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HIGH-AFFINITY NGF RECEPTOR IN THE RAT SPINAL CORD DURING ACUTE AND CHRONIC PHASES OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS: A POSSIBLE FUNCTIONAL SIGNIFICANCE

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INTRODUCTION

Experimental allergic encephalomyelitis (EAE) is a T cell-mediated autoimmune disease of the central nervous system, characterized by leukocyte infiltration, inflammation and demyelination. EAE can be induced in susceptible animals by immunization with CNS homogenate or specific myelin proteins or by adoptive transfer of neuroantigen-specific T cells. EAE in many aspects resembles the human disease multiple sclerosis (MS) and is thus recognized as a good experimental model (33).

Recently, evidence has accumulated about the role of the Nerve Growth Factor (NGF) in EAE and MS (2, 4, 6, 7, 9, 11, 16, 26, 27, 28). Reduction in the severity of EAE by NGF infusion has also been reported (34, 45). The biological effects of NGF are primarily mediated via the high affinity receptor – TrkA (see 5). Thus, knowledge about its expression and changes during EAE is particularly important in view of potential receptive sites for neurotrophin action.

The existing data about NGF receptor expression in multiple sclerosis and in EAE, in which the glia contribution has been revealed, mainly concern brain structures and in greater part p75 (9, 11, 13, 29, 44). Only very few data are available about NGF receptors in the spinal cord during EAE (7, 11, 32). Our recent data have shown the up-regulation of NGF receptors in reactive astrocytes in rat spinal cord during the acute phase of EAE. The magnitude of this up-regulation seemed to be related to the intensity of inflammation in sick animals (32). Our preliminary observations also indicated a contribution of oligodendroglia in NGF receptor up-regulation in the spinal cord of EAE rats (31).

While it is widely recognized that EAE is associated with prominent responses of non-neuronal cells in the spinal cord, alterations in neuronal components have also been reported (10, 43). Regarding expression of NGF receptors in neuronal components in the spinal cord during EAE, no data are available. Indications concerning

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their expression in the normal spinal cord neurons, particularly ventral motoneurons, are limited and controversial (12, 48). A recent study proves the expression of TrkA in the large soma cells in Rexed lamina IX (25).

The present study was undertaken to evaluate the possible changes in TrkA expression in neuronal components of the rat spinal cord and to widen our preliminary investigations on TrkA immunoreactivity (TrkA-IR) changes in oligodendroglia during the acute phase of EAE. In view of our aforementioned observations that the extent of astroglial changes in TrkA expression in the spinal cord was related to the intensity of inflammation in sick animals (32), we have decided to expand our investigations of neuronal and non-neuronal TrkA changes to include the chronic phase of the disease. Part of these results have been reported at Meetings (31, 46).

MATERIAL AND METHODS

Animals, EAE induction and clinical assessment

Adult (2 months old) female Lewis rats were used for immunization. Animal care procedures, both in Rome and Warsaw laboratories, were conducted in conformity with Intramural Committee and Institutional guidelines in accordance with National and International laws and policies. Rats were injected intradermally into each footpad with 0.1 ml of the following emulsion: 1:1 (1 g-1 ml) guinea pig spinal cord – Complete Freund's Adjuvant (CFA, Sigma, St.Louis, MO) containing 2 mg/ml of Mycobacterium tuberculosis. Rats were weighted and examined for grading of clinical signs. Rats were sacrificed at 14 day after immunization (acute stage of EAE) or 12 months postimmunization (chronic stage of EAE). As controls, rats injected with CFA or untreated rats of the same sex and of age matching the appropriate age of EAE rats were used. The number of rats analyzed in each experimental group was 5.

Tissue preparation

The animals were sacrificed by transcardial perfusion (under ether anaesthesia) with 0.1 M PBS plus heparin (6250 units/l) followed by 2% paraformaldehyde. The lumbar part of the spinal cord was surgically removed, postfixed in the same fixative and cryoprotected in 30% sucrose in 0.1 M PBS for 48h at 4°C. Frozen 20 µm sections were cut in coronal plane and collected in PBS with 0.1% sodium azide. Free floating, adjacent sections were then processed as described below.

Immunocytochemical and staining procedures

To identify inflammatory sites and gross cellular morphological alterations, the sections were stained with toluidine blue. For immunocytochemistry a standard avidin/biotin procedure was used, as described in detail before (21). For visualization of TrkA polyclonal rabbit anti-TrkA (Santa Cruz, Biotechnology, 1:200) was used. To identify various cell types, the following primary antibodies were used: for neurons – mouse anti-neuronal nuclei (NeuN) monoclonal antibody (Chemicon, 1:2000); for astrocytes – monoclonal mouse anti-glial fibrillary acidic protein (GFAP) (Boehring, Mannheim, 1:1000); for oligodendrocytes – monoclonal mouse anti – myelin associated glycoprotein (MAG) (1:100, Boehringer, Mannheim) or polyclonal anti-galactocerebroside (GAL-C) (Sigma, 1:50); for microglia/macrophages – monoclonal mouse anti-rat ED1 (Serotec, 1:100). For development of the reaction 0.1% 3`,3` - diaminobenzidine tetrahydrochloride (DAB) (Sigma) in the presence of 0.01% hydrogen peroxide was used. In order to ascertain the cellular localization of TrkA positive cells double immunostaining procedures were performed: for visualization of the investigated antigens DAB (brown color) and DAB plus 0.04% nickel (black color) were used. In control experiments primary or secondary antibodies were omitted resulting in unstained sections. For TrkA specificity test sections were also run with the control peptide (Santa Cruz), resulting in negative staining.

RESULTS

Clinical signs and inflammatory infiltrates in the spinal cord of EAE rats

At the acute stage of the disease similar EAE clinical signs occurred practically in all rats designated to both experimental groups: acute and chronic. At 14th day (the peak of the disease), in the group designed to be sacrificed on that day, the average clinical score was 3.8 and in the group intended to survive for 12 months, it was 3.6. The body loss in both groups had also similar time course and at 14th day postimmunization on average was 15 and 14%, respectively. Similarity of typical clinical EAE signs in both groups at the peak of the disease allowed to assume that the changes accompanying the clinical symptoms in these groups at the peak of the disease were also similar. Virtually no signs of the disease were observed in CFA injected groups of rats at 14th day, other than inflammation in both foot pads.

At the time of death of the chronic group of EAE animals only slight neurological signs of the disease (score 1-1.5) but still decreased weight (about 13%) were observed. Periodical exacerbations of neurological signs during the twelve month` observations were noted, confirming the previous findings (27) that Lewis rats

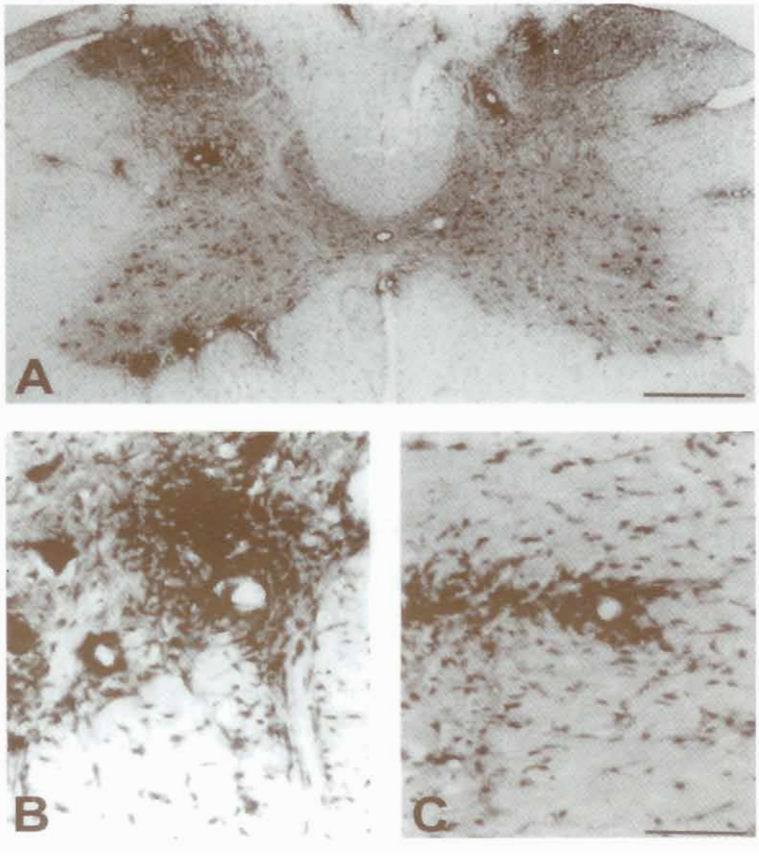

Fig. 1. - *Photomicrographs showing inflammatory foci in the lumbar spinal cord of EAE rat 14 days postimmunization, as visualized by toluidine blue staining.* (A): high magnifications of inflammatory foci in the gray (B) and white (C) matter. Bars: A – 480 μm, B,C – 77 μm.

actively immunized with guinea pig spinal cord homogenate are characterized also by relapsing remitting phases.

Multiple foci of perivascular and intraparenchymal inflammation were very intense in the EAE group sacrificed at 14 day postimmunization. The severity of inflammation differed somehow between the animals and was basically parallel to the clinical signs. Figure 1 illustrates example of inflammatory foci, as revealed by toluidine blue, both in white and gray matter of the spinal cord. No noticeable inflammation was found in CFA injected groups, as well as in the chronic EAE group.

Cellular expression of TrkA immunoreactivity

Controls: TrkA immunoreactivity was expressed in motoneurons located in Rexed lamina IX (Figures 2 C and 3 C). In neurons located in other laminae, the reaction

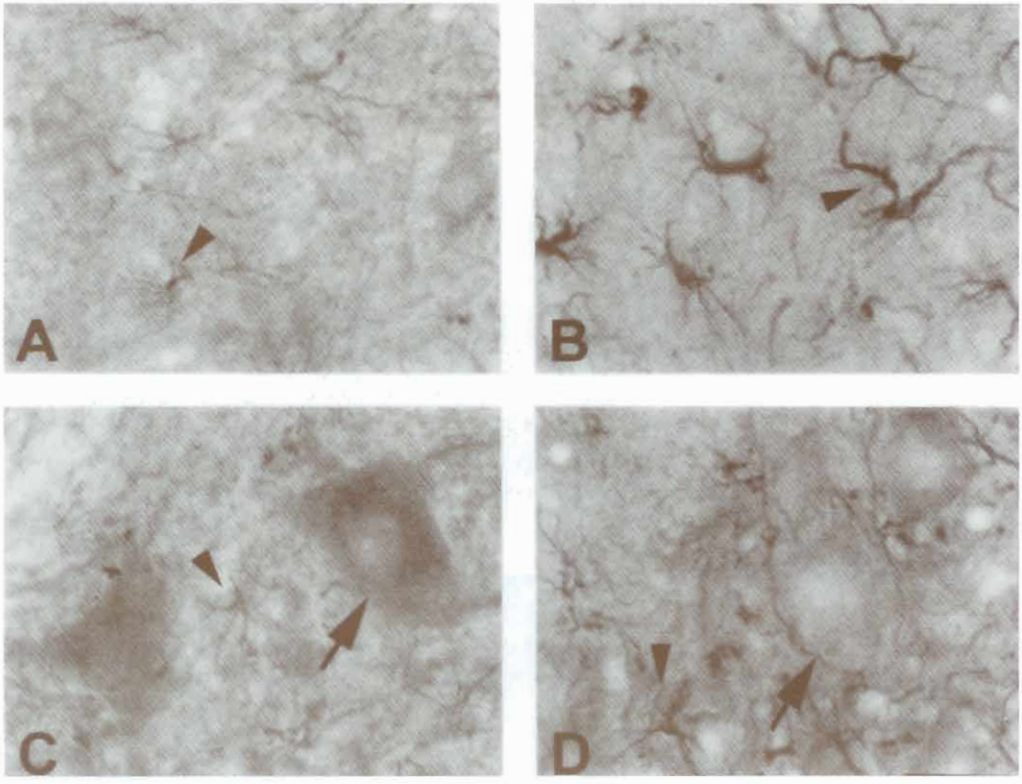

Fig. 2. - *Photomicrographs showing TrkA immunoreactivity in the gray matter of the lumbar spinal cord of control (A,C) and EAE at 14th postimmunization day (B,D) rats at the two levels of spinal cord: Rexed laminal IV-V (A,B) and Rexed lamina IX (C,D).*
Note the presence of TrkA immunoreactivity in control rat in motoneurons of laminae IX (C) (black arrows) and its strong decline (almost disappearance) in motoneurons of EAE rat (D).In the laminae IV-V neuronal TrkA-IR is almost invisible both in control (A) and in EAE rat (B). Note also the presence of TrkA-IR in astroglia at two control levels of the spinal cord (A,C) and its upregulation in the EAE rat (B,D) (black arrowheads). Bar: 38 μm.

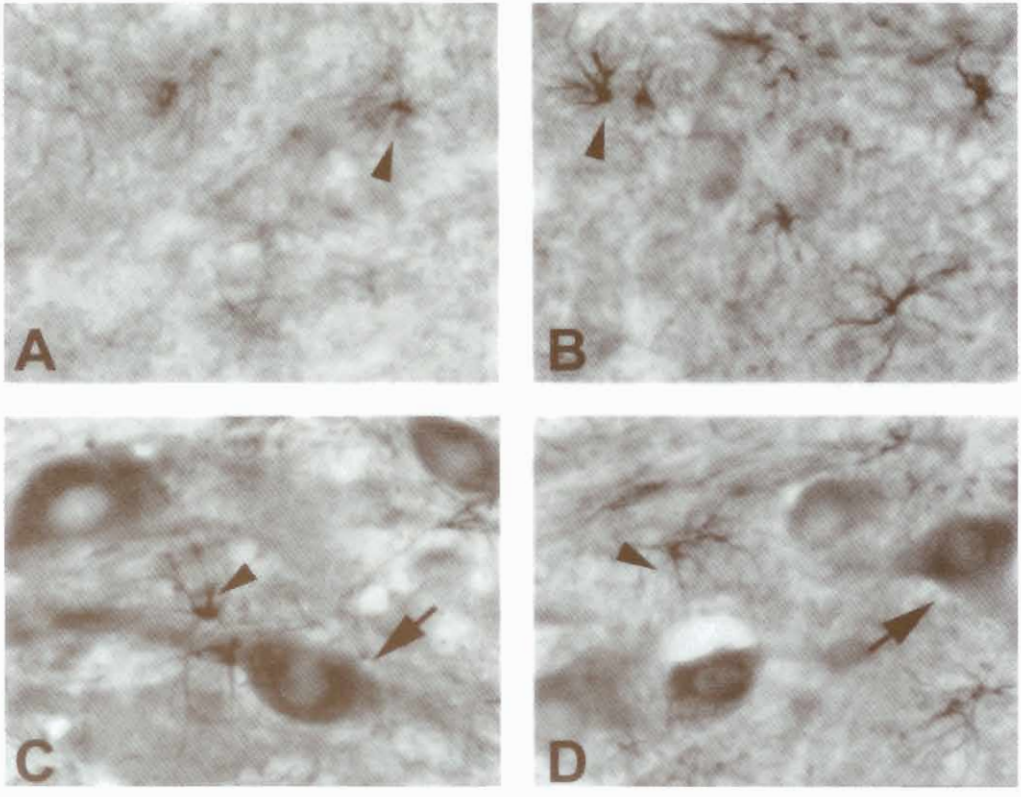

Fig. 3. - *Photomicrographs showing TrkA immunoreactivity in the gray matter of the lumbar spinal cord of control (A,C) and EAE at 12th postimmunization month (B,D) rats at the two levels of spinal cord: Rexed laminae IV – V (A,B) and Rexed lamina IX (C,D).*
Note the presence of TrkA-IR in motoneurons of both control (C) as well as EAE (D) rats (black arrows). In laminae IV-V (A,B) neuronal TrkA-IR is almost invisible. TrkA-IR in astroglia (black arrowheads) is of similar intensity in control and in EAE rats. Bar: 38 μm.

was almost not visible, as indicated by double immunostaining data with NeuN and TrkA (Figure 4). No conspicuous differences were observed between the immunoreactivity of motoneurons in the two control groups: mature and aged. Besides, TrkA immunoreactivity was in single non-neuronal cells in the white matter, morphologically resembling oligodendroglia (Figure 5 A and C; compare with Figure 6). Again, a similar pattern characterized both age control groups. It does not appear likely that a scarce population of ED1 immunoreactive cells present in the white matter of control rats also represented the cell source of TrkA. On the other hand, confirming our previous observations (32), TrkA immunoreactivity was seen in astroglia, both in the gray and white matter (Figures 2 A, C and 5 A) of mature rats. Astroglial expression in aged controls was confirmed by double immunostaining of TrkA and GFAP (not shown). TrkA-IR in astrocytes of aged rats was apparently expressed to a greater extent than in mature rats (Figures 2 A and 3 A, Figure 5 A and C). In CFA injected rats, no noticeable differences from native controls were noted.

Acute and chronic EAE rats. At the14th day, TrkA immunoreactivity in motoneurons, particularly in the Rexed lamina IX, was strongly decreased in comparison with controls and sometimes almost disappeared (Figure 2 D). As revealed by staining with toluidine blue and immunostaining for NeuN, some motoneurons in this area were visibly shrunk (Figure 4). On the other hand, this region was covered with more numerous astroglia cells with much stronger TrkA immunoreactivity than in controls (Figure 2 D). Confirming our previous observations (32) the increase of TrkA immunoreactivity in astroglia in gray matter of the spinal cord in EAE rats at the 14th day was correlated with astrogliosis, as revealed by an increase in GFAP immunostaining (not shown).

In contrast to the acute stage of the disease, in the chronic EAE group, it was found that TrkA immunoreactivity in motoneurons returned almost to the control level, suggesting the transient nature in the decline of TrkA immunoreactivity (Figure 3). No observable shrinkage of motoneurons were seen at the chronic stage

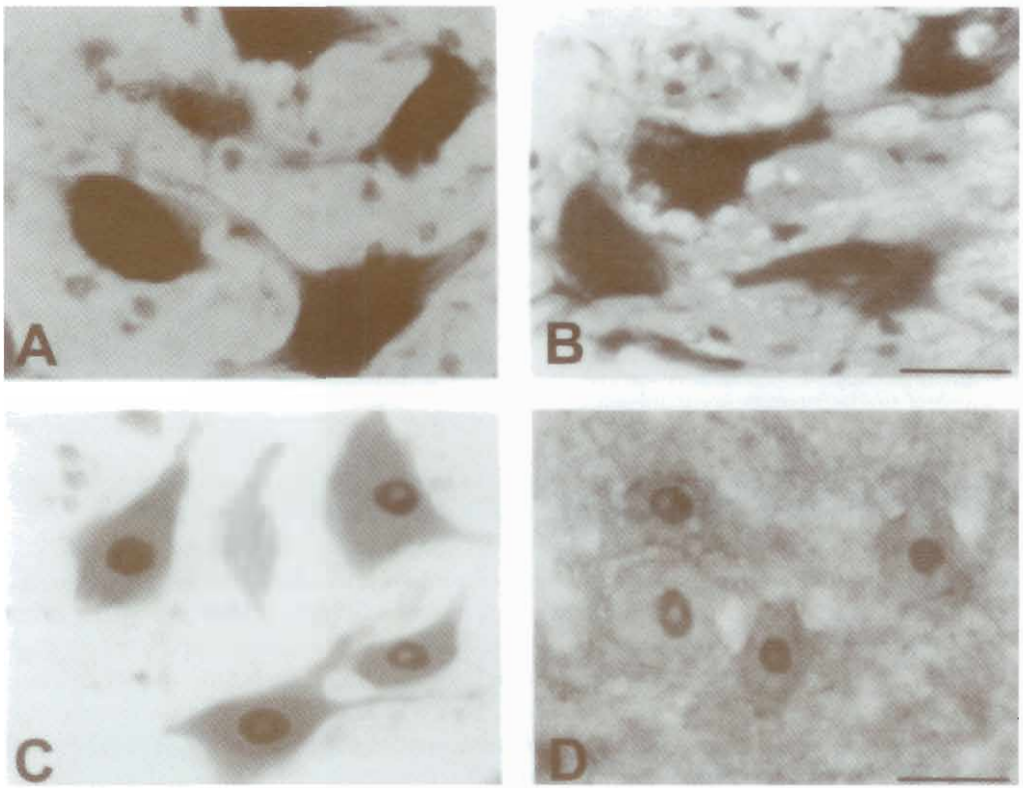

Fig. 4. - *Photomicrographs showing motoneurons in the Rexed laminae IX in the control rat (A,C) and EAE rat at 14th day postimmunization (B,D) as revealed by toluidine blue staining (A,B) and immunostaining for NeuN (C,D).*
Note that some motoneurons in EAE rats are shrunken.
Bar: 38 μm.

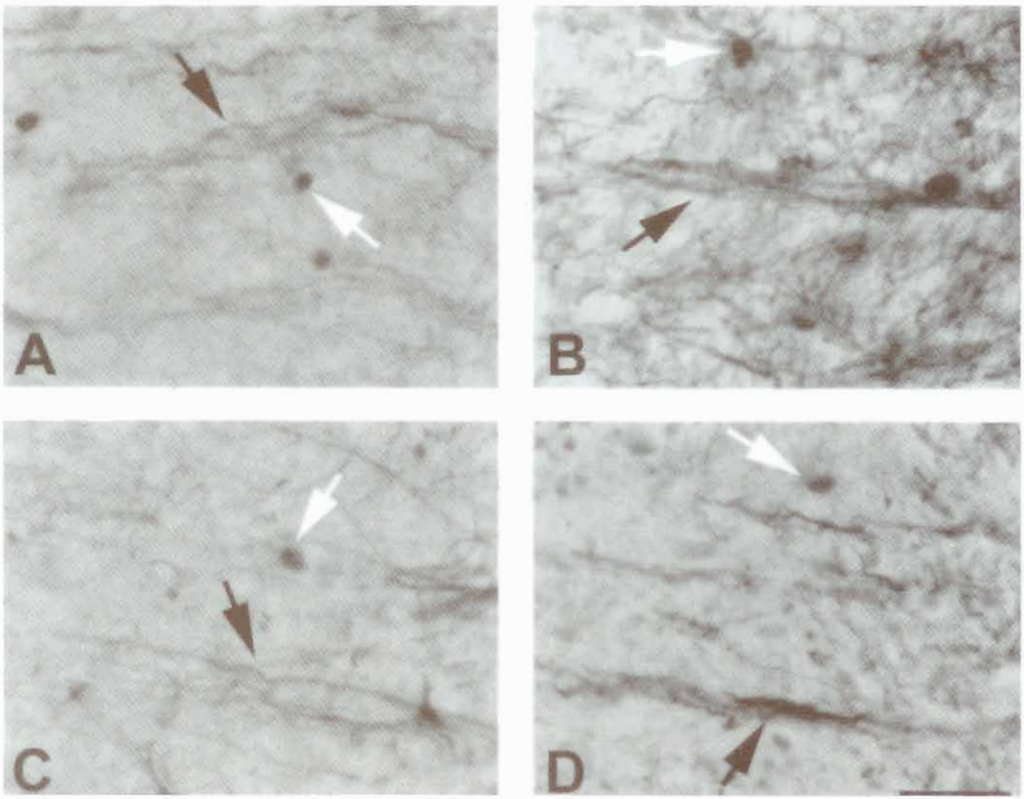

Fig. 5. - *Photomicrographs showing TrkA immunoreactivity in the dorsolateral part of the white matter in EAE rats at 14th day (B) and 12th month (D) postimmunization and in respective controls (A,C).* Note the presence of TrkA-IR in the round cells morphologically resembling oligodendroglia (white arrows) and in radially oriented astroglia (black arrows). Note strong up-regulation of TrkA-IR in radial astroglia in EAE rat at 14th postimmunization day and some up-regulation of TrkA in oligodendroglia at this time. Bar: 38 μm.

of EAE. Only a very slight up-regulation, or practically no elevation in comparison to the control level, of TrkA immunoreactivity in astroglia both in the gray and white matter of the spinal cord were noticed at the chronic stage of the disease (Figures 3 and 5). Virtually no astrogliosis at the 12th month postimmunization as revealed by GFAP immunostaining was noted.

At the 14th day, it appears that there are more glia cells morphologically resembling oligodendroglia expressing TrkA immunoreactivity (Figure 5 B) in the white matter than in controls. The increase in the number of the immunoreactive cells was particularly evident in the white matter dorsal columns. At the chronic stage of EAE, the TrkA immunoreactivity in oligodendroglia was less intense than at the acute phase (Figure 5 D). Double immunostaining experiments for TrkA and the oligodendroglial marker MAG have proved that oligodendroglia are the cellular source of TrkA (Figure 6).

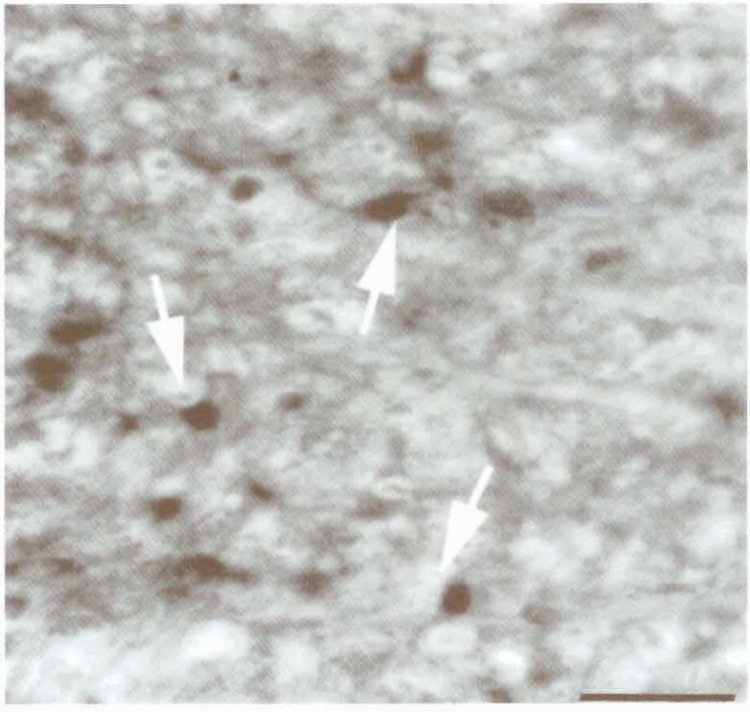

Fig. 6. - *Photomicrographs showing double immunostaining of TrkA and MAG expressing cells from the white matter of the lumbar spinal cord of EAE rat at 14th postimmunization day.*
TrkA-IR was visualized with DAB (brownish color) and MAG-IR with DAB + nickel (black color). Note colocalization of both immunoreactivities in several cells (white arrows). Bar: 38 μm.

DISCUSSION

In the present study, we extended our previous findings concerning TrkA expression in the spinal cord under EAE conditions (31, 32, 46). We confirmed that, in the acute phase of the disease, besides the strong up-regulation of TrkA immunoreactivity in astroglia there is also an up-regulation of this receptor in the population of oligodendroglia in the white matter. Moreover, we have found, that simultaneously with the up-regulation of TrkA-IR in non-neuronal elements, there was a reduction of this immunoreactivity in motoneurons of the Rexed lamina IX. We have also shown that both non-neuronal (astroglial and oligodendroglial) as well as neuronal TrkA changes had a transient character, related to the inflammatory stage, since in the chronic phase of the disease the pattern of TrkA-IR resembled largely that in the control conditions.

The novel findings are in the data concerning the immunocytochemical localization of TrkA in the motoneurons of the spinal cord of naïve rats as well as its decrease in the acute phase of EAE. The potential significance of the presence of mRNA of TrkA in the Rexed laminae IX in the spinal cord of the naïve rat recently reported by Liebl *et al.*, 2001 (25) has now been supported for the first time by immunocytochemical localization. Our findings indicating low or no of TrkA-IR in neuronal populations of laminal IV and V are also in line with their data demonstrating low or none mRNA TrkA in these neurons. On the other hand, it has to be

mentioned that, while the appearance of TrkA on motoneurons during development has been widely recognized (12, 15, 22, 48), its presence in the adult rat as well as human spinal motoneurons has been in the past questioned (12, 48). Di Stefano *et al.*, 1992 (12) for instance, reported that NGF showed no specific retrograde transport to ventral spinal cord motoneurons of the adult rat, which was interpreted as an indication of the TrkA absence on motoneurons. The reasons for the controversy of the reported data possibly depend on methodological differences like whether the binding sites, mRNA or imunocytochemistry was investigated, the more so as the existence of various isoforms of TrkA (5) and in particular in the spinal cord (25) have been reported.

As opposed to other pathological conditions, like crushing of the peripheral motor nerve or rhizotomy (17, 18, 36, 37) as well as in amyotrophic lateral sclerosis (ALS) (30, 40) where up-regulation of TrkA was reported, we found a decline of TrkA in the acute EAE phase. Although it is rather believed that NGF, in contrast to other neurotrophins, particularly BDNF, does not promote survival of spinal motoneurons, evidence is also provided suggesting a role for NGF for these neurons (see 39). While earlier data indicated rather a lack of NGF in spinal motoneurons (see 39) there is a possibility that an as-yet unidentified NGF isoform in motoneurons utilizes the TrkA receptor. Our unpublished data indicate that this may be the case. The existence of NGF isoforms, particularly of high molecular weight have been reported in various cells (35, 41).

Other novel findings concern our data demonstrating the up-regulation of TrkA in oligodendroglia, particularly in the white matter of the spinal cord during the acute phase of EAE. These data are in line with the very scarce data describing the up-regulation of TrkA in other nervous system structures during EAE (9) and MS (26, 44). More data show the up-regulation of the low affinity NGF receptor, p75 receptor in oligodendroglia, both in MS and EAE (13, 26, 28, 44). Our preliminary data also indicate such up–regulation in spinal oligodendroglia (31). It has to be mentioned here, however, that studies on the expression of Trka in glial cells have provided conflicting results (see 3). Most information, however has come from studies in vitro, where oligodendrocytes were reported to display TrkA-negative or TrkA-positive phenotype. The reason for the conflicting evidence of TrkA expression in oligodendrocytes is not yet clear. Our present data, along with some other positive results concerning the presence of TrkA in oligodendroglia and its up-regulation during the autoimmune disease, indicate that these cells are receptive to the neurotrophin. In fact, in experiments in which radioactive NGF was used it has been shown that oligodendroglia may uptake this neurotrophin (1).

Our data clearly show that all changes in TrkA immunoreactivity pattern, both in neuronal as well as in glial (oligodendroglia and astroglia) cells, are correlated with inflammation, which emerged in the acute stage and was not seen twelve months postimmunization in the chronic stage of the disease. The decline of neuronal TrkA may be dependent on the general deteriorating effects of inflammation upon neuronal cells (10, 43), also manifested in our present studies as a shrinkage of motoneurons, and as a decrease of immunoreactivity of other bioactive molecules

(unpublished data). On the other hand, the up-regulation of TrkA both in oligodendroglia as well as in astroglia may be dependent on some inflammatory mediators such as interleukin 1beta, which is known to be engaged in the stimulation of NGF production (see eg. 38). Our results are in line with previous reports on the correlation of NGF receptor up-regulation in glial cells with the degree of inflammation both in MS (44) and EAE (32). Several lines of evidence point to the involvement of autocrine/paracrine mechanisms in the up-regulation of NGF receptors. NGF is known to upregulate both p75 and TrkA in glial and neuronal cells (19, 20, 23). In line with other data concerning the increase of NGF in glial cells in some brain structures in EAE (9, 11, 28), our preliminary data (31) also indicated that, during the inflammatory phase in EAE, there was an increase of NGF immunoreactivity in oligodendroglia. As it previously reported (32), and at variance with some brain structures (9, 28), astroglia in the spinal cord during EAE do not up-regulate NGF. Thus, astroglial TrkA, as discussed before (32), could either be induced by NGF released by other cells, and/or by NT-3, which was found in astroglial populations of the rat spinal cord (14) and was reported to up-regulate NGF receptors in the central nervous system, also in glia cells (42).

What may be the functional significance of TrkA receptor upregulation on glial cells during the acute phase of EAE?

Increased TrkA in glial cells means an increase of receptive sites for NGF. It has recently been reported that continuous intracerebroventricular infusion of recombinant human NGF reduced the severity of EAE in a marmoset model, which shows striking clinical and pathological similarities to MS (45). Various mechanisms may be responsible for such a beneficial effect of NGF. As discussed by Villoslada *et al.*, 2000 (45), NGF could partially down-regulate the inflammatory processes by changing the cytokine profile from detrimental to neuroprotective in glial and inflammatory cells which upregulate TrkA. Evidence that NGF can exert an anti-inflammatory activity has been recently reported (16, 24). There are also data suggesting that NGF contributes to repairing demyelinated areas, acting on TrkA in oligodendroglia (see 3). On the other hand, the increase of TrkA in radial astroglial cells, may, as we hypothesized before (32), facilitate the migration of oligodendroglia progenitors, serving as a scaffold for them. The migratory activity of precursor cells of the subventricular zone in the CNS of EAE rats has recently been described and the involvement of NGF in this activity postulated (8). Our hypothesis has recently been strengthened by the finding that many NG2 positive cells, identified as oligodendroglia progenitors and activated in EAE, were closely spatially associated with the radially oriented astroglial cells (47).

Taken in concert, the present findings showing the upregulation of TrkA in glial cells indicate that these cells may be an important target for pharmacological manipulations particularly for exogenously administered NGF.

DEDICATION

This paper is dedicated to Professor Rita Levi-Montalcini with our deepest esteem, affection and thanks for her continuous interest in our work, encouragement, inspiring discussions and invaluable suggestions.

SUMMARY

The biological effects of Nerve Growth Factor (NGF) are primarily mediated via its high affinity receptor-TrkA. In the present study, we examined the effect of experimental autoimmune encephalomyelitis (EAE) upon the expression of TrkA in neuronal and non-neuronal cells of the spinal cord of Lewis rats during the acute (14 days postimmunization) and chronic (12 months postimmunization) phases of the disease. In the normal spinal cord, both of mature and aged rats, we found TrkA immunoreaction (TrkA-IR) in the motoneurons of the Rexed lamina IX and in both oligo- and astroglia cells. In the acute phase of the disease, we found a reduction of TrkA immunoreactivity in motoneurons and its up-regulation in oligodendroglia, mainly in the white matter. We also confirmed our previous findings concerning the up-regulation of TrkA-IR in astroglia. Both neuronal and non-neuronal changes of TrkA immunoreactivity had a transient character: they were not seen in the chronic phase of the disease. Our results suggest that both neuronal and glial TrkA expression changes depend on inflammation. Moreover, our data indicate that, during the acute phase of EAE, the glial cells become more receptive to NGF, pointing to glia as an important target for pharmacological manipulations, particularly for exogenously administered NGF.

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