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Selegiline enhances NGF synthesis and protects central nervous system neurons from excitotoxic and ischemic damage

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Abstract

It has been previously demonstrated that selegiline, an irreversible monoamine oxidase B (MAO-B) inhibitor, potentiates glial reaction to injury and possesses some 'trophic-like' activities which do not depend on the inhibition of MAO-B and which are probably associated with the induction of astrocyte-derived neurotrophic substances. Based on these findings, we tried to find out whether selegiline is able to modify the expression of nerve growth factor (NGF) and to protect central nervous system (CNS) neurons from excitotoxic and ischemic damage. Selegiline (10 pM–1 nM) induced NGF messenger RNA (mRNA) expression in cultured rat cortical astrocytes as determined by reverse transcription-polymerase chain reaction (RT-PCR) followed by a corresponding increase in NGF protein content measured by two-site NGF-enzyme-linked immunosorbent assay (ELISA) in astrocyte-conditioned medium. Additionally, exposure of hippocampal cultures containing neuronal and glial cells to this drug at the same concentrations enhanced significantly the content of NGF measured in the culture medium after 6 h of incubation. We hypothesize that selegiline could rescue hippocampal neurons from injury by induction of astrocyte-derived NGF in this cell culture system. To test this hypothesis, an excitotoxic damage was induced in the same type of cells by exposure to 0.5 mM L-glutamate for 1 h. Selegiline (10 pM–1 nM) present in the growth medium 6 h before until 18 h after induction of injury (the point of glutamate-toxicity measurement) protected hippocampal neurons from excitotoxic death. Furthermore, administered intraperitoneally (i.p.) (8 × 15 mg/kg per day) this drug enhanced the expression of NGF message in intact rat cerebral cortex and protected rat cortical tissue from ischemic insult due to permanent occlusion of the middle cerebral artery (MCA). The neuroprotective activity of selegiline (5 × 10 mg/kg per day i.p.) was also demonstrated in a mouse model of focal cerebral ischemia. The present data show that selegiline induced NGF expression in cultured rat cortical astrocytes. In mixed primary cultures of hippocampal neuronal and glial cells, selegiline increased NGF protein content and protected hippocampal neurons from excitotoxic degeneration. In vivo, this drug induced NGF gene expression in cerebral cortex from intact rats and protected rat and mouse cortical tissue from ischemic insult after occlusion of the MCA. Our results indicate that the induction of astrocyte-derived NGF could contribute to the neuroprotective activity of selegiline demonstrated both in vivo and in vitro and can explain, in part, the 'trophic-like' properties of this compound which has been observed by others.

Keywords: Selegiline; Nerve growth factor (NGF); Astrocyte, cultured; Hippocampal cell; Excitotoxicity; Protection; Cerebral ischemia

1. Introduction

The excessive activity of excitatory amino acids, such as L-glutamate and L-aspartate, followed by elevation of intracellular free Ca^{2+} concentration and accumulation of free radicals has been postulated to underlie the neurodegeneration that occurs after ischemic insults and trauma (Simon et al., 1984). Additionally, an excitotoxic component has been shown to play an important role in the pathogenesis of chronic neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and Huntington's disease, which are characterized by progressive loss of neuronal elements (Greenamyre and Young, 1989; Olney et al., 1990). This cell loss may be prevented by the localized delivery of neurotrophic factors. Among other neurotrophic substances, nerve growth factor (NGF) has been characterized as a survival and growth-promoting protein for certain neuronal populations in the central nervous system (CNS) and in the periphery. In the CNS, NGF exerts neurotrophic actions on the cholinergic neurons of the basal forebrain (Whittemore and Seiger, 1987) and protects them against

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axotomy-induced neurodegeneration and age-related atrophy (Hefti et al., 1984) but the effects of NGF are probably not restricted to these neuronal populations (Shelton and Reichardt, 1986). Recently, it has been shown that NGF protects rat hippocampus from ischemic insult (Pechan et al., 1995) and attenuates excitotoxic degeneration of cultured hippocampal neurons (Mattson et al., 1995). It suggests that NGF may have protective functions on this brain region which is particularly vulnerable to cerebral ischemia and chronic neurodegenerative disorders (e.g. Alzheimer's disease). Under physiological conditions, NGF synthesis in the brain is regulated by hormone and neurotransmitter receptor activation (for a review, see Mocchetti, 1991) and some information about pharmacological induction of NGF is available (for a review, see Carswell, 1993). The synthesis of NGF in the brain is upregulated after different kinds of damage (Lorez et al., 1989; De Kosky et al., 1994), suggesting that this may be a protective mechanism to maintain neuronal survival. By manipulating endogenous regulatory systems, the upregulation of NGF after CNS damage might be further elevated and/or sustained. Thus, induction of NGF by pharmacological intervention during the critical period of injury may ameliorate the extent of neuronal damage after acute events, such as stroke and trauma, and may have therapeutic benefits for Alzheimer's disease.

To examine this issue, we have selected selegiline, an irreversible monoamine oxidase B (MAO-B) inhibitor used for the treatment of Parkinson's disease. It has been shown that selegiline protects damaged with *N*-methyl-4-phenylpyridinium (MPP^+) dopaminergic neurons in vitro and attenuates excitotoxic as well as ischemic injury to the CNS (Koutsilieri et al., 1994; Knollema et al., 1995). The neuroprotective benefits of selegiline have been related to the enhancement of dopaminergic transmission and to the inhibition of free-radical production via MAO-B-induced catabolism of dopamine to dihydroxyphenylacetic acid. However, recent studies have shown that selegiline exerts some neurorescuing and 'trophic-like' activities that do not depend on the inhibition of MAO-B (Finnegan et al., 1990; Tatton, 1993; Tatton et al., 1994b). Some clinical studies suggest a therapeutic effect of selegiline in Alzheimer's disease (Stoll et al., 1994) and show that this drug prolonged the life span of patients (Birkmayer et al., 1985) and aged rats (Zeng et al., 1995). The basis for MAO-B-independent activities of selegiline is unknown. It has been reported that this drug alters gene expression and protein synthesis in astrocytes (Biagini et al., 1993; Tatton et al., 1994b; Seniuk et al., 1994; Ju et al., 1994). Based on these findings, it has been suggested that selegiline can promote the neuronal survival after CNS injury by the induction of neurotrophic substances in activated astrocytes (Biagini et al., 1994). In line with this hypothesis, we have found that selegiline enhances NGF protein content in medium from cultured rat cortical astrocytes (Semkova and Krieglstein, 1995). We hypothesize that the pharmacological basis for MAO-B-independent activities of selegiline could also be associated with enhanced synthesis of NGF. Therefore, we tried to find out whether selegiline is able to modify the expression of NGF and to protect CNS neurons against excitotoxic or ischemic damage.

2. Materials and methods

2.1. Animals

Neonatal (P1) Fischer 344 rats, male NMRI mice (20–30 g; Charles River, Sulzfeld, Germany) and Long-Evans rats (225–275 g; Denmark) were used. The animals had free access to food (Altromin, Lage, Germany) and water and were kept under environmental standardized conditions (12 h dark/light cycle, 23 ± 1°C and 55% relative humidity).

2.2. Cell culture agents and other substances

Eagle's minimum essential medium (MEM) (containing 2 mM L-glutamine, 28 mM of glucose and 22 mM of sodium bicarbonate), Dulbecco's modified MEM (DMEM), Leibovitz L-15 medium (containing 2 mM L-glutamine), fetal calf serum, penicillin-neomycin-streptomycin solution and NU serum were obtained from Gibco (Eggenstein, Germany). Sodium L-glutamate, cytosine β-D-arabinofuranoside, papain, trypsin inhibitor and poly-L-lysine hydrobromide were purchased from Sigma (Deisenhofen, Germany).

Anti-β (2.5S, 7S) NGF monoclonal antibody (clone 27/21), anti-β (2.5S, 7S) NGF-β-galactosidase (clone 27/21), NGF-β purified from mouse submaxillary glands, used as a standard, chlorophenol red-β-galactopyranoside, antibodies against glial fibrillary acidic protein (GFAP), anti-galactocerebroside antibodies and chemiluminescent substrate for alkaline phosphatase CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo-[3.3.1.1.^{3,7}]decan}-4-yl) phenyl phosphate were obtained from Boehringer Mannheim (Mannheim, Germany). Antisera to neuron-specific enolase was obtained from Polysciences (USA). RNeasy kit was purshased from Qiagen (Hilden, Germany), oligo (dT) primer from MWG Biotech (Ebersberg, Germany), PCR buffer and $MgCl_2$ from Amersham Buchler (Braunschweig, Germany), Maloney murine leukemia virus (MMLV)-reverse transcriptase, biotin-7dATP, dNTP mix (dATP, dCTP, dGTP and dTTP), dithiothreitol and agarose from Gibco, *Taq* polymerase from Dianova (Hamburg, Germany), Rnase-inhibitor and PCR marker were obtained from Promega (Heidelberg, Germany). Streptavidine-alkaline phosphatase complex was purchased from Sigma.

2.3. Primary cultures of rat cortical astrocytes

Primary cultures of astrocytes were prepared from cerebral cortices of post-natal Fischer 344 rats (PD 1–2) as described previously by McCarthy and DeVellis (1980)

with minor modifications. Briefly, the animals were decapitated under sterile conditions, the brains were removed and stripped of meninges. Cerebral cortices were isolated and dissected in DMEM containing penicillin-neomycin-streptomycin solution. The cortical tissue pieces were passed through a stainless steel monofilament mesh (pore size 190 μm). The dissociated cells were centrifuged at 1000 rpm for 5 min. The supernatant was removed and the pellet was resuspended with a fire-polished Pasteur pipette in an appropriate volume of media containing 10% fetal calf serum and penicillin-neomycin-streptomycin solution. The cells were seeded on culture flasks (175 cm^2, Corning) and cultivated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The culture medium was exchanged twice per week. Oligodendrocytes and microglial cells growing on the top of the astrocyte monolayer were removed by shaking and washing with cold phosphate-buffered saline. Astrocyte cultures obtained after one passage with the enzymatic treatment were used for the present study.

2.3.1. Preparation of astrocytes for RNA extraction

Confluent astrocytes were passaged and plated at a density of 1×10^4 cells/cm^2 on culture flasks (25 cm^2; Falcon, France) and cultivated in DMEM supplemented with 10% fetal calf serum and penicillin-neomycin-streptomycin solution till the confluence was reached. The cells were rinsed twice with Hank's balanced salt solution and were exposed for 24 h to serum-free DMEM. Afterwards, the astrocytes were treated with selegiline (10 pM–1 nM). The control sister cultures received the vehicle only. After 6 h of incubation, the cells were rinsed with Hank's balanced salt solution and harvested by scraping from each flask into lysis buffer (Qiagen) supplemented with β-mercaptoethanol (10 μl/ml). The samples were stored at −70°C until use.

2.3.2. Preparation of astrocyte-conditioned medium

Astrocyte-conditioned medium was prepared after the seeding of astrocytes on 24-well plates (Multiwell; Nunc, Denmark) at a density of 1×10^4 cells/cm^2 and cultivated in DMEM supplemented with 10% fetal calf serum and penicillin-neomycin-streptomycin solution till the confluence was reached. The culture medium was exchanged twice a week. The confluent cultures were rinsed twice with Hank's balanced salt solution and exposed for 24 h to serum-free DMEM. Afterwards, the astrocytes were incubated with different concentrations of selegiline for 6 h. The control sister cultures received the same volume of saline. After 6 h of incubation, the medium was collected and centrifuged to remove the cell debris. 20 μg/ml aprotinin was added and the supernatants were stored at −20°C until use.

2.4. Primary rat hippocampal cultures

Mixed primary cultures of hippocampal glial and neuronal cells were prepared from neonatal (P1) Fischer 344 rats. The animals were decapitated under sterile conditions and the hippocampi were isolated and incubated for 20 min at 37°C in Leibovitz's L-15 medium supplemented with 1 mg/ml papain and 0.2 mg/ml bovine serum albumin. The supernatant was removed and the tissue pieces were triturated in MEM containing 10% serum. The supernatant was transferred to another tube and the undispersed pieces were triturated again through a fire-polished Pasteur pipette. The supernatants were combined and trypsin inhibitor solution (1% in MEM containing 10 mg/ml bovine serum albumin) was layered under the cell suspension. The mixture was centrifuged at 600 rpm and 20°C for 10 min. The cells were resuspended in MEM containing 10% serum and seeded on poly-L-lysine-coated 35-mm culture dishes (3×10^5 cells/dish). The cells were cultivated in MEM supplemented with 10% serum, 20 U/ml penicillin and 20 μg/ml streptomycin, and were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. After 2 days in culture, cytosin-β-D-arabinofuranoside (1 μM) was added to the medium for 24–36 h to minimize glial cell proliferation. The medium was changed every 3–4 days.

For the measurement of NGF protein content, cultures grown for 14 days were exposed overnight to serum-free MEM and incubated with different concentrations of selegiline for 6 h. The control sister cultures received the same volume of saline. In parallel, the cell viability was assessed by Trypan blue staining after 6 h of incubation with selegiline and saline in serum-free MEM. The culture medium was collected and centrifuged to remove the cell debris. The supernatants containing 20 μg/ml aprotinin were stored at −20°C until use.

2.5. Immunocytochemistry

Hippocampal cells and rat cortical astrocytes cultivated on glass coverslips for 14 and 18 days, respectively, were used for immunocytochemistry. After fixation of the cells for 15 min at 37°C with 4% formaldehyde in phosphate-buffered saline containing 0.1% Triton (pH 7.4), the immunostaining with monoclonal antibody directed against neuron-specific enolase (dilution 1:2000), GFAP (dilution 1:4) and galactocerebroside (1:20) was performed according to the protocol recommended by the manufacturer. As a negative control, the immunostaining was performed without the first antibody.

2.6. RNA extraction and RT-PCR

For measurement of NGF messenger RNA (mRNA) in cerebral cortex, Long-Evans rats were treated with selegiline (15 mg/kg per day intraperitoneally, i.p.) or saline for 8 consecutive days. Cortical brain tissue was dissected out 6 h after the last injection of the drug and the samples were stored at −70°C until use. Total cellular RNA was

extracted from cultured astrocytes and brain tissue using the RNeasy Kit according to the manufacturer's instructions (Qiagen). RNA concentration was calculated by absorbance at 260 nm and purity was demonstrated by A_{260}/A_{280} ratios between 1.7 and 2.0 (Sambrook et al., 1989). 1 μg total RNA was analyzed by reverse transcription (RT) and followed polymerase chain reaction (PCR) (RT-PCR). RT was performed at a volume of 45 μl. RT solution contained: 1 × PCR buffer, 2.2 mM $MgCl_2$, dNTP mix (dATP, dCTP, dGTP and dTTP, 0.2 mM each), 4 mM dithiothreitol, 20 U Rnase-inhibitor, oligo-(T)-primer and Rnase-free water. The probes were heated to 65°C for 5 min and chilled on ice. 50 μl MMLV-reverse transcriptase was used for total RNA derived from cultured astrocytes. For total RNA extracted from cortical homogenates, 100 U MMLV-reverse transcriptase per probe was used. RT was performed at 37°C for 60 min followed by a 5-min enzyme denaturation at 95°C. For subsequent PCR, 10 μl cDNA solution was used.

PCR was performed at a volume of 50 μl. PCR solution contained: 1 × PCR buffer, 2.0 mM $MgCl_2$, dNTP mix (dATP, dCTP, dGTP and dTTP, 0.2 mM each), 0.3 μM from each primer (sense and antisense) and 0.1 U of *Taq* polymerase. PCR was carried out in a thermal cycler (Omni; Gene, Hybaid, Teddington, UK). Thirty cycles of PCR were performed (for NGF: initial template denaturation at 95°C for 30 s; annealing with NGF primers at 58°C for 60 s; primer extension at 72°C for 120 s; for β-actin: denaturation at 95°C for 30 s; annealing at 42°C for 60 s; primer extension at 72°C for 120 s). The 30th cycle was followed by a final extension step at 72°C for 3 min.

PCR products were analyzed using 2% agarose gels and visualized using Ethidium bromide staining and UV transillumination. Amplified cDNA was transferred from the gels to a nylon membrane (Hybond N^+, Amersham). Biotin-labeled PCR products were detected using a streptavidine-alkaline phosphatase complex. Chemiluminescence was produced by the reaction of CSPD with alkaline-phosphatase. The bands were visualized using an autoradiography film. The integrated optical density of the bands was measured (IBAS 2; Kontron, Eching, Germany). The oligonucleotide primers for NGF was designed according to the published sequence with a predicted product size of 391 bp:

Sense 5′ – ctggactaaacttcagcattc – 3′

Antisense 5′ – tgttgttaatgttcacctcgc – 3′

In all experiments, RT-PCR of β-actin was carried out concurrently (30 cycles) to control for sample loading. Primers for β-actin were as shown below with a predicted product size of 380 bp:

Sense 5′ – atttggcaccacactttctaca – 3′

Antisense 5′ – tcacgcacgatttccctctcag – 3′

To rule out amplification of any genomic DNA, all RT-PCR experiments were routinely controlled by conducting PCR without RT reaction.

2.6.1. Semiquantitative measurements of the PCR products

NGF cDNA was amplified using different amounts of total RNA (2, 1, 0.5, 0.25 and 0.125 μg). The linear correlation between the integrated optical density of the PCR products and the amount of total RNA was calculated. The dilution series was performed with saline- and selegiline (1 nM)-treated astrocyte cultures. Since a linear correlation coefficient between integrated optical density and the amount of total RNA of untreated and drug-treated astrocytes was calculated (8470 vs. 8509), semiquantitative comparisons of the amounts of NGF mRNA of saline- and selegiline-treated astrocytes were possible.

2.7. NGF protein measurement

A two-site enzyme immune assay for NGF was performed as described by Korsching and Thoenen (1983) with minor modifications. Monoclonal anti-NGF antibodies (clone 27/21) specific for mouse, rat, beef and human NGF were used. The wells of microtiter plates (MaxiSorb, Nunc 96-well) were coated with buffer solution (50 μl/well) containing 50 mM $Na_2CO_3/NaHCO_3$ buffer, 0.05% sodium azide, pH 9.6, and 0.5 μg/ml anti-NGF-β monoclonal antibodies for 2 h at 37°C. Non-specific binding sites were saturated with 300 μl/well blocking solution containing 1% bovine serum albumin. The wells were washed 3 times with buffer solution (50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, 0.1% Triton X-100, 0.05% sodium azide, pH 7.0). The samples and the standard solution (0–1 ng/ml of NGF) were added and the microtiter plates were incubated overnight at 4°C. After washing 3 times, anti-NGF-β monoclonal antibodies conjugated to β-galactosidase (clone 27/21) diluted to 0.2 U/ml with buffer solution (50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, 1% bovine serum albumin, 0.05% sodium azide, 1% Triton × 100, pH 7.0) were added at a volume of 50 μl/well. The microtiter plates were incubated for 5 h at 37°C and then washed 3–4 times. The wells were filled with freshly prepared substrate solution of chlorophenol red-β-galactopyranoside (2 mg/ml, 60 μl/well) diluted in substrate buffer (100 mM Hepes, 150 mM NaCl, 2 mM $MgCl_2$, 1% bovine serum albumin, 0.1% sodium azide, pH 7.0) and the color developed after incubation at 37°C for 4 h was measured photometrically by an enzyme-linked immunosorbent assay (ELISA) plate reader at 574 nm. Standard curves were prepared with 2.5S NGF-β standard purified from mouse submaxillary glands by assaying parallel wells containing increasing amounts of NGF-β (0–1 ng/ml). The amount of NGF-β in the samples was calculated from the standard curve.

2.8. Induction of excitotoxic neuronal damage by glutamate

Rat hippocampal neurons were damaged with L-glutamate after 14 days in vitro as described previously (Koh

and Choi, 1988). The cultures were washed with serum-free MEM and then exposed to serum-free MEM containing 0.5 mM L-glutamate for 1 h. Afterwards, glutamate was washed away twice with serum-free MEM and then fresh serum-free MEM was added to the cultures for 18 h (the point of mesurement of glutamate toxicity). Cultures that were not exposed to L-glutamate were also washed with serum-free MEM and switched to serum-free MEM without glutamate. The percentage of damaged neurons was determined by Trypan blue exclusion. The identification of neuronal phenotype was based on the standard morphological criteria, such as phase-bright fusiform and pyramidal cell bodies, which extend one or more processes. Neurons stained with Trypan blue and possessing fragmented neurites were regarded as non-viable. The non-stained neurons with intact neurites and soma were considered viable.

The effect of selegiline on glutamate-induced neurodegeneration was investigated at concentrations ranging between 1 pM and 10 nM. The drug was present in the medium from 6 h before until 18 h after excitotoxic injury, including the period of exposure to glutamate.

Control cultures received the vehicle only.

2.9. Permanent focal cerebral ischemia in rats

Permanent middle cerebral artery (MCA) occlusion was performed in male Long-Evans rats as described by Tamura et al. (1981) with minor modifications. Animals were anesthetized with a mixture of 68.5% N_2O/30% O_2 that contained 1.5% halothane. A vertical incision was performed between the left orbit and the ear, and the skull was exposed. A craniotomy was performed to approach the left MCA. The stem of the MCA was irreversibly occluded by microbipolar electrocoagulation. Mean arterial blood pressure and plasma glucose concentrations as well as arterial pH, pCO_2, pO_2 were monitored (Corning 178; Corning, Gießen, Germany) up to 1 h after administration of the drug. Body temperature was maintained at 37 ± 1°C with an IR lamp during the operation. Afterwards, the animals were kept at an environmental temperature of 30°C for 2 h and then in their home cages at 20°C for 2 days after MCA occlusion. After 7 days, rats were anesthetized with chloral hydrate (400 mg/kg i.p.) and decapitated. Brains were removed from the skull and frozen in isopentane (Fluka, Buchs, Switzerland) on dry ice. Transversial sections of 20 μm thickness were taken every 500 μm using a cryomicrotome (Frigocut; Reichert-Jung, Nussloch, Germany). The sections were stained with 0.5% Cresyl violet to differentiate between intact and damaged brain tissue. The infarct volume (mm^3) was calculated from the infarct area of each section and the distance between succeeding sections. Selegiline was administered i.p. at a dose of 15 mg/kg in two experimental schedules: once 2 h prior to MCA occlusion and for 8 consecutive days (15 mg/kg per day) before induction of ischemia.

2.10. Permanent focal cerebral ischemia in mice

Permanent MCA occlusion was performed in male NMRI mice according to the method modified from Welsh et al. (1987). The mice were anesthetized with tribromoethanol (600 mg/kg i.p.). A small hole was drilled above the MCA after surgical exposure to the skull. The stem of the MCA and both branches were permanently occluded by a microbipolar electrocoagulator. Body temperature was maintained at 37 ± 1°C with a heating lamp during the operation. Afterwards, the mice were kept at an environmental temperature of 30°C for 120 min and then in their home cages at 20°C for 2 days after MCA occlusion. During this period of time the behavior of the animals was observed.

For histological evaluation, the mice were anesthetized again with tribromoethanol and perfused i.p. with a 1% solution of Neutral red (0.5 ml) 2 days after MCA occlusion. The brains were removed and stored in a fixative (4% formalin in phosphate buffer solution, pH 7.4) for 24 h. The Neutral red-unstained tissue on the brain surface was determined as infarcted surface area (in mm^2) by means of an image-analyzing system (IBAS 2; Kontron) according to Backhauß et al. (1992). Selegiline was administered i.p. at a dose of 10 mg/kg once 5 h before induction of ischemia and in other series of experiments for 5 consecutive days (10 mg/kg per day) prior to vessel occlusion.

2.11. Statistics

All values are means ± S.D. or ± S.E.M. of n experiments. One-way analysis of variance (ANOVA) combined with Scheffé's test or Duncan's test were used for multiple comparisons. Homogeneity of errors was determined using Bartlett's test.

3. Results

3.1. Immunocytochemistry

Immunostaining performed in cultured hippocampal cells after 14 days in vitro showed that under our experimental conditions these cultures contained both neurons and astroglia positive for GFAP. The neuronal phenotype of the cells was identified by antisera to neuron-specific enolase. The neurons grew on the feeder monolayer of flat-type astrocytes as shown by immunocytochemistry performed after 14 days in vitro. The neurons were easily distinguishable from astrocytes by their specific cell morphology, pyramidal cell body and one or more extended major processes.

Immunostaining performed in astrocyte cultures showed that after selection by adherence, shaking and washing with cold phosphate-buffered saline, > 98% of cells in

culture were positive for the astrocyte-specific marker GFAP. No cells were positive for neuron-specific enolase and galactocerebroside indicating that neurons and oligodendrocytes, if any, were present below the levels of detection.

3.2. Expression of NGF mRNA and NGF protein in cultured astrocytes

NGF mRNA levels were determined after exposure of rat cortical astrocytes to selegiline (10 pM–1 nM) for 6 h by RT-PCR. Fig. 1 represents a photograph of the RT-PCR products visualized after electrophoresis in 2% agarose gel containing ethidium bromide. The bands showing the expression of NGF mRNA in the samples are located between 300 and 500 bp of the size standard (expected size of RT-PCR product 391 bp). The exposure of astrocytes to selegiline (0.01–1 nM) increased the expression of NGF message in comparison with vehicle-treated cultures. The most pronounced effect was observed at a concentration of 1 nM as shown in a Fig. 1. The semiquantitative measurement of the PCR product shows that selegiline at a concentration of 1 nM increased approximately 3-fold the expression of NGF message in cultured astrocytes in comparison to the saline-treated cells. In all performed experiments, the expression of mRNA for β-actin was not influenced by selegiline.

The increase in NGF mRNA was followed by a corresponding increase (also 2.5–3-fold) of NGF protein secreted into the culture medium as measured by NGF-ELISA (Fig. 2). Unstimulated astrocytes release detectable amounts of NGF protein ranging between 35 and 55 pg/ml which were significantly enhanced after exposure to selegiline (Fig. 2).

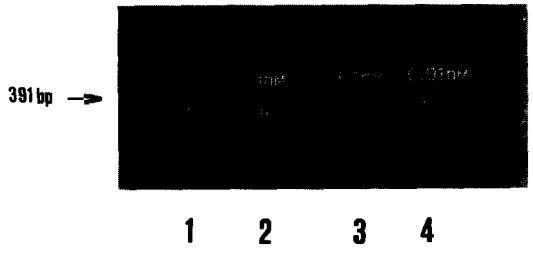

Fig. 1. Expression of NGF mRNA in cultured rat cortical astrocytes after exposure to selegiline (10 pM–1 nM) for 6 h in serum-free medium. The control sister cultures received the same volume of saline. Equivalent amounts of total RNA were used as a starting material and subjected to RT-PCR as described in Section 2. The PCR products were electrophorezed in 2% agarose gel, stained with ethidium bromide and photographed under UV light. The photograph represents a NGF mRNA expression in the samples. Line 1c-NGF mRNA in controls exposed to saline; lines 2s–4s represent the expression of NGF message in astrocytes treated with selegiline at concentrations of 1, 0.1 and 0.01 nM, respectively, for 6 h. The results were reproduced with 3 different astrocyte cultures.

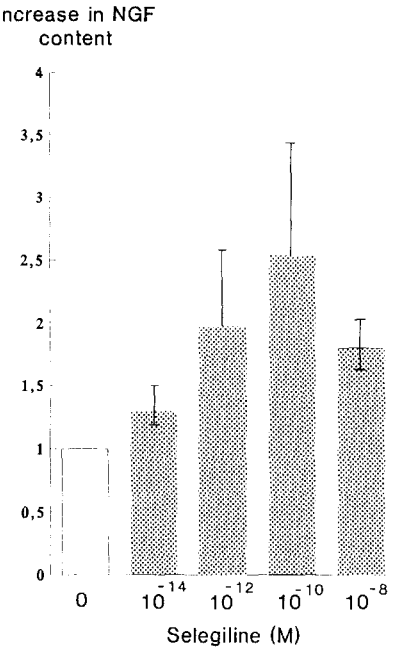

Fig. 2. Selegiline enhances NGF protein content in the medium from rat cortical astrocytes. The cells were incubated for 6 h in serum-free medium with various concentrations of selegiline. The control sister cultures received the same volume of saline. NGF protein content was determined directly in the culture medium by NGF-ELISA. NGF content in the medium is present as a fold increase in selegiline-treated cultures in comparison to the controls. Each point is the mean ± S.D. from 5 different experiments.

3.3. Effect of selegiline on NGF protein content in hippocampal cultures

A two-site enzyme immunoassay was used to measure NGF protein level in the medium of rat hippocampal cells. The culture medium was assayed directly in the ELISA system. An increase in NGF concentration in the medium was observed after exposure of these cells to selegiline (10 pM–1 nM) for 6 h (Fig. 3).

3.4. Effect of selegiline on glutamate-induced excitotoxic damage

To test whether selegiline can protect hippocampal neurons from excitotoxic degeneration, we exposed hippocampal cultures grown for 14 days to 0.5 mM L-glutamate for 1 h. It induced pronounced neurotoxicity evaluated morphologically and by Trypan blue staining 18 h later. In glutamate-exposed cultures neurites were decreased in number and some neurons had desintegrated into the debris. Approximately 70% of neurons lost their membrane integrity and became stained with Trypan blue 18 h after exposure to glutamate. Treatment of the hippocampal cells with selegiline (10 pM–1 nM) increased significantly the number of viable neurons (Fig. 3). The

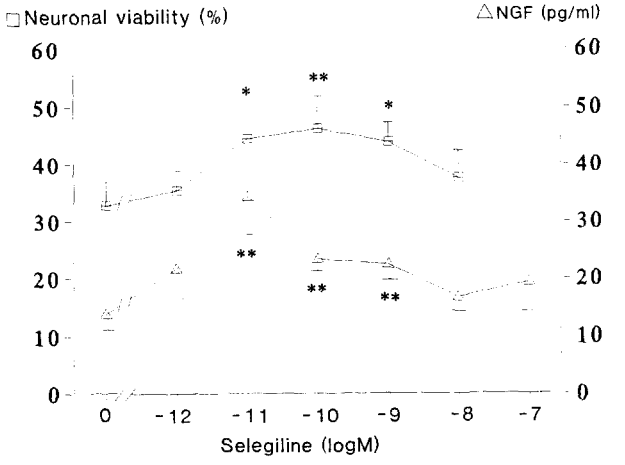

Fig. 3. Selegiline enhances NGF content in medium from rat hippocampal cells and protects hippocampal neurons against excitotoxic injury. Mixed neuronal/glial hippocampal cultures were grown for 14 days and then treated with various concentrations of selegiline for 6 h in serum-free medium. NGF content was determined directly in the culture medium by NGF-ELISA. Values are means ± S.D. from 5 different experiments. Different from controls: $^{**}P < 0.01$ by Scheffé's test. The sister cultures were exposed to 0.5 mM L-glutamate for 1 h in serum-free medium. Neuronal damage was determined 18 h later by Trypan blue exclusion. Selegiline was present in the culture medium 6 h before up to 18 h after excitotoxic injury, including the period of exposure to glutamate. Control cultures received the vehicle only. The values are means ± S.D. from 5 different experiments. Different from glutamate-free controls: $^{###}P < 0.001$; and from glutamate-exposed cells: $^{*}P < 0.05$ and $^{**}P < 0.01$ by Scheffé's test.

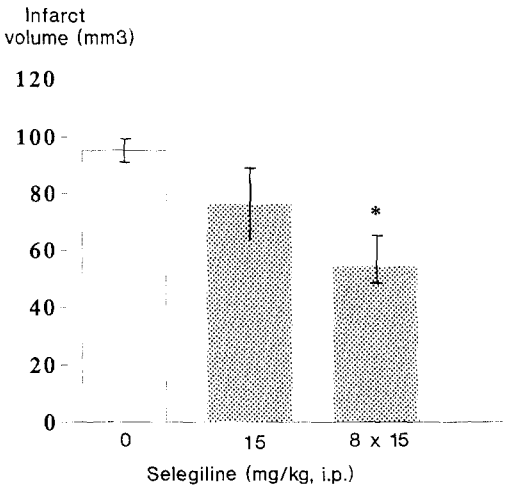

Fig. 4. Selegiline reduces the infarct volume in the rat model of focal cerebral ischemia. Permanent MCA occlusion was performed in male Long-Evans rats. 7 days after induction of ischemia, the damage was evaluated after staining of brain sections with Cresyl violet. The unstained cortical tissue was determined as cortical infarct area (mm^3) by means of an image-analyzing system. The ischemic damage is expressed as an infarct volume (mm^3) calculated from the infarct area of each section and the distance between succeeding sections. The animals (9/group) were treated i.p. with selegiline (15 mg/kg) once for 1 h or for 8 consecutive days (15 mg/kg per day) prior to occlusion of the MCA. Control animals received the vehicle only. Data are means ± S.E.M. Different from control: $^{*}P < 0.05$ by Duncan's test.

drug was present in the culture medium for 6 h before and up to 18 h after the induction of excitotoxic injury.

3.5. Effect of selegiline on brain damage in a rat model of focal cerebral ischemia

The neuroprotective activity of selegiline was investigated in a rat model of focal cerebral ischemia. Administration of selegiline (15 mg/kg i.p.) for 8 days prior to MCA occlusion significantly reduced cortical infarct volume (Fig. 4). However, treatment with a single dose of selegiline (15 mg/kg i.p.) 2 h prior to the induction of ischemic injury did not influence significantly the infarct volume of the rat brain.

3.6. Expression of NGF mRNA in rat cerebral cortex after treatment with selegiline

We investigated whether the systemical administration of selegiline (15 mg/kg per day) for 8 consecutive days can modify the expression of NGF mRNA in rat cerebral cortex. We found that this drug, systemically administered induced a marked increase in the level of NGF message in rat cerebral cortex in comparison with saline-treated animals as determined by RT-PCR (Fig. 5).

3.7. Effect of selegiline on the ischemic damage induced by MCA occlusion in mice

Permanent occlusion of the MCA in mice causes ischemic cortical injury evaluated 2 days later as infarcted brain surface area (mm^2). The pre-treatment of the mice

Fig. 5. Selegiline enhances NGF synthesis in rat cerebral cortex. The animals (5/group) were treated with selegiline (15 mg/kg per day i.p.) or saline for 8 consecutive days. Cortical brain tissue of both ipsi- and contralateral hemispheres was dissected out 6 h after the last injection of the drug. Total RNA was extracted from the samples and equivalent amounts were subjected to RT-PCR as described in Section 2. Lines 1 and 2 represent the expression of NGF mRNA in intact cerebral cortex 'ipsi-' and 'contralateral' side after administration of selegiline. Lines 3 and 4 represent NGF message in cerebral cortex of vehicle-treated animals. The size standard (5th line) is (top to bottom) 50, 150, 300, 500, 750 and 1000 bp.

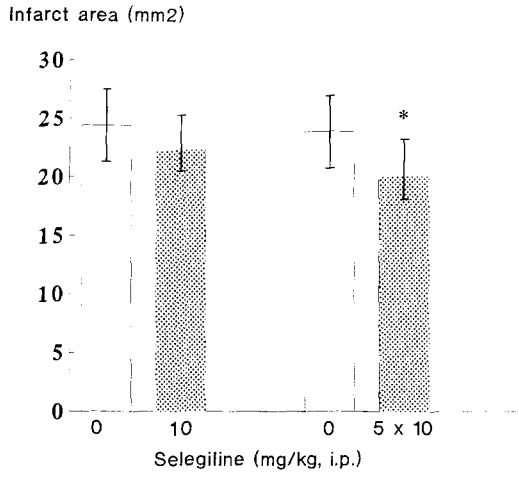

Fig. 6. Selegiline decreases the infarct area in the mouse model of focal cerebral ischemia. Permanent MCA occlusion was performed in male NMRI mice. 48 h after MCA occlusion, Neutral red was injected i.p. to stain the brain tissue. The unstained region on the brain surface was determined as infarct surface area (mm^2) by means of an image-analyzing system. The animals were injected i.p. with 10 mg/kg selegiline once for 5 h before induction of ischemia and in other series of experiments for 5 consecutive days (10 mg/kg per day) before MCA occlusion. Controls received the vehicle only. Data are means ± S.E.M. Different from control: * $P > 0.05$ by Duncan's test.

for 5 days with selegiline (10 mg/kg per day i.p.) reduced significantly the ischemic damage ($P < 0.05$ vs. controls, Duncan's test) (Fig. 6). Administration of a single dose of selegiline (10 mg/kg i.p.) 5 h prior to induction of ischemia did not influence the extent of the cortical infarct area (Fig. 6).

4. Discussion

The decrease of trophic support to neurons is associated with neuronal death and the appearance of neurodegenerative diseases with ages (Sofroniew and Cooper, 1993). On the other hand, the neurotrophic substances can serve as survival factors for the neurons under acute damage, such as stroke and trauma (for a review, see Mattson and Scheff, 1994). Therefore, the pharmacological upregulation and delivery of neurotrophic factors to the affected areas could become an elegant approach for the treatment of both acute (e.g. stroke) and chronic neurodegenerative disorders (e.g. Alzheimer's disease).

The dramatic increase of NGF synthesis in hippocampus and cerebral cortex in response to seizures (Gall and Isackson, 1989), trauma (De Kosky et al., 1994), hypoxic brain injury (Lorez et al., 1989) and lesions (Lärkfors et al., 1987) suggests that this may be a protective mechanism to maintain neuronal survival in these brain regions which are particularly vulnerable to acute and chronic neuronal damage. An increasing number of evidence shows that NGF exerts protective activities on the hippocampal neurons (Cheng and Mattson, 1991; Pechan et al., 1995). Therefore, by pharmacological intervention the upregulation of NGF synthesis after CNS damage might be further elevated or sustained and this can attenuate the degree of neuronal injury. To investigate the effects of drugs which systemically administered can stimulate NGF synthesis in the brain, we used selegiline, an irreversible MAO-B inhibitor, which has been shown to have some therapeutical benefits in the treatment of Parkinson's and Alzheimer's diseases. It has been reported that selegiline exerts 'trophic-like' activities since this drug was able to rescue rat motoneurons after facial nerve transection (Salo and Tatton, 1992; Ju et al., 1994) and to enhance neurite outgrowth in cultured rat spinal ventral horn neurons (Iwasaki et al., 1994) in a manner similar to several neurotrophic factors. Additionally, there is evidence that selegiline enhances both survival and neurite outgrowth of MPP^+-damaged dopaminergic neurons (Koutsilieri et al., 1994) at concentrations not sufficient to cause MAO-B inhibition (Tatton and Greenwood, 1991). Based on these findings, it has been suggested that selegiline can rescue damaged neurons by activation of survival-promoting and trophic processes, like induction of neurotrophic factors (Biagini et al., 1994; Tatton et al., 1994a). In line with this hypothesis, we have found that selegiline enhances NGF protein concentrations in culture medium from rat cortical astrocytes (Semkova and Krieglstein, 1995). In the present work, we investigate whether selegiline can induce NGF synthesis in this cell culture system. We found that this drug at very low concentrations ranging between 0.01 and 1 nM enhanced the expression of NGF message 6 h after treatment. The semiquantitative measurements of the PCR-products showed that selegiline at a concentration of 1 nM increased approximately 3-fold the expression of NGF message in cultured astrocytes. The increase in NGF mRNA was followed by a corresponding increase (also 2.5–3-fold) of NGF protein secreted into the culture medium as measured by NGF-ELISA. This indicates that selegiline exerts a positive control on the expression of NGF in astrocytes. It has been previously demonstrated that cultured astrocytes synthesize and secrete NGF (Houlgatte et al., 1989). Unstimulated astrocytes under our experimental conditions released detectable amounts of NGF ranging between 30 and 55 pg/ml which were significantly elevated after treatment with selegiline. The mechanism by which selegiline induces NGF synthesis in cultured astrocytes is not clear but it does not seem to depend on the inhibition of MAO-B since concentrations of < 10 nM were not sufficient to block this enzyme (Fowler et al., 1978). Interestingly, it has been reported that process bearing astrocytes respond to this drug with increased process growth and enhanced expression of ciliary neurotrophic factor (CNTF) message (Seniuk et al., 1994) also at concentrations insufficient to cause MAO-B inhibition. Additionally, there is evidence that in vivo selegiline can alter gene expression and protein synthesis in astrocytes (Biagini et al., 1993, 1994; Ansari et al.,

1993; Ju et al., 1994). It has been demonstrated that selegiline increased the process of reactive astrogliosis after mechanical lesions in vivo (Biagini et al., 1993; Ju et al., 1994) and enhanced both the lesion-induced expression of GFAP and basic fibroblast growth factor (bFGF) (Biagini et al., 1994). The mechanism by which selegiline augments the glial hypertrophy after injury is not clear but it seems to be related to the enhanced expression of bFGF known to be potent glial cell mitogen (Eclancher et al., 1990). Interestingly, we observed that selegiline can induce NGF synthesis and release in quiescent astrocytes, probably via direct modulation of astrocyte gene expression and activation of signal-transducing pathways not well understood till now. However, the exact mechanism by which selegiline enhanced NGF expression in cultured astrocytes remains to be determined. The present report also provides evidence that cultured rat cortical astrocytes, containing under our experimental conditions mainly flat-type astrocytes, are targets for selegiline. It has been previously shown that selegiline can influence CNTF gene expression only in process-bearing astrocytes (Seniuk et al., 1994) which morphologically resemble type-2 astrocytes (Levison and McCarthy, 1991). We observed that flat-type astrocytes described also as type-1 astrocytes respond to selegiline with increased NGF synthesis and release.

Furthermore, we attempted to determine whether selegiline could modify the expression of NGF in another cell culture system – primary hippocampal cultures containing neuronal and glial cells obtained from newborn rats. There is evidence that neuronal-glial interactions play an important role in regulating NGF synthesis and secretion in response to stimulation (Schmalenbach and Müller, 1993). Under our conditions, unstimulated hippocampal cells released small but detectable amounts of NGF into the culture medium. However, the incubation with selegiline (10 pM–1 nM) increased significantly NGF protein content in the medium as measured by NGF-ELISA, suggesting stimulation of NGF synthesis and release from NGF-producing cells. Since cultured astrocytes respond to selegiline with enhanced NGF synthesis, we suggest that the treatment of hippocampal cultures containing both neurons and astrocytes with this drug might cause an increased synthesis and release of NGF from activated astrocytes. However, it has been shown that cultured hippocampal neurons also synthesized and secreted NGF (Ayer-LeLievre et al., 1988; Houlgatte et al., 1989). In vivo studies have indicated that NGF gene expression was localized only to neuronal cells (Whittemore et al., 1988). Therefore, the exact cellular type responsible for the increased NGF concentration measured in the culture medium after exposure to selegiline remains to be determined.

The hippocampal neurons are vulnerable to different kinds of damage, including ischemic and traumatic damage to the CNS. We suggested that selegiline can protect hippocampal neurons from damage by enhanced expression of NGF in these cell cultures. To investigate this hypothesis, we exposed cultured hippocampal cells to the excitotoxin glutamate. It has been postulated that an 'excitotoxic' form of neuronal injury and death underlies the pathophysiology of cerebral ischemia and chronic neurodegenerative disorders. Hippocampal neurons possess glutamate receptors and are vulnerable to excitatory amino-acid stimulation. Glutamate-induced excitotoxicity in hippocampal neurons is associated with a massive Ca^{2+} influx through NMDA-receptors since NMDA receptor antagonists are able to attenuate the degree of neuronal injury (Simon et al., 1984). The glutamate-damaged neurons showed cell body swelling within a few minutes after exposure to this neurotoxin and approximately 70% of them became stained with Trypan blue 18 h later. Selegiline was able to protect hippocampal neurons from glutamate-induced degeneration reducing significantly the number of damaged neurons. When this drug (10 pM–1 nM) was present in the medium 6 h before until 18 h after induction of injury, the hippocampal neurons were greatly protected from glutamate-induced degeneration. It has been reported that hippocampal neurons contain MAO-B (Lique et al., 1995) but selegiline attenuated the severity of excitotoxic neuronal injury at concentrations too low to inhibit this enzyme. The neuroprotective activity of selegiline was demonstrated at the same concentrations (10 pM–1 nM) which increased NGF content in the culture medium, suggesting that NGF might underlie the neuroprotective action of this compound. Interestingly, it has been reported that this drug at the same concentration range protects NGF-dependent pheochromocytoma (PC12) cells from apoptotic cell death (Tatton et al., 1994a). We did not observe neuroprotection at concentrations of > 1 nM, this may be because NGF protein levels were not enhanced and probably because of the toxic effects of (−)-methamphetamine and (−)-amphetamine, the major metabolites of selegiline. Our results agree with the previously reported neuroprotective activity of selegiline in cultured dopaminergic neurons against MPP^+ damage (Koutsilieri et al., 1994) since an excitotoxic component of neuronal degeneration has been shown to underlie this type of injury (Akaneya et al., 1995). Interestingly, Koutsilieri et al. (1994) have observed that selegiline not only rescues MPP^+-injured dopaminergic neurons but also exhibits 'trophic-like' activities stimulating morphological differentiation, suggesting that the effects of this compound on these neurons are not restricted to the increased dopaminergic transmission. Additionally, the effects of selegiline are also not restricted to the substantia nigra dopaminergic neurons since it has been shown that this drug enhances neurite outgrowth in cultured spinal ventral horn neurons (Iwasaki et al., 1994) and increased the number of survival motoneurons after axotomy (Salo and Tatton, 1992; Ju et al., 1994) in a manner similar to CNTF and bFGF (Grothe and Unsicker, 1992; Sendtner et al., 1990). Additionally, we observed that selegiline can protect hippocampal neu-

rons from excitotoxic damage, probably by induction of NGF. We hypothesize that enhanced synthesis of NGF might contribute to the neuroprotective and 'trophic-like' activities of selegiline observed also by others. Since it has been reported that selegiline augments the astrocyte hypertrophy after damage, we suggest that astrocyte-derived cytokines and neurotrophic substances could mediate the neuroprotective activity of selegiline and NGF is one of them. Therefore, the pharmacological basis of MAO-B-independent activities of selegiline could also be associated with enhanced synthesis and release of NGF from activated astrocytes. There is evidence that NGF can rescue hippocampal neurons from ischemic insult (Yamamoto et al., 1992; Pechan et al., 1995) and excitotoxic damage (Mattson et al., 1995) even though the high affinity TrkA receptors for NGF have not been detected on hippocampal neurons (Ip et al., 1993). However, very recently, Cellerino (1996) reported that hippocampal neurons express mRNA coding for the NGF receptor TrkA detected by using highly sensitive in situ hybridization and RT-PCR. Additionally, these neurons possess low affinity p75 receptors for NGF (Cheng and Mattson, 1991) which may contribute to the neuroprotective activity of NGF (Rabizadeh et al., 1994; Lee et al., 1995). Furthermore, it has been shown that astrocytes express both TrkA and p75 receptors for NGF (Hutton and Perez-Polo, 1995). Therefore, we cannot exclude the possibility that in the mixed neuronal/glial hippocampal cultures the response of neurons to NGF could be mediated by activated glial cells which synthesize and secrete NGF and might respond to this factor in an autocrine fashion.

In vivo, it has been previously demonstrated that selegiline ameliorated neuronal damage in the CA1 subfield of the hippocampus in a gerbil model of transient forebrain ischemia (Sivenius et al., 1994). The present study shows that this drug can protect cortical neurons against ischemic insult induced by permanent occlusion of the MCA. The neuroprotective activity was observed both in a rat and mouse model of focal cerebral ischemia after repetitive administration of selegiline. Our results are in agreement with previously reported neuroprotective activity of this drug against ischemic/hypoxic damage in the rat striatum and thalamus (Knollema et al., 1995). The mechanism by which selegiline diminishes neuronal damage after ischemic insult to the CNS is unclear. Interestingly, we found that repetitive systemical administration of selegiline leads to a sustained increase in NGF mRNA in the cerebral cortex of intact adult rats, suggesting that induced NGF synthesis in this brain region might underlie the observed neuroprotection. Additionally, we observed that a single i.p. injection of this drug was not sufficient to reduce the severity of neuronal injury in both animal models, suggesting that the neuroprotective activity of selegiline needs accumulation of NGF to appear. It has been shown that exogenous NGF protected rat hippocampus from ischemic injury (Yamamoto et al., 1992; Pechan et al., 1995). In view of the importance of NGF in protecting the cerebral cortex from ischemic insult it remains to be determined whether selegiline can induce NGF expression in ischemic rat brain and whether the rise of NGF mRNA is followed by the availability of NGF protein in this brain region. We suggest that selegiline enhanced NGF message in astrocytes since in vitro astrocytes respond to this drug with enhanced synthesis and release of NGF. Additionally, it has been shown that selegiline augmented the process of reactive astrogliosis after different kinds of damage to the CNS (Biagini et al., 1993, 1994; Ju et al., 1994). The increased reactive gliosis has been related to the increased neuronal survival after injury probably due to the enhanced supply of astroglially derived neurotrophic factors (Giulian et al., 1993). Therefore, we suggest that astrocyte-derived NGF after administration of selegiline could protect cortical neurons from ischemic insult. However, we can not exclude that expression of other trophic substances (bFGF and CNTF) and activation of antioxidant enzymes, such as superoxide dismutase and catalase (Carrillo et al., 1992), could also contribute to the neuroprotective activity of selegiline observed both in vitro and in vivo.

In conclusion, the present study indicates that selegiline induced NGF synthesis and release in cultured rat cortical astrocytes. In hippocampal cultures containing both neurons and glial cells this drug enhanced NGF protein concentration in the culture medium and protected hippocampal neurons from glutamate-induced excitotoxic damage. Selegiline induced NGF gene expression in cerebral cortex from intact rats and protected rat and mouse cortical tissue from ischemic damage due to permanent MCA occlusion. The present results show that the induction of NGF by selegiline causes neuroprotection and could explain at least in part the 'trophic-like' activities and the therapeutical benefits of this compound for the treatment of Alzheimer's disease.

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