Cognitive deficit associated with cholinergic and nerve growth factor down-regulation in experimental allergic encephalomyelitis in rats

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Clinical symptoms in multiple sclerosis include cognitive dysfunction. Difficulties in learning and remembering new information represent the most common cognitive deficit and are associated with a general and progressive brain pathology. Possible pathogenetic mechanisms for neuronal damage such as neuroprotective strategies are under active investigation also in experimental allergic encephalomyelitis, the most widely used experimental model for multiple sclerosis. In this paper we demonstrate that a selective deficit in learning and memory performance, as investigated by the Morris water maze test, is a consistent feature in rat encephalomyelitis, which correlates with a decline in choline acetyltransferase activity and nerve growth factor mRNA level in cerebral cortex, hippocampus, and basal forebrain. Treatment aimed to restore acetylcholine content through chronic administration of selective acetylcholinesterase inhibitors (rivastigmine and donepezil) restores cognitive performance, choline acetyltransferase activity, and nerve growth factor mRNA expression.

choline acetyltransferase | multiple sclerosis | learning and memory | water maze | acetylcholinesterase inhibitors

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system (CNS), which begins as an immune-mediated inflammatory disease and evolves with a wide spectrum of oligodendrocyte and neuronal damage (1). MS causes a wide variety of clinical features with many different characteristic signs and a few virtually pathognomonic symptoms. It is characterized by relapses and remissions of neurological defects and occurs as "acute episodes," or in a "relapsing–remitting" or "secondary progressive" form (2). Sensory symptoms are the most common, such as optic neuritis and diurnal fatigue pattern.

There is a general consensus regarding the presence of axonal and neural damage in different areas of the CNS and of the spinal cord (3–6), and MRI studies have indicated that the degree of atrophy in the cerebrum, corpus callosum, and spinal cord, as well as the ventricular size, correlate directly with severity of disability (7, 8). The axonal loss and neuronal degeneration occur early in MS and are considered as the key factors responsible for chronic disabilities, thus evolving as primary pathogenetic mechanisms in later stages of the disease (9–11).

Clinical symptoms in MS include sensory and motor disabilities but also concern cognitive and affective behaviors. Cognitive dysfunction affects about half of individuals with MS (12), and difficulties in learning and remembering new information represent the most common cognitive deficit (13, 14). Verbal memory deficits are observed in the progressive form of the disease, and visuospatial memory deficits are observed in the relapsing–remitting form (15). Moreover, there is a correlation between the extent of MRI alterations in the cerebral white matter and the severity of cognitive deficit (16).

Therefore, it has been suggested that neuroprotection needs to be considered as a new target for MS therapy. Possible pathogenetic mechanisms for neuronal damage such as neuroprotective strategies are under active investigation also in experimental allergic encephalomyelitis (EAE), the most widely used experimental model for MS. We recently demonstrated that long-lasting neurological disabilities in EAE correlates with neurochemical phenotype changes in spinal motoneurons involving neurotransmitter enzymes [choline acetyltransferase (ChaT)], peptides (calcitonin gene-related peptide), and neurotrophin receptors (low-affinity receptor p75), matching those observed after axotomy (17). This damage is accompanied by axonal pathology (18) and a dramatic drop in nerve growth factor (NGF) content in the spinal cord (19, 20), which recovers after therapies, which also induce a rise in NGF content (18).

The aim of this investigation was, if a cognitive deficit is present in EAE rats, to try to elucidate a molecular substrate in view of therapeutic intervention. We demonstrate that a selective deficit in learning and memory performance, as investigated by the Morris water maze test, is a consistent feature in rat EAE, which correlates with a decline in ChaT activity and the NGF mRNA level in cerebral cortex, hippocampus, and basal forebrain. Treatment aimed to restore acetylcholine content through chronic administration of selective acetylcholinesterase inhibitors restores cognitive performance, ChaT activity, and NGF mRNA expression.

Materials and Methods

Animals, Treatments, and Schedule of the Experiments. Female, pathogen-free Lewis rats (Charles River) were used. A group of rats from each strain was sensitized with a medium containing 0.15 g/ml guinea pig spinal cord tissue in complete Freund's adjuvant (CFA, Sigma), 50% vol/vol, to which 5 mg/ml heat-inactivated *Mycobacterium* (Difco H37Ra) was added. Immunization was performed by injecting 100 μl of this emulsion in each hind paw. Uninjected and CFA-injected rats were used as controls. Clinical disease was scored according to the following scale: 1, loss of tail tone; 2, weakness in one or both hind legs or middle ataxia; 3, ataxia or paralysis; 4, severe hind leg paralysis; 5, severe hind leg paralysis accompanied by urinary incontinence. Animals were randomly assigned to the different experimental groups, and treatment was administered blindly. Groups of control and EAE animals were treated with the selective cholinesterase inhibitors rivastigmine and donepezil. Drugs were added to the food pellets up to a final dosage of 0.5 mg per kg of body weight (rivastigmine) and 1.5 mg per kg of body weight (donepezil), treatment was started on day 0 and continued until the rats were killed (see Fig. 1*A*). Drug doses were chosen on

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Abbreviations: AD, Alzheimer's disease; ChaT, choline acetyltransferase; dpi, days postimmunization; EAE, experimental allergic encephalomyelitis; MS, multiple sclerosis; NGF, nerve growth factor.

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the basis of our experiments (see *Results*) and the literature, to achieve a significant acetylcholinesterase inhibition with reduced side effects (21). Data presented in this paper are from three separate experiments: experiment 1, in which alteration of the cholinergic system in EAE was investigated (behavioral, immunohistochemical, biochemical, and molecular experiments); experiment 2, in which the effect of rivastigmine treatment on behavioral, biochemical, and molecular parameters was investigated; and experiment 3, in which the effect of donepezil treatment on molecular parameters was investigated. For behavioral experiments, 10 animals were included in each group; for immunohistochemical, biochemical, and molecular experiments, 5 animals were included in each group for each set of experiments. Pharmacological treatments were all tested in both EAE and control groups. Animal care and treatments were performed in accordance with the European Community Council Directives of November 24, 1986 (86/609/EEC) and approved by our intramural committee and the Italian Ministry of Education, University and Research, in compliance with the guidelines published in *Guide for the Care and Use of Laboratory Animals* (National Research Council).

Behavioral Task. Tasks were run in a circular pool (185 cm diameter, with visible or hidden platform and targets on the walls), recorded, and analyzed by using SMART software (PanLab, Barcelona). Latency, speed, and strategy used to reach platform were analyzed according to the following schema: first day, three blocks (two trials each, 90 sec each trial) with visible platform. Each trial started from random points of the pool, and animals had 90 sec to reach the platform. The trial was ended if the animal remained on the platform for 15 sec. On the second day, the same scheme was repeated with the hidden platform. The trials were separated by 15-sec pauses. Twenty-four hours later animals were tested with a single trial without platform starting from a random position. Time spent in the target quadrant was measured. These tests, developed to investigate even fine alterations in the cholinergic system (22), can evaluate learning and short-term memory.

ChaT Activity. Tissue samples were dissected from both cerebral hemispheres, immediately frozen in liquid N_2, and stored at −80°C until the day of the assay. Tissue homogenates were prepared by using a 10 mM Hepes/1 mM DTT (pH 7.5) lysing buffer, containing a protease inhibitor mixture (Sigma). Supernatants were suitably diluted, incubated at 37°C for 15 min with a reaction mix composed of 0.4 M NaCl, 1 mM EDTA, 0.4 mM eserine hemisulfate (Sigma), 0.15 M Choline chloride (Sigma), 1.6 mM acetyl CoA (Sigma), and ^{3}H-acetyl CoA (Amersham Pharmacia Bioscience) to a final specific activity of 181 GBq/mmol. The reaction was then stopped by transferring the samples to an ice bath and adding 1.5 ml of ice-cold stop solution (3.12 mM Na_2HPO_4/2.5 mM NaH_2PO_4/0.312 mM EDTA, containing 6.4 mM sodium tetraphenylborate in acetonitrile), and radioactive acetylcholine was extracted by using a scintillation liquid for organic solutions and quantified with a Beta-counter.

Immunohistochemistry. Brains were fixed (4% paraformaldehyde), and indirect immunofluorescence procedures were used to visualize the following antigens in the horizontal diagonal band nucleus in the basal forebrain: anti-ChaT 1:75 (goat polyclonal, Chemicon), anti-p75^{NTR} (mouse monoclonal, clone IgG192, generously supplied by E. M. Johnson, Washington University, St. Louis), anti-CD11b (OX42 1:200, Sera-Lab, Crawley Down, U.K.), and anti-GFAP (glial fibrillary acid protein, rabbit polyclonal 1:300, Eurodiagnostica, Bologna, Italy). Confocal laser scan microscopy (Olympus FV500, Ar/HeNe lasers and appropriate filters for green and red fluorescence) was used for imaging.

RNA Isolation, Reverse Transcription, and Semiquantitative PCR. mRNA was prepared by using a kit from Roche Molecular Biochemicals based on base-pairing between the poly(A) residues at the 3′ ends of the mRNAs and the biotin-labeled oligo(dT)$_{20}$ probe, which will be immobilized on a streptavidin solid support (magnetic particles). In brief, tissues were homogenated in a guanidine thiocyanate lysis buffer (4 M GTC/0.1 M Tris·HCl, pH 8/0.1% DTT/0.5 mg/ml laurylsarcosine) and centrifuged. The supernatant was diluted with GTC dilution buffer (0.1 M Tris·HCl, pH 8/0.4 M LiCl/0.02 M EDTA) to which 150 pmol of biotin-labeled oligo(dT)$_{20}$ probe was added and incubated with the magnetic particles at 4°C for 5 min. Once the particles were properly washed, the elution of the mRNA was performed in molecular-biology-grade water by heating for 2 min at 65°C. The concentration of mRNAs obtained was determined spectrophotometrically at A_{260} nm. Equal amounts of mRNA from the different samples were used for the reverse transcription. mRNA was subjected to DNase treatment to avoid possible genomic DNA contamination. Fifty nanograms of mRNA of each sample was incubated with 1 units/μl DNase (Roche Molecular Biochemicals), in the presence of 10 mM DTT (GIBCO/BRL) and 4 units/μl RNase inhibitor (Promega), at 37°C for 30 min. Heating at 95°C for 5 min terminated the incubation. First-strand cDNAs were obtained according to the specifications of the Moloney murine leukemia virus (MuLV) reverse transcriptase (GIBCO/BRL) enzyme. In brief, 50 ng of each mRNA was reverse-transcribed in the presence of 1× first-strand buffer, 0.5 mM each d(NTP)s (Roche Molecular Biochemicals), 50 μM p(dN)$_6$ random primers (Roche Molecular Biochemicals), and 200 units of the enzyme MuLV reverse transcriptase (GIBCO/BRL), at 37°C for 50 min, followed by a termination step at 70°C for 15 min.

Statistical Analysis. In the descriptive analysis data are expressed as mean ± SEM. Statistical analysis was carried out with one univariate and multivariate; for repeated measure, the *P* value is reported. Between-group comparisons were made with Tukey's test. All probability values were two-tailed. Student's *t* test was also used when appropriate. The probability level was set at 5% (two-tailed) (PRISM software package for Macintosh, GraphPad, San Diego).

Results

Experimental Animals and Learning and Memory Performance. Fig. 1*A* reports the schedule of the experiment and clinical neurological score of experimental animals. As previously described (17, 19, 23, 24), the severity of EAE, as evaluated by strength deficit, gradually increased, reaching its peak between 10 and 14 days postimmunization (dpi), and then partially recovered. In our experience (24), which was confirmed in this experiment, the disease relapses in the Lewis strain with lower severity in 60% of animals. Only nonrelapsing animals were included in this study (Fig. 1*B*). To perform the Morris water maze test, animals were allowed to fully recover from the motor point of view. This was tested by clinical score and by measuring the swim speed, which was normalized at 90 dpi (Fig. 2*B*). We monitored behavioral performance in experimental animals by using visible and hidden platforms to identify any early impairment in learning and memory performance. At 90 dpi, EAE animals' performance in the water maze test was slightly but significantly impaired compared with control animals, as illustrated by the latency value in the test with the hidden platform (Fig. 2*A*). In addition, a defect in the spatial orientation test is present, as illustrated by the percentage of time spent in the trained quadrant (Fig. 2*C*).

The Cholinergic System of the Basal Forebrain. To investigate possible cellular and molecular mechanisms underlying cognitive impairment in EAE rats, we analyzed morphological, biochemical, and molecular parameters of the cholinergic basal forebrain system, which is mainly involved in learning and memory (25, 26). The

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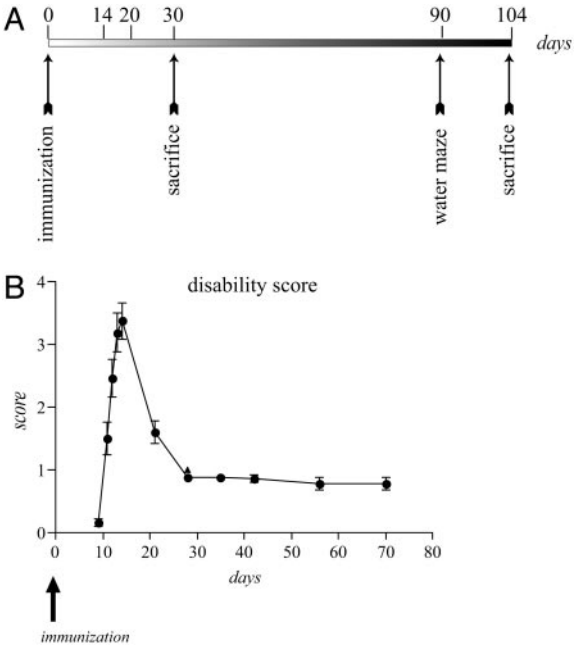

Fig. 1. Design of the study (*A*) and clinical score of EAE in Lewis female rats (*B*) included in experiment 1. Animals were tested for behavioral performance (Morris water maze test) at the indicated time, and all animals were then killed for biochemical, molecular, and morphological analysis. Animals developed severe EAE 10–14 dpi. Only nonrelapsing animals were included in the study.

cholinergic neurons were visualized by immunohistochemistry with ChaT, $p75^{NTR}$, and trkA at 90 dpi. Inflammation and gliosis were also investigated with OX42 as a marker for activated microglial cells, and GFAP as a marker for astrocytes. Although OX42-immunostaining in EAE was indistinguishable from control animals (Fig. 3 *A* and *B*), a slight increase in GFAP immunostaining around ChaT-positive neurons was found in the basal forebrain (Fig. 3 *C* and *D*). $p75^{NTR}$-immunoreactivity is strongly up-regulated in the cholinergic neurons of the basal forebrain, as illustrated by dendrite staining (Fig. 3 *E* and *F*).

We investigated ChaT activity at 30 and 90 dpi in the basal forebrain and in its target areas, e.g., the cerebral cortex and the hippocampus. As shown in Fig. 4, where results are expressed as a percentage difference with respect to control, adjuvant-injected rats, EAE animals have a lower ChaT activity at both 30 and 90 dpi in all investigated areas.

Finally, we measured NGF mRNA expression in the cerebral cortex by semiquantitative RT-PCR (Fig. 5). In EAE rats, we observed a dramatic drop in NGF mRNA compared with control animals.

Acetylcholinesterase Inhibitor Treatment in EAE Rats. Dosage and method of administration of rivastigmine and donepezil has been defined in a preliminary series of dose–response experiments to avoid cholinomimetic side effects (hypothermia, tremor, fasciculation, diarrhea, and altered velocity in water) and to obtain true acetyl esterase inhibition. The dosages used were devoid of the indicated side effects and were able to induce a mean 40% reduction of plasma acetyl esterase activity (data not shown). Effects of rivastigmine treatment on cognitive performance with the hidden platform and ChaT activity levels in EAE animals are shown in Figs. 2*C* and 4, respectively. In these experiments, we also confirmed the learning deficit in EAE animals, as indicated by the deficit in the spatial orientation test. Rivastigmine treatment reduced this impairment, even though performance was not fully

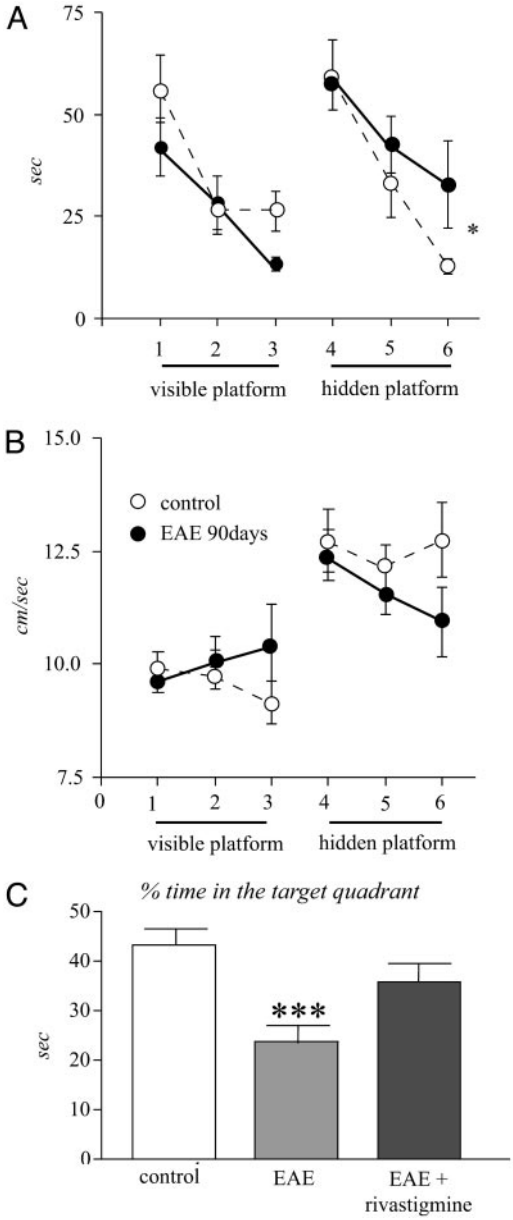

Fig. 2. The graphs report performances in the water maze task, as indicated by latency (*A*) and velocity (*B*) in the visible and hidden platform trials, and in the spatial orientation trial (*C*) in control, EAE, and EAE+rivastigmine animals at 90 dpi. In *A* and *B*, each point represents the average over the two trials within each block. In *C*, the percentage of time spent in the target quadrant is reported. Repeated overall ANOVA measures between EAE and control animals indicated a significant group effect ($F = 5.66$, $P = 0.0365$) that was also confirmed in the spatial orientation trail. Rivastigmine treatment restores behavioral capability in the spatial orientation test (Student's *t* test; *, $P < 0.05$).

restored. In addition, the ChaT activity level was significantly increased by rivastigmine treatment in EAE animals, even if this effect is observed in the basal forebrain but not in the cerebral cortex and hippocampus. Finally, NGF mRNA expression in the cerebral cortex, which is dramatically lower in EAE than in control animal, is restored by rivastigmine treatment (Fig. 5). In a different experiment, we also tested the effect of donepezil on NGF mRNA expression. We confirmed the severe reduction of NGF mRNA expression in the cerebral cortex and the restored levels in donepezil-treated rats.

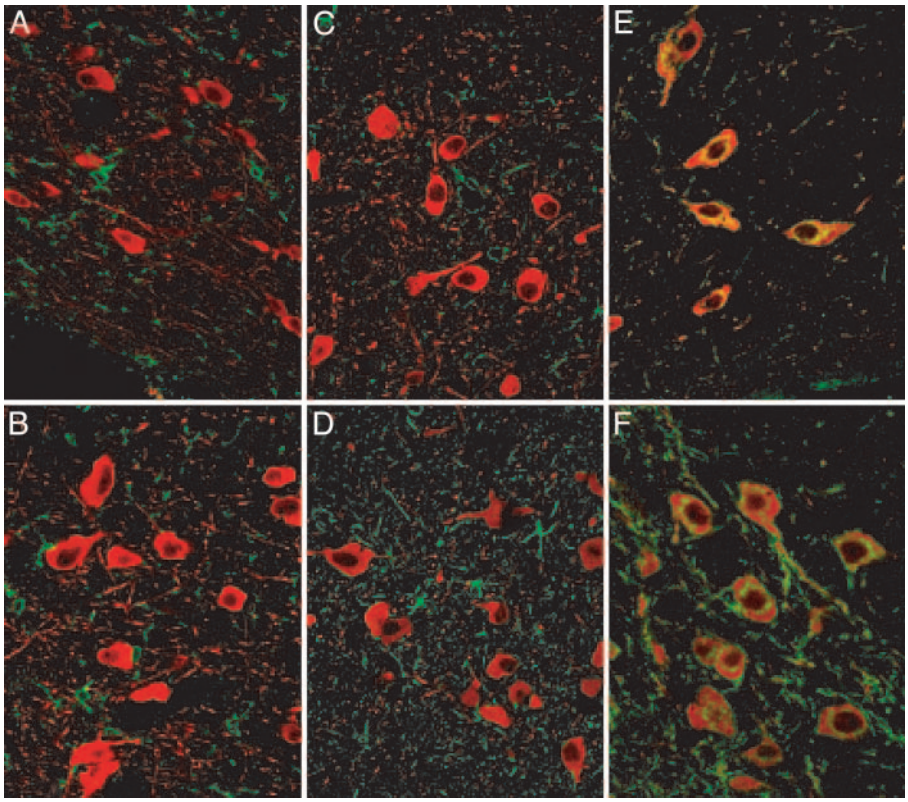

Fig. 3. Morphological study of cholinergic neurons in the basal forebrain (horizontal diagonal band nucleus). (*A–D*) Immunohistochemical visualization of ChaT-positive neurons to show codistribution with microglial cells (OX42-positive; *A* and *B*) and astrocytes (GFAP-positive; *C* and *D*) in control (*A* and *D*) and EAE (*B* and *D*) 90-dpi animals. A slight astrocytosis is observed in EAE animals. (*E* and *F*) Colocalization of $p75^{NTR}$ in ChaT-positive neurons. A wider expression of $p75^{NTR}$ is observed in dendrites in EAE animals (*F*; 90 dpi) compared with control animals (*E*), suggesting an increased expression of the receptor. Representative images ($n = 5$ per group) obtained by confocal laser microscopy are shown.

Discussion

This investigation demonstrated that a cognitive impairment in EAE rats 90 dpi occurs when motor recovery is complete. This impairment consists in a longer latency in the Morris water maze test with the hidden platform and in a defect in the spatial orientation task. This is associated with a decrease in the ChaT activity in the brain areas (basal forebrain, cerebral cortex, and hippocampus) involved in the acetylcholine control of memory acquisition and maintenance. We also describe a significant decrease in NGF mRNA expression in the cortex, further pointing to a vulnerability of cholinergic neurons during EAE. This process is quite specific because it occurs in a brain region involved in learning ability and in the release and/or utilization of endogenous acetylcholine. Moreover, it was found that treatment with acetylcholinesterase selective inhibitors almost completely restores behavioral performance and ChaT activity levels and also reduces NGF mRNA defects. One important implication of these observations is that these drugs could also have a neuroprotective effect.

CNS Inflammation and the Cholinergic System of the Basal Forebrain. A possible role of brain inflammation in degeneration of selective neuronal population, including the cholinergic system of the basal forebrain, represents a working hypothesis for Alzheimer's disease (AD) (26). In AD and other types of neurodegenerative diseases (27), a major role is attributed to microglia activation, which generates a primary inflammatory state (28). Leukocyte invasion is a secondary phenomenon (29, 30). Although the initial step differs, AD and MS share common feature in the cascades of inflammatory events observed in these diseases. Here, we report that a cholinergic vulnerability, i.e., impairment in learning tasks and decline in ChaT activity and NGF mRNA

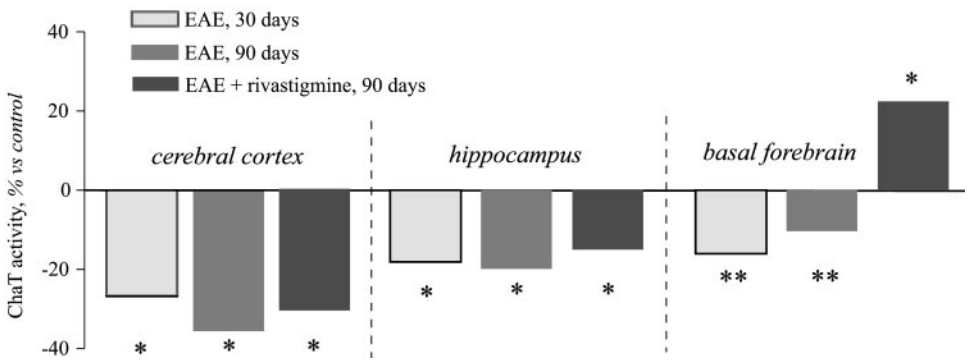

Fig. 4. Decline of ChaT activity in different brain areas (basal forebrain, cerebral cortex, and hippocampus) in EAE rats, expressed as the percentage variation compared with control animals at two different times (30 and 90 days) after immunization. There is a significant decrease in the ChaT activity at both 30 and 90 dpi in all investigated areas. The recovery in rivastigmine-treated EAE rats is also shown. Statistical analysis was by one-way ANOVA and Dunnet's test ($P < 0.01$).

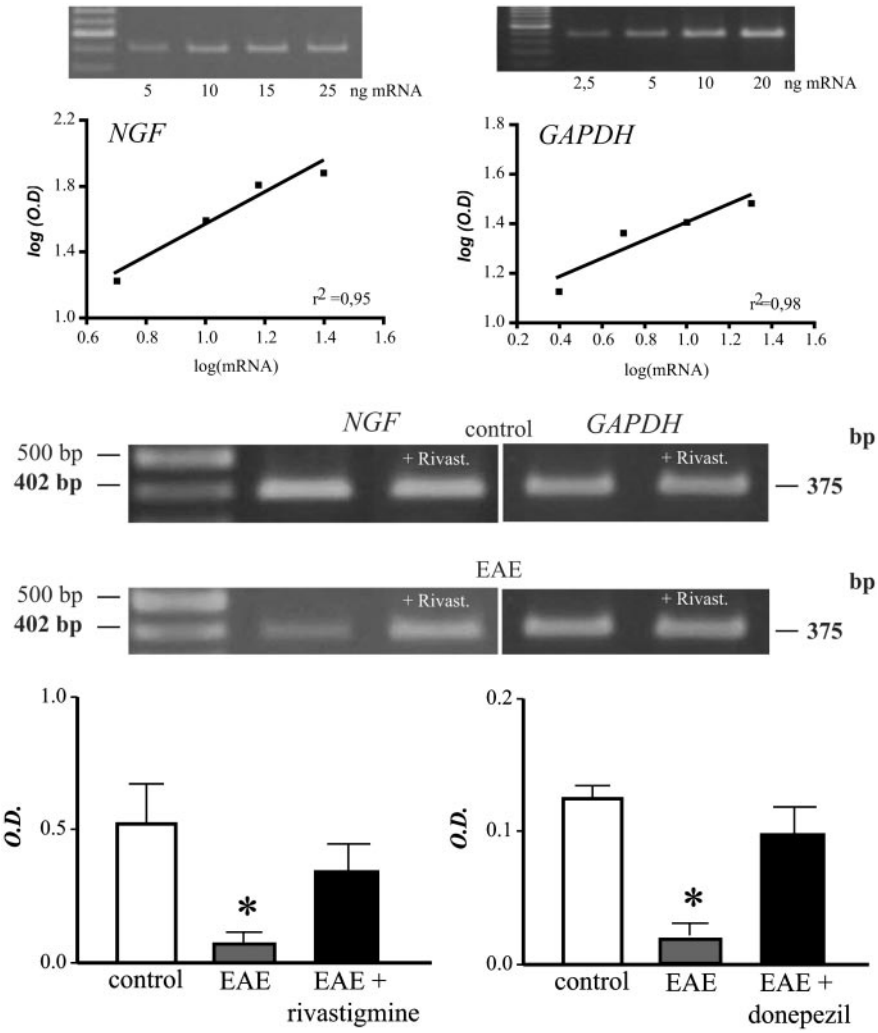

Fig. 5. NGF mRNA significantly decreases in the cortex of EAE animals as measured by RT semiquantitative PCR. Rivastigmine and donepezil induce a partial recovery in NGF mRNA level. Linear regressions for NGF and GAPDH housekeeping genes indicate the linear relationship between the cDNA amplified and the PCR product obtained. Data obtained from three to four animals per groups were statistically analyzed by one-way ANOVA and Dunnett's test (*, $P < 0.05$).

expression, is present in a model of EAE showing a single acute episode. These defects are early and long-lasting, even after resolution of the acute inflammation. Conversely, the decline in ChaT mRNA expression observed in spinal cord motor neurons, associated with other neuron phenotype regulation due to axonal lesion (e.g., expression of $p75^{NTR}$ and up-regulation of CGRP mRNA expression), is transient and limited to the acute stage (17). The different responsiveness of these two populations of cholinergic neurons to inflammatory insult suggests that the cholinergic neurons of the basal forebrain are particularly vulnerable to inflammatory insult. Indeed, chronic infusion of TNF-α significantly decreases cortical ChaT activity, reduces the number of ChaT-immunoreactive cells, and increases the number of activated astrocytes and microglia within the basal forebrain (31), and similar results are obtained in different models of brain inflammation, including excitotoxin (32) and lipopolysaccharide administration (33).

Myelin Breakdown, NGF Transport, and Cognitive Impairment. EAE is associated with a myelin breakdown, which is followed by partial or complete remyelination, depending on the animal species, strain, gender, age, immunogen, immunization strategy, etc. In all cases, there is interplay between demyelination and remyelination by oligodendrocytes and oligodendrocyte precursor cells (OPC). Recently, is has been suggested that an age-related imbalance between these two processes, together with the high sensitivity of oligodendrocyte and OPC to a variety of insults, could be a severe risk factor for cognitive decline in AD as well (34), or a trigger (35) or precipitating event (36) in AD. Axonal dysfunction and degeneration observed in both EAE and MS impair retrograde transport (37, 38), thus affecting the cellular availability of neurotransmitter and trophic factor, including NGF. It is well known that survival and proper function of cholinergic neurons in the adult brain is NGF-dependent (39–41). Therefore, both changes in NGF level in selective brain areas (19, 20) and its retrograde transport are impaired in EAE animals, thus influencing the responsiveness of cholinergic neurons to inflammatory attack. It has been reported that the NGF level and synthesis during MS and EAE varies according to the stage of the disease and CNS area (19, 20), leading to the general concept of increased neuronal requirement of NGF during EAE. Here, we indicate that the NGF synthesis defect in the target area (cerebral cortex) is associated with a biochemical and functional impairment of the cholinergic system of the basal forebrain. Thus, damage and/or degeneration of the cholinergic system can be associated with altered NGF uptake and retrograde transport, as is also found in a mouse model of Down's syndrome (Ts65Dn), which is characterized by a progressive, age-dependent degeneration of the cholinergic system (42).

Neuroprotection as a Therapeutic Target for MS. Neural damage in MS could be directly due to inflammation (43) or result from loss of trophic support normally provided to axons by myelin or glia. Consequently, neuroprotection is considered a novel target for MS, and different drugs and molecules, including statins, green tea epigallocatechin-3-gallate, and erythropoietin (44–46), have been indicated as effective in experimental models of the disease. Here, we indicate that chronic administration of selective acetylcholinesterase inhibitors that are approved for AD treatment, at a dosage effective in increasing acetylcholine release in the hippocampus and cortex of aged rats (21), restores ChaT activity and performance in the water maze test in EAE rats. An up-regulation of ChaT activity in the brain has been obtained with different acetylcholinesterase inhibitors, such as ENA 713 (47, 48), tacrine (49), and TAK-147 (50). Mechanisms through which acetylcholinesterase inhibitors up-regulate ChaT activity are not known. Possible explanations include a presynaptic protection of cholinergic neurons (48), leading to a better function of cholinergic pathways also favoring glucose utilization (51). In our experiments, this effect is associated with a strong up-regulation of NGF mRNA expression in the target areas of the cholinergic neurons, thus suggesting that this regulation is directly involved in protection of the cholinergic system. Interestingly, a synergic action of NGF and the acetylcholinesterase inhibitor TAK-147 in potentiating ChaT activity has been shown in cultured rat septal cholinergic neurons (50).

Furthermore, a proper neurotrophin signaling from axons seems to be necessary for effective myelination (52). *In vitro* study have indicated that NGF promotes myelination in Schwann cells and inhibits conversion of premyelinating into myelinating oligodendrocyte in a cellular system derived from embryonic dorsal root ganglia (53). However, it is unknown whether the same observations are applicable to remyelination after injury. Moreover, treatment with recombinant human NGF inhibits the development of inflammation and demyelination in the nonhuman primate model of EAE (54), and a better remyelination is obtained by treatment that restores NGF content to control values in chronic EAE in rat, where NGF levels are dramatically lower (18, 20).

Finally, a recent randomized clinical trial (single center) indicated that the selective acetylcholinesterase inhibitor donepezil improved memory in MS patients with initial cognitive impairment (55), and rivastigmine acutely administered facilitates adaptative brain plasticity in patients with MS, favoring performance in a task requiring prefrontal cortex activation (56).

Thus, MS is recognized as a heterogeneous disease. Inflammation, demyelination and remyelination, axonal damage and loss, and brain atrophy are intertwined mechanisms in a pathogenic cascade during different phases of this long-lasting disease (6, 57) and a possible target for combinatory therapy (58). Another important aspect that should be taken into consideration is that human and experimental demyelinating-related diseases also include relevant cognitive deficit and neuronal degeneration. Our results suggest that these deficits in EAE correlate with a deficit of ChaT and, most likely, NGF. In conclusion, a particular effort should be devoted to neuroprotection, not only to prevent axonal pathology and neuron degeneration but also to favor myelination through proper axonal function and molecular signaling. The present findings in EAE could aid in the understanding of the pathogenic mechanism and, it is hoped, in the identification of additional therapeutic strategies.

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