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CIRCULATING NGF ANTIBODY ALTERS THE DISTRIBUTION OF NG2 AND CD56 POSITIVE CELLS IN THE BRAIN OF AN ANIMAL MODEL OF INFLAMMATORY DISORDER

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INTRODUCTION

One important limiting factor in the replacement of damaged cells and in promoting mechanisms of repair is the difficulty to identify and stimulate resident immature cells of the central nervous system (CNS). Undifferentiated cells able to give rise to glial cells and neurones have been detected in specific regions of developing and adult brain (40). The knowledge of the events and molecules involved in stem cell proliferation and differentiation could be helpful to develop repair strategies for brain disorders where damage and/or loss of cells occurs. A number of extracellular signals and growth factors (17, 23, 33) regulate stem cell proliferation, cell fate determination, progenitor migration and differentiation, including Nerve Growth Factor (NGF) (29).

NGF is the first and best-characterised member of the family of structurally and functionally related neurotrophins (22, 27), which include brain-derived neurotrophic factor (BDNF) and neurotrophins 3/5 (NT-3/5) (7, 8). NGF is produced and released by a variety of tissues within and outside the central nervous system (CNS) of mammals and plays a crucial role during neuronal development and after injury (27). In addition to its neurotrophic activity, NGF exerts broad biological activities on other cell types, including cells of the innate and adaptive immune responses, such as mast cells, basophils and lymphocytes (2). The biological effect of NGF is mediated by binding to two classes of receptors: trkA, the tyrosine kinase receptor, and p75, which lack intrinsic kinase activity (7, 16). NGF seems to participate to the compensatory mechanisms intervening in response to inflammation in the central nervous system (CNS) of EAE rats (13, 30, 31), the experimental model of the human disease Multiple Sclerosis (MS) (18).

We have previously shown that the levels of NGF in the cerebrospinal fluid (CSF) of patients affected by MS significantly increase during the acute phase of the disease (11). Moreover we observed that NGF injected into the brain ventricle of rats with Experimental Allergic Encephalomyelitis (EAE) is taken up by SVZ cells and transported into brain parenchyma (3, 13). Indeed, neuroepithelial cells lying in the subventricular zone (SVZ) of amphibians (26) and rodents express NGF-receptors and are responsive to the action of NGF (3).

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Since EAE is a neuroinflammatory disorder characterised by loss of brain and immune cells, the possibility that NGF can exert its protective/reparative effect acting on as yet unidentified progenitor cells cannot be excluded. In a previous report, it was hypothesized that the increased levels of NGF in the CSF of MS patients (11) might have an effect on stimulating growth and differentiation of immature cells localised in the SVZ in order to repair the damaged brain tissue (3, 13, 36).

The evidence that hematopoietic cells can generate nerve cells, that brain progenitor cells can generate blood cells (9) and that hematopoietic stem cells (12) and immature nerve cells express the NGF-receptor TrkA (13, 27) reinforces the hypothesis that unidentified precursors can be one of the target for the reparative/protective effect prospected for NGF.

A major aim of this study was therefore to investigate whether cell types expressing immunological and/or neuronal markers are the targets for the action of NGF. Accordingly, using NGF-autoimmune EAE-rats, we have examined the brain regions of EAE rats with circulating NGF-antibodies and analysed the distribution of brain cells expressing NG2, a marker that identifies Ols progenitors (OPC) (15, 41, 43) and CD56, a cell surface marker expressed by SVZ cells during remyelination (35) and also by activated immune cells during EAE (5).

We also investigated whether or not NGF-autoimmunization affects NGF level and trkA expression in cells of the frontal cortex, which are one of the major brain source of NGF and are also NGF receptive cells.

METHODS

Animals

Pathogen-free, female Lewis rats weighing approximately 200 gr were purchased from Charles River (Como, Italy), housed in group of two per cage (18x30x24 cm) and allowed free access to food and water. Animal care procedures were conducted in conformity with the Intramural Committee and Institutional guidelines and the National and International laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

Induction and Assessment of EAE

EAE was induced in 120-days old female rats (n = 30). The rats were deeply anaesthetised and injected in each hind pad with 300 µl of a 1:1 (vol:vol) mixture of 29 mg of Guinea Pig Spinal Cord (GPSC) in saline (0.9% NaCl) and Complete Freund's Adjuvant (CFA, Difco Detroit, MI) 1 mg/ml M. Tuberculosis bacteria enriched (H37Ra, Difco), as previously reported (31). Another group of rats (n = 30) was injected with CFA as control for EAE induction.

Beginning on day 4 post-inoculation animals were daily weighed, assessed for clinical signs of disease and graded according to described criteria. Specifically: *0*, no clinical signs; *1*, loss of tail tonus; *2*, weakness in one or both hind legs or middle paresis; *3*, severe paresis or paralysis of both hind legs; *4*, severe paralysis of complete lower part of the body; *5*, death due to aggressive EAE. Scores *3* and *4* were often accompanied by urinary and faecal incontinence (47).

NGF-autoimmune EAE rats

Sixty-day old female rats (n = 30) were anaesthetised and treated for stimulating the production of endogenous anti-NGF antibodies following a protocol previously reported (21). Briefly, each rat was injected in dorsal skin with 300 µl of emulsion containing 200 µg/ml of purified 2.5S NGF, prepared as described by Bocchini and Angeletti (10), in 150 µl of complete Freund's adjuvant (Sigma, St. Louis, USA) and 150 µl of physiological saline (0.9% NaCl); booster of the same dose

of emulsion was given four weeks later. Following this procedure, rats produced anti-NGF antibodies directed against their own NGF. Circulating levels of anti-NGF antibodies were detected using a Doubleday and Robinson ELISA assay slightly modified (20) and biological activity of anti-NGF was assessed with a previous described bioassay (21). To obtain NGF-autoimmune rats affected by EAE, rats treated as above were used for induction of EAE (32). No mortality was observed in NGF-autoimmune EAE rats.

Tissue dissection

NGF-autoimmune EAE rats, EAE rats and control-CFA rats were sacrificed with an overdose of Nembutal at 15 days after EAE induction. The SVZ and the frontal cortex (Figure 1) were then surgically removed following the method reported by Cuello (19).

NGF Assay

The levels of NGF were measured by a modified highly sensitive two-site immunoenzymatic assay (ELISA), using anti-NGF antibodies (11, 46). This assay specifically recognises human and murine NGF and does not cross react with brain-derived neurotrophic factor (10). Briefly, polystyrene 96-well microtubes immunoplates (Nunc, purchased from Sial, Italy) were coated with affinity purified polyclonal goat anti-NGF antibody (Boehringher Mannheim, Germany), diluted in 0.05 M carbonate buffer (pH 9.6). Parallel wells were coated with purified goat IgG (Zymed, San Francisco, CA, USA), in order to evaluate the non-specific signal. Following overnight incubation at room temperature and 2 hours incubation with a blocking buffer (0.05 M carbonate buffer, pH 9.5, 1% BSA), plates were washed three times with Tris-HCl, pH 7.4 50 mM, NaCl 200 mM, 0.5% gelatine, 0.1% Triton X-100. After extensive washing of the plates, the samples and the NGF standard solutions were diluted with sample buffer (0.1% Triton X-100, 100 mM Tris-HCl, pH 7.2, 400 mM NaCl, 4 mM EDTA, 0.2 mM PMSF, 0.2 mM benzethonium chloride, 2 mM benzamidine, 40 U/ml aprotinin, 0.05% sodium azide, 2% BSA and 0.5% gelatine), distributed among the wells and left to stand at room temperature overnight. The plates were then washed three times and incubated with 4 mU/well anti-NGF-b-galactosidase (Boehringer Mannheim) for 2 hours at 37°C. After further washing, 100 µl of 4 mg/ml of chlorophenol red substrate solution (Boehringer Mannheim) in substrate buffer (100 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 0.1% sodium azide and 1% BSA) were added to each well. After an incubation of 2 hours at 37°C, optical density was

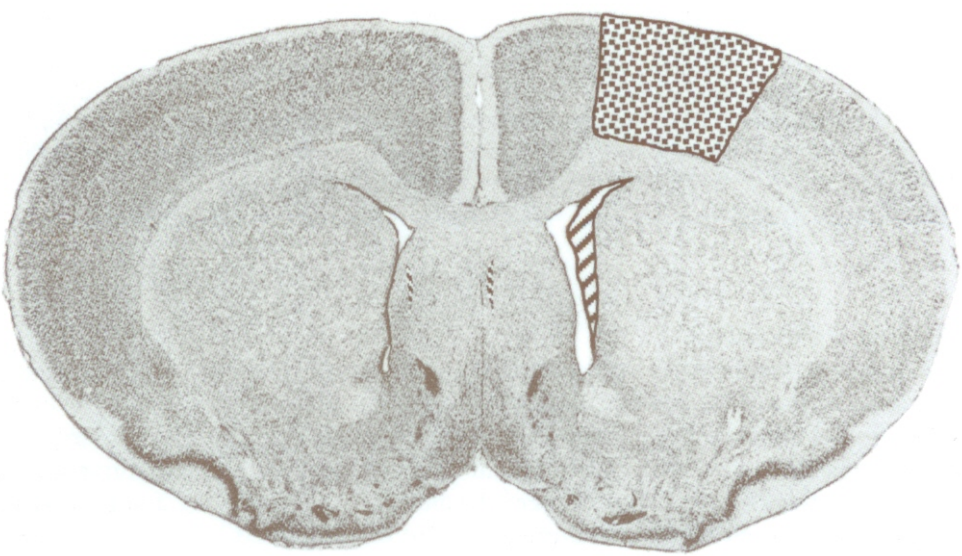


Fig. 1. - *Brain coronal section illustrating the examined areas: the SVZ (dashed area) and the frontal cortex (pointed area); scale bar = 2 mm.*

measured at 575 nm, using an ELISA reader (Dynatech MR5000, PBI International), and the values of standards and samples were corrected by taking non-specific binding into consideration. Under these conditions the sensitivity was 3 pg/gr and the recovery of NGF in our assay ranged from 80 to 90%. Data were presented as pg/gr (wet weight) and all assays were performed in triplicate.

Histological and immunohistochemical analysis

NGF-autoimmune EAE rats, EAE rats and control-CFA rats were deeply anaesthetised and perfused through the ascending aorta with saline solution followed by 4% paraformaldehyde in 0.1 M pH 7,4 phosphate buffer saline (PBS). The brains were then removed, immersion fixed for a further hour in the same fixative and cryoprotected for 24 hours in the same buffer containing 30% sucrose. Coronal brain sections were cut at 25 μm thick and stained with toluidine blue or immunostained with antibodies as indicated below.

Serial sections, starting from plate 11 to plate 20 of Paxinos and Watson (37), were processed for immunohistochemical localisation of trkA, NG2 and CD56. Briefly, free-floating sections were pre-treated with 3% H_2O_2-10% methyl alcohol (Carlo Erba, Italy) to quench endogenous peroxidase activity and then incubated with 10% normal serum and 5% BSA for 1 hour before overnight incubation at 4°C with the primary antiserum diluted in PBS 0.1 M containing 0.1% Triton X-100 (Sigma).

Antibodies were used at the following dilutions: 1:200, rabbit polyclonal anti-trkA antibodies (Santa Cruz Biotechnology, CA, USA); 1:300, rabbit polyclonal anti-NG2 antibodies (Chemicon International, Inc., Temecula, USA); 1:50, mouse monoclonal anti-CD56 antibodies (Novocastra, New Castle, U.K.). Sections were then incubated with biotinylated goat anti-rabbit or horse anti-mouse IgG (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, USA) for 2 hours, rinsed in PBS and then incubated with avidin-biotin peroxidase complex (Vectastain Elite ABC, Kit). Diaminobenzidine (0.6 mg/ml, DAB, Sigma) and 0.02% H_2O_2 were used to reveal the presence of the immunocomplex. To detect non-specific binding, control immunohistochemistry was performed by replacing the primary antibody with non-specific purified immunoglobulins (IgG). A Zeiss Axiophot microscope equipped with a x40 objective and connected to a computerized Image Analysis System (IAS 2000, Delta Sistemi, Rome, Italy) was used to evaluate the expression of trkA in the frontal cortex and of NG2 and CD56 in the SVZ.

Statistical analysis

Immunopositive cells localized in the entire SVZ and in the frontal cortex (four different fields for each section, n = 15/each animal) of NGF-autoimmune EAE, EAE and control-CFA rats were counted and compared. The mean values from all sections of each group were calculated and used for statistical analysis. The statistical package used was StatView II for Macintosh computer (Abacus Concepts. Inc. USA). One-way ANOVA followed by Tukey-Kramer post-hoc was used to compare immunopositive cell number and NGF levels in the SVZ and in the frontal cortex. A probability of 0.05 or less was considered statistically significant.

RESULTS

General observations

Adult Lewis rats injected with GPSC homogenate developed EAE clinical signs following a typical time course, as described by several authors (28, 42). The most severe clinical signs of EAE were observed between 8 and 12 days post induction (dpi), whereas the most severe histological changes (perivascular and intraparenchimal inflammations) were found between 10 and 14 dpi in the white and grey matter. Both clinical signs and brain inflammation were more marked in NGF-autoimmune EAE brains (32).

NGF-autoimmune EAE rats: NGF levels and trkA expression

To assess whether circulating anti-NGF antibodies influence brain NGF levels, we measured the levels of NGF in the frontal cortex, which, along with the hippocampus, produces the largest amount of NGF in the brain.

As reported in Figure 2, the concentration of NGF in the frontal cortex of NGF-autoimmune EAE rats was lower compared to control-CFA rats (45 ± 40 *vs.* 280 ± 80, pg/gr; $p < 0.05$). Although the NGF levels in the frontal cortex of NGF-autoimmune EAE rats were lower than in the frontal cortex of EAE rats, it did not reach the statistical significance. As shown in Figure 3, the number of trkA-positive cells significantly decreased in the frontal cortex of both NGF-autoimmune EAE ($p < 0.01$) and EAE rats ($p < 0.01$) as compared to control-CFA rats. Moreover, trkA-positive cells in NGF-autoimmune EAE rats were less numerous than in EAE (14.6 ± 8.0 *vs.* 105.6 ± 13.7; $p < 0.01$). These observations indicate that cortical cells are receptive to the action of NGF and that circulating levels of anti-NGF antibodies can affect both NGF levels and trkA receptor expression in this brain region.

NGF level and trkA expression in the SVZ

As reported in Fig. 4, the levels of NGF in the SVZ of EAE rats increased compared to control-CFA rats. In NGF-autoimmune EAE rats the amount of NGF in the SVZ was lower than in EAE rats (193 ± 57 *vs.* 570 ± 55, pg/gr; $p < 0.01$), reaching values comparable to control-CFA rats. This latter observation indicates that endogenous anti-NGF antibodies reduce the enhanced NGF levels that have been observed

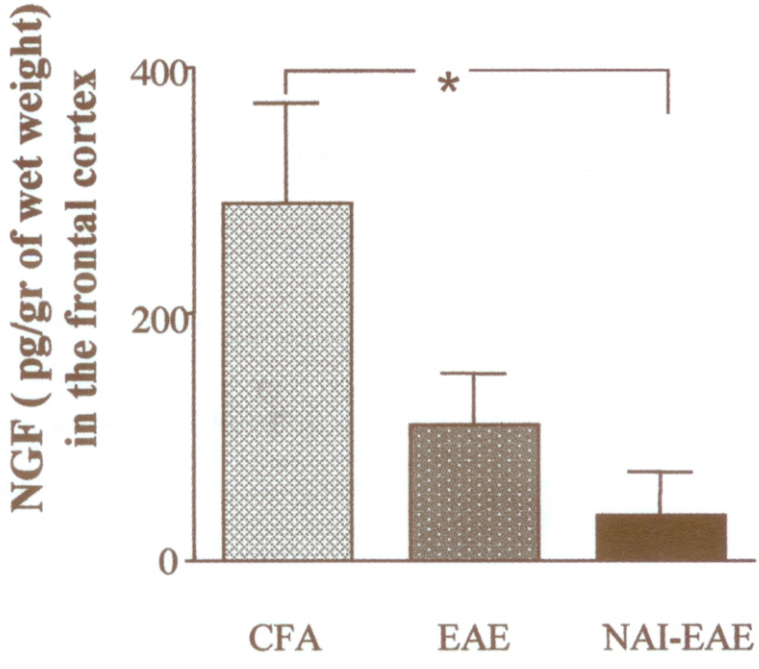


Fig. 2. - *NGF content of the frontal cortex expressed as pg/gr (wet weight) in CFA, EAE and NGF-autoimmune EAE rats (NAI-EAE).*
No changes of NGF levels were found in the frontal cortex of EAE-NGF-autoimmune, while in EAE rats the levels were significantly reduced. Statistical analysis was performed with ANOVA and Tukey-Krumer post-hoc,* $p < 0.05$.

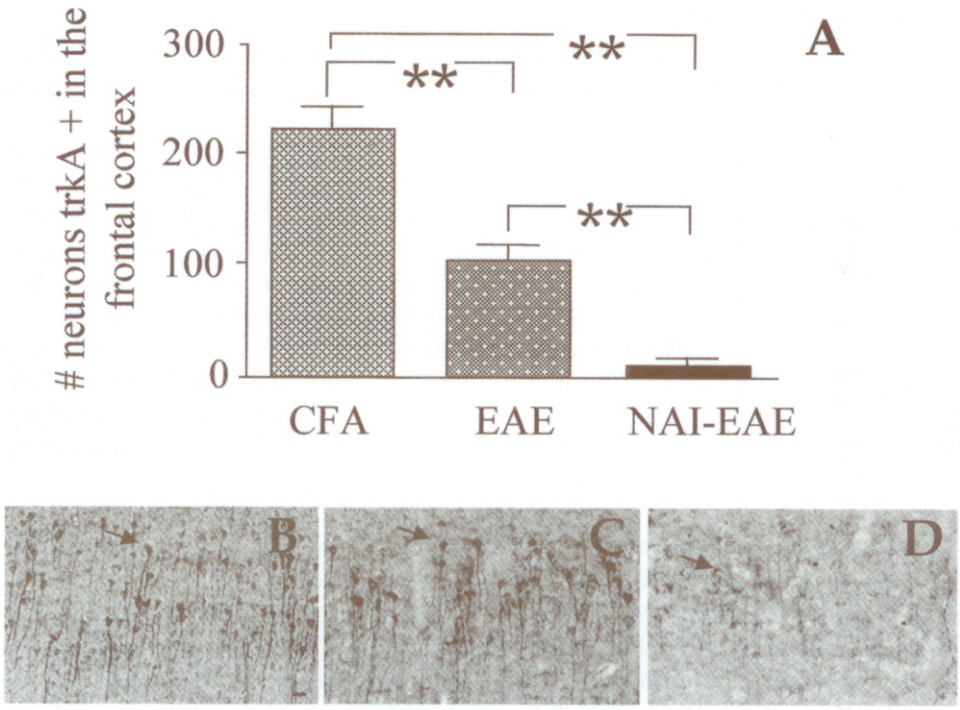


Fig. 3. - *The mean number of trkA-positive cells in the frontal cortex of CFA and EAE rats is showed in pannel A.*
The photos show the distribution of trkA-positive cells in frontal cortex of CFA (B), EAE (C) and NGF-autoimmune EAE rats (D); scale bar = 30 μm. Statistical analysis was performed with ANOVA and Tukey-Kramer post-hoc, $^{**}p < 0.01$.

in EAE. The effect of NGF-antibodies in NGF-autoimmune EAE rats was confirmed by the observation that SVZ cells express trkA in EAE, but not in NGF-autoimmune EAE rats (data not shown).

NG2 expression in the SVZ

To further characterize the effect of NGF-antibodies on EAE progenitor cells, brain sections of NGF-autoimmune EAE rats, EAE and control-CFA rats were immunostained for NG2, a proteoglycan marker of OPC. NG2-positive cells were localized within and in proximity of the SVZ. A significant decrease in the number of cells expressing NG2 was found in the SVZ of NGF-autoimmune EAE rats compared to control-CFA rats (67.0 ± 12.4 *vs.* 109.8 ± 8.5; $p < 0.05$). No significant differences were observed between NGF-autoimmune EAE and EAE, and between EAE and CFA rats (Figure 5).

CD56-positive cells in the SVZ

It has been previously shown that undifferentiated cells of the SVZ are able to migrate in response to intracerebroventricular injection of NGF (12). To assess

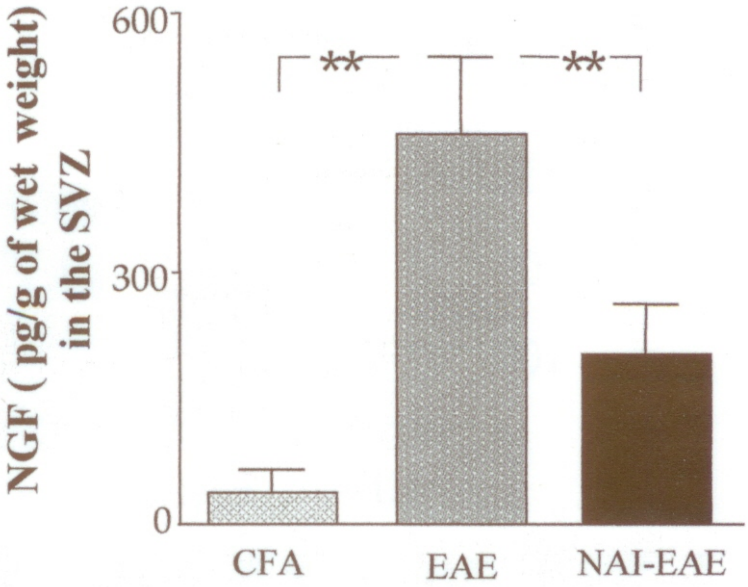
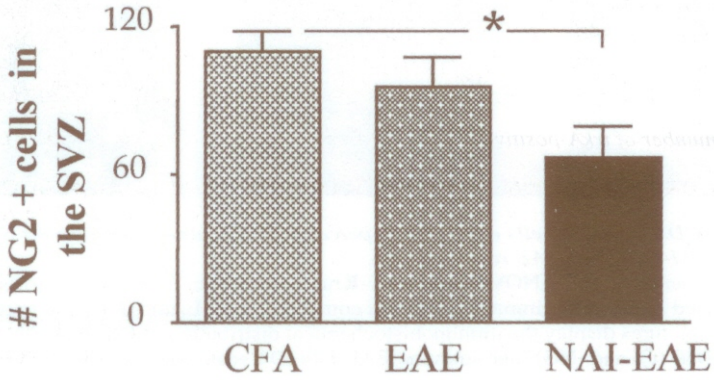


Fig. 4. - *NGF levels expressed as pg/gr (wet weight) in the SVZ of CFA, EAE and NGF-autoimmune EAE rats (NAI-EAE).*
NGF was increased in SVZ of EAE rats and was significantly reduced in NGF-autoimmune EAE rats compared to EAE rats. Statistical analysis: ANOVA and Tukey-Krumer post-hoc, ** $p < 0.01$.



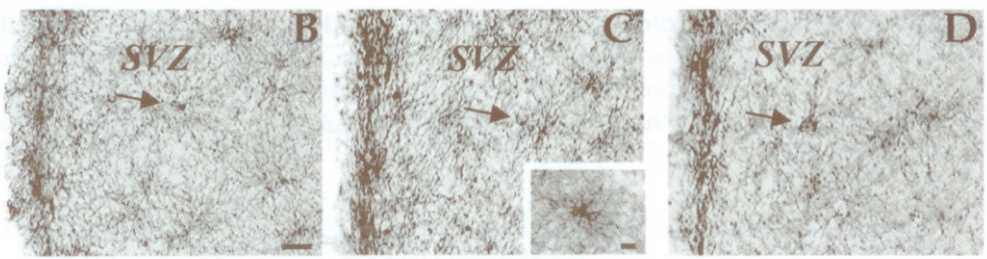


Fig. 5. - *The graph (panel A) shows the mean number of NG2-positive cells (OPC) in the SVZ of CFA, EAE and NGF-autoimmune EAE rats (NAI-EAE).*
Statistical analysis: ANOVA and Tukey-Krumer post-hoc, * $p < 0.05$. OPCs decrease in NGF-autoimmune EAE rats, but not in EAE, indicating that the reduced number of OPC is due to NGF-autoimmunization more than to EAE induction. The immunohistochemistry shows the presence of OPC (indicated by the arrow) in the SVZ of CFA (B), EAE (C) and NGF-autoimmune EAE rats (D). Scale bar for B, C, D = 20 μm. Scale bar for islet in C = 7 μm.

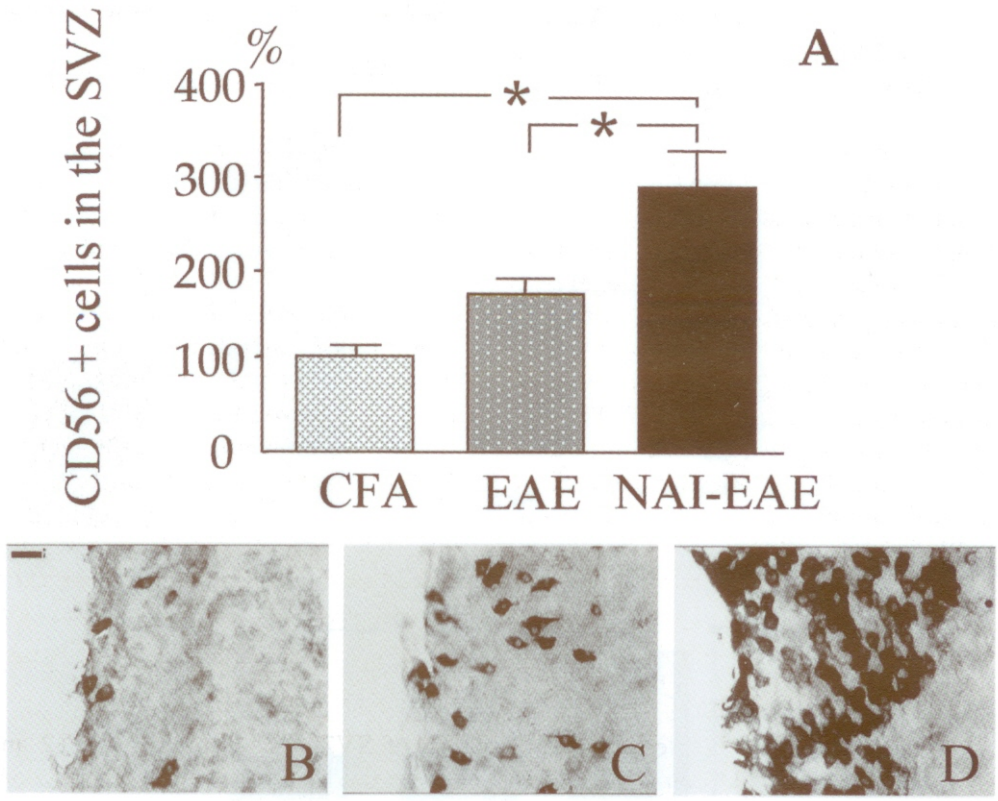


Fig. 6. - *Number of CD56-positive cells expressed as percentage of control (100%) in the SVZ of NGF-autoimmune EAE (NAI-EAE) and EAE rats (panel A).*
Statistical analysis – performed by ANOVA and Tukey-Krumer post-hoc, ** $p < 0.01$ – shows that CD56-positive cells increased in NGF-autoimmune EAE rats compared to EAE, and in EAE compared to control – CFA rats. The pictures display the immunohistochemical distribution of CD56 positive cells in the SVZ of CFA (B), EAE (C) and NGF-autoimmune EAE rats (D); scale bar = 20 μm.

whether NGF-antibody and/or low presence of NGF affect the activity of SVZ cells, brain sections of EAE rats were immunostained with CD56 antibodies. It was found that the number of CD56 positive cells (Figure 6) in the SVZ of NGF-autoimmune EAE rats was significantly higher compared both to EAE ($p < 0.01$) and to control-CFA rats ($p < 0.01$).

DISCUSSION

Our study demonstrates that EAE rats are characterised by reduced number of trkA-immunopositive cells in frontal cortex and altered amounts of NGF in both frontal cortex and SVZ. More dramatic effects on brain NGF and trkA expression, as well as in the severity of clinical signs were observed in NGF-autoimmune EAE

rats indicating that the reduced NGF availability might be associated with the worsening of EAE clinical markers.

Chiefly, this study shows that NGF levels in SVZ of NGF-autoimmune EAE rats decrease compared to EAE rats. As we have previously hypothesised, the NGF increase in the cerebrospinal fluid (CSF) of patients affected by Multiple Sclerosis (MS) during the acute phase of the disease might have effects on SVZ cells implicated in mechanisms leading to brain repair (11). However, the cell types that respond to the changes of NGF level in this brain region remain to be elucidated. To address this question we have carried out immunohistochemical analysis on serial brain sections to analyse whether the distribution of NG2 and CD56-positive cells in SVZ is influenced by changes of brain NGF levels. NG2 is a marker of OPC and is expressed in the white and grey matter of the normal adult brain and in the lesions of patients affected by MS (15, 43), while CD56 is expressed by neuronal and glial cells during development and after brain lesions (4). CD56 positive-cells increase after cortical lesion (44), during CNS remyelination (34) in the SVZ and following demyelination of the adult spinal cord (33).

We have found that the reduced amount of NGF in SVZ of NGF-autoimmune EAE rats causes a decrease in the number of NG2-positive cells. In line with other in vitro (6) and in vivo observations (3,13) this data suggests that NGF might act on SVZ population as a survival and/or differentiating factor for OPC.

We also demonstrated a marked increase of CD56 cells in SVZ of NGF-autoimmune EAE rats compared with both CFA and EAE rats. The fact that reduced amounts of brain NGF affects the distribution of SVZ cells expressing NG2 and CD56 provides an additional evidence supporting the hypothesis that NGF plays a role on progenitors cells of the SVZ.

In line with this hypothesis, the increased number of CD56-positive cells – in presence of reduced concentration of brain NGF in EAE rats – indicates that NGF can influence negatively the migratory activity of cells of the SVZ. An anti-migratory effect of NGF has been previously reported in the peripheral nervous system (1). In CNS, cell migration may take place with the participation of radial glial cells (14), which are particularly active during development and increase during neuropathological conditions, including EAE (35, 36).

Thus, it is likely to hypothesise that, in NGF autoimmune EAE rats, radial cells may also play an active role in modulating the migration and/or differentiation of SVZ cells.

Since large molecules have limited ability to cross the blood-brain barrier (BBB), one important question raised by our observation is how a large circulating molecule, such as anti-NGF antibody, can reach the CNS and affect progenitor cell behaviour. However, while this may be true under normal conditions, modification of the BBB might be possible during pathological conditions such as EAE, permitting the passage of large molecules from the periphery to the brain (25). Evidences that NGF can cross the BBB have also been reported (38, 39).

The findings reported here strengthen the hypothesis that NGF plays a role in EAE and probably in MS. Our observations also suggest that NGF acts on cell pop-

ulations having the ability to migrate and/or differentiate into Ols. The hypothesis of a role of NGF on SVZ cells is consistent with evidences that:

(a) increased levels of NGF have been found in patients affected by MS (11);
(b) radiolabelled NGF injected into the brain ventricles of EAE rats is taken up by SVZ cells and transported into the brain parenchyma (3, 26);
(c) NGF released into the nervous system improves clinical manifestations and delays neuropathological deficits of autoimmune neuritis (24);
(d) clinical signs of EAE are exacerbated by NGF-autoimmunization (32).

Based on the available evidence, we hypothesize that NGF is an important molecule for the survival and migration of SVZ cells, which may replace damaged cells in EAE brain. The findings that intracerebroventricular administration of NGF ameliorates clinical symptoms of marmosets affected by EAE support this hypothesis (45).

SUMMARY

Using an animal model of endogenous Nerve Growth Factor (NGF) deprivation, we have investigated the effect of this molecule on the distribution of NG2 and CD56-positive cells in the brain of normal and of Experimental Allergic Encephalomyelitis (EAE) rats. We found that the number of these cells is significantly altered in the SubVentricular Zone (SVZ) and in other brain regions. These findings indicate that NGF might be implicated in the regulation of the antigen expressed by oligodendrocyte progenitors (NG2) and of the neural cell adhesion molecule (CD56/NCAM). Both antigens are associated with mechanisms of brain repair thus providing additional evidence for a major role of NGF in the brain response to pathological conditions such as EAE.

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