Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 3368–3373, April 1997
Neurobiology

Time-course changes of nerve growth factor, corticotropin-releasing hormone, and nitric oxide synthase isoforms and their possible role in the development of inflammatory response in experimental allergic encephalomyelitis

LAURA CALZÀ*†‡, LUCIANA GIARDINO§, MONICA POZZA†, ALESSANDRA MICERA¶, AND LUIGI ALOE¶

*Institute of Human Physiology, University of Cagliari, 09124 Cagliari, Italy; †Pathophysiology Center for the Nervous System, Hesperia Hospital, 41100 Modena, Italy; §Institute of Otolaryngology II, University of Milano, 20122 Milan, Italy; and ¶Institute of Neurobiology, Consiglio Nazionale delle Ricerche, 00137 Rome, Italy

Communicated by Rita Levi-Montalcini, Institute of Neurobiology, Consiglio Nazionale delle Ricerche, Rome, Italy, January 2, 1997 (received for review November 2, 1996)

ABSTRACT **In this paper we report a time-course study of development of experimental allergic encephalomyelitis in Lewis rats, by monitoring neuroendocrine regulation of the hypothalamus–pituitary–adrenal axis through corticotropin-releasing hormone mRNA expression, inflammatory cellular infiltrate, macrophagic and neuronal nitric oxide synthase, nerve growth factor (NGF), and NGF p75 and trkA receptors in the brain and spinal cord. We analyzed animals during 20 days after immunization, a time interval that corresponds to the acute immunological phase. We have described a severe, early fall of corticotropin-releasing hormone mRNA expression, which could account for the decreased response of the hypothalamus–pituitary–adrenal axis to inflammatory stress. During this period, an increase of neuronal nitric oxide synthase was observed in the cerebral cortex and spinal cord, and macrophagic nitric oxide synthase positive cells were found in the inflammatory cellular infiltrate, which was abundant in perivascular and submeningeal areas 20 days after immunization. Concomitantly, we found a dramatic up-regulation of NGF receptors on the wall of blood vessels and adjacent neurons in perivascular areas. NGF content also had increased in some brain areas, such as the thalamus, while it had decreased in others, like the spinal cord and medulla oblongata, at time points in which the most serious cellular infiltrate was found.**

Experimental allergic encephalomyelitis (EAE) is a neuroinflammatory disease that also comprises immune components that have been widely used as an experimental model for human multiple sclerosis. It is caused by active sensitization of the animal after peripheral injection of myelin basic protein suspended in complete Freund's adjuvant or after passive transfer of myelin basic protein reactive cells. Animals develop characteristic limp tail, muscle weakness, and paralysis within 8–12 days after injection, followed by a partial recovery within 20 days. According to different authors, a second and even third exacerbation characterized by a slower, but more severe and long-lasting, neurological deterioration then takes place, and the animals partially recover, in this case too, after a long period (4–5 months). In this second chronic stage, signs of demyelinization have been described in terms of histopathological hallmark (1), nuclear magnetic resonance studies (2), functional tests, and evoked potentials (3). Acute exacerbation is mainly determined by immunological responses resulting in damage to the blood–brain barrier (BBB) together with parenchimal inflammatory infiltration. The latter triggers the chronic phase that includes tissue damage (4).

A decreased response of the hypothalamus–pituitary–adrenal (HPA) axis to inflammatory and immune mediators confers susceptibility to the development of inflammatory/immune diseases, including EAE and arthritis (5). For example, EAE develops well in Lewis rats, a strain characterized by the animals' inability to adequately stimulate the HPA axis through inflammatory mediators (6). Reciprocal interactions between immune, neural, and endocrine cells seem to be relevant for both physiological and pathological processes (7). Over the last few years, much attention has been paid to the investigation of molecules interacting with all these cell types, crossing body barriers, i.e. BBB, and disrupting structural units, i.e. oligodendrocyte-myelin complex, in physiological or pathological conditions. The reciprocal interactions between body compartments in terms of local and long-distance molecular circuits (7) also have been indicated as a key mechanism for body homeostasis.

The relative role of inflammatory and immune components and mediators in clinical and histopathological findings in EAE (and also in multiple sclerosis), such as the primary or secondary role of the HPA axis dysfunction in the pathogenesis of the disease, is still not clear. Moreover, the molecules responsible for central nervous system (CNS) lesions in EAE are still unknown. To study the above, we investigated the development of EAE by monitoring nerve growth factor (NGF), nitric oxide synthase (NOS), and corticotropin-releasing hormone (CRH) mRNA in the CNS. NGF and NO are critical molecules in the functional regulation of immune, neural, and endocrine systems. NGF levels are altered during inflammation (8), such as in multiple sclerosis patients (9), and this has been regarded as either a pivotal or reactive phenomenon (10). The involvement of NO in inflammatory/immune demyelinizating diseases has been suggested by clinical (11) and emphiric data (12, 13). Moreover, NO is one of the potential mediators of interleukin 1 (IL1)-induced toxicity (14). In this study we have demonstrated an increased production of inducible and neuronal isoforms of NOS during EAE, which correlates with a severe fall of CRH mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus. NGF content and NGF receptor immunostaining also change according to

Abbreviations: EAE, experimental allergic encephalomyelitis; HPA axis, hypothalamus–pituitary–adrenal axis; CRH, corticotropin-releasing hormone; iNOS, macrophagic nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NGF, nerve growth factor; BBB, blood–brain barrier; CNS, central nervous system; NOS, nitric oxide synthase; PVN, paraventricular nucleus; IL1, interleukin 1; CFA, complete Freund's adjuvant.

‡To whom reprint requests should be addressed at: Pathophysiology Center for the Nervous System, Hesperia Hospital, Via Arquà 80/A, 41100 Modena, Italy. e-mail: cefisnmo@mbox.vol.it.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

0027-8424/97/943368-6$2.00/0
PNAS is available online at **http://www.pnas.org**.

the clinical stage of the disease and to the inflammatory cell infiltrate.

MATERIALS AND METHODS

Animals. A total of 250 female, pathogen-free Lewis rats (Charles River, Italy) were used in this study. Ninety-six rats were sensitized with a medium containing 29 mg of guinea pig spinal cord tissue in complete Freund's adjuvant (CFA; Sigma) to which 2 mg/ml heat-inactivated *Mycobacterium* (Difco H37Ra) was added. Sensitization was performed in both hind pads. Ninety-six rats were injected in both hind pads with CFA. Fifty-eight uninjected rats were used as controls. For NGF measurement and *in situ* hybridization experiments, animals were killed 2, 4, 6, 8, 10, 12, and 20 days after sensitization or CFA, whereas for histopathological and immunocytochemical observations animals were killed 3, 7, 14, and 20 days after treatment. Rats were observed daily for signs of EAE and were scored as follows: 1, loss of tail tonicity; 2, weakness in one or both hind legs or middle ataxia; 3, severe ataxia or paralysis; and 4, severe hind leg paralysis accompanied by urinary incontinence. All animal protocols described here were approved by our intramural committee, and the animals were cared for in accordance with the guideline published in the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Immunocytochemistry. The day they were killed, the rats were anesthetized and perfused through the ascending aorta with saline solution followed by 4% paraformaldehyde. After 25–30 min of perfusion, the brains were removed and immersed for 24 h in the same ice-cold fixative. The brains were then rinsed for 48 h in ice-cold 0.1 M Sorensen's buffer containing 5% sucrose, and then cut on a Vibratome (thickness of section 50 μm) and immediately processed for the ABC procedure on free-floating sections. The sections were pretreated with H_2O_2 to quench endogenous peroxidase activity and then incubated with 1.5–2.0% normal serum, followed by overnight incubation at 4°C with the primary antisera diluted in PBS containing 0.3% Triton X-100 (vol/vol). The following antisera were used: monoclonal anti-p75 antibody (clone IgG192, generously supplied by E. M. Johnson, Washington University, St. Louis); polyclonal anti-trkA (Santa Cruz Biotechnology); and polyclonal anti-iNOS (Transduction Laboratories, Lexington, KY). Staining specificity was assessed by the *in vitro* overnight preincubation of the antiserum with the respective antigen (100 mg/ml). This treatment prevented the staining. After the sections had been rinsed, they were incubated with biotinylated goat anti-mouse or anti-rabbit immunoglobulin (Dako), rinsed in PBS, and incubated using streptavidin biotinylated horseradish peroxidase complex (Amersham) 1:250. Diaminobenzidine was used to detect the immunocomplex. Sections (20 μm) also were stained for histological examination (toluidine blue and hematoxylin/eosin staining).

NGF Determination. For NGF measurement, rats were sacrificed by decapitation. NGF levels were measured using a two-site immunoenzymatic assay (ELISA) as previously described (9). Briefly, polystyrene 96-well microtitel immunoplates were coated with affinity-purified polyclonal goat anti-NGF antibody. Parallel wells were coated with purified goat IgG (Zymed) for evaluation of nonspecific signal. After overnight incubation at room temperature and 2 h of incubation with the coating buffer (50 mM carbonate buffer, pH 9.5, in 2% BSA) plates were washed with 50 mM Tris·HCl, pH 7.4/200 mM NaCl/0.5% gelatin/0.1% Triton X-100. After extensive washing, the diluted samples and the NGF standard solutions, ranging from 0 to 1 ng/ml, were distributed in each plate and left at room temperature overnight. The plates then were washed and incubated with 4 milliunits of anti β-NGF-galactosidase per well (Boehringer Mannheim). After an incubation of 2 h at 37°C, the plates were washed and incubated with 100 μl of substrate solution (4 mg of chlorophenol red per ml of substrate buffer: 100 mM Hepes/150 mM NaCl/2 mM $MgCl_2$/0.1% sodium azide/1% BSA) that was added to each well. After an incubation of 2 h at 37°C, the optical density was measured at 575 nm using an ELISA reader (Dynatech). The recovery was estimated by adding to the tissue samples a known amount of purified NGF. The yield of the exogenous NGF was calculated by subtracting the amount of exogenous NGF from the values of the exogenous ones. Under these conditions the recovery of NGF from the tissue samples was about 80–90%. The values were corrected by subtracting the relevant background value to nonspecific binding. Specificity for NGF also was assessed by using a recombinant human brain-derived neurotrophic factor (Genentech).

***In Situ* Hybridization.** Oligonucleotide probes with sequences complementary to mRNA encoding neuronal NOS (nNOS, amino acids 151–164) and CRH (nucleotides 64–111) were labeled at the 3′-end with deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (New England Nuclear) using terminal deoxynucleotidyltransferase (Amersham) in a buffer containing 10 mM $CoCl_2$, 1 mM DTT, 300 mM Tris base, and 1.4 M potassium cacodylate (pH 7.2). The labeled probes were purified through Nensorb-20 columns, and DTT was added to a final concentration of 10 mM. The specific activity obtained ranged from 1 to 4×10^6 dpm/ng oligonucleotide. Sections were covered with a hybridization buffer containing 50% formamide, 4× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1× Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% BSA, and 0.02% Ficoll), 1% sarcosyl, 0.02 M phosphate buffer (pH 7.0), 10% dextran sulfate, 500 μg/ml heat-denatured salmon sperm DNA, and 200 mM DTT and 40 ng/μl of the labeled probes. The slides were incubated for 15–20 h at 50°C, rinsed in 1× SSC at 55°C for 1 h and at room temperature for 1 h. Finally, the slides were rinsed in distilled water and 60% and 95% ethanol (2 min each), air dried, and then dipped in NTB2 nuclear track emulsion (Kodak) for 3 weeks before being developed. The quantitative analysis was performed on emulsion-dipped sections slightly counter stained by toluidine blue using AIS Imaging Research (Ontario, Canada) grain-counting software. nNOS mRNA expression was measured in the lamina X of the spinal cord and in the cerebral cortex by choosing three matching sections in six rats for each experimental group at each time point. In all sections, 8 to 10 cells were measured. The average values from each animal were used for a statistical analysis.

Histopathology. Toluidine blue and hematoxylin-eosin staining were used for histological studies in paraformaldehyde-fixed animals. All brains and spinal cord sectioned for *in situ* hybridization studies also were analyzed for the presence of cellular infiltrates.

Statistical Analysis. ANOVA and Dunnett's test (vs. control uninjected rats) were used to compare data at each experimental time.

RESULTS

The clinical profile and body weight of experimental animals are reported in Fig. 1. The severity of EAE gradually increases

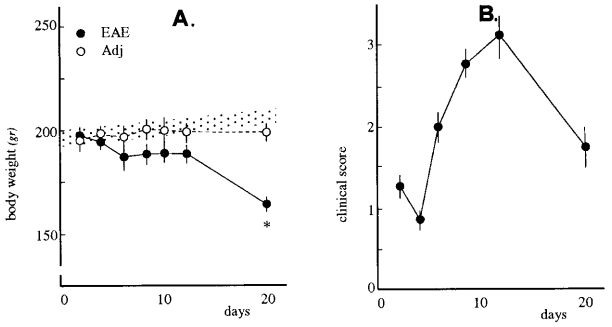

FIG. 1. Body weight of experimental rats (*A*) and clinical score of EAE rats (*B*). Statistical analysis: ANOVA and Dunnett's test; *, $P < 0.05$.

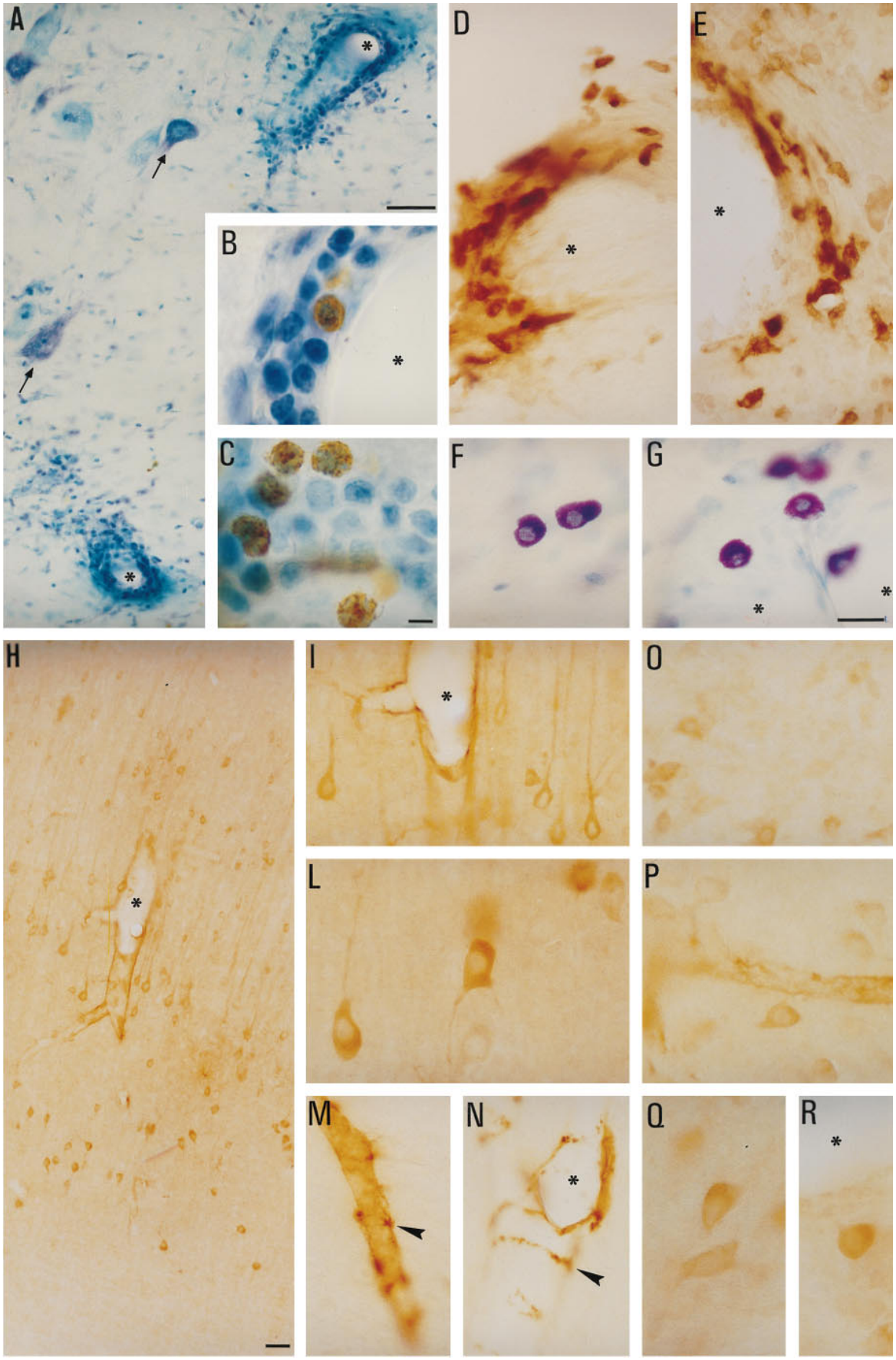

FIG. 2. (*A*) Toluidine blue stained section from mesencephalon of EAE rat 20 days after immunization. Arrows indicate large neurons in the mesencephalic nucleus of the V nerve. Intense perivascular cellular infiltrates were found in this area. Numerous diaminobenzidine-positive elements, probably neurophils (*B* and *C*) and iNOS-like positive cells (*D* and *E*) were observed in perivascular and intraparenchymal inflammatory cellular infiltrates.

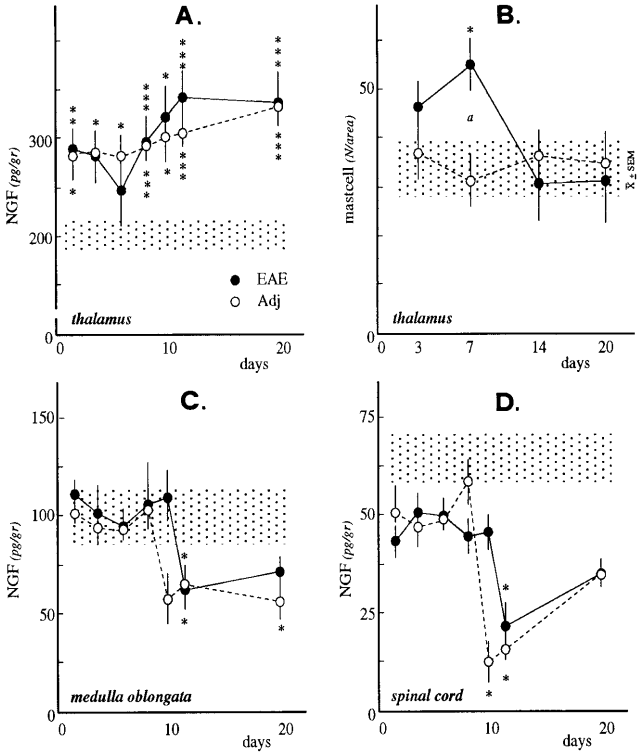

FIG. 3. (*A*, *C*, and *D*) NGF content in different brain areas during development of EAE and CFA-induced inflammation. (*B*) Number of thalamic mast cells in the thalamus during EAE. Mast cells have been counted in 5 matching sections from the thalamus of each animal, and the relative number has been normalized according to the sampled area. Statistical analysis: ANOVA and Dunnett's test; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$; a, Student's *t* test, $P < 0.05$.

and reaches its peak between 8 and 12 days after immunization. The animals then partially recover 20 days after immunization, when a severe weight loss is observed.

Histopathological Findings. Multiple confluent foci of inflammation appear in many areas of the brain and spinal cord between 14 and 20 days after myelin immunization (Fig. 2). In particular, numerous small blood vessels in the cervical spinal cord, medulla oblongata, pons, ventral mesencephalon, hypothalamus, and septal nuclei showed inflammatory perivascular infiltrates, including mononuclear cells. Small intraparenchimal infiltrates were observed all over the brain and spinal cord, and perivascular infiltrates were found in the white matter, too (i.e. corpus callosum and cerebellar peduncoli). Submeningeal infiltrates also were observed in the medulla oblongata, pons, and spinal cord. Mononuclear and polymorphonuclear, probably neutrophil diaminobenzidine-positive, cells were present in all foci of inflammation 20 days after sensitization. Almost all EAE animals showed a histological picture scored as 4 according to Hickey *et al.* (15). Several iNOS-positive cells were found in perivascular and intraparenchimal infiltrates. Only in a few rats did we observe small scattered areas of inflammation (1–2 according to Hickey *et al.*, ref. 15) 14 days after immunization. No significant cellular infiltrates were found in EAE rats 3 and 7 days after immunization, and at no time in rats injected with CFA.

NGF and NGF Receptors. NGF content was measured in the medulla oblongata and cervical spinal cord, where a severe cellular infiltrate appears during EAE and in the thalamus. The NGF content significantly increased in the thalamus in

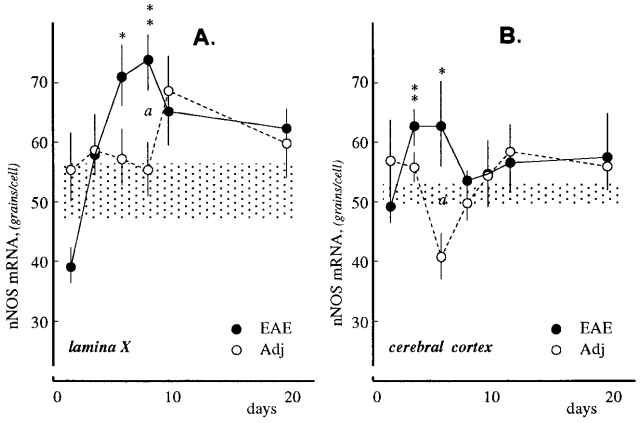

FIG. 4. nNOS mRNA expression in single neurons in the spinal cord (*A*) and cerebral cortex (*B*) during EAE, CFA-induced arthritis, and in control rats. Statistical analysis: ANOVA and Dunnett's test, *, $P < 0.05$, **, $P < 0.01$; a, Student's *t* test, $P < 0.05$.

EAE and CFA-injected animals as soon as clinical signs of central or peripheral inflammation appeared (Fig. 3*A*). An increase in the number of mast cells adherent to the intraparenchimal blood vessel also was observed in EAE 7 days from immunization (Fig. 3*B*). On the contrary, NGF content was unchanged in the early and critical stages of clinical inflammation in the cervical spinal cord and medulla oblongata of both experimental groups, and it dropped dramatically after 12 and 20 days from treatment (Fig. 3 *C* and *D*). In EAE rats, p75- and trkA-like perivascular positivity appeared in several CNS areas between 14 and 20 days after immunization, coinciding with an increased severity of perivascular cellular infiltrates, also in brain areas where infiltrates were scanty (Fig. 2). For example, in the parietal and pyriform cortex, such as in the amygdaloid complex, a strong p75- and trkA-like positivity was observed on blood vessels walls 14 days after immunization. Small, stellar cells (Fig. 2) and positive fibers were found around blood vessels (Fig. 2). Neurons adjacent to labeled blood vessels were also positive, and staining intensity gradually decreased moving away from positive vessels. Twenty days after immunization, blood vessels tended to be p75 and trkA negative, whereas a large area of cortex showed p75-like immunoreactive neurons. Moreover, few trkA-positive elements were still present. A similar phenomenon was observed in CFA-injected rats, but staining was less intense in both blood vessels and adjacent neurons.

nNOS mRNA Regulation. nNOS mRNA expression was investigated in lamina X of the spinal cord, where NOS is constitutively expressed in neurons around the central channel, and in the cerebral cortex, where NO-producing neurons are scattered in all areas and layers. A significant increase in nNOS mRNA expression was found early after immunization in both areas during EAE. However, there was a return to normal when the clinical signs of the disease diminished (Fig. 4). No variations in nNOS mRNA expression were found at any of the investigated times in CFA-injected rats.

CRH Regulation of the HPA Axis. CRH mRNA expression in the PVN fell dramatically 4 days after immunization in EAE rats (Fig. 5*A*). A temporary recovery was observed after 6, 8, and 10 days, and a second fall was observed after 20 days. A similar profile also was found in early phases of inflammation in CFA-injected rats. However, the CRH mRNA recovery in this group was slower, complete, and stable until the end of the

(*F* and *G*) Mast cells stained metachromatically violet by toluidine blue in the thalamus. (*H–L*) p75-immunoreactive neurons in the cerebral cortex of EAE rats 20 days after immunization. Small, stellar, p75-positive cells adherent to the wall (*M*), and fibers around blood vessels (*N*) were observed all over the brain and spinal cord. (*O–R*) trkA-immunoreactive cells in the cerebral cortex of EAE rats 20 days after immunization. Asterisks indicate the lumen of blood vessels. (Bars: *A*, *I*, and *O*, 50 μm; *B* and *C*, 25 μm; *D–G*, *L–N*, and *P–R*, 25 μm; *H*, 10 μm.)

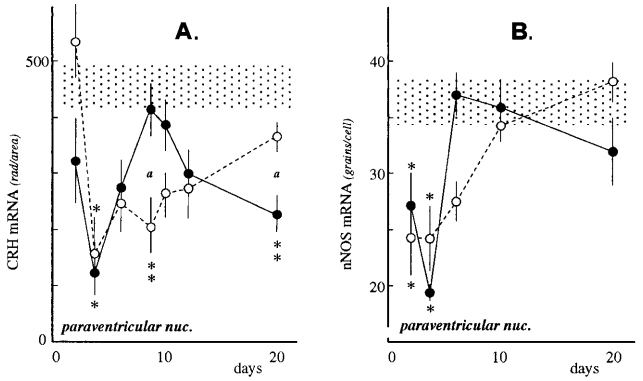

FIG. 5. (*A*) CRH and (*B*) nNOS mRNA expression in the PVN during EAE (●), CFA-induced arthritis (○), and in control rats (stippled band). Statistical analysis: ANOVA and Dunnett's test, *, $P < 0.05$, **, $P < 0.01$; a, Student's *t* test, $P < 0.05$.

experiment. NOS mRNA expression in the PVN also decreased in the early phases of both central (EAE) and peripheral (CFA) inflammation, but NOS mRNA expression already had returned to control level in both experimental groups and kept steady after 6–8 days (Fig. 5*B*).

DISCUSSION

In the EAE time-course experiments reported in this study, we have correlated neuroendocrine events to variations of NGF, NGF receptor, and inducible and neuronal isoforms of NOS, compared with arthritic and control rats. iNOS-positive cells were found in the inflammatory cellular infiltrate in perivascular and submeningeal areas in EAE during transition from the acute to chronic phase. mRNA expression for the neuronal isoform of NOS also was up-regulated in the cerebral cortex and spinal cord during EAE. We have demonstrated opposite modifications of NGF content in different brain areas in EAE, i.e., increased content in the thalamus and decreased content in the spinal cord and medulla oblongata at specific time points. Moreover, up-regulation of NGF receptors in different brain areas and the spinal cord was found in EAE. Similar, but reduced, changes in NGF content and NGF receptor regulation also were observed in CFA-injected rats. A paradoxical down-regulation of CRH mRNA expression in the PVN preceded EAE-induced brain inflammation and chronic exacerbation of EAE.

Stress response of the HPA axis plays a crucial role in health, because all HPA hormones are involved in the regulation of the body homeostasis, the immune system, and neuron survival (7). In particular, inflammatory and immune mediators, including IL1, IL2, and tumor necrosis factor, stimulate the HPA axis at hypothalamic level (5), and this is a crucial mechanism in response to inflammation. Here we have demonstrated that a defect in CRH-mediated regulation of the HPA axis precedes the peak of clinical severity in acute exacerbation of EAE and also the chronic-relapsing phase. On the contrary, the initial fall in CRH mRNA expression gradually is restored in arthritic rats, when they recover from the disease. A defect of biosynthesis of CRH, which could account for the defective HPA axis responses to inflammation, has been described in Lewis rats (6, 16), in which IL1α is unable to induce CRH release from explanted hypothalami (6). The impairment of CRH up-regulation observed during critical phases in EAE could affect inflammatory/immune response through peripheral hormones (ACTH and glucocorticoids) or through CRH itself. In fact, it has been reported, that cerebral administration of CRH affects natural killer cell cytotoxicity via the autonomic nervous system (see ref. 17). CRH receptors are also present on monocytes and CRH suppress leukocyte chemotaxis (see ref. 17). nNOS mRNA in the PVN follows CRH mRNA modification during inflammatory stress. A role for NO in modulating CRH release induced by IL1β (18, 19) and IL2 (20) has been suggested by *in vitro* experiments, and parallel regulation of CRH and NOS mRNAs in the PVN has been described in different stress paradigms (21, 22).

We have described interesting regulation of NOS isoforms, which might be involved in the evolution from acute to chronic EAE. In many systems and in different pathological conditions, the free-radical NO derives from different cellular sources, forming networks of paracrine communication (23). We have found that the transition from the acute to chronic phase of EAE is characterized by the presence of numerous iNOS positive cells, presumably macrophages, in perivascular, submeningeal, and intraparenchimal inflammatory cellular infiltrates. The appearance of iNOS in the brain has been described during systemic inflammation caused by LPS (24), different viral infections, and EAE itself (12). NO produced close to the BBB can be toxic for endothelium of brain vessel, as demonstrated in the respiratory epithelium (25) and in the development of widespread microvascular injury in toxic conditions (26). In fact, inhibition of NOS attenuates BBB disruption during experimental meningitis (27) and also attenuates clinical signs of EAE, although it has no effect on the histological lesions (28). Thus, these iNOS-positive cells could have a role in the breakdown of the BBB, which is a critical event in transition from the acute to chronic phase of EAE. iNOS-mediated generation of NO also could be implicated in the demyelination and destruction of oligondendrocytes (29).

Moreover, we also have shown an increase of nNOS mRNA in neurons that normally express nNOS. NOS containing neurons are presumed to be resistant to neurodegeneration and neurotoxicity, i.e. during focal cerebral ischemia (30), but neuronal overproduction of NO may facilitate or mediate neurotoxicity during ischemia (31), as also has been confirmed using transgenic mice lacking NOS activity (32). An overproduction of neuronal NO could represent a molecular mechanism for lesion of the axon. It has been suggested that axonal loss could be prominent during EAE, and that demyelination may be a secondary consequence of axon damage, rather then a primary event (see ref. 4). We also have described an increase in nNOS mRNA expression in sensory ganglia during chronic arthritis, when damage of joint structures, presumably including fine nerve fibers, takes place (L.C., M.P. & E. Manzini, unpublished data).

One major finding in this study is the dramatic regulation of NGF content and NGF receptors expression in different areas of the brain and spinal cord in EAE. A general concept that can help to explain this result refers to the cytokine regulation of neurotrophins function (33, 34). Cytokines involved in inflammation and immune responses during EAE, such as IL-1β and tumor necrosis factor-α (35, 36) are potent inductors of NGF synthesis in peripheral tissues (37, 38) and in the CNS (34, 39, 40). During EAE, higher levels of NGF are found in certain brain areas, like the thalamus and cerebral cortex (41), and a strong up-regulation of p75- and trk-A-like immunoreactivity also has been found. NGF receptor-like immunoreactivity appears in perivascular areas, on neurons, and probably on glial cells. On the other hand, here we also have described a drop in NGF content in the spinal cord and medulla oblongata, where the most serious inflammatory cellular infiltrate was found. A reciprocal regulation of NGF content and receptors has been described in different pathological conditions (42, 43). In our experimental conditions, we presume that locally produced or peripheral derived cytokines induce an increase in NGF content and the resulting receptor up-regulation. A strong request in brain areas with severe inflammation could account for the drop in NGF content in the spinal cord and medulla oblongata.

NGF content significantly increases in the thalamus during EAE, and this also could lead to pain and hyperalgesia during central inflammation (44). Peripheral and central mechanisms account for NGF-mediated hyperalgesia (45). Peripheral mech-

anisms probably are triggered by mast cells degranulation, whereas central mechanism could take place at primary afferent entry level (45, 46). We found a higher number of thalamic mast cells in EAE and also an increase in mast cell degranulation (41). Because mast cells synthesize NGF (47), they can be a source of thalamic NGF in EAE, and this mechanism also could give rise to central pain during multiple sclerosis. However, we also should consider the possibility of peripheral sources of NGF, in view of BBB breakdown during EAE (48), or glial origin, because astrocytes can synthezise NGF (49). A likely consequence is that thalamic NGF also is involved in the development of central hyperalgesia during EAE.

We should mention that some of the NGF and NGF-receptor alterations that we found during EAE also have been found during CFA-induced arthritis. This is not surprising considering that arthritis is also an inflammatory disease involving central processes, like thermal regulation and hyperalgesia. However, NGF receptor up-regulation in perivascular areas is much stronger in EAE than in arthritis, and this could be due to the severity of the diseases (EAE is a chronic-relapsing disease, whereas CFA-induced arthritis recovers within 20 days), or to the localization of inflammation areas (in the brain during EAE, in the periphery during CFA-induced arthritis), or to the relative importance of autoimmune components.

In conclusion we suggest that the development of EAE requires a peculiar neuroendocrine profile of the HPA axis and the coordinated presence of molecules that can be produced and interact with different cell types and body compartments. NGF and NO could be candidates for this. The CRH-mediated deficit of HPA axis response encourages the early immunological stage of EAE, which is characterized by increased levels of cytokines. During this stage, the up-regulation of nNOS could represent a protection strategy of certain neuronal populations, but it also could indicate a molecular mechanism causing axonal damage preceding demyelinization. Cytokines induce NGF accumulation around vessels and, consequently, up-regulation of NGF receptors. NGF itself could act as an immunomodulator. A second fall in CRH mRNA expression favors the massive perivascular and intraparenchimal cellular infiltration that supports the chronic phase of EAE. iNOS positive cells present here could lead to the physical breakdown of BBB. Our data support the hypothesis that acute symptoms are sustained by inflammatory and immune events that do not require recruitment of peripheral cells into the brain and spinal cord, whereas the chronic, long-lasting phase is characterized by severe morphological modifications including demyelinization. Thus, diagnostic and therapeutic efforts must be directed to molecules involved in the evolution from the acute to chronic phase.

We gratefully acknowledge Prof. Rita Levi-Montalcini for her invaluable support, suggestions, and assistance. This work has been supported by Associazione Italiana Sclerosi Multipla (L.C.), BIOMED 2 BMH4-CT95-0172 (L.C.), and Istituto Superiore di Sanità, Progetto Sclerosi Multipla (L.A.).

1. Lassmann, H., Kitz, K. & Wisniewski, H. M. (1980) *Acta Neuropathol.* **51,** 191–201.
2. Namer, I. J., Steibel, J., Poulet, P., Armspach, J. P., Mauss, Y. & Chambron, J. (1992) *Magn. Reson. Med.* **24,** 325–334.
3. Duckers, H. J., Van Dokkum, R. P., Verhaagen, J., Lopes Da Silva, F. H. & Gispen, W. H. (1996) *Neuroscience* **71,** 507–521.
4. Scolding, N. J., Zajicek, J. P., Wood, N. & Compston, D. A. S. (1994) *Prog. Neurobiol.* **43,** 143–173.
5. Gaillard, R. C. (1994) *Trends Endocrinol. Metab.* **5,** 303–309.
6. Sternberg, E. M., Hill, J. M., Chrousos, G. P., Kamilaris, T., Listwak, S. J., Gold, P. W. & Wilder, R. L. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2374–2378.
7. Besedovsky, H. O. & Del Rey, A. (1996) *Endocr. Rev.* **17,** 64–102.
8. Bracci-Laudiero, L., Aloe, L., Levi-Montalcini, R., Galeazzi, M., Schilter, D., Scully, J. L. & Otten, U. (1993) *NeuroReport* **4,** 563–565.
9. Bracci-Laudiero, L., Aloe, L., Levi-Montalcini, R., Buttinelli, C., Schilter, D., Gillessen, S. & Otten, U. (1992) *Neurosci. Lett.* **147,** 9–12.
10. Levi-Montalcini, R., Aloe, L. & Alleva, E. (1990) *Prog. Neuroendocrinoimmunology* **3,** 1–10.
11. Bagasra, O., Michaels, F. H., Zheng, Y. M., Bobroski, L. E., Spitsin, S. V., Fu, Z. F., Tawadros, R. & Koprowski, H. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 12041–12045.
12. Koprowski, H., Zheng, Y. M., Heber-Katz, E., Fraser, N., Rorke, L., Fu, Z. F., Hanlon, C. & Dietzschold, B. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3024–3027.
13. Mitrovic, B., Ignarro, L. J., Montestruque, S., Smoll, A. & Merril, J. E. (1994) *Neuroscience* **61,** 575–585.
14. Moncada, S., Palmer, R. M. J. & Higgs, E. A. (1991) *Pharmacol. Rev.* **43,** 109–142.
15. Hickey, W. F., Cohen, J. A. & Burns, J. B. (1987) *Cell. Immunol.* **109,** 272–282.
16. Sternberg, E. M., Young, W. S. III, Bernardini, R., Calogero, A. E., Chrousos, G. P., Gold, P. W. & Wilder, R. L. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4771–4775.
17. Dunn, A. J. & Berridge, C. W. (1990) *Brain Res. Rev.* **15,** 71–100.
18. Costa, A., Trainer, P., Besser, M. & Grossman, A. (1993) *Brain Res.* **605,** 187–192.
19. Sandi, C. & Guaza, C. (1995) *Eur. J. Pharmacol.* **274,** 17–23.
20. Karanth, S., Lyson, K. & McCann, S. M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3383–3387.
21. Calzà, L., Giardino, L. & Ceccatelli, S. (1993) *NeuroReport* **4,** 627–630.
22. Kishimoto, J., Tsuchiya, T., Emson, P. C. & Nakayama, Y. (1996) *Brain Res.* **720,** 159–171.
23. Schmidt, H. H. H. W. & Walter, U. (1994) *Cell* **78,** 919–925.
24. Wong, M.-L., Rettori, V., Al-Shekhlee, A., Bongiorno, P. B., Canteros, G., McCann, S. M., Gold, P. W. & Licinio, J. (1996) *Nat. Med.* **2,** 581–584.
25. Nixon Heiss, L., Lancaster, J. R., Jr., Corbett, J. A. & Goldman, W. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 267–270.
26. Laszlo, F., Whittle, B. J. R., Evans, S. M. & Moncada, S. (1995) *Eur. J. Pharmacol.* **283,** 47–53.
27. Boje, K. M. K. (1996) *Brain Res.* **720,** 75–83.
28. Zhao, W., Tilton, R. G., Corbett, J. A., McDaniel, M. L., Misko, T. P., Williamson, J. R., Cross, A. H. & Hickey, W. F. (1996) *J. Neuroimmunol.* **64,** 123–133.
29. Sparrow, J. R. (1995) *J. Mol. Neurosci.* **5,** 219–229.
30. Zhang, Z. G., Chopp, M., Gautam, S., Zaloga, C., Zhang, R. L., Schmidt, H. H. H. W., Pollock, J. S. & Forstermann, U. (1994) *Brain Res.* **654,** 85–95.
31. Dalkara, T., Yoshida, T., Irikura, K. & Moskowitz, M. A. (1994) *Neuropharmacology* **33,** 1447–1452.
32. Huang, Z., Huang, P. L., Panahian, N., Dalkara, T., Fishman, M. C. & Moskowitz, M. A. (1994) *Science* **265,** 1883–1885.
33. Aloe, L., Skaper, S. D., Leon, A. & Levi-Montalcini, R. (1994) *Autoimmunity* **19,** 141–150.
34. Otten, U. & Gadient, R. A. (1995) *Int. J. Dev. Neurosci.* **13,** 147–151.
35. Navikas, V. & Link, H. (1996) *J. Neurosci. Res.* **45,** 322–333.
36. Simmons, R. D. & Willenborg, D. O. (1990) *J. Neurol. Sci.* **100,** 37–42.
37. Lindholm, D., Heumann, R., Meyer, M. & Thoenen, H. (1987) *Nature (London)* **330,** 658–659.
38. Steiner, P., Pfeilschifter, J., Boeckh, C., Radeke, H. & Otten, U. (1991) *Am. J. Physiol.* **261,** F792–F798.
39. Gadient, R. A., Cron, K. C. & Otten, U. (1990) *Neurosci. Lett.* **117,** 335–340.
40. Friedman, W. J., Larkfors, L., Ayer-LeLievre, C., Ebendal, T., Olson, L. & Persson, H. (1990) *J. Neurosci. Res.* **27,** 374–382.
41. Micera, A., De Simone, R. & Aloe, L. (1995) *Arch. Ital. Biologie* **133,** 131–142.
42. Knusel, B. & Gao, H. (1996) *Life Sci.* **58,** 2019–2027.
43. Calzà, L., Giardino, L. & Aloe, L. (1997) *Exp. Neurol.* in press.
44. Lewin, G. R. & Mendell, L. M. (1993) *Trends Neurosci.* **16,** 353–359.
45. Lewin, G. R., Rueff, A. & Mendell, L. M. (1994) *Eur. J. Neurosci.* **6,** 1903–1912.
46. Woolf, C. J., Ma Q.-P., Allchorne, A. & Poole, S. (1996) *J. Neurosci.* **16,** 2716–2723.
47. Leon, A., Buriani, A., Dal Toso, R., Fabris, M., Romanello, S., Aloe, L. & Levi-Montalcini, R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3739–3743.
48. Poduslo, J. F. & Curran, G. L. (1996) *Mol. Brain Res.* **36,** 280–286.
49. Lee, T.-H., Kato, H., Kogure, K. & Itoyama, Y. (1996) *Brain Res.* **713,** 199–210