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Regulation of Nerve Growth Factor Release by Nitric Oxide through Cyclic GMP Pathway in Cortical Glial Cells

HUABAO XIONG, KIYOFUMI YAMADA, HUSSAM JOURDI, MEIKO KAWAMURA, NOBUYUKI TAKEI, DAIKEN HAN, TOSHITAKA NABESHIMA, and HIROYUKI NAWA

Department of Molecular Neurobiology, Brain Research Institute, Niigata University, Niigata, Japan (H.X., H.J., M.K., N.T., H.N.); and Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University School of Medicine, Nagoya, Japan (K.Y., D.H., T.N.)

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ABSTRACT

In the present study, we found that *S*-nitroso-*N*-acetyl-DL-penicillamine, a spontaneous nitric oxide (NO) generator, dose-dependently inhibited basal nerve growth factor (NGF) release from mixed glial cells. To elucidate the function of endogenous NO in the regulation of NGF release, the mixed glial cells were stimulated with lipopolysaccharide (LPS) or LPS plus interferon-γ (IFNγ). The results showed that LPS alone induced NGF release and moderate NO production. However, costimulation with LPS plus IFNγ greatly enhanced NO production but significantly suppressed LPS-induced NGF release. When N^G-monomethyl-L-arginine, an NOS inhibitor, was added to the culture, the suppression of NGF release by IFNγ was significantly reduced. Quantitative reverse transcription-polymerase chain reaction demonstrated *S*-nitroso-*N*-acetyl-DL-penicillamine was also able to inhibit the LPS-induced NGF mRNA expression. To understand the different contributions of astroglia and microglia to this phenomenon, both cell types were purified. We found purified astroglia produced high amounts of NGF but low amounts of NO. However, purified microglia produced a large amount of NO but very low amounts of NGF after stimulation with LPS or LPS plus IFNγ. Our data also indicated the second messenger cyclic GMP, but not cyclic AMP, was able to inhibit basal NGF release. In vivo experiments confirmed that NGF protein level was significantly enhanced in rats treated with L-N^ω-nitro-arginine methyl ester and in endothelial NO synthase mutant mice. Taken together, we conclude NO derived mainly from microglia down-regulates NGF release from astroglia at the transcriptional level by stimulating cyclic GMP pathway.

Nerve growth factor (NGF) is a target-derived neurotrophic factor that has distinct functional effects on the developing nervous system. It belongs to the neurotrophin family and is essential for the development, survival, and differentiation of the peripheral sympathetic and sensory neurons (Levi-Montalcini, 1987). In the central nervous system (CNS), NGF is produced in distinct areas, including the hippocampus (Korsching et al., 1985; Large et al., 1986), and it exerts a trophic influence on the septal cholinergic neurons projecting to the hippocampus (Hefti, 1986). In situ hybridization experiments have shown that NGF mRNA in unlesioned brain is predominantly localized in neurons (Bandtlow et al., 1990; Ernfors et al., 1990). However, cultured glial cells also synthesize NGF mRNA (Furukawa et al., 1986), whose levels are regulated by various cytokines, growth factors, and bacterial components, including fibroblast growth factor, interleukin-1 (Yoshida and Gage, 1991), tumor necrosis factor (Hattori et al., 1993), transforming growth factor (Lindholm et al., 1990), and bacterial lipopolysaccharide (LPS; Galve-Roperh et al., 1997).

Nitric oxide (NO) is an important intercellular messenger with many diverse actions in the nervous, vascular, and immune systems (Schuman and Madison, 1991; Nussler and Billiar, 1993; Bredt and Snyder, 1994; Nathan and Xie, 1994; Garthwaite and Boulton, 1995). This molecule is produced by NO synthases, which oxidize the guanidine nitrogen of arginine to form citrulline and a short-lived radical gas, NO. A family of related NOS proteins are produced from different genes and referred to as: inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS). Glial cells, when stimulated with LPS and/or inflammatory cytokines such as interferon-γ (IFNγ), interleukin 1, and tumor necrosis factor, begin to express iNOS and produce a certain amount of NO, which may play a contributory role in CNS inflammation (Parkinson et al., 1997). Glial cells are also a source of various neurotrophic factors. Inflammatory cytokines or LPS,

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ABBREVIATIONS: NGF, nerve growth factor; EIA, enzyme immunoassay; CNS, central nervous system; cGMP, cyclic GMP; cAMP, cyclic AMP; IFN, interferon; GFAP, antiglial fibrillary acidic protein; LPS, lipopolysaccharide; iNOS, inducible NOS; nNOS, neuronal NOS; eNOS, endothelial NOS; NAME, N^ω-nitro-arginine methyl ester; TBST, Tween 20-containing Tris-buffered saline; NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; PCR, polymerase chain reaction; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; TNF, tumor necrosis factor.

when applied alone or in combination, can act as inducers of the synthesis of the neurotrophic factor NGF (Galve-Roperh et al., 1997) both in vitro and in vivo (Pluss et al., 1995; Hattori et al., 1996).

Glial cells are able to produce both NGF and NO, which are believed to play many roles in CNS, therefore, we wondered whether endogenous NO could regulate NGF mRNA expression and its protein release. In the present study, we report that NO can down-regulate NGF release in the CNS.

Materials and Methods

Reagents. N^G-Monomethyl-L-arginine (NMMA), *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), and L-N^ω-nitro-arginine methyl ester (L-NAME) were purchased from Wako Pure Chemical industries, Ltd. (Osaka, Japan). LPS was obtained from Difco Laboratories (Detroit, MI).

Cell Culture. Primary cultures of glial cells were prepared from postnatal rats. Briefly, whole striata or neocortices of P3 rats were mechanically dissociated and plated onto poly(D-lysine)-coated dishes. Dissociated cells were first grown for 7 days with Dulbecco's modified Eagle's medium containing 2 mM purified glutamine and 10% horse serum. Then, cells were subcultured with the same culture medium for 5 days. For stimulation experiments, the cells were cultured with Dulbecco's modified Eagle's medium supplemented with nutrient mixture N2 (100 μg/ml transferrin, 5 μg/ml bovine insulin, 100 nM putrescine, 30 nM sodium selenite, and 20 nM progesterone), 100 μg/ml BSA, 1 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin.

After 7 to 10 days in culture, microglia that grow loosely attached on top of mixed glial cultures were isolated by mechanical shaking of the culture flasks for 30 min at 200 rpm on a gyratory shaker. Harvested cells were transferred to fresh culture dishes. This procedure still left behind firmly attached microglial cell population. The purity of the cultures was more than 95%, as tested by morphological criteria and by their reactivity toward MRC OX-42 (CD11b) (determined by immunocytochemistry).

The original cultures were fed with fresh medium, equilibrated in a CO_2 incubator, and shaken for an additional 16 h at 250 rpm to separate the phase-dark, round oligodendrocyte progenitors that grow on top of a confluent layer of astrocytes. This procedure was repeated as needed. The remaining cultures, substantially depleted of oligodendrocyte progenitors and microglia, were subcultured into 6-well culture dishes and used as astrocyte-enriched cultures. The purity (more than 95%) was confirmed by labeling with anti-glial fibrillary acidic protein (GFAP, an astrocyte marker) antibodies.

Immunocytochemistry. Cultured cells were fixed with methanol for 30 min and then immunostained with polyclonal antibodies directed against astroglial (GFAP) and microglial (CD11b) markers. Cells were then incubated with a fluorescein isothiocyanate-conjugated swine anti-mouse IgG antibody. After washing for three times with PBS, cells were observed under a microscope.

Nitrite Determination and NOS Enzyme Assay. Nitrite concentration in culture, a measurement of NO synthesis, was assayed by a standard Griess reaction adapted to microplates, as described previously (Green et al., 1982). The Griess reagent was prepared by mixing equal volumes of sulfanilamide (1.5% in 5% H_3PO_4) and naphthylethylene diamine dihydrochloride (0.1% in H_2O). A volume of 100 μl of reagent was mixed with 100 μl of supernatant and incubated at room temperature for 10 min. Absorbance of the chromophore formed was measured at 540 nm in an automated microplate reader. Nitrite was quantified using $NaNO_2$ as standard, and the data were expressed as micromolar nitrite.

Cytosolic NOS activity was determined according to a modification of the method described by Rogers and Ignarro (1992). The enzymatic reaction was performed at 37°C for 30 min in 50 mM Tris, pH 7.5 containing 0.1 mM EDTA, 2 mM $CaCl_2$, 0.1 mM L-arginine, 0.5 mM tetrahydrobiopterin, and 0.004 mM flavin mononucleotide. The formation of nitrite and nitrate in the reaction mixture was measured by HPLC and expressed as NOS activity (pmol of nitrite and nitrate/min/mg protein).

NGF Enzyme Immunoassay. The NGF levels were determined by NGF enzyme immunoassay (EIA) as described previously (Narisawa-Saito et al., 1996). Briefly, EIA plates were coated with 100 ng of anti-NGF antibody/well in 0.1 M Tris buffer (pH 9.0) for 12 to 18 h and then blocked with EIA buffer (50 mM Tris, 0.3 M NaCl, 0.1% Triton X-100, 1% BSA, 1% gelatin, pH 7.5) at 4°C for more than 12 h. One hundred microliters of tissue extracts (duplicate) or NGF standards (1–300 pg; triplicate) in EIA buffer was loaded onto wells at room temperature for 12 to 18 h. After three washings with W-buffer (EIA buffer excluding BSA), 100 μl of biotinylated anti-NGF antibody (10 ng/ml) in EIA buffer was loaded to wells and incubated for 12 to 18 h at room temperature. Bound biotinylated secondary antibodies were detected by incubation with avidin-β-galactosidase (1:10,000; Sigma Chemical Co., St. Louis, MO) for 3 h. Unbound enzyme was removed by extensive washing with W-buffer followed by saline (pH 7.3). Then, the enzyme activity retained in each well was measured by incubation with a fluorogenic substrate, 200 μM 4-methylumbelliferyl-β-D-galactosidase (Sigma Chemical), in 50 mM sodium phosphate and 10 mM $MgCl_2$. The reactions were carried out in the dark at room temperature for 12 h. The amount of fluorescent product was monitored by a Perkin-Elmer fluorometer (model 650-40) with 364-nm excitation and 405-nm emission.

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total cellular RNA from glial cultures was extracted by a method as described previously (Xiong et al., 1996). Preparation of cDNA by RT was done as follows. Total RNA extracted (2 μg) was mixed with 4 μl of RT buffer, 2 μl of 0.1 M dithiothreitol, 0.5 μl of RNasin (Promega, Madison, WI), 1 μl of 10 mM dNTPs (Pharmacia), 2 μl of random primer (Pharmacia), 0.5 μl of reverse transcriptase (GIBCO-BRL, Life Technologies Inc., Gaithersburg, MD), and distilled water to give a final volume of 20 μl. The mixture was incubated at 42°C for 90 min and then boiled at 95°C for 3 min. Samples were kept at −20°C until use. The PCR mixture consisted of 2 μl of sample cDNA, 5 μl of PCR amplification buffer, 2 μl of 25 mM $MgCl_2$, 4 μl of 2.5 mM dNTPs, 0.3 μl of *Taq* DNA polymerase (5 U/μl, Promega), 2 μl of 20 μM primer, and 32.7 μl of double-distilled water to give a final volume of 50 μl. All the PCR primers were made to our order by Kurabo Biomedical (Osaka, Japan) according to our sequence design. The sequences of oligonucleotide primers used were as follows: 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′ for iNOS, 5′-CCAAGGACGCAGCTTTCTAT-3′ and 5′-CTCCGGTGAGTCCTGTTGAA-3′ for NGF, and 5′-CACAGCTGAGAGGGAAATCG-3′ and 5′-CACACAGAGTACTTGCGCTC-3′ for β-actin. PCR amplification was performed by using a TP cycler-100 (Toyobo, Osaka, Japan). One PCR cycle was run under the following condition: DNA denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and DNA extension at 72°C for 1 min. Samples were amplified at 15 to 30 cycles according to the most appropriate cycle number determined by a preliminary experiment. The PCR products were stored in the cycler at 4°C until they were collected. The PCR products were analyzed by agarose gel electrophoresis with a horizontal 1% agarose gel (low-melting-temperature agarose L; Wako Pure Chemicals, Tokyo, Japan) in 1× Tris-acetate-EDTA buffer supplemented with 0.005% ethidium bromide for DNA staining. Undiluted PCR product (8 μl) plus 2 μl of bromphenol blue were applied to each well. The PCR products were visualized on a UV transilluminator and photographed. For quantitative PCR, 15 cycles were used for β-actin, and 20 cycles were used for NGF and iNOS. Our experiment demonstrated that with these numbers of PCR cycles, PCR products for β-actin, iNOS, and NGF did not reach saturation. Then, the PCR product was analyzed by polyacrylamide gel electrophoresis and stained with SYBR Green I. The strength of fluores-

cence for PCR product was determined by FUJIFILM FLA-2000. The ratios of NGF and iNOS over β-actin were calculated.

Western Blotting. Total protein was extracted from cultures with 2% SDS and 50 mM Tris buffer, pH 6.8. Protein extracts as well as prestained molecular weight markers (New England BioLabs) were denatured in Laemmli's sample buffer (10% glycerol, 2% SDS, 0.1 M dithiothreitol, 65 mM Tris, 0.01 mg/ml bromophnol blue) at 90°C; separated by SDS-polyacrylamide gel electrophoresis with 1.0-mm-thick, 7.5% polyacrylamide slab gels; and transferred to a nitrocellulose membrane (0.45 μm pore, BA85; Schleicher & Schull) by electrophoresis in transfer buffer (25 mM Tris, 92 mM glycine, and 20% methanol). After the completion of protein transfer, the membrane was rinsed with Tween 20-containing Tris-buffered saline (TBST; 10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0) and treated with blocking solution (TBST plus nonfat dry milk). The membranes were incubated with anti-iNOS antibody (1:1000; Transduction Lab) at room temperature for 2 to 4 h. After extensive washing with TBST, the membrane was reacted with anti-mouse/rabbit Ig conjugated to peroxidase (1:10,000) diluted with the blocking solution. The immunoreactivity on the membrane was visualized by chemiluminescence reaction combined with film exposure (ECL Kit; Amersham, Buckinghamshire, UK).

Animal Procedures. Heterozygous nNOS or eNOS mutant mice were mated to produce homozygous mutants (Huang et al., 1993, 1995). The genotypes of their offspring were determined by PCR. Generally, both nNOS and eNOS mutants look normal compared with wild type.

Intraventricular injection into 9-week-old male Wistar rats (Nihon SCL, Shizuoka, Japan) was performed according to our previous report (Yamada et al., 1998). In brief, a guide cannula (0.5 mm in outer diameter) was implanted in the frontoparietal cortex (1.7 mm anterior to bregma, 3.7 mm lateral to midline, 1.5 mm below the surface of the skull) under pentobarbital (50 mg/kg i.p.) anesthesia according to the atlas of Paxinos and Watson (1982). The rats were allowed at least 24 h recovery time after the implantation of the guide cannula. Then, 50 mM L-NAME, 50 mM D-NAME, or saline (all 6 μl) was injected into the right cerebral cortex through the guide cannula. After the injection of L-NAME, the injection cannula was left in place for 5 min to allow adequate diffusion of the solution.

After decapitation, the brain was immediately removed from the skull and transferred to ice-cold saline. The brain was sliced with piled blades arranged with 1-mm intervals. The right parietal cortex around the cannula route (60–80 mg) was dissected from the slices and stored at −80°C until use.

Results

SNAP, an NO Donor, Inhibited Baseline NGF Release from Glial Cells. Primary mixed cultures of glial cells were prepared from rat cortex on postnatal day 3. Immunocytochemical analysis showed that astroglia occupied about 53% (SD = 4.6%), whereas microglia occupied about 19% (SD = 5.2%) of the cell population (Fig. 1). Generally, it is accepted that cultured glial cells secrete NGF at a basal level, which can be regulated by inflammatory factors such as LPS, TNFα, or IL-1 (Yoshida and Gage, 1991; Hattori et al., 1993; Galve-Roperh et al., 1997). These factors are also known as inducers of NO; therefore, we speculated that NGF release may also be regulated by NO. To explore this phenomenon, we directly applied exogenous NO to cell cultures. This was accomplished by using an NO donor, SNAP, which releases NO spontaneously and is widely used as a source of NO because it is less cytotoxic than other NO donors (Garg and Hassid, 1993). Various doses of SNAP (30 to 1000 μM) were added to the cultures, and the supernatants were collected for the determination of NGF protein after 24 h. Measurement of nitrite accumulation in the supernatants confirmed that the expected release of NO from the donor did occur

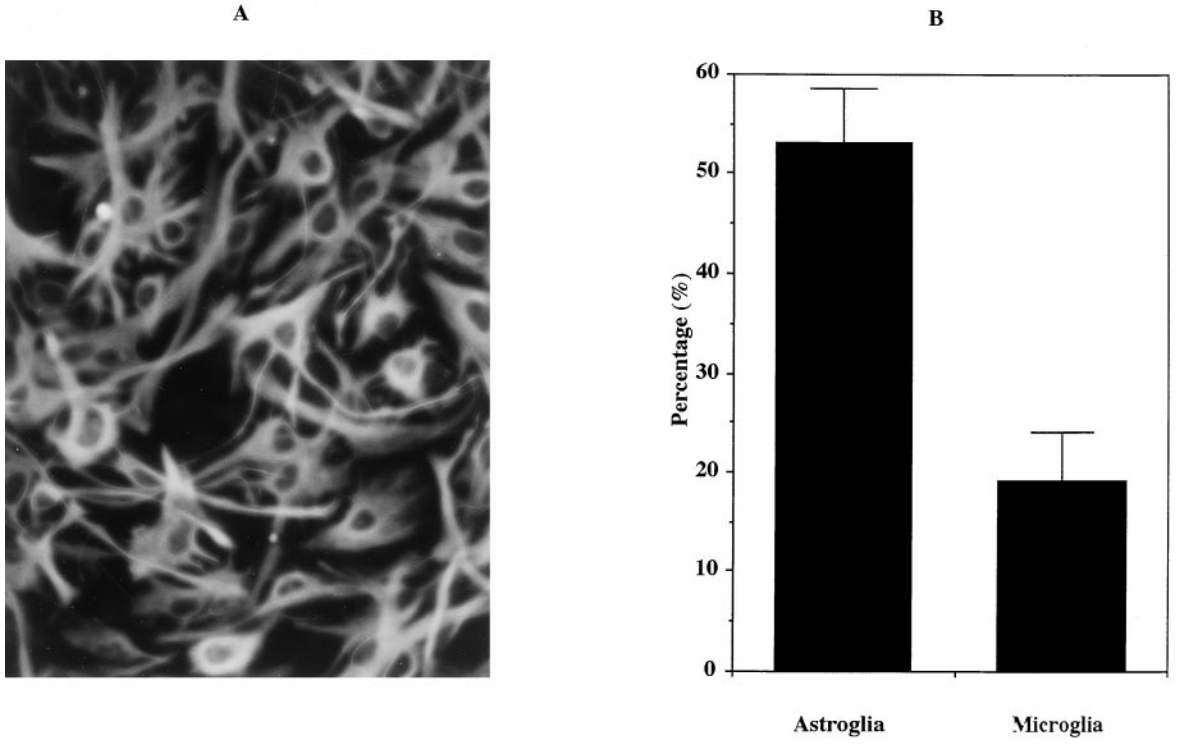


Fig. 1. Percentages of astroglial and microglial cells in primary mixed glial cell culture. Cells were immunnostained with rabbit anti-GFAP or mouse anti-rat CD11b antibodies, respectively. Then, the positive cells were counted under a fluorescence microscope, and the percentages of astroglia and microglia were calculated. A, immunostaining of cultured glial cells with rabbit anti-GFAP antibody. B, percentages of astroglia and microglia in the primary mixed glial cell culture. The data are representative of three consecutive experiments.

(data not shown). The results showed that SNAP inhibited basal NGF release in a dose-dependent manner (Fig. 2), which suggested that NO down-regulated NGF release from glial cells.

Cyclic GMP (cGMP) Inhibited Baseline NGF Release from Glial Cells. cGMP has been reported to be essential for NO signal transduction in several biological systems (Ignarro, 1991). To know whether cGMP is also involved in the down-regulation of NGF release by NO in our system, the primary mixed glial cells were incubated with various concentrations of dibutyryl-cGMP and dibutyryl-cyclic AMP (cAMP) for 24 h. Then, supernatants were determined for NGF protein level. The results showed that second messenger cGMP significantly inhibited the basal NGF release in a dose-dependent way as the NO donor did but that cAMP had no effect. The data suggested that NO suppressed NGF release, presumably through a cGMP-signaling pathway (Fig. 3).

Inhibition of NO Resulting in Enhancement of LPS-Induced NGF Release. The data above indicated that exogenous NO had a negative effect on NGF release in the primary mixed glial cell cultures. Now we wanted to know whether endogenous NO is involved in the regulation of NGF release. The primary mixed glial cells were stimulated with various doses of LPS or LPS plus IFNγ for 24 h; then, total cellular RNA and protein were extracted. RT-PCR was performed for the detection of NGF and iNOS mRNA expressions, and Western blotting was done for the detection of iNOS protein. The supernatants were collected for the determination of NGF protein levels and nitrite accumulation. Even at a very low concentration (0.01 μg/ml), LPS alone was able to induce NGF gene expression in a dose-dependent manner (Fig. 3C). In addition, LPS enhanced iNOS gene expression (Fig. 4A). Costimulation with LPS plus IFNγ similarly resulted in further enhancement of iNOS mRNA, its protein, and nitrite accumulation (Fig. 4, A–C). However, this combination significantly suppressed NGF mRNA expression and its protein level in the supernatants (Figs. 4D and 5). Nevertheless, IFNγ itself had no effect on NGF release (data not shown). In an attempt to elucidate the involvement of endogenous NO in the regulation of NGF release, NMMA, an NOS inhibitor was used in the system. We found that 1 mM NMMA could completely inhibit LPS- or LPS plus IFNγ-induced NO production; however, the inhibitory effect of IFNγ on LPS-induced NGF release was reversed (Fig. 5).

SNAP Inhibited LPS-Induced NGF mRNA Expression. The fact that inhibition of endogenously produced NO enhanced LPS-induced NGF release suggested that NO is involved in the regulation of NGF release from mixed glial cells. Next, we tested whether exogenous NO can influence LPS-induced NGF mRNA expression. Mixed glial cells were stimulated with LPS (1 μg/ml) in the presence of SNAP for 12 h. Then, total RNA was extracted from the culture, and quantitative RT-PCR was performed for the detection of NGF mRNA expression. The data showed that SNAP inhibited NGF expression in a dose-dependent manner and that cGMP inhibited NGF expression as well. However, NMMA moderately enhanced NGF mRNA expression (Fig. 6). The results suggested that NO did influence NGF release by regulating NGF expression at the transcriptional level.

Different Contributions of Astroglial and Microglial Cells to NGF Release and NO Production. The above data showed that NO can regulate NGF release in the mixed glial cell cultures. Next, we asked the question, "Which cell type is mainly responsible for NGF release and which cell type is responsible for NO production?" To answer the questions, we purified astroglial and microglial cells from mixed cultures and stimulated these cells with LPS or LPS plus IFNγ. After 24 or 48 h, supernatants were collected for determination of NGF release or nitrite accumulation. It was

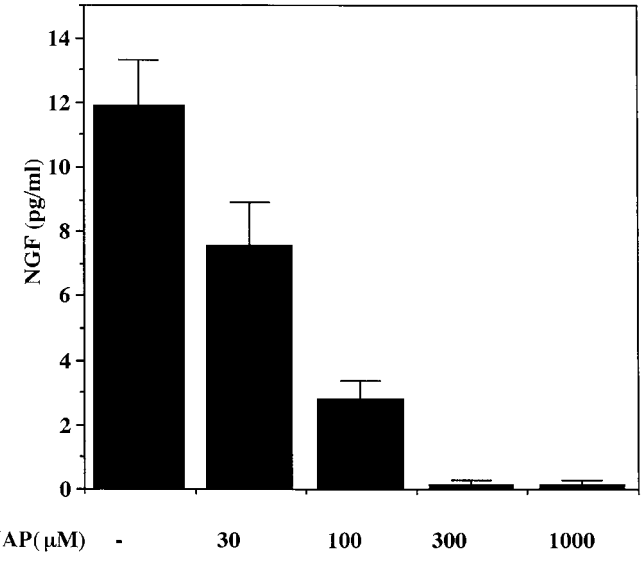


Fig. 2. SNAP inhibited basal NGF release in primary mixed glial cell culture. Cells were stimulated with SNAP at various concentrations (30 to 1000 μM) for 24 h. Then, supernatants were collected for the determination of NGF protein level by an NGF EIA. The data are representative of three similar experiments.

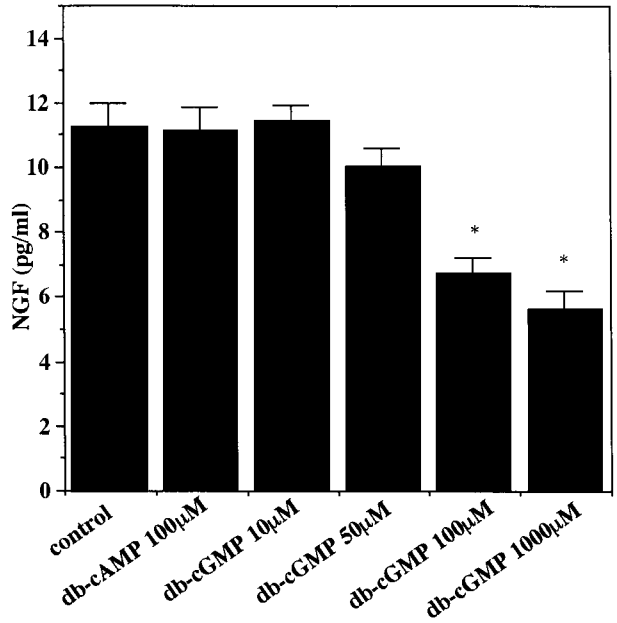


Fig. 3. cGMP inhibited basal NGF release from primary mixed glial cells. The cells were stimulated with various concentrations of dibutyryl-cGMP (10–500 μM) or dibutyryl-cAMP (100 μM) for 24 h. Then, the supernatants were collected for the determination of NGF protein levels by specific NGF EIA. The results are representative of three similar experiments. *$P < .05$ compared with control (ANOVA followed by Duncan's multiple range test).

shown that LPS greatly enhanced NGF release but resulted in just weak nitrite accumulation in the astroglial cell cultures. However, in the microglial cell cultures, LPS greatly induced NO production on determination of nitrite accumulation, but there was no significant change in NGF release in the supernatants (Fig. 7, A and B). The data indicated that astroglial cells were the major cell type responsible for NGF release and that microglial cells were mainly for NO production. The results suggested that NO derived from microglial cells down-regulated NGF release from astroglial cells.

L-NAME Enhanced NGF Protein Level In Vivo. L-NAME has been used for in vivo experiments and proved to be effective for NOS inhibition in the neocortex (Ayers et al., 1997). Thus, we chose L-NAME instead of NMMA and injected it into the anterior part of the cerebroventricle of adult rats to assess its effects on NGF protein level in vivo. We administered L-NAME twice within a 14-h interval to optimize the effects of the NOS inhibitor. D-NAME (an inactive stereoisomer of L-NAME) and saline were also injected as controls. Fourteen hours after the second injection, the cortical tissue was taken and cut into 1-mm-thick slices. NGF protein level was measured in the neocortical slice located at the position of the cannula, and the NOS enzyme activity was monitored in its adjacent section. In the brain tissue around the cannula route, NGF protein content was significantly increased by administration of L-NAME but not D-NAME or saline (Fig. 8A). Endogenous NOS activity was still significantly inhibited in the adjacent section by L-NAME even 14 h after its injection (Fig. 8B). In contrast, D-NAME had no apparent effect at that point compared with saline. This observation was consistent with the result obtained in vitro, suggesting that NO exerts the negative effect on NGF production in vivo.

NGF Protein Level in eNOS and nNOS Mutant Mice. To further confirm the regulatory role of NO on NGF protein level in vivo, both eNOS and nNOS mutant mice were investigated. NGF protein levels were determined by NGF EIA. The results showed that there were no significant changes in NGF protein levels in neocortex of nNOS mutant mice compared with wild-type control, but NGF protein levels were significantly elevated in eNOS mutant mice (Fig. 9). The results did suggest that NO regulates NGF protein release in the CNS, although the contribution of individual NOS isoform is controversial.

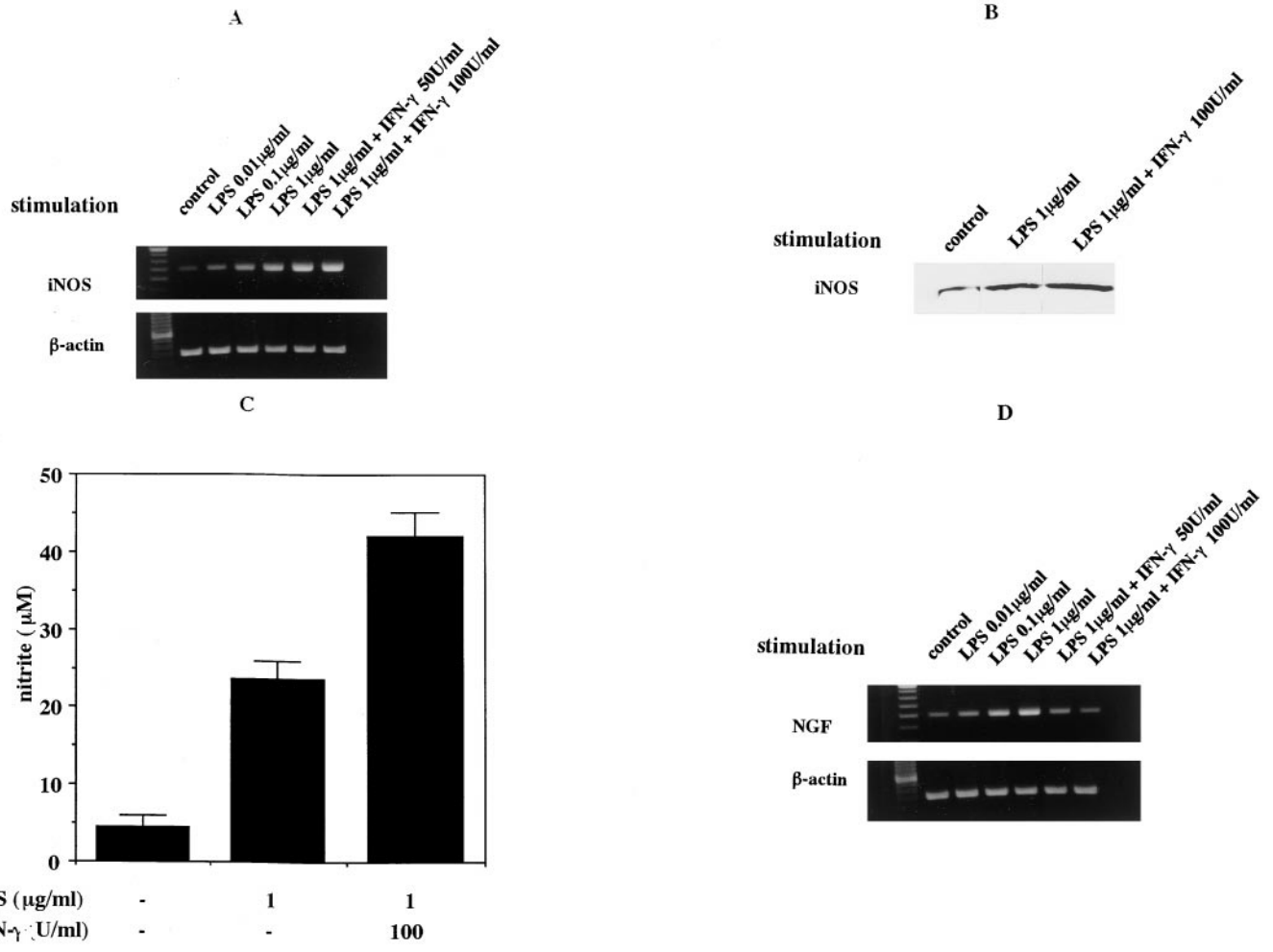


Fig. 4. Costimulation with LPS plus IFNγ enhanced iNOS expression but inhibited NGF expression. Cells were stimulated with LPS or LPS plus IFNγ at various concentrations for 12 h (RNA) or 24 h (protein). Then, total RNA and protein were extracted for RT-PCR and Western blotting. A, LPS plus IFNγ enhanced iNOS mRNA expression. B, LPS plus IFNγ enhanced iNOS protein expression. C, LPS plus IFNγ enhanced nitrite accumulation. D, LPS plus IFNγ inhibited NGF mRNA expression. The results are representative of three similar experiments.

Discussion

In the nervous system, NO is a gaseous modulator to which several diverse functions have been ascribed, including effects on synaptic transmission, plasticity, neurotoxicity, and development (Schuman et al., 1994; Bredt and Snyder, 1994; Garthwaite and Boulton, 1995). NGF is another diffusible bioactive molecule, which is mainly known as a neuronal survival factor, in both the peripheral nervous system and CNS (Varon et al., 1995). But so far, there has been no report about the mutual interaction between NO and NGF in the CNS. In the present study, we demonstrated that both endogenous and exogenous NO regulates NGF gene expression and its protein release via cGMP pathway. These observations suggest that there exist the potential interaction and cooperative function of these two signaling molecules in the brain.

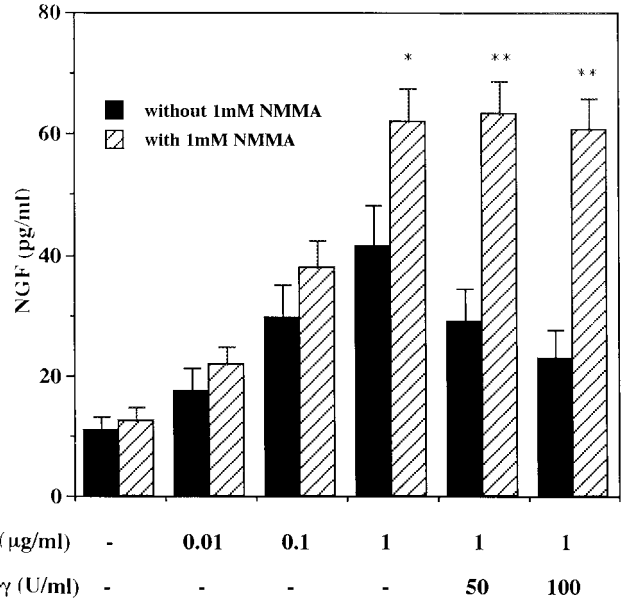
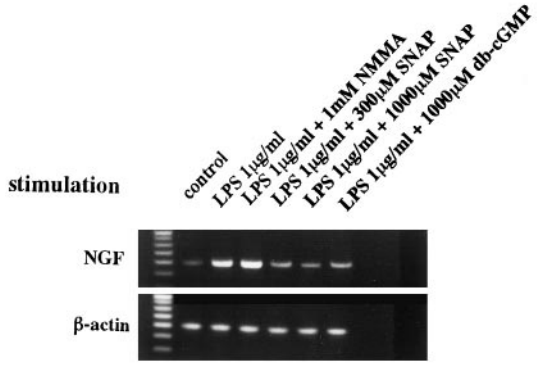


Fig. 5. Inhibition of endogenous NO enhanced NGF release in primary mixed glial cell culture. Cells were stimulated with LPS or LPS plus IFNγ in the presence of 1 mM NMMA to inhibit endogenous NO for 24 h. Then, supernatants were collected for the determination of NGF protein level by specific NGF EIA. The data are representative of three consecutive experiments. $*P < .05$, $**P < .01$ compared with those without NMMA (ANOVA followed by Duncan's multiple range test).

Various cells in the CNS, including both neurons and glial cells, are reported to have the ability to synthesize NO (Murphy et al., 1993). Glial cells and some hippocampal neurons can synthesize NO in a calcium-independent way but require the induction of iNOS expression. eNOS was found to be associated with the brain vasculature in the CNS. By contrast, nNOS is most abundant in intrinsic GABAergic neurons in the neocortex, and it initiates NO synthesis in response to calcium signaling (Bredt and Snyder, 1994). In the present study, we prepared mixed glial cell cultures from neonatal cortices and grew them in the medium containing 10% horse serum. In the mixed cultures, more than 50% of the cultured cells exhibited immunoreactvity for the astroglial marker GFAP, and about 19% of the cells showed immunoreactivity for the microglial marker CD11b. After stimulation with LPS or LPS plus IFNγ, the mixed glial cells expressed iNOS, which resulted in the enhanced production of nitrite, whereas there was almost no significant changes in nNOS and eNOS gene expressions (data not shown). These results indicate that NO produced endogenously in these cultures was mainly derived from iNOS and that the contributions of nNOS and eNOS were almost negligible.



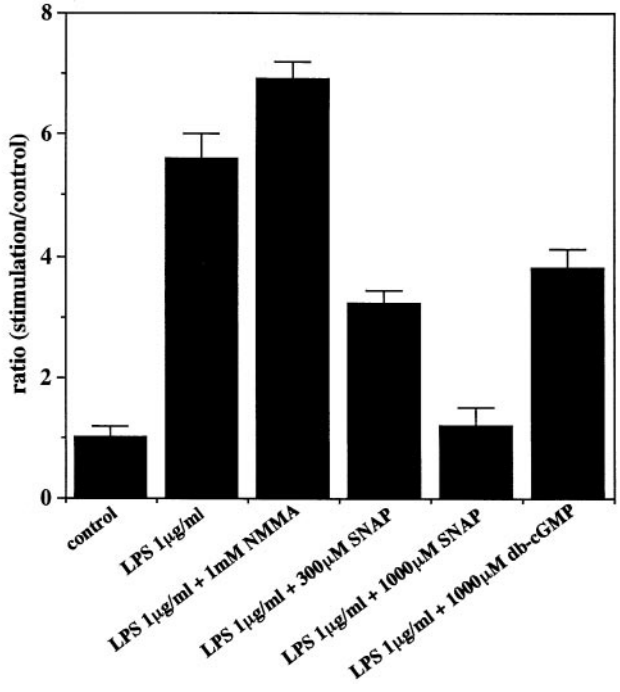


Fig. 6. SNAP suppressed LPS-induced NGF mRNA expression. Cells were stimulated with LPS in the presence of SNAP, dibutyryl-cGMP, and NMMA for 12 h. Then, total RNA was extracted, and cDNA was made for the performance of RT-PCR. To more accurately quantify mRNA levels, NGF cDNA amplified by nonsaturated PCR cycles ($n = 20$) was separated in an acrylamide gel and stained with the sensitive dye SYBR Green I (with a detectable range of 5–1000 pg DNA), and the fluorescence intensity of the products was quantified using a fluorescence image analyzer (Fuji FLA-2000; $n = 4$). The values were normalized with those of PCR products amplified from β-actin cDNA (15 PCR cycles).

There are several reports of the inhibitory effects of NO on the synthesis of growth factors and cytokines in various systems. In the ectodermal system, NO also influences the production of vascular endothelial growth factor to control endothelial integrity (Tsurumi et al., 1997). We also reported that NO down-regulates the production of the cytokine IFNγ by inhibiting T cell proliferation (Xiong et al., 1996). It has been suggested that the NOS gene serves as a growth arrest gene, initiating the switch to cytostasis during differentiation by unknown mechanism (Peunova and Enikolopov, 1995). In the CNS, we observed a similar effect of NO on NGF: NGF gene expression and protein release induced by LPS in mixed glial cells were consistently enhanced by an NOS inhibitor. The effect of the NOS inhibitor indicated that NO may func-

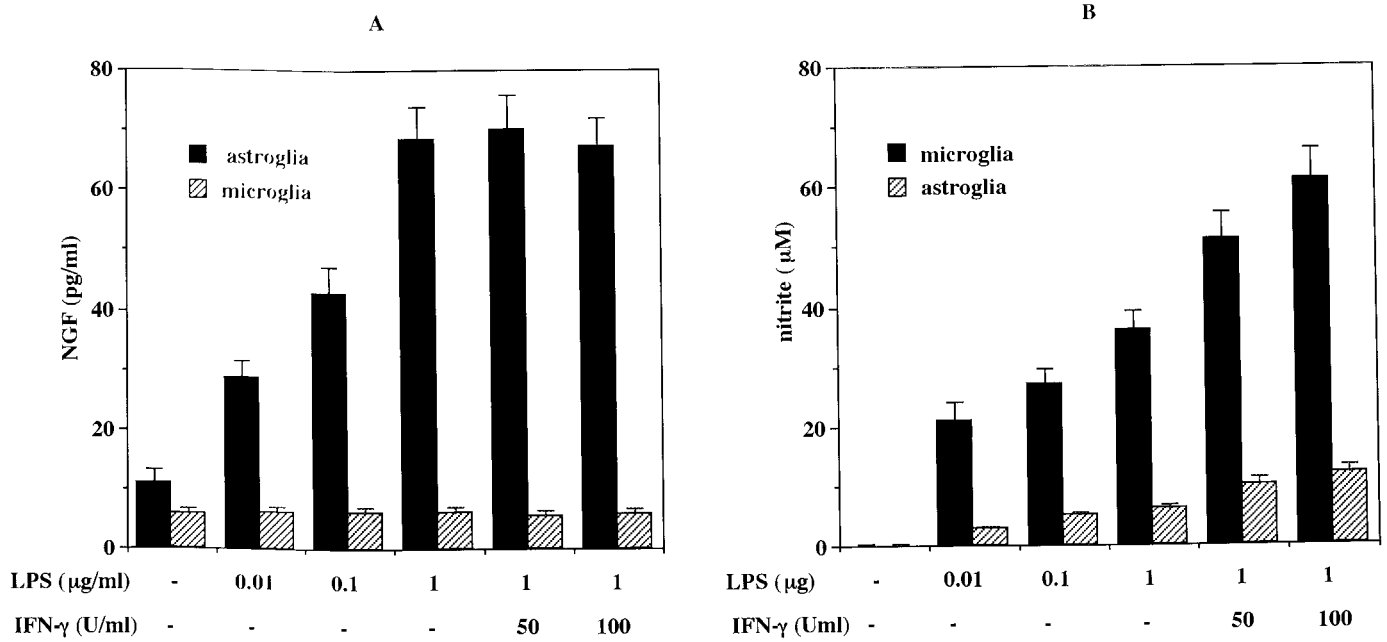


Fig. 7. Different contributions of astroglia and microglia to NGF release and NO production. Both purified astroglia and microglia were stimulated with LPS or LPS plus IFNγ for various time periods. Then, the supernatants were collected for the determination of NGF protein level and nitrite accumulation. A, different abilities of astroglia and microglia to release NGF. After stimulation with LPS or LPS plus IFNγ for 24 h, the supernatants were collected for the determination of NGF protein level by specific NGF EIA. B, different contributions of astroglia and microglia to NO production. After stimulation with LPS or LPS plus IFNγ for 48 h, the supernatants were collected for the determination of nitrite accumulation by Griess agents. The data are representative of three similar experiments.

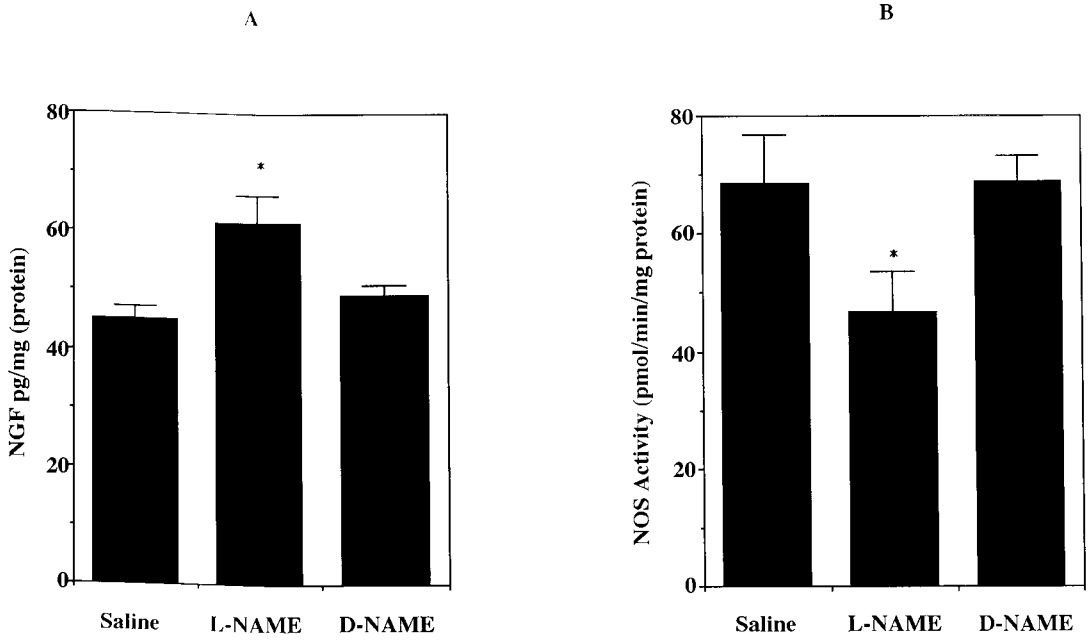


Fig. 8. Effects of intraventricular L- or D-NAMEs on NGF content in neocortex. Then, 50 mM L-NAME, 50 mM D-NAME, or saline (all 6 μl) was injected twice into the right lateral ventricle through the anterior frontal cortex of adult male rats with a 14-h interval. The brain section containing the cannula route was homogenized, and NGF was measured by a specific NGF EIA in A. To confirm the drug effect, the adjacent neocortical slice was also examined for its NOS enzyme activity as shown in B. $^*P < .05$ compared with control (ANOVA followed by Duncan's multiple range test).

tion as a negative regulator for NGF release. By contrast, after we stimulated glial cells with the combination of LPS and the NO donor, NGF gene expression was suppressed. To confirm these results in vivo, we administered L-NAME, another NOS inhibitor, directly into brain, and the NGF level around the injection site was determined by EIA. We found that the NGF level was enhanced only in the group treated with L-NAME. Both in vitro and in vivo results implied that NO suppressed NGF production at the transcriptional level.

In nNOS mutant mice, we did not find a significant difference of NGF protein level in neocortex compared with wild-type mice, but in eNOS mutant mice, the NGF protein level was significantly increased. It may be explained that eNOS, initially believed to be present only in endothelial cells, is the main isoform in CA1 pyramidal cells (Dinerman et al., 1994). It is quite possible that in nNOS mutant mice, NO production would be compensated for with eNOS; then, NGF protein level in the brain tissue did not change. But in eNOS mutant mice, NGF protein level was elevated, suggesting that NO derived from eNOS similarly contributed to the regulation of NGF release.

In the CNS, astroglial cells occupy a critical position in the regulation of neuronal function during development and in the mature brain. The function of astroglial cells in development and brain injury is ascribed to the ability of these cells to undergo a proliferative or reactive phase under certain conditions. Proliferative astroglial cells synthesize and secrete a number of diffusible neurotrophic factors; among them, NGF has been best characterized. In the present study, we hypothesized that astroglia may be the main cell source for NGF release that can be regulated by NO derived from microglia. To test this, we purified astroglial cells and microglial cells, respectively. When astroglial cells were stimulated with LPS or LPS plus IFNγ, a 6-fold increase in NGF in the supernatant was observed, instead there was only minor change of nitrite accumulation. When microglial cells were stimulated with LPS, nitrite production was greatly enhanced; instead only minor change of NGF release was observed. Taken together, it may be concluded that astroglial cells are the main cell types for NGF release, and NO derived from microglial cells regulates NGF release from astroglial cells.

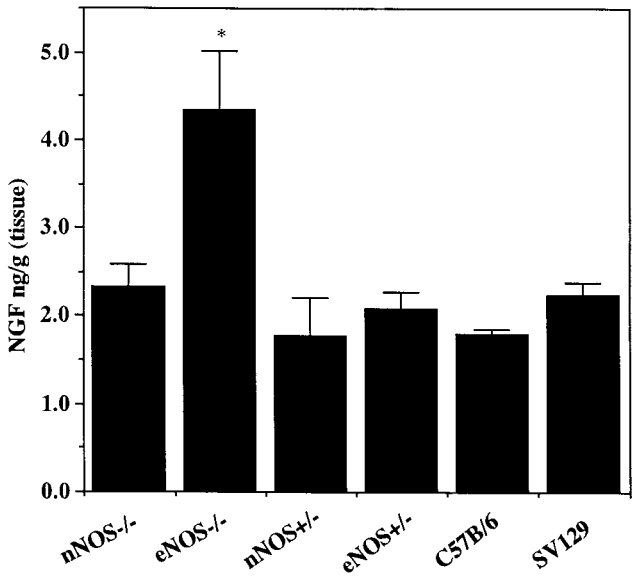


Fig. 9. The NGF protein levels in nNOS and eNOS mutant mice. Neocortices were dissected from both 7-week-old mutant and wild-type mice. After homogenization, 100 μl of each tissue extract was measured by a specific NGF EIA (see *Materials and Methods*). $^*P < 0.05$ compared with control (ANOVA followed by Duncan's multiple range test).

Although the target molecules, or receptors, of NO effects on various cells remain unclear, recent biochemical investigations suggest that there are multiple biological targets for NO with different sensitivities; such as the enzymes, guanylyl cyclase (Zhuo et al., 1994), ADP-ribosyltransferase (Schuman et al., 1994), and the *N*-methyl-D-aspartic acid receptor (Lei et al., 1992). Among those pathways, it is generally agreed that the NO/cGMP pathway involving NO-mediated activation of soluble guanylyl cyclase is needed to provoke its signal transduction (Ignarro, 1991). In the vasculature, NO/cGMP signaling is important for the regulation of blood pressure and platelet function (Moncada and Higgs, 1991; Moncada et al., 1991); in the brain, this pathway controls the release of neurotransmitters, such as glutamate and acetylcholine (Garthwaite and Boulton, 1995). In the present study, we found that the stimulation with cGMP inhibited basal NGF release. This means that the down-regulation of NGF may depend on cGMP pathway.

In conclusion, our results demonstrate that NO down-regulates NGF release both in vitro and in vivo. This inhibition may depend on cGMP pathway. In this regard, it may be reasonable to assume that NO, mainly produced by microglial cells, suppresses NGF derived from astroglial cells and serves as a negative feedback mechanism.

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Send reprint requests to: Huabao Xiong, M.D., Ph.D., Department of Molecular Neurobiology, Brain Research Institute, Niigata University, 1-757, Asahimachi-dori, Niigata 951-8585, Japan. E-mail: xionghbl@bri.niigata-u.ac.jp