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Effect of neurotropic virus infection on neuronal and inducible nitric oxide synthase activity in rat brain

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To elucidate the potential role of inducible nitric oxide synthase (iNOS) and neuronal constitutive nitric oxide synthase (cNOS) in the pathogenesis of virus-induced encephalopathy, the activities of both NOS isoforms were determined in the brains of rats infected with Borna disease virus (BDV) or rabies virus. iNOS activity strongly increased, whereas neuronal cNOS activity significantly decreased in a time-dependent manner after either BDV or rabies virus infection. Choline acetyltransferase activity in the brain remained unchanged during both virus infections, suggesting that the decrease in cNOS activity does not reflect a generalized neuronal loss. Immunohistochemistry and Northern blot analyses indicate that the decrease in neuronal cNOS activity is due to a decrease in cNOS protein and mRNA synthesis. These results suggest that both an excessive generation of NO by activated macrophages or microglia, as well as a decrease of NO production in neurons may contribute to the neuropathogenesis of neurotropic virus infections.

Keywords: inducible nitric oxide synthase; constitutive nitric oxide synthase; Borna disease virus; rabies virus

Introduction

The mechanisms by which virus infections cause neuronal damage are only understood in a few viral diseases of the central nervous system (CNS) in which there is evidence that the virus directly destroys the target cells. In many other cases of virus-induced encephalopathy, the virus does not directly destroy neuronal tissue, but causes indirect damage by altering neuronal functions (Lipkin *et al*, 1988a,b; Fu *et al*, 1993) or by triggering cell-mediated responses within the CNS (Doherty *et al*, 1976; Byrne and Oldstone, 1984; Carbone *et al*, 1988). Soluble factors, proinflammatory cytokines, proteases, free radicals, and neurotoxins produced by immune cells in particular are thought to play an important role in the process of neuronal destruction (Shankar *et al*, 1992; Koprowski *et al*, 1993).

Recently, attention has focused on the possibility that nitric oxide (NO), generated by nitric oxide synthases (NOS), directly damages neuronal cells (Koprowski *et al*, 1993; Murphy *et al*, 1993). In particular, the inducible NOS (iNOS), which can be induced by proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ, might play an important role in the process of neuronal damage (Nathan, 1992; Zheng *et al*, 1993). It has recently been shown that in Borna disease virus (BDV)-induced encephalopathy, the expression of interleukin-1 (IL-1), TNF, and IFN-γ in the brain (Shankar *et al*, 1992) correlates not only with the degree of inflammatory lesions in the CNS, but also with the expression of iNOS mRNA (Zheng *et al*, 1993). The localization of iNOS expression in brain lesions of BDV-infected rats, strongly suggests the involvement of NO in neuronal damage.

In addition to NO produced in activated macrophages or microglia, NO generated in neurons by the constitutive form of NOS (cNOS), which is believed to have important functions in long-term potentiation as well as long-term depression (Schuman and Madison, 1991; Shibuki and Okada,

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1991; Bliss and Collingridge, 1993; Zhuo *et al*, 1993), has also been suggested to be involved in neurodegenerative processes (eg excitotoxicity) (Dawson *et al*, 1991; Hyman *et al*, 1992).

To obtain a better understanding of the potential roles of iNOS and neuronal cNOS in the pathogenesis of neurodegenerative disease, we investigated the changes of both neuronal cNOS and iNOS activities in the brains of rats following BDV or rabies virus infection. Our results clearly show tht iNOS activity is strongly increased, whereas that of neuronal cNOS is significantly decreased in the brains of rats infected with BDV or rabies virus.

Results

Kinetics of cNOS, iNOS, and CoAT activities in BDV — or rabies virus-infected rat brain

The enzyme activities of both neuronal cNOS and iNOS were determined in the cerebrum and cerebellum of BDV-infected rats by quantitating the amount of [^{3}H] L-citrulline produced from [^{3}H] L-arginine by NOS in homogenates of rat brains. The activity of NOS that was not inhibited by the calmodulin inhibitor trifluoperazine was considered macrophage-type iNOS. In BDV-infected cerebrum, the time course changes in neuronal cNOS and iNOS activities were inversely related; whereas iNOS activity was induced and increased in a time-dependent manner (Figure 1a), cNOS activity decreased significantly after BDV infection (Figure 1b). Decreased neuronal cNOS activity which was already observed at 16 days post infection, declined to 51% of the activity found in brains of normal rats on day 20 post infection. A suppression of neuronal cNOS activity was also observed in rabies-infected cerebrum (Figure 2b) whereas iNOS activity marginally increased 6 days after rabies infection (Figure 2a). The decrease in cNOS activity in rat cerebrum was also confirmed by quantitative analysis of nitrite (data not shown). No significant changes in neuronal cNOS activity were observed in the cerebellum infected with either BDV (Figure 3) or rabies virus (data not shown).

To determine whether the decrease in neuronal cNOS activity during the course of BDV and rabies infection reflects a general neuronal dysfunction or neuronal death, CoAT activity in the cerebrum was measured. As shown in Figures 1c and 2c, CoAT activity remained almost unchanged in BDV-infected cerebrum and decreased only slightly in rabies-infected cerebrum on day 6 post infection.

Determination of expression of cNOS by immunohistochemistry and Northern blot analyses

To determine the level of cNOS expression in rat brain, adjacent coronal brain sections were reacted with either anti-cNOS (Figures 4 and 5) or anti-iNOS (Figure 6) antibodies. There is a general reduction of immunostaining for cNOS throughout the forebrain of BDV-infected (Figure 4a) rats as compared to control rats (Figure 4b). Areas with most pronounced reduction in cNOS immunostaining include the cerebral cortex and the caudate-putamen. The number of cerebrocortical and striatal neuronal cell bodies strongly staining for cNOS is markedly reduced and also intensity of cNOS immunostaining in neuronal cell bodies is reduced. The reduction in the intensity of neuronal cell body staining and in the number of cNOS positive cells is paralleled by a marked decrease in the number of cNOS positive neuronal processes and varicose nerve fibres (Figure 5). Cerebellar cNOS immunostaining appeared to be largely unaffected.

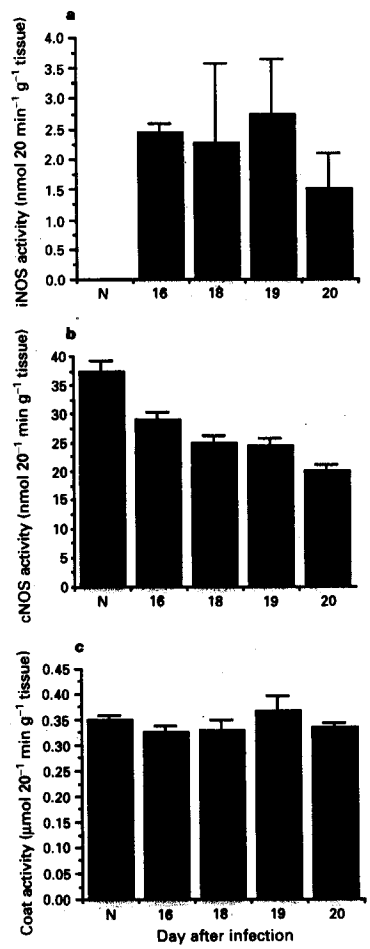


Figure 1 Time course of the activities of iNOS (a), cNOS (b), and CoAT (c) in the cerebrum of rats after BDV infection. Virus-infected brains were obtained 16, 18, 19 and 20 days after BDV infection. Homogenates were prepared from normal (N) and virus-infected cerebrums and subjected to enzymatic analyses of NOS and CoAT as described in Materials and Methods. Each column represents the mean value of enzymatic activities obtained from three cerebrums. Bars over columns represent standard errors. Statistical analysis (one-factor analysis of variance) revealed a significant decrease in cNOS activity in BDV-infected brains at 20 days post infection ($P = 0.001$).

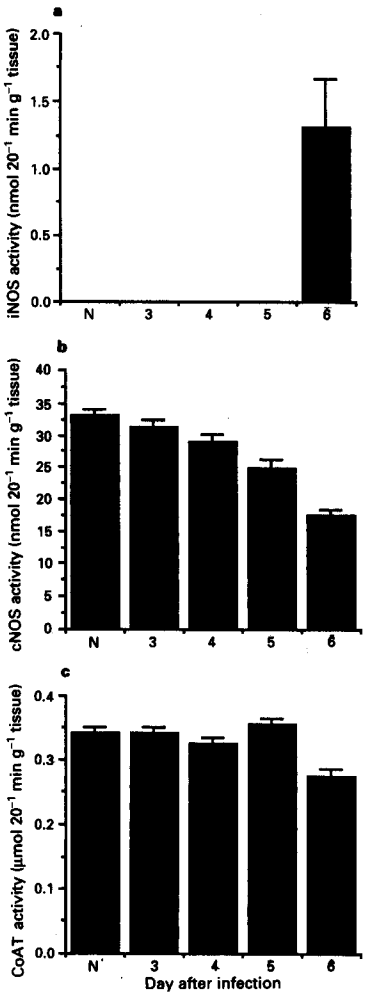


Figure 2 Time course of the activities of iNOS (a), cNOS (b), and CoAT (c) in the cerebrum of rats infected with rabies virus. The virus-infected brains were obtained 3, 4, 5, and 6 days after infection. The enzyme activities in the cerebrum homogenates were determined as described in Materials and Methods. Each column represents the mean value of enzymatic activities obtained from three cerebrums. Bars over columns represent standard errors. Differences in cNOS activity between normal and rabies virus-infected rats at 6 days post infection are significant ($P < 0.001$).

As reported previously (Zheng *et al*, 1993) iNOS immunostaining is absent from control brains (Figure 6a). In BDV-infected brains, numerous iNOS immunoreactive cells of non-ramified cytology are seen (Figure 6b). These cells are particularly frequent in perivascular regions and throughout the cortex of the forebrain with some higher concentrations in basolateral cortex where most of the damages is observed and in juxtameningeal patches.

Northern blot analysis (Figure 7) also showed a time-dependent decrease in expression of cNOS mRNA in BDV-infected brain. No obvious differences in G3PDH mRNA levels were detected between normal and BDV-infected brains. These

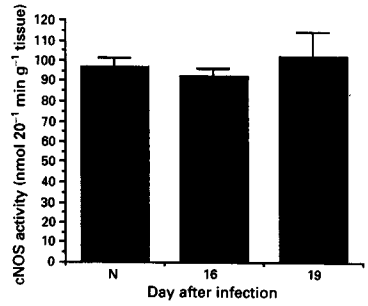


Figure 3 Time course of cNOS activity in the BDV-infected cerebellum. NOS activity was determined in normal and virus-infected cerebellum similar to the analysis described for cerebrum in Figure 1. Each column represents the mean value of enzymatic activities obtained from three cerebellums. Bars over columns represent standard errors.

results suggest that the decrease in neuronal cNOS activity during BDV infection is due to a decrease in neuronal cNOS mRNA expression.

Discussion

In this study, we report that the activity of iNOS is strongly increased whereas the neuronal cNOS activity is significantly decreased in the cerebrum of BDV — or rabies virus-infected rats. No significant change in cNOS activity was observed in cerebellum of BDV-infected rats, which is consistent with the low level of BDV infection in the cerebellum. The increase in iNOS activity is consistent with previous findings in BDV-infected brains using reverse transcriptase-polymerase chain reaction (RT-PCR) and *in situ* hybridization or immunohistochemistry. However, the finding that neuronal cNOS activity is decreased during BDV infection contrasts with our recently pubished data (Zheng *et al*, 1993) indicating an increase in cNOS mRNA expression in whole brain as determined by RT-PCR. This discrepancy might be the result of the RT-PCR amplification employed in the previous study being not exclusively specific for cNOS mRNA. Immunohistochemistry and Northern blot analysis revealed a decrease in expression of neuronal cNOS protein and mRNA, suggesting that the decrease in neuronal cNOS activity reflects either a reduction in cNOS mRNA synthesis or a selective destabilization of cNOS mRNA (Evans *et al*, 1993). However, we cannot exclude the possibility that post-translational modifications of NOS such as phosphorylation (Nakane *et al*, 1991; Bredt *et al*, 1992) or enhanced degradation of neuronal cNOS during viral infection contribute to the decrease in enzymatic activity. CoAt activity in the cerebrum remained almost unchanged during BDV and rabies virus infections, suggesting that the decrease in neuronal cNOS activity dose not reflect extensive neuronal death. Although it is possible that cNOS-

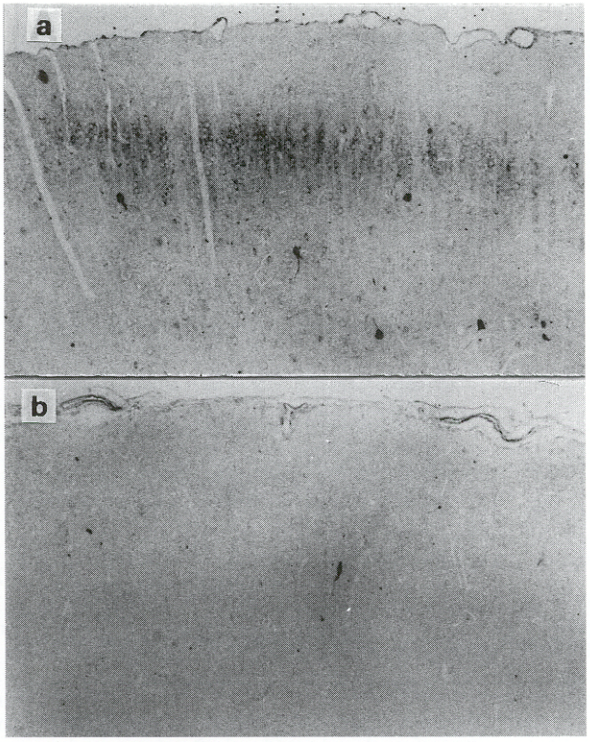


Figure 4 Comparison of immunostaining for neuronal cNOS in the parietal cortex of rats infected with Borna disease virus at 20 days post infection (b) as compared to controls (a). In BDV-infected brain, there is a marked reduction in the number of cNOS immunostained cell bodies and nerve fibres. The intensity of immunostaining in BDV is also reduced (magnification × 90).

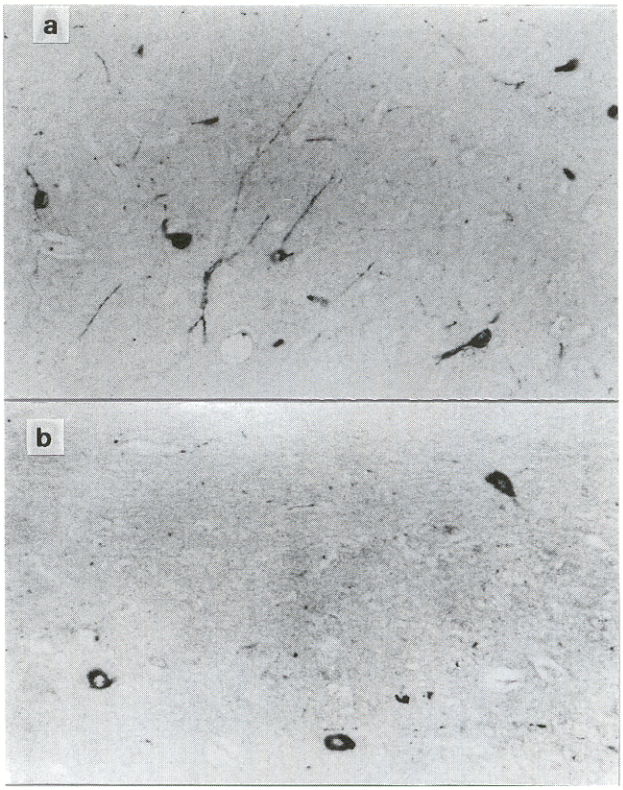
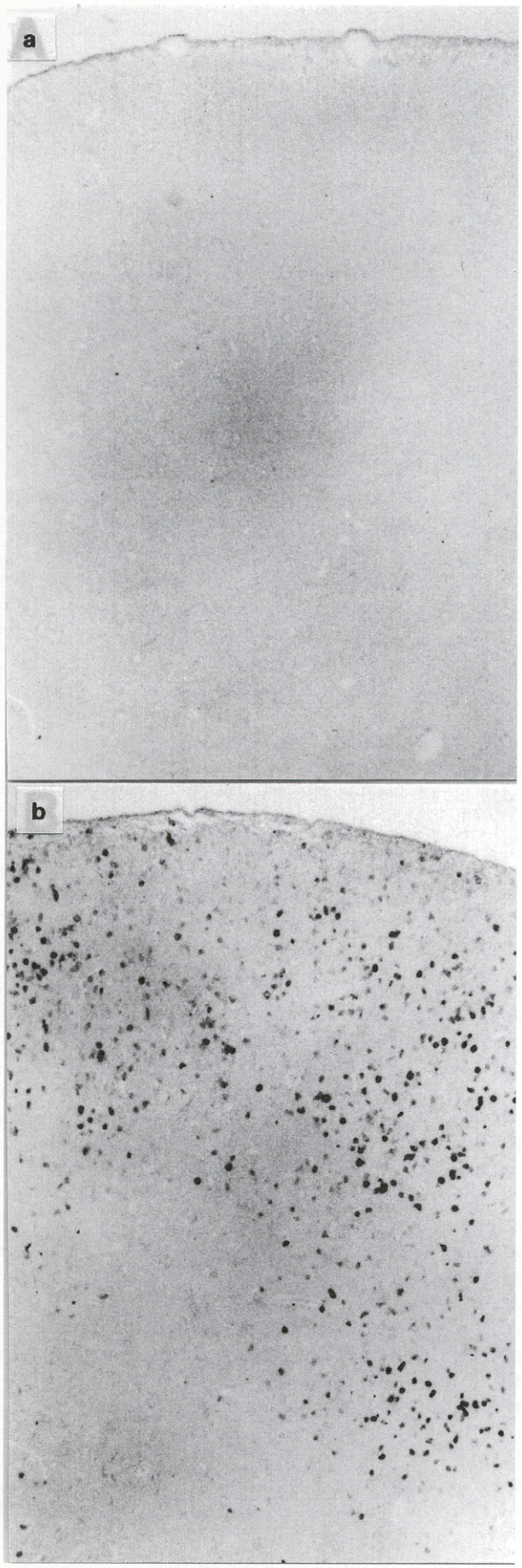


Figure 5 Reduction of immunostaining for neuronal cNOS in the caudate-putamen of rats with BDV at 20 days post infection (b) as compared to controls (a). Note the lower intensity of cNOS immunostaining in neuronal cell bodies and the marked rarefication of cNOS immunostaining in neuronal processes and varicose nerve fibres in BDV as compared to controls (magnification × 225).

positive neurons might exhibit a selective vulnerability to BDV or rabies virus infections, we believe that this possibility is remote because neuronal destruction in the BDV-infected brain is caused by relative unselective immune mechanisms.

Other factors might also account for the decrease of neuronal cNOS in the virus-infected brain. It has been reported that expression of iNOS mRNA can be downregulated by various soluble factors such as glucocorticoids and some cytokines such as IL-4 and TGF-β (Nathan, 1992). Furthermore, NO itself produced by NOS (Rogers and Ignarro, 1992) and activation of protein kinases (Nakane *et al*, 1991; Bredt *et al*, 1992) can lead to an inhibition of enzymatic activity of both NOS isoforms without affecting mRNA expression. However, so far there are no reports on downregulation of cNOS mRNA expression in the brain. We have previously shown that BDV or rabies virus infection can induce expression of transcriptional factors such as c-fos, junB, and egr-1 in brain neurons (Fu *et al*, 1993). Some of the transcriptional factors that are upregulated during virus infection may be involved in downregulating neuronal cNOS. The decrease in cNOS and increase in iNOS may both contribute to the pathogenesis of BDV infection. The action of NO is apparently paradoxical; it can be neurodestructive as well as neuroprotective (Lipton *et al*, 1993). The NO produced by macrophages may be toxic because its reaction with superoxide anion O_2^- leads to the formation of peroxynitrate ($ONOO^-$) which is a reactive oxidizing agent capable of causing tissue damage. Our recent finding that expression of xanthine oxidase, which provides a source of O_2^-, is also highly upregulated in the brain of BDV-infected rat brain (data not shown), supports the role of NO in causing tissue damage. On the other hand, endogenous NO produced by neurons might be neuroprotective. The neuroprotective activity of NO is thought to be caused by inactivation of the NMDA receptor through reaction of NO with thiol groups of the receptor (Lipton *et al*, 1993). NO produced by neurons could also be involved in the regulation of cerebral blood flow because NOS containing neuronal processes often form contact with blood vessels and it is possible that a reduction of NO could result in vasoconstriction and reduction of blood flow. Therefore the decrease in NO production by neurons could have a significant contribution to development of clinical signs in BD.



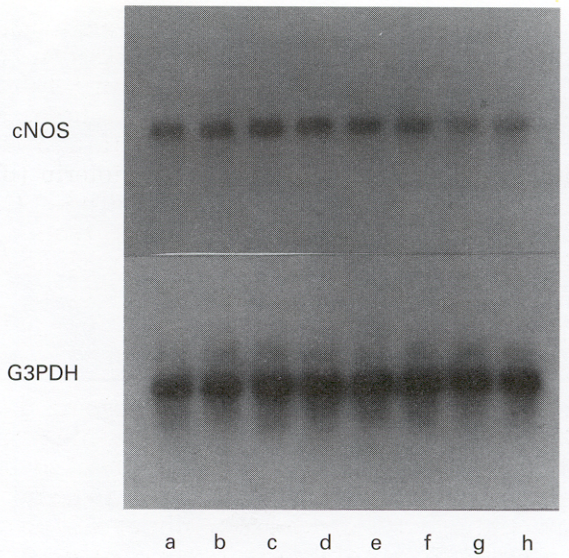


Figure 7 Northern blot analyses of neuronal cNOS mRNA and G3PDH mRNA from normal (a, b) and BDV infected cerebrum at 16 (c, d), 18 (e, f) and 20 (g, h) days post infection. RNA was denatured and electrophoresed in a 1.2% agarose gel containing 1.1 M formaldehyde and 10 mM sodium phosphate buffer (pH 7.4). RNA was transferred and covalently fixed onto nylon membranes and hybridized with cNOS and G3PDH specific probes as described (Fu *et al*, 1993).

Materials and methods

Virus infection in rats

Six-week-old female Lewis rats were anesthetized with methoxylflurane (Metofane; Pitman-Moor, Inc., Mundelen, IL), and infected intranasally with 30 µl of a 20% rat brain suspension containing 3×10^6 focus-forming units of BDV or 5×10^5 plaque-forming units of rabies virus strain CVS-24. At various time points after virus inoculation, rats were anesthetized with ketamine (100 µg kg^{-1}) and transcardially perfused with phosphate-buffered saline (PBS, pH 7.2) containing 5 g l^{-1} procaine-HCl. Control rats were treated identically, except that the inoculum did not contain BDV or rabies virus. After perfusion, the brain was removed, the cerebrum and cerebellum were separated, and the tissues frozen at –80°C.

Chemicals

[^{3}H] L-arginine and [^{14}C] acetyl coenzyme A were purchased from NEN DuPont (Boston, MA). Dowex AG50WX-8 (Na^+ form) and Dowex 1-X8 (Cl^- form) resins were obtained from Bio-Rad (Melville, NY).

Figure 6 Comparison of immunostaining for iNOS in the parietal cortex of rats infected with BDV at 20 days post infection (b) as compared to controls (a). Staining for iNOS is absent in control rats. In BDV-infected rats, a marked patchy accumulation of small non-ramified cells staining for iNOS is seen throughout the cortex (magnification × 90).

NADPH, dithiothreitol, [ethylenebis (oxyethylenenitrilo)] tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), soybean trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, and calmodulin were obtained from Sigma (St Louis, MO). Tetrahydro-L-bioterin (BH_4) was purchased from ICN (Irvine, CA). Trifluoperazine was purchased from LC Laboratories (Woburn, MA). All other chemicals were of the highest analytical grade commercially available.

Preparation of brain homogenates

After thawing, brain tissues were homogenized in 5 vol 20 mM HEPES buffer (pH 7.6) containing 1 mM dithiothreitol, 0.32 M sucrose, 10 μg ml^{-1} of soybean trypsin inhibitor, 100 μg ml^{-1} of PMSF, 10 μg ml^{-1} of leupeptin, and 10 μg ml^{-1} aprotinin at 4°C using a homogenizer (Tekmar; Tekmar Company, Cincinnati, OH). The homogenates were then centrifuged at 10,000 g for 10 min at 4°C, and the resulting supernatant was used for choline acetyltransferase (CoAT) enzyme assays. The cytosol fraction of brain tissue was obtained by ultracentrifugation of brain homogenate at 100,000 g for 1 h at 4°C.

Assays for NOS and CoAT in virus-infected brains

NOS activity was measured radiochemically by quantitating the amount of [^{3}H]L-citrulline derived from [^{3}H] L-arginine according to the method of Bredt and Snyder (Bredt and Snyder, 1989). The assay mixtures contained aliquots of each cytosol fraction (50 μl for cerebrum and 20 μl for cerebellum), 50 μM [^{3}H] L-arginine (specific activity, 80.9 mCi $mmol^{-1}$), 100 μM $CaCl_2$, 25 units ml^{-1} of calmodulin, 100 μM L-citrulline, 50 mM BH_4, and 1 mM NADPH in 250 μl of 10 mM HEPES buffer, pH 7.6. To differentiate the activity of macrophage type iNOS from that of neuronal cNOS, the calmodulin inhibitor, trifluoperazine (final concentration; 100 μM), was added to the assay mixture. After incubation for 20 min at 23°C, the reaction was stopped by the addition of 1 mM EGTA and 1 mM EDTA, and reaction mixtures were applied to a 1-ml column of Dowex AG50WX-8. L-Citrulline produced in the mixture was eluted twice with 1 ml of water, and [^{3}H] L-citrulline was quantitated by liquid scintillation spectroscopy of the flow-through from the resin column.

NOS activity was also determined by measuring the generation of nitrite, a major oxidized product of NO (Ignarro *et al*, 1993). The reaction mixture (500 μl) contained 250 μl of cytosol fraction of the brain homogenate, 100 μM L-arginine, 25 units ml^{-1} of calmodulin, 100 μM $CaCl_2$, 50 μM BH_4 and 1 mM NADPH in 10 mM HEPES buffer, pH 7.6. After incubation of the reaction mixture at 37°C for 30 min, nitrite produced in the reaction was quantitated by the diazocoupling reaction as described (Green *et al*, 1982).

CoAT activity was determined according to the method of Schrier and Schuster (Schrier and Schuster, 1967). Ten μl of the supernatant of the brain homogenate was incubated in a total volume of 200 μl of PBS containing 60 μM [^{14}C] acetyl coenzyme A (specific activity, 0.93 mCi $mmol^{-1}$), 50 mM choline chloride, and 60 μM neostigmine. After incubation for 20 min at 23°C, reaction mixtures were applied to a Dowex 1-X8 column, and synthesized [^{14}C] acetylcholine was eluted three times with 0.5 ml of water. The effluent was then analyzed by liquid scintillation counting.

Northern blot analysis of neuronal cNOS in virus-infected brains

Northern blot analysis for neuronal cNOS mRNA expression was performed as described (Auffray and Rougeon, 1980). For RNA extraction, brains were collected and immediately placed in 2 ml 4 M guanidine isothiocyanate containing 50 μM Tris-HCl (pH 7.2), 10 mM EDTA, and 1% β-mercaptoethanol. Total RNA was denatured with 10 mM sodium phosphate buffer, pH 7.4, 50% (vol/vol) formamide at 65°C for 15 min and electrophoresed in a 1.2% agarose gel containing 1.1 M formaldehyde and 10 mM sodium phosphate buffer (pH 7.4). RNA was transferred and covalently fixed onto nylon membranes (Fisher Scientific, Pittsburgh, PA). The hybridization probe for cNOS was a 27-mer antisense oligonucleotide previously described and the probe for G3PDH was prepared from G3PDH cDNA as described (Fu *et al*, 1993).

Immunohistochemistry

Rats were transcardially perfused with phosphate-buffered saline containing procaine-HCl (5g l^{-1}) and then with Bouin-Hollande fixation solution. Brains were dissected and divided into anterior, middle, and posterior parts, which were postfixed for 24 h in the same fixative. After dehydration in a graded series of 1-propanol, tissues were embedded in Paraplast Plus (Merck, Darmstadt, Germany) and cut into 5- to 7-mm-thick sections. Immunohistochemistry was performed on coronal serial sections. Briefly, sections were incubated with a rabbit antibody specific for neuronal cNOS or with a polyclonal rabbit antiserum against iNOS. Immunoreactions were visualized with biotinylated sheep anti-rabbit immunoglobulin G and streptavidin peroxidase complex (Amersham, Braunschweig, Germany) by using nickel-enhanced diaminobenzidine reactions as described elsewhere (Zentel *et al*, 1990; Zentel and Weihe, 1991).

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