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Intracerebral injection of human immunodeficiency virus type 1 coat protein gp120 differentially affects the expression of nerve growth factor and nitric oxide synthase in the hippocampus of rat

(apoptosis)

GIACINTO BAGETTA*†, M. TIZIANA CORASANITI*‡, LUIGI ALOE*§, LAURA BERLIOCCHI*, NICOLA COSTA*‡, ALESSANDRO FINAZZI-AGRÒ¶, AND GIUSEPPE NISTICÒ*||

*Department of Biology, "Mondino-Tor Vergata" Center for Experimental Neurobiology and ¶Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata," Via della Ricerca Scientifica, 00133 Rome, Italy

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ABSTRACT **We have studied the neuropathological characteristics of the brain of rats receiving daily intracerebroventricular administration of freshly dissolved human immunodeficiency virus type 1 recombinant protein gp120 (100 ng per rat per day) given for up to 14 days. Histological examination of serial brain sections revealed no apparent gross damage to the cortex or hippocampus, nor did cell counting yield significant neuronal cell loss. However, the viral protein caused after 7 and 14 days of treatment DNA fragmentation in 10% of brain cortical neurons. Interestingly, reduced neuronal nitric oxide synthase (NOS) expression along with significant increases in nerve growth factor (NGF) were observed in the hippocampus, where gp120 did not cause neuronal damage. No changes in NGF and NOS expression were seen in the cortex, where cell death is likely to be of the apoptotic type. The present data demonstrate that gp120-induced cortical cell death is associated with the lack of increase of NGF in the cerebral cortex and suggest that the latter may be important for the expression of neuropathology in the rat brain. By contrast, enhanced levels of NGF may prevent or delay neuronal death in the hippocampus, where reduced NOS expression may be a reflection of a subcellular insult inflicted by the viral protein.**

It has been proposed that human immunodeficiency virus type 1 (HIV-1) glycoprotein gp120 may be the etiologic agent of neuronal loss observed post mortem in the brain of AIDS patients because it causes death of several types of neurons in culture (see ref. 1). In fact, gp120 produces death of rodent hippocampal neurons, retinal ganglion cells (see ref. 1), and cerebellar granule cells (2). The mechanism by which gp120 produces cytotoxicity involves excessive Ca^{2+} entry into neurons via *N*-methyl-D-aspartate (NMDA) receptor-associated cation channels and through voltage-operated Ca^{2+} channels, since NMDA antagonists and Ca^{2+} channel blockers prevent neuronal death (1, 2). *In vitro* experiments have recently shown that exposure of cortical neurons to gp120 increases nitric oxide (NO), a radical species that in neuronal cells is produced by the Ca^{2+}-dependent enzyme NO synthase (NOS), and this seems to be involved in the mechanism of gp120-induced death because inhibition of NOS abolished the cytotoxic effects of the HIV-1 coat protein (3).

Recently it has been reported that gp120 induces DNA laddering in rodent brain cortical neurons maintained *in vitro*, suggesting that neuronal death caused by the viral protein is of the apoptotic type (4). Apoptosis is an active process underlying cell death that occurs during development and adult life (5, 6) and is also implicated in the pathogenesis of diverse neurodegenerative disorders (7). Thus, identification of biological mediators involved in apoptosis would be of great help in understanding the mechanisms regulating neurological diseases. Interestingly, nerve growth factor (NGF) and related neurotrophins seem to play a pivotal role in apoptosis during development, during adult life, and in some pathological conditions (see ref. 8). However, the knowledge about the role of NGF in gp120-induced neuronal degeneration is lacking.

Here we report that intracerebroventricular (i.c.v.) microinfusion of gp120 for 14 days produces DNA fragmentation and death of neuronal cells in the cerebral cortex of rats. In addition, gp120 *reduces* the expression of the constitutive, neuronal type (see ref. 9) of NOS and increases NGF in the hippocampus but not in the cortex, and these effects are seen in the absence of hippocampal cell death.

MATERIALS AND METHODS

Subjects. Male Wistar rats (250–280 g), housed in a temperature (22°C)- and humidity (65%)-controlled colony room, were anesthetized with chloral hydrate (400 mg/kg i.p.) for chronic implantation of a cannula (25 g) into one lateral cerebral ventricle (i.c.v.) under stereotaxic guidance (10). The animals were allowed 4 days to recover before treatment. Then, a single dose of gp120 was administered daily for up to 14 consecutive days to each rat with a 5-μl Hamilton syringe (volume of 1 μl; rate of 1 μl/min) connected via a Teflon tube to an injector that exceeded by 2 mm the length of the guide cannula.

Neuropathology. Twenty-four hours after the last injection, rats were anesthetized and perfused through the left ventricle of the heart with 60 ml of heparinized saline solution followed by 200 ml of paraformaldehyde (4%) dissolved in phosphate-buffered saline (PBS; pH 7.4). Serial coronal brain sections (14 μm) were cut from wax-embedded tissue blocks and processed for conventional light microscopy (Leitz Orthoplan). Quantitation of neuronal cells was performed in the CA1 and CA3 pyramidal cell layer of the dorsal hippocampus and neocortex as detailed elsewhere (see ref. 11).

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Abbreviations: NMDA, *N*-methyl-D-aspartate; HIV-1, human immunodeficiency virus type 1; NGF, nerve growth factor; NGFR, NGF receptor; NO, nitric oxide; NOS, nitric oxide synthase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling; BSA, bovine serum albumin; i.c.v., intracerebroventricular; GFAP, glial fibrillary acidic protein.

†Present address: Department of Neuroscience, University of Cagliari, Italy.
‡Present address: Faculty of Pharmacy and Institute of Biotechnology Applied to Pharmacology–Consiglio Nazionale delle Richerche, Catanzaro, Italy.
§Present address: Consiglio Nazionale Ricerche Institute of Neurobiology, Rome.
||To whom reprint requests should be addressed.

***In Situ* DNA Fragmentation Study.** Two hours after the perfusion procedure described above, the rat brain was removed from the skull, postfixed in 4% paraformaldehyde overnight, cryoprotected in sucrose (30%, wt/vol), immersed in nitrogen, and stored at −80°C until use. Cryostat brain coronal sections (14 μm) from rats treated with gp120 or bovine serum albumin (BSA) were mounted on polylysine-coated slides and processed for *in situ* analysis of DNA fragmentation according to the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) method (12) with minor modifications (13).

Immunocytochemistry for Glial Fibrillary Acidic Protein (GFAP). Cryostat brain sections were stained by a biotin–streptavidin-amplified detection system (B-SA; BioGenex Laboratories, San Ramon, CA). After rinsing in PBS, sections were incubated with 0.01% H_2O_2 in methanol to inactivate endogenous peroxidases; then nonspecific binding was prevented by incubating the slides with 4% (vol/vol) normal goat serum in PBS containing 0.1% Triton X-100. Sections were then incubated for 1 h at room temperature with a mouse monoclonal antibody to GFAP (BioGenex Laboratories) diluted 1:160 in PBS containing 4% normal goat serum and 0.1% Triton X-100. After washing in PBS, sections were incubated successively with biotinylated goat anti-mouse immunoglobulins and horseradish peroxidase-conjugated streptavidin (BioGenex Laboratories), each for 20 min. Peroxidase activity was revealed using 3,3′-diaminobenzidine.

NGF Assay. NGF was evaluated in supernatants of brain tissue homogenates by a two-site immunoassay (ELISA) as described (14) with minor modifications (15) using 96-microtiter plates coated with 50 μl of monoclonal mouse anti-NGF (0.02 μg/ml) diluted in 0.05 M carbonate buffer (pH 9.6) per well. To assess nonspecific binding, parallel wells were coated with equal amounts of purified mouse IgG. The optical density was measured at 575 nm using an ELISA reader (Dynatech), and the results were corrected by subtraction of background values (for further details see ref. 15).

Immunohistochemistry of Low-Affinity NGF Receptor ($p75^{NGFR}$)-Positive Neurons. Cryostat brain coronal sections (14 μm) were cut at 30-μm intervals as basal forebrain, including septum. Sections were stained with monoclonal antibodies against $p75^{NGFR}$ (clone IgG-192; kindly provided by E. J. Johnson, Department of Medicine, Washington University, St. Louis, MO). Coded brain sections of control and gp120-treated rats were simultaneously processed for the presence of $p75^{NGFR}$-positive cell bodies, fibers, and terminals immunolabeled according to a previously described procedure (16). Quantitative analysis (distribution and cross-sectional size) of $p75^{NGFR}$-immunoreactive neurons in the septum (n = 22 sections) was carried out using a computerized image-analysis system (Axiophot Zeiss microscope equipped with a Vidas Kontron system). The area of $p75^{NGFR}$-immunoreactive neurons in each experimental group was measured and compared. Only $p75^{NGFR}$-positive neurons ranging from 45 to 300 μm^2 were included in the analysis, and an average of 6500 neurons were compared for each rat. The results from each experimental group were pooled and evaluated statistically using the Wilcoxon–Mann (U) test.

RNA Isolation, Electrophoresis, and Hybridization. Hippocampal RNA isolation, electrophoresis, and hybridization were performed as described (see ref. 17). Briefly, 24 h after the last day of treatment, total RNA was isolated from individual rat hippocampi using Ultraspec RNA (Biotecx Laboratories, Houston), electrophoresed through a 1% agarose gel, and blotted onto a Hybond-N membrane (Amersham) by capillary transfer. The filters were hybridized to a 4.8-kb rat neuronal NOS cDNA probe spanning the whole gene (see ref. 17) and labeled with [^{32}P]dATP by random priming using a kit supplied by Boehringer Mannheim. The blots were then washed as detailed elsewhere (see ref. 17) and exposed for 72 h to Kodak X-Omat AR autoradiography film. Neuronal NOS mRNA signal intensities were evaluated by laser densitometry (LKB-Pharmacia Ultroscan Laser densitometer) as described (see ref. 17).

Determination of Hippocampal and Cortical NOS Activity. NOS activity was measured in individual rat hippocampal and cortical homogenates as reported (see ref. 17), and the results of six experiments per group were expressed as pmol per min per mg of protein of [^{3}H]citrulline formed (mean ± SEM).

Materials. Glycosylated recombinant HIV-1 gp120 IIIB (from baculovirus expression system; >90% pure) was from Medical Research Council Directed Reagent Project (National Institute of Biological Standards and Control, South Mimms, U.K.); all other chemicals were from Sigma (Milan).

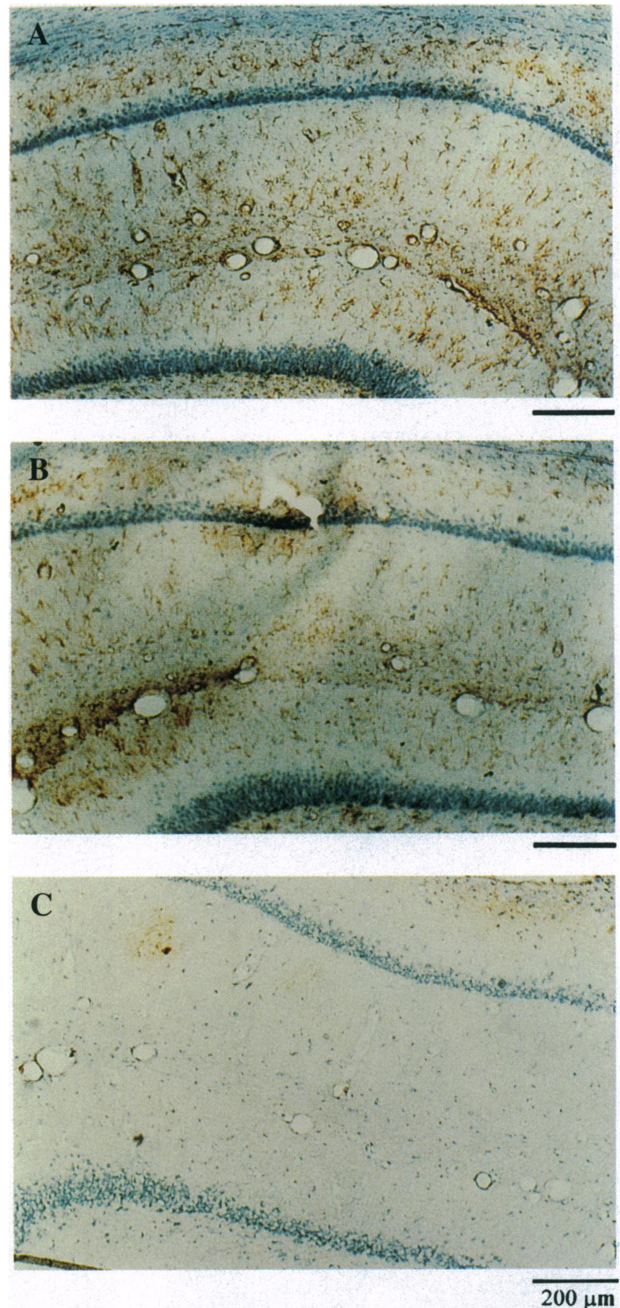

FIG. 1. Photomicrographs of GFAP-like immunoreactivity in the hippocampal formation [bregma level −3.3 mm according to the atlas of Paxinos and Watson (10)] of a BSA (300 ng i.c.v., given once daily for 14 consecutive days)-treated rat (*A*) and of a rat receiving a single daily injection (i.c.v.) of gp120 (100 ng) for 14 days (*B*). The specificity of staining is shown in *C*, a control section (from a gp120-treated rat; 14 μm) where the primary antibody was omitted. (Bars = 200 μm.)

RESULTS

Neuropathological Effects of gp120. Examination of brain coronal sections (10 μm; $n = 6$ per rat) of rats ($n = 6$ per group) receiving a single daily i.c.v. injection of gp120 (100 ng per rat per day) for up to 14 consecutive days did not reveal gross damage to the dorsal hippocampus. By contrast, dark-degenerating neurons and neuronal cell loss were observed in the neocortex of rats treated for 7 (mean cell number = 88.5 ± 2.6; $P > 0.05$ vs. contralateral, uninjected side) and 14 days (75.8 ± 2.7; $P > 0.05$ vs. contralateral, uninjected side). In the hippocampus and cortex of gp120-treated rats, GFAP immunoreactivity did not increase over control; a typical example of GFAP immunostaining in the hippocampus is shown in Fig. 1. Interestingly, *in situ* DNA fragmentation was observed in the neocortex of rats ($n = 6$ per group) receiving daily injection of gp120 for 7 and 14 days but not for 1 day. A typical example of neocortical cells positive to the TUNEL staining is shown in Fig. 2. The effects of gp120 were more evident in the neocortex of the treated side as compared to the control side (treated = 2.2 ± 0.3 vs. control = 1.2 ± 0.1, and treated = 6.0 ± 0.1 vs. control = 2.0 ± 0.3 TUNEL-positive cells at 7 and 14 days, respectively). In no instance was *in situ* DNA fragmentation observed in the hippocampal formation of rats receiving daily injection of gp120 for up to 14 consecutive days (Fig. 2). Lack of *in situ* DNA fragmentation (Fig. 2) or neuronal cell loss was observed in the neocortex (cell number = 82.1 ± 3.9; $P > 0.05$ vs. control side) and hippocampus (51.5 ± 3.2; $P > 0.05$ vs. control side of brain sections ($n = 6$ per rat) obtained from rats ($n = 6$) treated with BSA (300 ng per rat given i.c.v. once daily for 14 days).

gp120 Enhances Hippocampal but not Cortical NGF. In parallel experiments, it has been observed that i.c.v. microinfusion of gp120 (100 ng per rat per day) doubled the concentration of NGF in the rat ($n = 6$ per group) hippocampus after 7 days (control = 1834 ± 518 vs. gp120 = 3100 ± 51 pg/g of tissue) and 14 days (control = 1420 ± 189 vs. gp120 = 3538 ± 453 pg/g of tissue) of treatment; no consistent changes in NGF levels were observed in the cortex after 14 days of gp120 treatment (control = 889 ± 31 vs. gp120 = 804 ± 88 pg/g of tissue; $P > 0.05$). Interestingly, the increase in hippocampal NGF induced at day 7 by gp120 was accompanied by a significant decrease in the total number of $p75^{NGFR}$-immunopositive neurons (gp120, $n = 259 \pm 19.9$; control, $n = 393 \pm 55.8$; $P < 0.05$) in the range of 45–120 μm^2 in the septum (see Fig. 3 for a typical example), where a coincident increase in $p75^{NGFR}$ immunopositivity was observed in neurons (gp120, $n = 64 \pm 10.5$; control, $n = 28 \pm 5.4$; $P < 0.01$) of 200–300 μm^2; however, no signs of neuronal degeneration or death were observed in this area (data not shown).

gp120 Reduces Neuronal NOS Expression. Microinfusion of gp120 (100 ng per rat per day) for 14 consecutive days decreased the level of hippocampal neuronal NOS mRNA (Fig. 4*A*). Densitometric analysis of NOS mRNA signal yielded a 2-fold decrease at 14 days, but not at 1 day and 7 days ($n = 4$ rats per group of treatment), as compared to the effect of treatment with BSA (Fig. 4*B*). The effect of gp120 on neuronal NOS mRNA was paralleled by a significant decrease

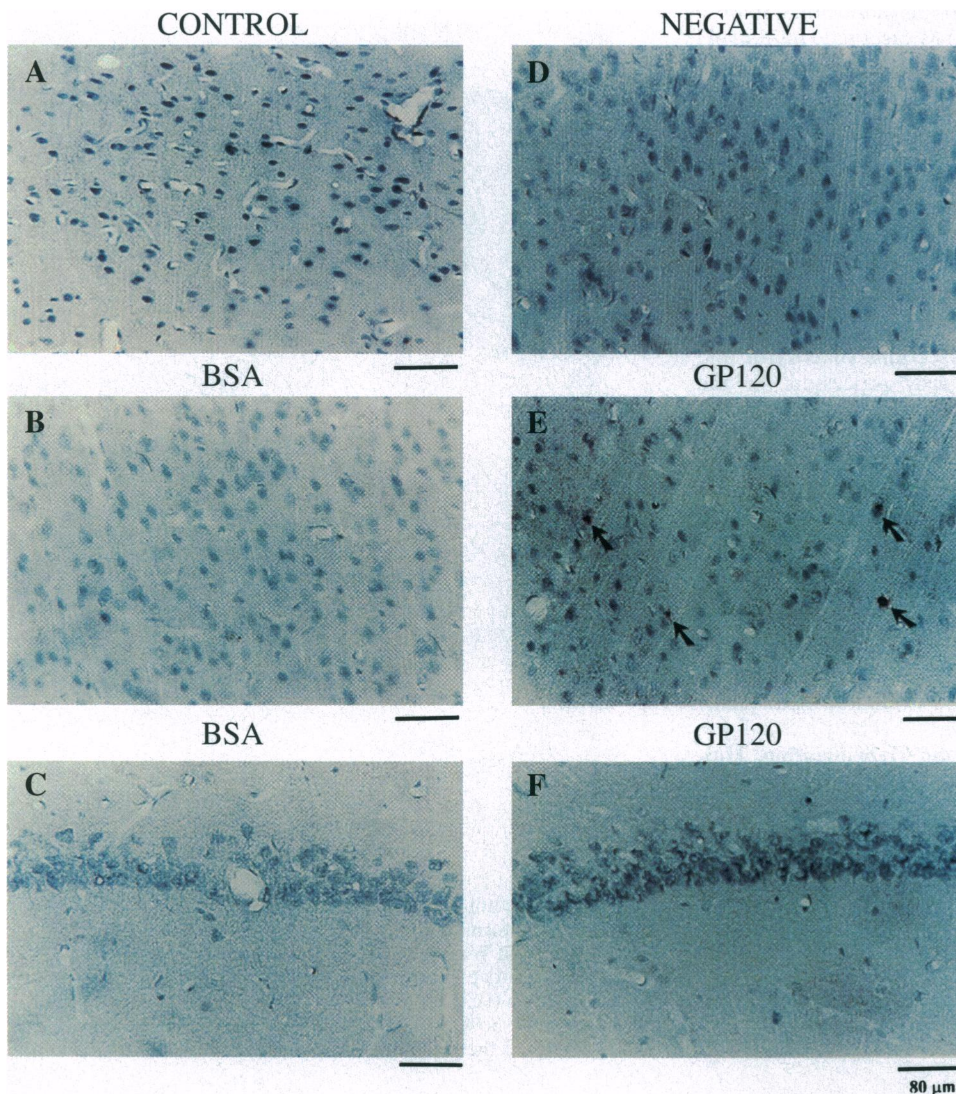

FIG. 2. *In situ* DNA nick end-labeling of brain tissue emi-sections (14 μm) of a rat treated with a single, daily dose (100 ng per rat i.v.c.) of gp120 given for 14 days. Note in *E* the presence of TUNEL-positive cells (arrows) in the brain cortex but not in the hippocampus (*F*) ipsilateral to the side of i.c.v. injection of gp120. Lack of nuclear labeling in the cortex (*B*) and in the hippocampus (*C*) is evident in brain tissue emi-sections from a rat treated with BSA (300 ng per rat i.c.v.) for 14 days. (*D*) Rat brain tissue emi-section adjacent to the emi-section in *E* stained in the absence of terminal deoxynucleotidyltransferase for negative control (see *Materials and Methods* for details); note the lack of nuclear labeling. (*A*) Lack of TUNEL-positive cells in a cortical brain tissue emi-section from an untreated (control) rat. (Bars = 80 μm.)

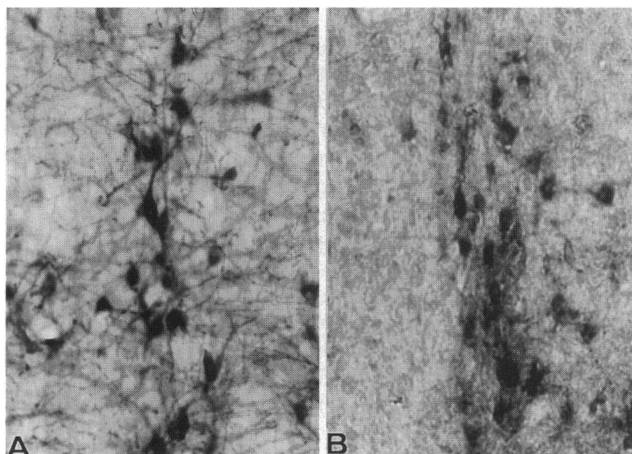

FIG. 3. p75^{NGFR}-immunoreactive neurons in the medial septum of BSA-treated (*A*) (control) and gp120-treated (*B*) rats. BSA (300 ng i.c.v.) and gp120 (100 ng i.c.v.) were administered once daily for 7 consecutive days. Twenty-four hours after the last injection, the animals were anesthetized and perfused, and brain coronal sections were immunostained for detection of the low-affinity NGFR. (×140.)

of Ca^{2+}-dependent (data not shown) [^{3}H]citrulline formation in hippocampal but not cortical homogenates (Table 1). In control experiments, gp120 (100 pM) was applied *in vitro* to hippocampal and cortical tissue homogenates from untreated animals, and this did not produce significant changes in NOS activity (Table 1). The observed increase in hippocampal NGF does not account for the reduction of NOS activity in the same brain structure, because in rats treated with NGF (20 μg, given i.c.v. for up to 14 days) the enzyme activity assayed in hippocampal homogenates did not change significantly (Table 1).

DISCUSSION

The present experiments show that i.c.v. administration of HIV-1 gp120 produces cell death in the cerebral cortex though this involved ≈10% of the neurons counted per microscopical field. *In situ* DNA fragmentation was detected in a similar proportion of cortical cells, suggesting an apoptotic type mechanism of death. The induction by gp120 of DNA fragmentation in cerebral cortical neurons *in vivo* (present data and ref. 13) and *in vitro* (4) is in line with the neuronal cell loss reported *post mortem* in the neocortex of AIDS patients (see ref. 18) and suggests that the latter might be the consequence of apoptosis.

Spontaneous and drug-induced apoptotic cell death has often been described in cultured neurons upon removal of NGF from the culture medium (8, 19). An important finding of the present study is that i.c.v. injection of gp120 for 7 and 14 days increased NGF levels in the hippocampus where no signs of neuronal degeneration or death were seen (present data and ref. 11); by contrast, no significant changes in NGF content were observed in the brain cortex, though it cannot be excluded that a reduction of this neurotrophin might have occurred in a very limited area of the cortex. It is conceivable, therefore, that the lack of apparent change in cortical NGF in the presence of a persistent gp120-induced neuronal insult may correspond to a relative reduced availability of this growth factor with consequent neuronal cell death.

In rat pups, systemic administration of gp120 produces brain cortical neuron dystrophic changes and alterations in developmental behaviors (20), and these neuropathological changes may be compatible with a relative decrease of cortical NGF (see ref. 21).

Hippocampal NGF originates from neurons, though glial production has also been reported under certain experimental

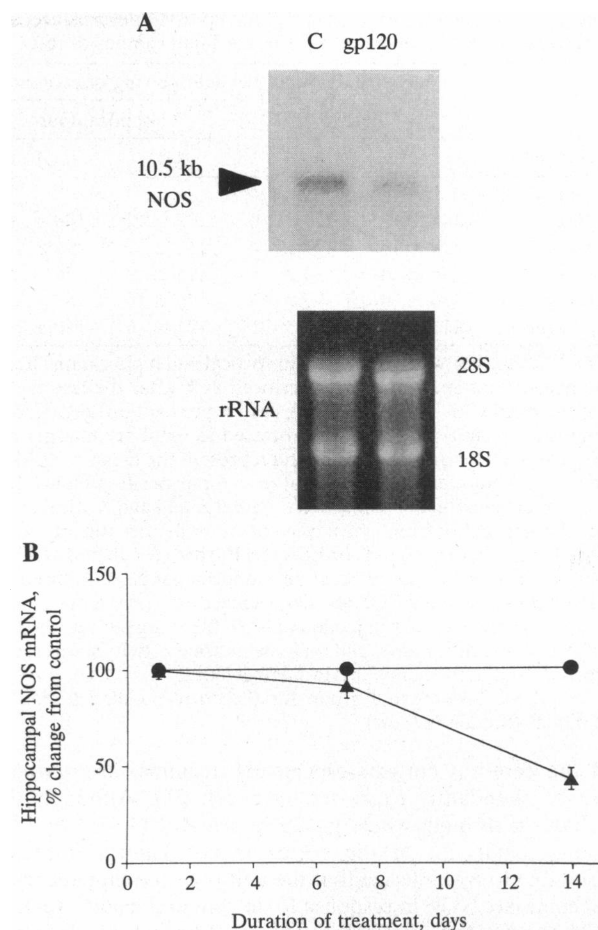

FIG. 4. (*A*) Typical example of the effect of gp120 on the NOS mRNA level in the rat hippocampus. Total RNA was extracted from the hippocampus of a control rat (lane C) and from a rat treated with gp120 (100 ng per rat per day, given i.c.v. daily for 14 continuous days) and analyzed by Northern hybridization to a rat brain NOS cDNA probe (*Upper*). The 10.5-kb NOS mRNA signal is indicated by an arrowhead. (*Lower*) Ethidium bromide staining of total RNA loaded (20 μg per lane as estimated from the absorbance at 260 nm) on a gel as a control. (*B*) Densitometric analysis of the NOS mRNA signals obtained from rats (n = 4 for each data point) treated daily with gp120 (100 ng per rat per day, given i.c.v.; ▲) or with BSA (300 ng per rat per day; ●) for 1, 7, and 14 continuous days. The data are expressed as the mean ± SEM percentage changes vs. control (set to 100%), BSA-treated rats.

conditions (21, 22). The lack of an obvious increase in GFAP immunoreactivity in the hippocampal formation of gp120-treated rats, while demonstrating the absence of inflammatory processes, indicates that enhanced NGF may not originate from astroglial cells (22). A more attractive explanation would be that increases in hippocampal NGF may form part of a protective and/or an adaptive response to the neurotoxic insult inflicted by gp120. This hypothesis is supported by the observed decrease in the number of p75^{NGFR}-immunopositive cells of 45–120 μm^2 in the septal nucleus whose nerve fibers terminate in the hippocampus. The plastic nature of this response is further confirmed by the coincident increase in the p75^{NGFR} immunoreactivity of 200–300 μm^2 neurons in the septum, where no apparent neuronal death was seen (data not shown). In the rat brain, p75^{NGFR} is expressed in cholinergic neurons of the medial septal and diagonal band nuclei (see ref. 23). To produce its trophic effects, NGF binds to p75^{NGFR} and, along with trkA, is internalized and transported to the neuronal cell body (see ref. 24). The septum and diagonal band share robust, reciprocal connections with the hippocampus

Table 1. i.c.v. microinfusion of gp120 for up to 14 days reduces the activity of Ca^{2+}-dependent NOS in the hippocampus of rats

	NOS activity, pmol per min per mg of protein			
	Cortex		Hippocampus	
Treatment	C	T	C	T
i.c.v. gp120				
1 day	142 ± 12	128 ± 11	123 ± 12	108 ± 8
7 days	115 ± 10	107 ± 7	111 ± 7	99 ± 7
14 days	96 ± 4	93 ± 5	115 ± 8	76 ± 5*
In vitro gp120	116 ± 4	97 ± 13	76 ± 16	88 ± 21
i.c.v. NGF	111 ± 11	133 ± 12	124 ± 6	110 ± 13

The NOS activity was determined in cortical and hippocampal brain homogenates obtained from rats sacrificed 24 h after the last day of i.c.v. treatment with gp120 (T; 100 ng per rat per day) or BSA (C; 300 ng per rat per day). The data are expressed as pmol per min per mg of protein of [^{3}H]citrulline formed and represent the mean ± SEM of six determinations, each from one rat (n = 6 rats per treatment). For *in vitro* determination of NOS activity, hippocampal and cortical brain tissue homogenates from naive rats (n = 6 per treatment) were exposed to gp120 (T; 100 pM) or BSA (C; 100 pM) for 20 min at 37°C. To ascertain whether the increase in hippocampal NGF induced by gp120 could account for NOS activity decrease, rats (n = 6 per group) received a single daily i.c.v. injection of NGF (T; 20 μg per rat) or BSA (C) for 14 consecutive days, and then the enzyme activity was assayed in cortical and hippocampal brain homogenates.
*$P < 0.01$ vs. BSA-treated group for the corresponding period of treatment (Student's t test).

and the cerebral cortex, respectively, terminal areas where NGF is abundantly expressed (see ref. 21). Although the mechanism through which gp120 increases NGF in the hippocampus but not in the cortex is not known, it seems reasonable to hypothesize that the ability of the hippocampus to accumulate NGF in response to detrimental insults, such as gp120, renders this plastic structure of the brain less vulnerable than the cerebral cortex.

In vitro experiments have recently shown that inhibition of NOS prevents neurite outgrowth elicited by NGF, thus involving NO in the neurotrophic mechanisms of this growth factor (25). NOS and $p75^{NGFR}$ colocalize in cholinergic neurons of medial septal and diagonal band nuclei (23), and NOS immunoreactive neurons are present in the hippocampus, though they are not numerous (26). Here we have observed that a gp120-induced increase in NGF was accompanied by a reduced expression of neuronal NOS in the hippocampus in the absence of neuronal cell loss. These data, together with the observed decrease in $p75^{NGFR}$-immunoreactive neurons in the septum, suggest that gp120 may be detrimental for the septo-hippocampal pathway, though the increase in NGF may prevent or delay the appearance of gross damage.

The reduction in hippocampal NOS expression is likely to be accompanied by a parallel decrease of the gene product. This is reflected by a significant decrease of Ca^{2+}-dependent NOS activity detected in the same region of the rat brain (present data) and by a reduction in neuronal NOS immunoreactive cells (G.B., unpublished observation). Dawson *et al.* (3) have reported that inhibitors of NOS prevent death of cortical neurons in primary culture exposed to gp120 and external glutamate, suggesting that NMDA receptor-mediated excessive production of NO might be involved. These data seem to contrast with our present results; however, it has been shown that gp120 inhibits radioligand binding to rat forebrain NMDA receptors, NMDA-evoked currents, and cytotoxicity in cultured cerebellar granule cells and provides partial protection against NMDA-induced lethality *in vivo* (27). Therefore, it is conceivable that in the hippocampus, an area endowed with a high density of NMDA receptors (28), the observed reduction of NOS activity (present data) may reflect a persistent gp120-mediated blockade of NMDA-gated Ca^{2+} entry into neurons, though this does not sufficiently explain the observed reduction in NOS mRNA expression. Under normal conditions, neuronal NO mediates important physiological processes including long-term potentiation and depression of synaptic transmission (29), nociception (30), and cerebral blood flow (31), and some NO species have been shown to be endowed with neurotoxic as well as neuroprotective properties (32). Therefore, regardless of the mechanism through which gp120 reduces the expression of hippocampal NOS, this should be considered a neurotoxic event *per se.*

In conclusion, the observation that gp120 induces apoptotic cell death in the rat cortex *in vivo* suggests that this mechanism may underlie the well-established brain cortical neuronal loss described in AIDS patients (18); in addition, the associated dementia syndrome (see ref. 1) may well be due to insufficient production of NGF with consequent neuronal loss in this brain region together with reduced NOS activity in the hippocampus, an important area for memory formation.

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