**Journal of NeuroVirology (1998) 4,** 445–450

http://www.jneurovirol.com

*Short Communication*

# Expression of inducible nitric oxide synthase in the brains of scrapie-infected mice

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**The neuronal cell damage caused by inducible nitric oxide synthase (iNOS) in brain has been reported to be associated, at least in part, with many neurodegenerative diseases including Alzheimer's disease. We recently observed vacuolation and astrocytosis in the brains of ME7 scrapie strain-infected C57BL mice. To investigate if these phenomena might have a relationship to iNOS, the level of iNOS expression was measured immunohistochemically and molecular biologically in the brains of scrapie-infected C57BL mice. The number and size of astrocytes were increased and immunoreactivity of glial fibrillary acidic protein (GFAP) was significantly enhanced. iNOS immunoreactivity was observed in the astrocytes of the scrapie-infected group, but not in the control group. iNOS mRNA levels were increased in scrapie-infected mice compared to the levels in non-infected mice of the same age. Our results suggest that iNOS induction in reactive astrocytes is a part of the neurodegenerative mechanisms in scrapie infection.**

**Keywords:** scrapie; TSE; iNOS; astrocyte

## Introduction

Scrapie is a transmissible neurodegenerative disease of sheep and goats which can be transmitted experimentally to small rodents (Carp *et al*, 1994). The symptoms of scrapie include ataxia, weakness, lethargy, and eventual immobility and death. Histopathological changes in the brain include neuronal cell loss, spongiform changes, and astrocytosis. The pathogenesis of scrapie, the animal prototype of human transmissible spongiform encephalopathy (TSE), is associated with the conversion of a normal protein, $PrP^{C}$, into an abnormal form of protease resistant protein, $PrP^{Sc}$, by a post-translational process that probably involves conformational changes (Prusiner, 1987). This abnormal isoform, $PrP^{Sc}$, accumulates in astrocytes and neurons and polymerizes into amyloid plaques. Deposition of insoluble amyloids in astrocytes and reactive microglia is the hallmark of neurodegenerative disease including TSE (Kim *et al*, 1990; Manuelids, 1994; 1997; Wisniewski *et al*, 1994). The free radical nitric oxide (NO) is an inorganic gas that mediates a variety of biological functions, including vasodilation, neurotransmission, neuromodulation, and cytotoxicity (Bredt and Snyder, 1994; Moncada *et al*, 1991; Nathan, 1992). NO synthase (NOS) produces NO from L-arginine, and at least three isoforms of NOS have been characterized. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, but they produce NO at low levels (Moncada *et al*, 1991; Nathan, 1992). However, an inducible, calcium-independent isoform of NOS, termed iNOS, is expressed in cells of various origins, e.g., macrophages, microglial cells, and reactive astrocytes when these cells are exposed to pro-inflammatory cytokines or microbial products *in vitro* (Lowenstein and Snyder, 1992; Marletta, 1993). Recent studies demonstrated that NO produced by iNOS causes damage to brain cells, a process which has been demonstrated in many neurodegenerative diseases. NO synthesized by iNOS is known to exert numerous toxic effects in a wide variety of mammalian cell targets (Korproski *et al*, 1993). Thus, it is possible that brain cell damages in TSE might be associated with increased levels of NO which would be related to enhanced expression of iNOS. To investigate the induction of iNOS in ME7 scrapie-infected brain, the protein and mRNA levels

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Received 16 September 1997; revised 4 December 1997; accepted 18 December 1997

of iNOS were analyzed. Immunohistochemical staining for iNOS was observed in astrocytes of scrapie-infected mice, but not in controls. iNOS mRNA was significantly increased in the scrapie-infected mice. Thus, iNOS induction in scrapie may be involved in the pathological changes induced in brain cell.

## Results

*GFAP immunoreactivity in the hippocampus*

In order to assess the neuropathological characteristics of scrapie-infected and control mice, brains were stained with anti-GFAP antibody; GFAP is an astrocyte-specific protein. After a 150 day incubation, astrocytosis and vacuolation were detected in the hippocampus of scrapie-infected C57BL mice. In contrast, there were far fewer immunoreactive cells in the hippocampus sections of control animals, and the intensity of GFAP immunoreactivity in the perinuclear region and cytoplasmic processes of astrocytes was minimal (Figure 1a, c). In the scrapie-infected group, GFAP immunoreactivity was significantly increased in intensity compared to control mice; staining of cell bodies and cytoplasmic processes of GFAP-positive astrocytes was increased markedly in brains from scrapie animals (Figure 1b, d).

*iNOS immunoreactivity in the hippocampus*

In control animals, there were no iNOS immunoreactive cells in the hippocampus (Figure 2a, c). In contrast, in scrapie-infected mice, iNOS immunoreactivity was markedly increased in the hippocampus (Figure 2b, d). The distribution and the shape of iNOS-positive cells yielded a pattern similar to that seen for GFAP-positive cells.

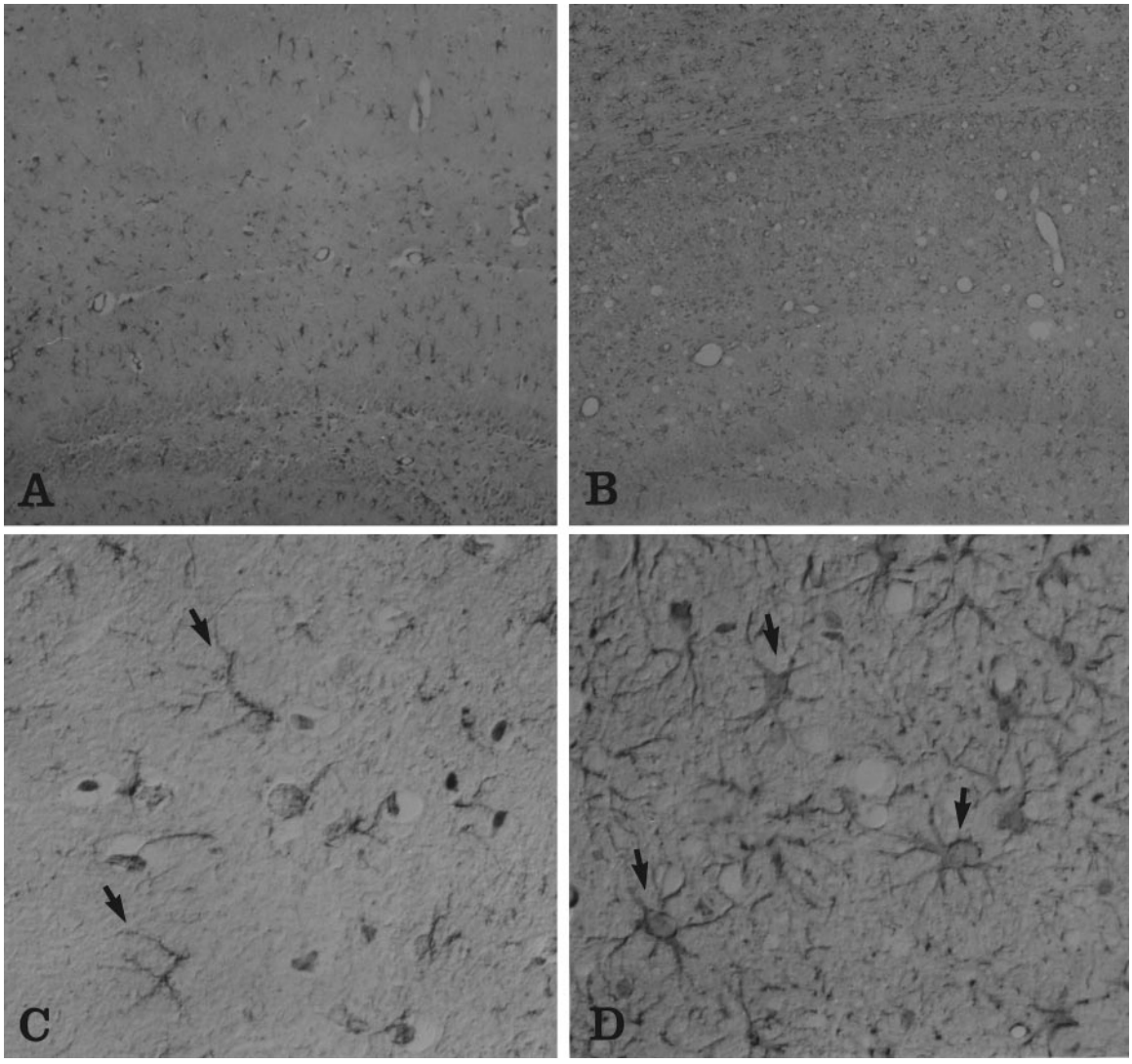


**Figure 1** GFAP immunoreactivity after infection of mice with ME7 scrapie agent. Sections from control (**a, c**) and scrapie infected mice (**b, d**) were immunostained for GFAP. Immunoreactivity of astrocytes (black arrows) in scrapie infected mice appears to be increased compared with staining in control mice. **a**, **b** × 100; **c**, **d** × 400.

*Co-localization of GFAP and iNOS in the hippocampus*

To investigate whether iNOS expression occurs in GFAP-positive cells, we performed double immunostaining for both GFAP and iNOS. There were no cells doubly stained for both iNOS and GFAP in control brains (data not shown), but in the scrapie-infected group, some GFAP-positive cells were immunoreactive for iNOS (Figure 3a, b), indicating that many of the reactive astrocytes induced by scrapie infection express iNOS. It is possible that some of the perivascular cells that are immunostained for iNOS are microglia.

*Induction of iNOS expression in the brain*

To determine the induction of iNOS mRNA in the brains of scrapie-infected mice, RT–PCR and Southern blot analysis were performed. iNOS mRNA was expressed at a high level in scrapie-infected mice as compared to the control and uninfected SPF mice (Figure 4); in the latter groups, single faint bands were seen, representing the basal level. GPDH mRNA levels as constitutively expressed controls were virtually identical in scrapie, control and uninfected SPF mice (Figure 4).

## Discussion

*In vitro* iNOS expression in the central nervous system has been investigated mainly in astrocytes, microglia, and recently in neuronal cells by activating the respective cells with cytokines, such as interferon-$\gamma$ (IFN-$\gamma$), tumor necrosis factor-$\alpha$ (TNF-$\alpha$),

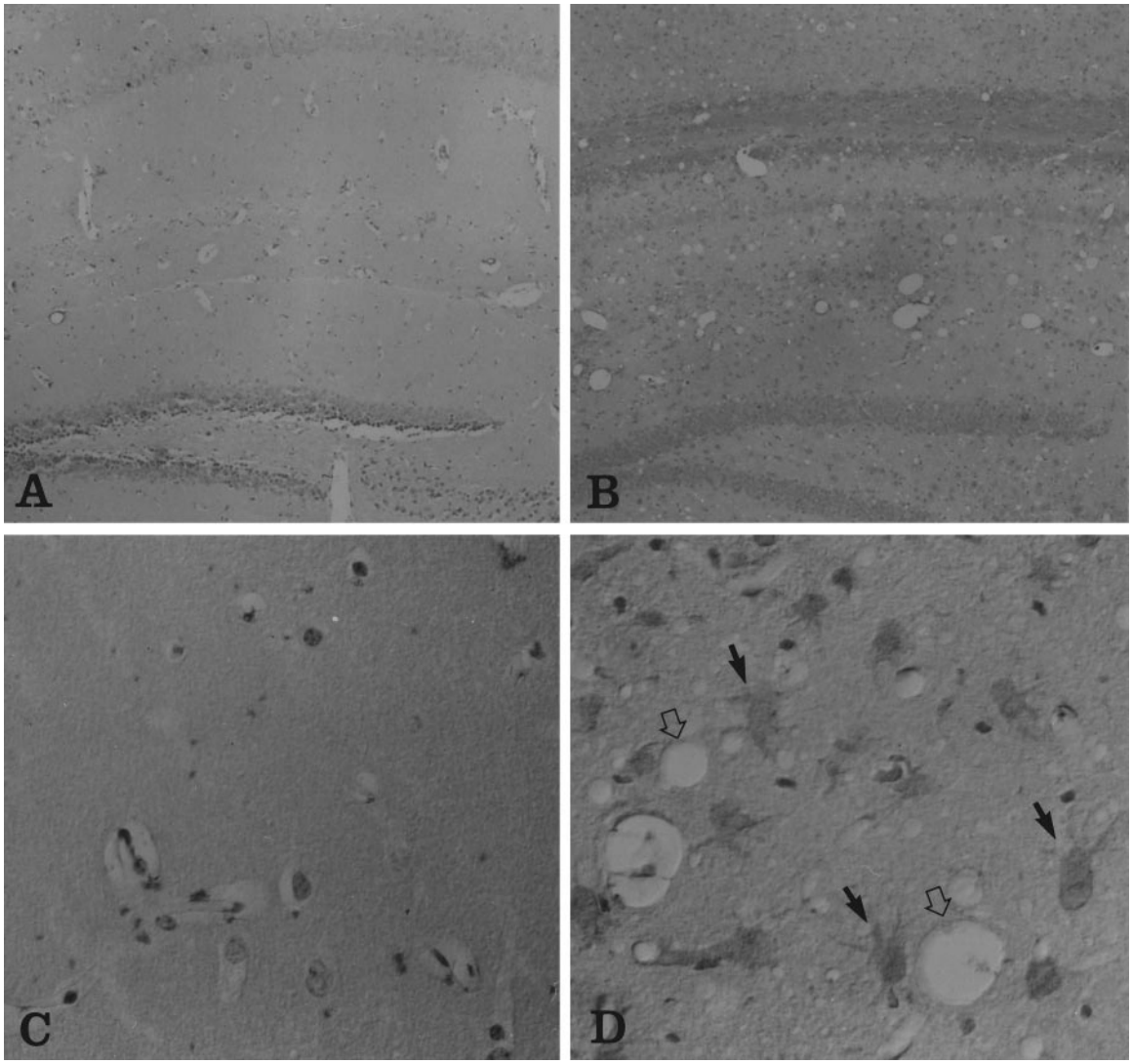


**Figure 2** iNOS immunoreactivity after infection of mice with ME7 scrapie agent. Sections from control (**a, c**) and scrapie infected mice (**b, d**) were immunostained for iNOS. There is no immunostaining in hippocampus of control sections. Strong immunoreactivity of iNOS associated with astrocytes (black arrows) of the hippocampus was detected in the scrapie infected mice. Loss of neurons is also evident (vacuolation, open arrows). **a**, **b** $\times 100$; **c**, **d** $\times 400$.

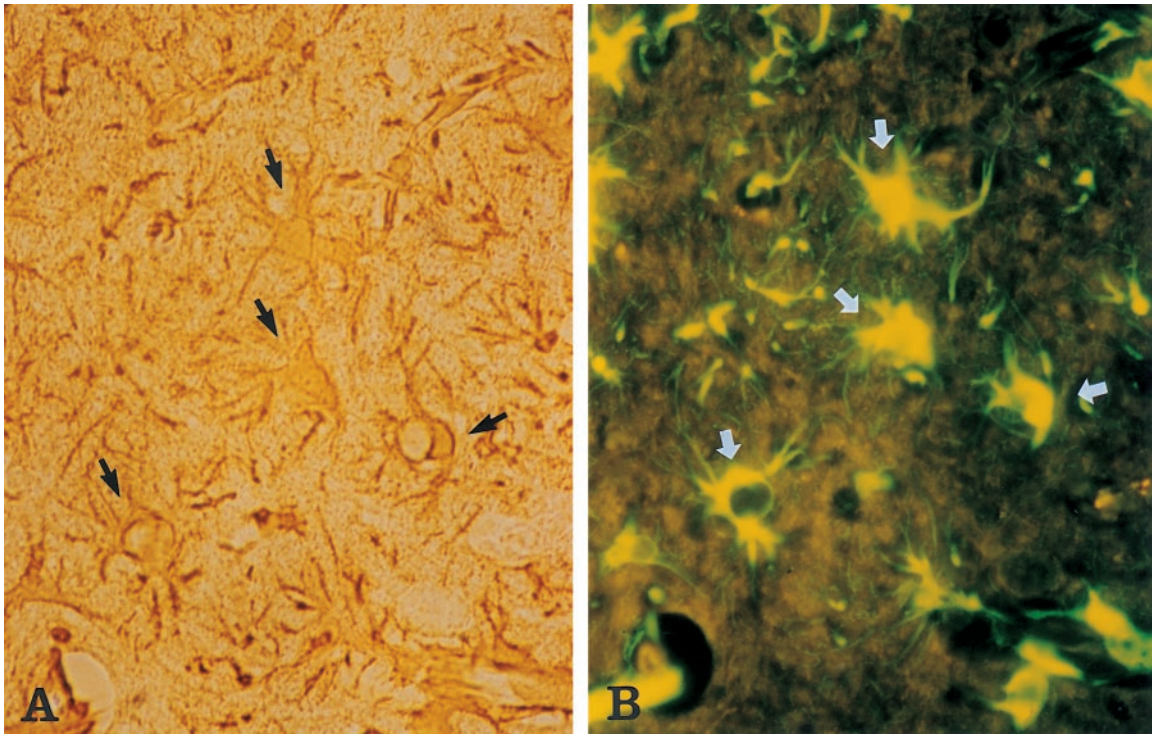


**Figure 3** iNOS expression in astrocytes after infection of mice with ME7 scrapie agent. Sections from scrapie infected mice (**a, b**) were immunostained for both GFAP and iNOS. GFAP immunoreactivity was revealed by DAB (black arrows) (**a**); iNOS immunoreactivity was revealed by FITC (white arrows) (**b**). ×400.

or lipopolysaccharide (LPS). Astrocytes treated with LPS, IFN-$\gamma$, interleukin-1$\alpha$ (IL-1$\alpha$), IL-1$\beta$ or TNF-$\alpha$ induced iNOS (Selmaj *et al*, 1990; Vigne *et al*, 1993). Sean *et al* (1993) reported that about 20% of cultured astrocytes were strongly stained with the anti-iNOS polyclonal antibody for iNOS when they were activated with LPS. The majority of iNOS-positive cells were proven to be astrocytes, since more than 95% of cultured cells expressed GFAP. In our studies, vacuolation and astrocytosis were detected in the brains of ME7 scrapie-infected C57BL mice. Immunohistochemical staining for iNOS was clearly observed in astrocytes of scrapie-infected mice, but not in the controls. Analysis of iNOS mRNA expression showed that induction occurred in scrapie-infected mice, whereas noninfected mice of the same age showed only a low basal level of iNOS expression. Other investigators could not detect any expression of iNOS in healthy brain (Korproski *et al*, 1993). In our study, the brains of uninfected SPF mice contained iNOS mRNA at the same level as that of the C57BL mice injected with control homogenates. Thus, results demonstrated that iNOS was expressed at a basal level in the brain, but the level of iNOS mRNA was dramatically augmented when mice were infected with scrapie. Increased expression of GFAP was detected in the hippocampus of scrapie-infected mice; astrocytosis may be the result of astrocyte proliferation or hypertrophy. Enhanced activation of

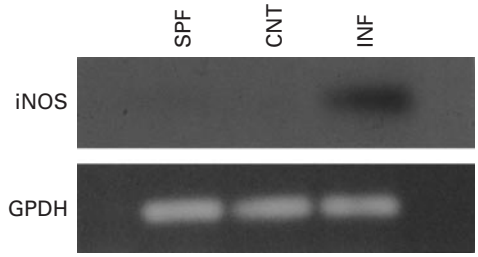


**Figure 4** Detection of iNOS mRNA expression by RT–PCR and Southern blot analysis in total RNA from whole brains from ME7 infected mice (INF) and in control (CNT) and uninfected SPF mice. As a control, RT–PCR products were standardized by analysis of constitutive expression of GPDH.

astrocytes suggests the possibility that astrocytes may be involved in the formation and deposition of insoluble amyloid plaque (Diedrich *et al*, 1991). One hypothesis is that $PrP^{Sc}$ deposited in astrocytes induces cytokines which in turn increase the synthesis of iNOS; the resulting NO would then cause neurodegeneration. This hypothesis is supported, in part, by the following results: The infection of mice with scrapie results in significant increases in the expression of the IL-1$\alpha$, IL-1$\beta$, and TNF-$\alpha$, whose pattern correlates with the onset and development of molecular and clinical pathologic changes (Campbell

*et al*, 1994). Recent studies indicated that TNF-$\alpha$ and IFN-$\gamma$ induced iNOS in astrocytes *in vitro* (Kong *et al*, 1996). Although nNOS reduction in scrapie-infected mice and hamsters has been reported, there has been no previous studies of iNOS induction in scrapie-infected mice. Ovadia *et al* (1996) demonstrated that nNOS activity was markedly inhibited in brains of mice and hamsters infected with scrapie. The activity of nNOS was totally abrogated in neuroblastoma cells infected with scrapie. Ye and Carp (1994) observed that some of the NADPH histostaining neurons in scrapie-infected hamsters appeared to be atrophic and that there were fewer NADPH histostaining neurite networks in scrapie-infected animals than in controls. On the other hand, NO activated by iNOS is induced to a high level in reactive astrocytes, activated macrophages and microglial cell in the CNS. Overexpressed NO exerts a neurotoxic effect (Nathan, 1992). Our results are consistent with the concept that iNOS induction in astrocytes of scrapie-infected mice may be involved in neuronal cell damage in this disease. Studies of vacuolation, astrocytosis, and expression of iNOS in scrapie-infected brains may elucidate the pathogenic mechanisms of scrapie infection in mice.

## Materials and methods

*Mouse strain and scrapie strain*
Female C57BL mice, 4–6 weeks of age, were obtained from the Experimental Center of Hallym University and specific pathogen free (SPF) mice of the same age were obtained from Korea Research Institute of Bioscience and Biotechnology (Daejun, Korea). The ME7 scrapie strain was kindly provided by Dr Alan Dickinson (Edinburgh, UK). The animals were inoculated intracerebrally with 30 $\mu$l of 1% brain homogenate in 0.01 M phosphate-buffered saline (PBS). The brain homogenate was prepared from ME7-infected C57BL mice. Control inoculum was prepared from brains of uninfected C57BL mice.

*Preparation of tissue*
After the scrapie incubation of 150 days, at least five mice were sacrificed for immunohistochemistry. Animals were anesthetized with 16.5% urethane and then perfused transcardially with cold PBS followed by cold 4% paraformaldehyde in PBS. The brains were immediately removed, cut into blocks, postfixed in the same fixative for 1 h at 4°C, rinsed with PBS, dehydrated with ethanol and embedded in polyester wax (Polyscience, USA). Coronal sections of the brain (5 $\mu$m thick) were cut with a microtome. For reverse transcription of RNA followed by the polymerase chain reaction (RT–PCR) and Southern blot analysis, unfixed brains were immediately removed from anesthetized mice and stored at −80°C until analysis.

*Immunohistochemistry*
Brain sections were immunostained by the indirect immunoperoxidase procedure. Briefly, the sections were dewaxed with xylene and hydrated through graded ethanol. Endogenous peroxidase activity was blocked by placing the sections in 1.4% $H_2O_2$ in methanol for 15 min. For better permeability, sections were incubated in 0.05% Triton X-100 and rinsed in PBS. To stain for GFAP or inducible nitric oxide synthase (iNOS), the sections were incubated in the following order, 10% normal goat serum (Vector, USA) in PBS for 1 h at 22°C, primary antibody (see below) for 14 h at 4°C, peroxidase conjugated goat anti-rabbit IgG (1 : 50, Vector, USA) for 2 h at 22°C and 0.05% diaminobenzidine (DAB, Sigma, USA)/$H_2O_2$ for 3 min. Sections were counterstained with hematoxylin (Sigma, USA). The primary antibodies were rabbit anti-GFAP (1 : 300, Zymed, USA) or rabbit anti-mouse macrophage NOS (1 : 300, UBI, USA). To double stain for GFAP and iNOS, the sections were incubated in the following order, 10% normal goat serum for 1 h, anti-GFAP antibody (1 : 300) overnight at 4°C, peroxidase conjugated goat anti-rabbit IgG as a secondary antibody for 2 h, 0.05% DAB/$H_2O_2$ for 3 min, 10% normal goat serum for 1 h, anti-mouse macrophage NOS antibody (1 : 300) overnight at 4°C, and then FITC conjugated goat anti-rabbit IgG (1 : 50, Vector, USA) for 1 h.

*RT–PCR and Southern blot analysis*
Total RNA from whole mouse brain was isolated by a single-step guanidinium thiocyanate phenol-chloroform method (Chomczynski and Sacchi, 1987). RT–PCR and Southern blot analysis were performed as described (Shankar *et al*, 1991; Southern, 1975). The PCR was performed in a thermal cycler (Perkin Elmer Cetus, USA) for 30 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 90 s, and polymerization at 72°C for 3 min, followed by a final polymerization step of 10 min at 72°C. The nucleotide sequences of the oligonucleotide primers used for RT–PCR was the iNOS antisense 30mer, 5′-GTCGACGAGCCTCGTGGCTTTGGGCTCCTC-3′, and iNOS sense 30mer, 5′-GTCGACCT-CCGA-AGTTTCTGGCAGCAGCG-3′. The nucleotide sequence of the hybridization probe for Southern blot analysis was the 30mer, 5′-ACGTTCAGGAC-ATCCTGCAAAAGCAGCTGG-3′ (Korproski *et al*, 1993). The specific primers 5′-TGGTATCGTGGA-AGGACTCATGAC-3′ and 5′-ATGCCAGTGAGCTT-CCCGTTCAGC-3′ for glyceraldehyde-3-phosphate dehydrogenase (GPDH) have been used to quantify the amount of PCR products (Rainer *et al*, 1994).

## Acknowledgements

We thank Dr Seung-Uon Shin and Dr Jae-Hong Kim for their helpful comments and critical

reading of the manuscript. This work was supported by the SPECIAL FUND for UNIVERSITY RESEARCH INSTITUTE, Korea Research Foundation (1994), Ministry of Education and the 1996 Good Health R & D Project (#HMP 96-M-3-1045), Ministry of Health & Welfare, Republic of Korea.

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