κB. However, ASOs against N-SMase, but not A-SMase, suppressed Aβ+IL-1β-induced DNA-binding activity of NF-κB (Fig. 6A). On the other hand, under similar condition, ScOs had no effect on the DNA-binding activity of NF-κB (Fig. 6A). To confirm these results further, we used chemical inhibitors of N-SMase and A-SMase. Consistent to antisense results, N-SMase inhibitor GW4869, but not A-SMase inhibitor imipramine, markedly suppressed Aβ+IL-1β-induced DNA-binding activity of NF-κB (Fig. 6B,C). These results clearly indicate that the activation of NF-κB in human astrocytes also depends on N-SMase, but not A-SMase.

Fibrillar Aβ_{1-42} peptides induce the activation of N-SMase and the production of ceramide in vivo *in the cortex of male C57BL/6 mice*
Using various approaches, the studies presented above demonstrate that fibrillar Aβ-activated astroglia kill neurons via

N-SMase and that fibrillar Aβ also activates astroglia via N-SMase. Next, to address whether these findings could be reproduced *in vivo* in the cortex of mice, fibrillar Aβ_{1-42} peptides were microinjected into the cortex of male C57BL/6 mice (10–12 weeks old) (Fig. 7A). First, we examined whether Aβ peptides were able to induce the activation of N-SMase and the production of ceramide *in vivo* in the cortex. As evident from Figure 7B, microinjection of Aβ_{1-42} peptides led to marked increase in the activation of N-SMase compared to saline microinjection. A more than threefold increase in the activity of N-SMase was observed at 6 h of microinjection and it increased further at 12 h (Fig. 7B). However, activity of N-SMase decreased at 24 h of microinjection (Fig. 7B). In contrast, reverse peptides (Aβ_{42-1}) were unable to increase the activity of N-SMase *in vivo* in the cortex compared to saline microinjection (Fig. 7B). Whereas after 12 h of microinjection, Aβ_{1-42} increased the activity of N-SMase by >300% compared to saline, Aβ_{42-1} remained unable
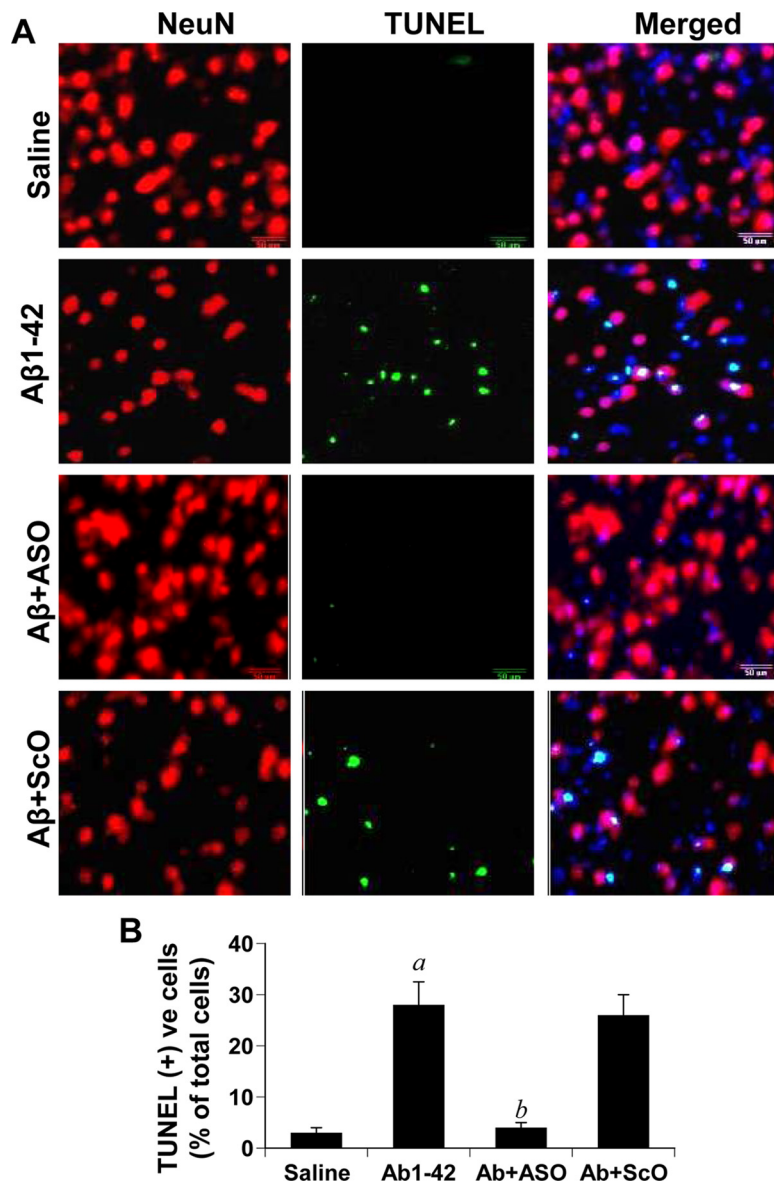
Figure 9. Antisense oligonucleotides against N-SMase protect neurons from fibrillar Aβ toxicity *in vivo* in the cortex of C57BL/6 mice. One microgram of either ASO or ScO against N-SMase dissolved in 2 μl of saline was stereotaxically injected into the frontal cortex. After 24 h of microinjection, 1 μg of fibrillar Aβ$_{1-42}$ in a 2 μl volume was again microinjected at the same site. ***A***, After 24 h of the final microinjection, mice were perfused and double immunofluorescence for NeuN (red), and TUNEL (green) was performed. Five mice (n = 5) were used in each group. ***B***, TUNEL-positive cells as well as total 4′,6′-diamidino-2-phenylindole-positive cells were counted in three cortical sections (2 images per slide) of each of five different mice with an Olympus IX81 fluorescence microscope using the MicroSuite imaging software. Results are expressed as the number of TUNEL-positive cells per 100 cells. $^a p <$ 0.001 versus saline; $^b p <$ 0.001 versus Aβ.

Does antisense knockdown of N-SMase inhibit fibrillar Aβ-mediated astrogliosis in vivo in the cortex of C57BL/6 mice?
At first, we examined efficacy and specificity of ASOs against mouse N-SMase (mN-SMase) *in vivo* in the cortex. Sequences of ASOs and ScOs against mN-SMase were as follows: ASO, 5′-CAG CGA GCC GGT CCA CCA GCC-3′; ScO, 5′-CAC GCG TCC GAC GCC GCA CGA-3′.

It is clearly evident from supplemental Figure 4*A* (available at www.jneurosci.org as supplemental material) that fibrillar Aβ$_{1-42}$ increased the activity of N-SMase *in vivo* in the cortex by more than fourfold compared to saline. However, premicroinjection of mN-SMase ASOs, but not ScOs, was capable of suppressing Aβ$_{1-42}$-induced activation of N-SMase *in vivo* in the cortex (supplemental Fig. 4*A*, available at www.jneurosci.org as supplemental material). We also observed an ~35% increase in the activity of A-SMase after Aβ microinjection (supplemental Fig. 4*B*, available at www.jneurosci.org as supplemental material). However, either mN-SMase ASOs or mN-SMase ScOs did not influence the activity of A-SMase in fibrillar Aβ-microinjected cortex (supplemental Fig. 4*B*, available at www.jneurosci.org as supplemental material).

Glial activation and neuronal apoptosis are two hallmarks of AD. We investigated whether fibrillar Aβ$_{1-42}$ peptides were capable of inducing glial activation and neuronal apoptosis *in vivo* in the cortex. To understand whether both are occurring simultaneously or the one is preceding the other in fibrillar Aβ$_{1-42}$-insulted cortex, we analyzed glial expression of iNOS and neuronal TUNEL bodies in cortical sections after 6 and 24 h of microinjection. Astroglia are the major cell type in the brain and increase in GFAP expression represents an important way to monitor astroglial activation (Brahmachari et al., 2006). After activation, astrocytes express iNOS and produce NO, and we demonstrated previously that NO plays a critical role in the upregulation of GFAP in astroglia (Brahmachari et al., 2006). Similarly, activated microglia also express considerable amount of iNOS, and we have demonstrated that NO is an important factor in increasing microglial surface markers required for gliosis (Roy et al., 2006). Therefore, we monitored the expression of iNOS in both GFAP- and CD11b-positive cells in cortical sections. Fibrillar Aβ$_{1-42}$ peptides induced the expression of iNOS in GFAP-positive astrocytes and CD11b-positive microglia within 6 h of microinjection (data not shown). However, fibrillar Aβ peptides were unable to induce any apoptosis in neurons (NeuN-positive cells) within this time period (data not shown). On the other hand, at 24 h of microinjection, both

to increase its activity significantly at the same time point (Fig. 7*B*). However, under same experimental setting, microinjection of saline, Aβ$_{1-42}$, or Aβ$_{42-1}$ was unable to increase the activity of A-SMase significantly in the cortex (Fig. 7*C*). Then, we monitored the level of ceramide in the cortex. Fibrillar Aβ$_{1-42}$ peptides caused an approximately threefold increase in the ceramide level after 6 h and an approximately fourfold increase in the ceramide level after 12 h, compared to an ~30% increase in ceramide level in saline-microinjected cortex at 6 h (Fig. 7*D*). Together, these findings suggest that fibrillar Aβ$_{1-42}$ peptides induce the generation of ceramide *in vivo* in the cortex and that ceramide production is attributable to the activation of N-SMase, but not A-SMase.

glial expression of iNOS and neuronal apoptosis were observed (data not shown). These results clearly suggest that in the Aβ-microinjected model, glial activation is followed by neuronal apoptosis.

Therefore, first we investigated the effect of N-SMase ASO on glial activation. As evident from Figure 8*A*, the level of iNOS was almost undetectable, and the level of GFAP was also very low in saline-microinjected mice. On the other hand, marked increase in the expression of iNOS and GFAP was observed in Aβ-injected mice, and many iNOS signals colocalized with GFAP-positive astroglia after both 6 h (Fig. 8*A*) and 24 h (data not shown) of microinjection. Similarly, Aβ microinjection also induced microglial activation as evidenced from increased iNOS staining in CD11b-positive microglia after 6 h (Fig. 8*A*) and 24 h (data not shown) of microinjection. However, antisense knockdown of N-SMase dramatically reduced the expression of iNOS, GFAP, and CD11b *in vivo* in the cortex of Aβ-microinjected mice at both 6 and 24 h of microinjection. On the other hand, as expected, N-SMase ScOs had no effect on the expression of iNOS, GFAP, and CD11b (Fig. 8). Because there was no neuronal apoptosis *in vivo* in the cortex after 6 h of microinjection, our results clearly suggest that knockdown of N-SMase is capable of abrogating the inflammatory response in glial cells without involving neuronal apoptosis.

Antisense knockdown of N-SMase protects neurons from Aβ toxicity in vivo *in the cortex of C57BL/6 mice*
Next we investigated whether fibrillar Aβ$_{1-42}$ peptides induced neuronal apoptosis *in vivo* in the cortex. Cortical sections were double labeled for TUNEL and NeuN. As evident from Figure 9, fibrillar Aβ$_{1-42}$ markedly induced the apoptotic death of neurons *in vivo* in the cortex as evidenced by the increase in TUNEL signal in NeuN-positive cells. We wondered whether fibrillar Aβ also induced apoptosis in the other three cell types in the cortex. In contrast to marked apoptosis of neurons, we did not observe any apoptosis in either GFAP-positive astroglia or Iba1-positive microglia (data not shown). However, TUNEL bodies colocalized with O4-positive oligodendroglia and oligodendroglial progenitors (data not shown). This is consistent with the finding of Lee et al. (2004) that Aβ peptides induce oligodendroglial apoptosis. Next we investigated whether antisense knockdown of mN-SMase was capable of protecting neurons *in vivo* in the cortex from Aβ toxicity. As evident from Figure 9, *A* and *B*, the increase in the apoptotic death *in vivo* in the cortex was blocked by mN-SMase ASOs. On the other hand, mN-SMase ScOs did not ameliorate cell death in Aβ injected mice under similar experimental conditions, suggesting the specificity of the protective effect (Fig. 9).

Discussion

Although the disease mechanisms that cause AD are poorly understood, recent studies strongly support the role of inflammation in neurodegeneration in this disease (MacKenzie, 1996;
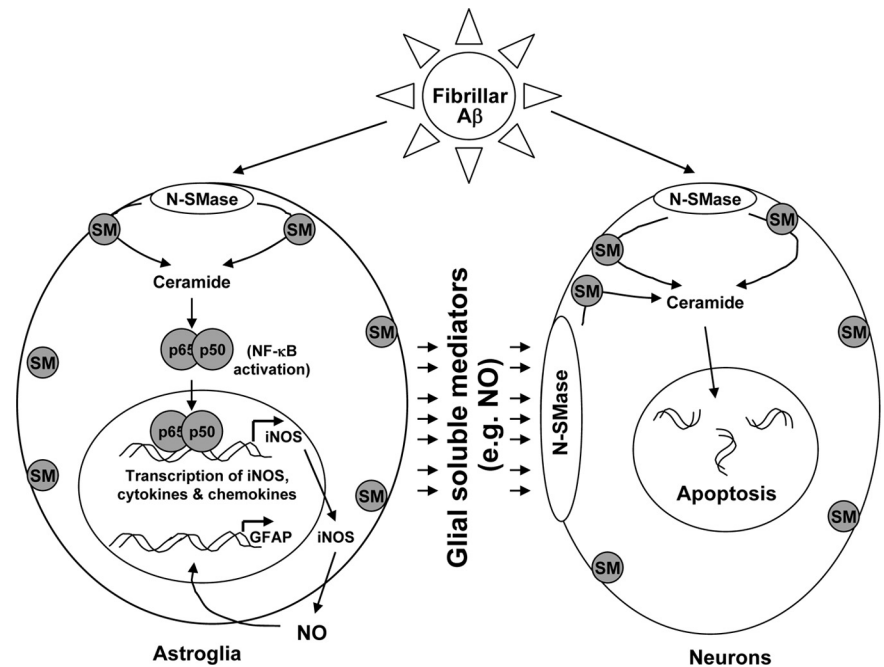
Figure 10. Schematic presentation of the summary of our work. Fibrillar Aβ alone is capable of activating the N-SMase–ceramide pathway in neurons, which leads to apoptosis and cell death (Jana and Pahan, 2004a,b). In astroglia, fibrillar Aβ-mediated activation of the N-SMase– ceramide pathway is responsible for the activation of NF-κB and the expression of different proinflammatory soluble mediators including NO. Once NO is produced, it can induce the expression of GFAP in astrocytes leading to astrogliosis. On the other hand, these glial-derived soluble mediators (e.g., NO) also induce neuronal apoptosis via the N-SMase– ceramide pathway. It appears that multiple neurotoxic signaling pathways converge on N-SMase. SM, Sphingomyelin.

Akiyama et al., 2000; McGeer and McGeer, 2001; Walsh and Aisen, 2004; Heneka and O'Banion, 2007; Rogers, 2008; Rezai-Zadeh et al., 2009). First, early intervention with nonsteroidal anti-inflammatory drugs slows disease incidence (in t' Veld et al., 2001; Szekely et al., 2004, 2007). Second, significant astroglial and microglial activation occurs in close proximity to neuritic plaques containing Aβ. Third, overexpression of astrocytic S100B leads to brain inflammation and exacerbation of Alzheimer's disease pathology in a mouse model of the disease (Mori et al. 2010). Fourth, the concentration of NO$_2$– (nitrite), a metabolite of NO, increases in the CSF of patients with AD compared to age-matched controls (Luth and Arendt, 1998; Tohgi et al., 1999). It has been shown that glial cells in the cortex from postmortem AD samples express considerable amounts of inducible iNOS, whereas those from age-matched controls do not (Luth et al., 2002; Fernandez-Vizarra et al., 2004) Fifth, a variety of proinflammatory cytokines including TNF-α, IL-1β, IL-6, eicosanoids, and other immune neurotoxins are found in either CSF or affected brain regions in AD (Sun et al., 2003; Tarkowski et al., 2003; Cacquevel et al., 2004). Sixth, concentrations of various chemokines, such as IL-8, monocyte chemoattractant protein-1, and interferon gamma-induced protein-10 increase in CSF of patients with mild cognitive impairment and AD compared to age-matched control (Sun et al., 2003; Galimberti et al., 2006). Seventh, NF-κB is considered as a proinflammatory transcription factor because of its involvement in the transcription of almost all proinflammatory molecules (Muller-Ladner et al., 2002). It has been found that in AD brains, NF-κB immunoreactivity is higher in astrocytes surrounding the plaque region. Eighth, in addition to releasing nitric oxide, proinflammatory cytokines, and chemokines, activated glia also produce reactive oxygen species and eicosanoids capable of damaging neurons. Together,

these studies demonstrate the utmost importance of unraveling mechanisms of glial activation and activated glia-mediated neuronal death for providing significant insight into the pathophysiology of AD and developing treatment paradigms.

Intracellular signaling mechanisms involved in Aβ-induced glial activation are poorly understood. Recent studies have shown that Aβ activates three subtypes of mitogen-activated protein kinases (MAPKs) in glial cells, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase (Bodles and Barger, 2005). Out of the three MAPKs, p38 and JNK, but not ERK1/2, have been shown to play important roles in mediating the activation of glia (Xie et al., 2004; Bodles and Barger, 2005). Here we have found that fibrillar Aβ_{1-42} peptides activate human astroglia via neutral sphingomyelinase (N-SMase). First, knockdown of N-SMase by either chemical inhibitor or antisense oligonucleotides suppresses Aβ-induced production of nitrite and the expression of iNOS and proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in primary human astrocytes. On the other hand, knockdown of A-SMase had no effect on Aβ-induced activation of human astroglia, suggesting the specificity of the effect. Previous studies have also shown the involvement of N-SMase in the induction and/or stimulation of iNOS in C6 rat glioma cells (Won et al., 2004) or oligodendrocytes (Zeng et al., 2005). Second, inhibition of N-SMase, but not A-SMase, attenuated the activation of NF-κB in fibrillar Aβ-stimulated human astrocytes. Our result is in direct contrast with previous reports where TNF-α has been shown to induce the activation of NF-κB via A-SMase (Van Antwerp et al., 1996; Wang et al., 1996). Whereas TNF-α requires the TNF receptor 1–A-SMase pathway for the activation of NF-κB, fibrillar Aβ couples different receptors (receptor for advanced glycation end products, scavenger receptor, etc.), which may prefer N-SMase for transducing signals for the activation of NF-κB in human astroglia. Third, antisense knockdown of N-SMase also inhibited the expression of iNOS in vivo in fibrillar Aβ-intoxicated mouse cortex. We demonstrated previously that fibrillar Aβ kills human neurons via activation of N-SMase (Jana and Pahan, 2004b). Here we demonstrate that fibrillar Aβ activates human astroglia in culture and mouse astroglia in vivo in the cortex via N-SMase. Previously, Pahan et al. (1998) showed that ceramide stimulates the activation of NF-κB and the expression of iNOS in primary rat astrocytes. Recently, we demonstrated that NO is an important signal for the upregulation of GFAP in astrocytes. Therefore, it appears that the activation of N-SMase–ceramide pathway is one of the earlier events for the initiation of NF-κB–iNOS–NO–GFAP pathway, which ultimately culminates into astroglial activation and astrogliosis. Here we have also delineated a nice feedback regulation of N-SMase by NO as well. Although proinflammatory cytokines (TNF-α and IL-1β) and reactive oxygen species are known to induce N-SMase, it was not known whether NO is capable of activating N-SMase. Our results suggest that NO produced from activated glial cells could activate N-SMase on its own in neurons.

Once glial cells are activated, in addition to producing NO, these activated cells produce diverse set of molecules, such as proinflammatory cytokines, proinflammatory chemokines, reactive oxygen species, and arachidonic acid, which could damage neurons on their own. Therefore, it is important to identify a common molecule where various signaling pathways emanating from different neurotoxic molecules converge. Several lines of evidence presented in this manuscript clearly indicate that Aβ-activated primary human astroglia kill primary human neurons via N-SMase. First, in a transwell setting where astrocytes were not in direct contact with neurons, fibrillar Aβ-activated astroglia induced the activation of N-SMase, the production of ceramide, and cell death in neurons. However, antisense knockdown of neuronal N-SMase, but not A-SMase, protected these cells against fibrillar Aβ-activated astroglia as evidenced by decrease in TUNEL-positive cells, amelioration in LDH release, and restoration of MTT metabolism. Consistently, treatment of neurons with N-SMase inhibitor GW4869, but not A-SMase inhibitor imipramine, also led to protection of neurons from activated astroglia insult. Second, stereotaxic injection of fibrillar Aβ_{1-42} peptides, but not soluble Aβ_{42-1} reverse peptides, increased the activity of N-SMase, the production of ceramide, and the apoptosis of neurons in mouse cortex. However, antisense knockdown of N-SMase protected cholinergic neurons in vivo in the cortex from fibrillar Aβ toxicity.

In vivo in the brain of AD patients, fibrillar Aβ peptides exert effect on neurons as well as glial cells. Although according to our earlier work (Jana and Pahan, 2004b) knockdown of N-SMase protects neurons from direct toxic effects of fibrillar Aβ, glial-derived neurotoxic factors may kill neurons independent of N-SMase, thereby limiting the therapeutic potential of inhibitors of N-SMase in AD. Therefore, we performed this study. Here we demonstrate that fibrillar Aβ activates glial cells via N-SMase and that N-SMase is also involved in activated glial cell-induced neuronal death (Fig. 10). These results suggest that N-SMase is the converging point of multiple neurotoxic signaling pathways (Fig. 10). Although the in vitro situation of human fetal neurons and astroglia in culture does not truly resemble the in vivo situation of neurons in the brain of AD patients, our results suggest that specific targeting of N-SMase may be an important therapeutic avenue to halt neuronal damage in AD and other neurodegenerative disorders.

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