ELSEVIER

European Journal of Pharmacology 306 (1996) 81–88

ejp

Marked diversity in the action of growth factors on *N*-methyl-D-aspartate-induced neuronal degeneration

Jochen H.M. Prehn *

Department of Pharmacology and Toxicology, FB 16, Philipps-University, Ketzerbach 63, D-35032 Marburg, Germany

Received 16 October 1995; revised 7 March 1996; accepted 12 March 1996

Abstract

Neuronal degeneration was induced in cultured rat hippocampal neurons by a 20-min exposure to the glutamatergic agonist, *N*-methyl-D-aspartate (NMDA; 100 μM), and the neuroprotective activity of a set of growth factors and cytokines was compared. During the early stages of degeneration, NMDA induced changes that were characteristic of neuronal necrosis, including swelling and darkening of the neuronal soma and swelling of neurites, leading to the formation of beaded varicosities ('blebs'). These changes were followed by nuclear pyknosis, formation of double-stranded DNA breaks and loss of membrane integrity. Only transforming growth factor-β1 (TGF-β1; 1–10 ng/ml) and tumor necrosis factor-α (TNF-α; 30 ng/ml) protected the hippocampal neurons against NMDA neurotoxicity after short-term (60 min) pre-treatments. Interleukin-1β (10–100 ng/ml) and fibroblast growth factor-2 (FGF-2; 50 ng/ml) were clearly effective when administered 24 h prior to the NMDA exposure, but not when given 60 min before the insult. Interestingly, the protective effect of interleukin-1β was significantly reduced in the presence of a neutralizing antibody to TGF-β. Of note, short-term pre-treatment with brain-derived neurotrophic factor (BDNF; 5–50 ng/ml) significantly potentiated NMDA-induced neurodegeneration. These experiments demonstrate marked diversity in the actions of growth factors on NMDA-induced neuronal degeneration.

Keywords: Growth factor; Cytokine; Neuroprotection; Excitotoxicity

1. Introduction

Neuronal growth factors are promising agents for the treatment of a variety of neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and stroke (Eide et al., 1993; Lindsay et al., 1993; Thoenen et al., 1993). Drugs that mimic their molecular action or that are able to modulate their expression and release represent important alternative therapeutic strategies (Carswell, 1993). Although there is an increasing number of peptide growth factors and cytokines that have been shown to exert some neurotrophic or neuroprotective activity, little is known as to which particular factor is the most efficacious agent in a given pathophysiological condition.

Inappropriate activation of glutamate receptors ('excitotoxicity') is believed to contribute to the neurodegeneration observed in stroke, trauma, epilepsy and Parkinson's disease (Choi, 1988). NMDA receptors in particular have been shown to be largely responsible for glutamate-induced cell death in many in vitro and in vivo systems (Rothman and Olney, 1987; Choi, 1988). This is due to the fact that these receptors are highly permeable to Ca^{2+} (MacDermott et al., 1986), and that overactivation can lead to toxic Ca^{2+} overloading (Hartley et al., 1993; Prehn et al., 1994). Neuroprotective effects of growth factors and cytokines against glutamate- and *N*-methyl-D-aspartate (NMDA)-induced neurotoxicity have been reported by numerous laboratories. Protective factors include fibroblast growth factors (FGFs) (Mattson et al., 1989; Fernandez-Sanchez and Novelli, 1993; Louis et al., 1993; Nozaki et al., 1993), brain-derived neurotrophic factor (BDNF) and related neurotrophins (Cheng and Mattson, 1994; Shimohama et al., 1993; Lindholm et al., 1993; but see Koh et al., 1995), epidermal growth factor (EGF) (Pauwels et al., 1989), transforming growth factor-βs (TGF-βs) (Prehn et al., 1993a, 1994), tumor necrosis factors (TNFs) (Cheng and Mattson, 1994) and interleukin-1β (Strijbos and Rothwell, 1995). In the present study I directly compared the effects of these factors on NMDA neurotoxicity in cultured rat hippocampal neurons and found a marked diversity in the neuroprotective activity of these factors.

* Tel.: (49) (6421) 28 13 11; fax: (49) (6421) 28 89 18.

PII S0014-2999(96)00225-7

2. Materials and methods

2.1. Cell culture

Primary hippocampal neurons were isolated from newborn (P1) Fischer 344 rats, dissociated and plated onto poly-L-lysine-coated glass coverslips as described previously (Prehn et al., 1993b). Cells were cultured in minimum essential Eagle's medium (MEM with Earle's salts; Gibco, Paisley, UK) supplemented with 10% (v/v) NU serum (Gibco), L-glutamine, (2 mM), glucose (5 g/l) and sodium bicarbonate (2.2 g/l). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 2–4 days in culture, the cells were treated with 10 μM cytosin β-D-arabinofuranoside (Sigma, Deisenhofen, Germany) in order to inhibit the proliferation of glia cells. After one week, cells were switched to serum-free MEM with N2.1 supplements (Gibco). The cells were used between 10 and 12 days in vitro.

2.2. Induction of neuronal injury

Excitotoxic neuronal injury was induced in cultured rat hippocampal neurons by a 20-min exposure to the excitatory amino acid, NMDA. For this purpose, cultures were washed twice in HEPES-buffered saline (HBS; 146 mM NaCl, 10 mM Hepes, 2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose; pH 7.4) and were then switched to Mg^{2+}-free HBS supplemented with 100 μM NMDA (Sigma) and 100 nM glycine. Exposure to NMDA was carried out at room temperature. Afterwards, the cultures were rinsed twice with HBS and returned to the serum-free MEM/N2.1 culture medium. Control cultures were exposed to Mg^{2+}-free HBS devoid of NMDA.

2.3. Assessment of neuronal viability

Neuronal viability was assessed 24 h after exposure to NMDA. For this purpose, the cells were washed once with HBS and subsequently incubated with Trypan blue (0.4% in HBS) for a period of 5 min. Afterwards, the cells were fixed in phosphate-buffered formalin (4%) at 37°C for 15 min. A total of 300–400 neurons were counted per coverslip by means of light microscopy. Only dark-stained neurons were considered damaged. Cell counts were performed in a blinded fashion. For comparison, cell viability is given as a percentage of the controls. Cell viability of control cultures ranged from 79 to 92%.

2.4. Terminal deoxynucleotidyl transferase-based dUTP-biotin nick-end labelling (TUNEL) technique

Cultures were fixed in phosphate-buffered formalin (4%) at 37°C for 15 min. Double-stranded DNA breaks were visualized using terminal deoxynucleotidyl transferase-based labelling of 3′-OH-DNA nick ends with digoxigenin-11-dUTP and immunocytochemical detection according to the manufacturer's instructions (Apoptag-Peroxidase Kit, Oncor, Gaithersburgh, MD, USA). 3,3′-Diaminobenzidine and hydrogen peroxide were used as the chromogenic system.

2.5. Treatments

Recombinant human EGF, TGF-β1 and TNF-α and recombinant murine interleukin-1β were purchased from R&D Systems (Minneapolis, MN, USA), recombinant human FGF-2 was from Sigma, and recombinant human BDNF came from Promega (Madison, WI, USA). Factors were prepared as 100 or 1000× stock solutions in phosphate-buffered saline (pH 7.4) supplemented with 0.1% ovalbumin (Sigma).

Pan-specific neutralizing rabbit antiserum to TGF-β was obtained from R&D Systems (A-100-AB, Lot E003). The antiserum was used in a concentration of 25 μg/ml, which is five times the half-maximal neutralization dose (ND_{50}) of this antibody required to inhibit the maximal biological activity of TGF-β1 in a growth inhibition assay (inhibition of interleukin-4-dependent [^{3}H]thymidine incorporation by HT-2 cells; manufacturer's information). Mouse monoclonal antibody to the β-subunit of NGF (2.5S- and 7S-form) was purchased from Boehringer Mannheim (Germany; clone 27/21). The antibody was used in a concentration of 0.5 μg/ml, which is approximately 10 times the concentration required to inhibit 50 ng/ml of mouse-NGF (7S-form; manufacturer's information). Mouse monoclonal antibody to rat ciliary neurotrophic factor (CNTF) was also from Boehringer Mannheim (clone 5/3/6B) and was used in a concentration of 5 μg/ml.

Two different treatment schedules were carried out, a long-term and a short-term pre-treatment. For long-term pre-treatments, cultures received the factors 24 h prior to the exposure. For short-term pre-treatments, factors were added to the cultures 60 min before the exposure. Treatments were continued after the NMDA exposure. Controls were treated with an equivalent volume of the vehicle. I did not investigate longer periods of pre-treatment, since such treatments may significantly change the morphological and biochemical phenotype of neurons (for example, see Ventimiglia et al., 1995).

2.6. Statistics

All data are given as means ± S.E.M. For statistical comparison, one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test was employed. P values smaller than 0.05 were considered to be statistically significant.

3. Results

3.1. Characterization of NMDA neurotoxicity in cultured rat hippocampal neurons

A 20-min exposure of cultured rat hippocampal neurons to NMDA (100 μM) in Mg^{2+}-free, Hepes-buffered saline decreased neuronal viability by approximately 50%. In contrast, the cell viability of cultures exposed to saline devoid of NMDA was comparable to that of cultures that remained in the original culture medium (data not shown).

During the NMDA exposure, cells exhibited a darkened, granulated soma which frequently was moderately swollen. These somatic changes were accompanied by swelling of neurites, leading to the formation of beaded varicosities ('blebs'; Fig. 1). Thus, NMDA induced changes characteristic for neuronal necrosis during the early stages of degeneration (Wyllie et al., 1980). While swelling of the cell body recovered soon after removal of NMDA, both the granulated appearance of the soma and swelling of neurites persisted hours after the NMDA exposure. Subsequently, nuclear pyknosis and formation of double-stranded DNA breaks visualized with the TUNEL technique became evident (Fig. 1). Neurons degenerated with shrinkage of the neuronal soma and loss of membrane integrity, as indicated by positive Trypan blue staining.

3.2. Interleukin-1β and FGF-2 inhibited NMDA neurotoxicity after long-term, but not after short-term pre-treatments

A 24-h pre-treatment with interleukin-1β (10–100 ng/ml) significantly protected the hippocampal neurons against NMDA- induced degeneration (Fig. 2A, Table 1). In contrast, interleukin-1β (1–100 ng/ml) was ineffective if the treatment was started 60 min before the NMDA exposure (Fig. 2A, Table 1).

Similar effects were observed in FGF-2-pre-treated cultures. FGF-2 (50 ng/ml) clearly protected the hippocampal neurons after a 24-h pre-treatment, but did not show a protective effect in the case of the 60-min pre-treatment schedule (Fig. 3).

3.3. TGF-β1 and TNF-α inhibited NMDA neurotoxicity after both short-term and long-term pre-treatments

In contrast to interleukin-1β and FGF-2, TGF-β1 (10 ng/ml) was equally effective when given 60 min or 24 h prior to the NMDA exposure (Fig. 4). Interestingly, protective effects of TGF-β1 after a 60-min pre-treatment were even observed with a concentration as low as 1 ng/ml (Table 2).

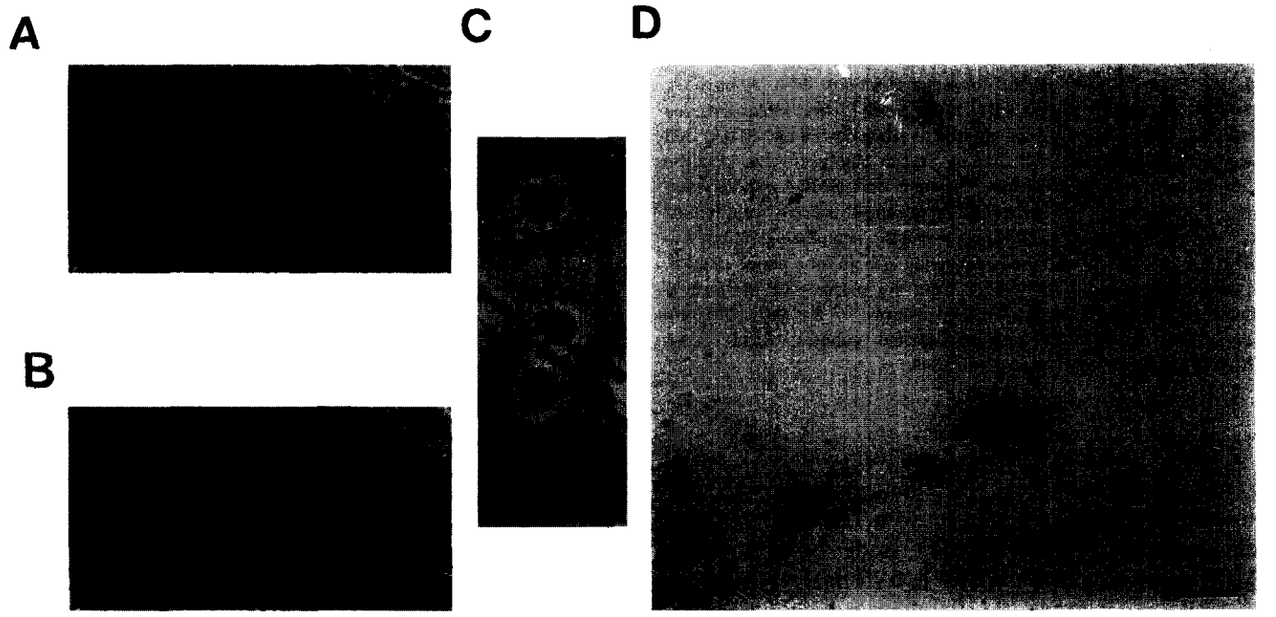

Fig. 1. Characterization of NMDA neurotoxicity on cultured rat hippocampal neurons. After 10 days in vitro, cultured rat hippocampal neurons were exposed to 100 μM NMDA in Mg^{2+}-free Hepes-buffered saline for a period of 20 min. A,B: Phase-contrast micrographs of hippocampal neurons before (A) and 4 h after (B) the NMDA exposure. Note the formation of pyknotic nuclei (arrows) and granulated neuronal somata in B. Bar = 20 μm. C: Higher magnification of changes in neurites 4 h after the NMDA exposure. Note the selective swelling of neurites, leading to the formation of beaded varicosities ('blebs'). Bar = 10 μm. D: NMDA-induced neuronal death is accompanied by the appearance of double-stranded DNA breaks. Twelve hours after the NMDA exposure, cells were stained for DNA fragmentation using the TUNEL technique in conjunction with diaminobenzidine staining (see Materials and methods). Note intense staining indicative of DNA fragmentation in degenerating cells. Bar = 40 μm.

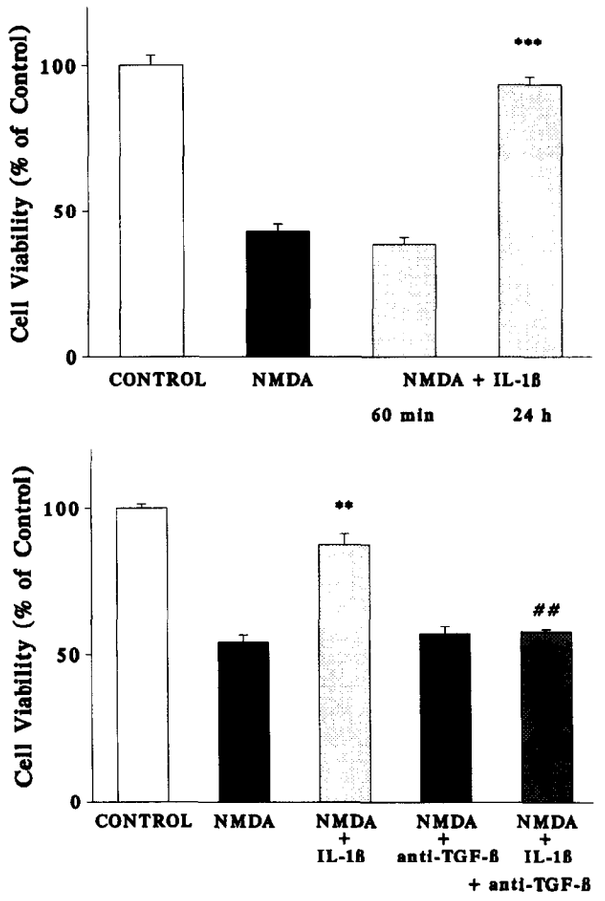

Fig. 2. Long-term pre-treatment with interleukin-1β protected cultured rat hippocampal neurons against NMDA-induced excitotoxic injury: involvement of TGF-β. A: Cultured rat hippocampal neurons were treated with interleukin-1β (100 ng/ml) for either 60 min or 24 h. Afterwards, cells were exposed to NMDA (100 μM) in Mg^{2+}-free Hepes-buffered saline for 20 min. Controls were exposed to saline devoid of NMDA. Cell viability was determined 24 h later by Trypan blue exclusion. Data are means $\pm$ S.E.M. from 4–5 cultures. Different from NMDA-exposed controls: *** $P < 0.001$. B: Cultured rat hippocampal neurons were treated with interleukin-1β (100 ng/ml), a pan-specific neutralizing antibody to TGF-β (25 μg/ml) or with the combination of both for a period of 24 h, and were then exposed to NMDA. Data are means $\pm$ S.E.M. from 4 cultures. Different from NMDA-exposed controls: ** $P < 0.01$. Different from NMDA-exposed, interleukin-1β-treated cultures: ## $P < 0.01$ (ANOVA and Student-Newman-Keuls test).

Similarly, while a 24-h pre-treatment with TNF-α (30 ng/ml) provided complete protection against NMDA neurotoxicity, a 60-min pre-treatment with TNF-α (30 ng/ml) still increased neuronal viability from 49.5 $\pm$ 2.4% in NMDA-exposed controls to 85.5 $\pm$ 0.7% in TNF-α-treated cultures ($n = 4$ cultures; $P < 0.001$).

3.4. Protective effect of interleukin-1β mediated by TGF-β1

Interleukin-1β has previously been shown to increase the expression of TGF-β1 in cultures of rat cortical astrocytes (Da Cunha et al., 1993). To determine whether the neuroprotective effect of interleukin-1β against NMDA toxicity could be related to stimulation of expression of a factor, such as TGF-β1, I treated cultured rat hippocampal neurons for 24 h with both interleukin-1β (100 ng/ml) and a pan-specific neutralizing antiserum to TGF-β (25 μg/ml). While treatment with this antibody itself did not influence NMDA neurotoxicity, it significantly inhibited the neuroprotective activity of interleukin-1β (Fig. 2B). A

Table 1
Dose-response relationship for the protective effect of interleukin-1β on NMDA-induced neuronal degeneration. Hippocampal cultures were treated with interleukin-1β (1–100 ng/ml) for periods of either 24 h or 60 min. Afterwards, cells were exposed to 100 μM NMDA in Mg^{2+}-free Hepes-buffered saline for 20 min. Controls were exposed to saline devoid of NMDA. Cell viability was determined 24 later by Trypan blue exclusion. Data are means $\pm$ S.E.M. from n cultures

Treatment	Interleukin-1β (ng/ml)	Cell viability (% of control)	n
Experiment 1: 24 h pre-treatment			
Control	0	100.0 $\pm$ 2.4	4
	10	99.0 $\pm$ 0.6	3
	100	98.3 $\pm$ 2.5	3
NMDA	0	59.0 $\pm$ 1.3	5
	1	66.4 $\pm$ 6.1	4
	10	86.0 $\pm$ 4.6 [a]	4
	100	89.4 $\pm$ 2.8 [b]	4
Experiment 2: 60-min pre-treatment			
Control	0	100.0 $\pm$ 2.4	4
NMDA	0	60.4 $\pm$ 2.0	4
	1	60.1 $\pm$ 2.9	4
	10	67.1 $\pm$ 4.9	4
	100	62.5 $\pm$ 2.7	4

[a] Different from respective NMDA-exposed controls: $P < 0.01$. [b] $P < 0.001$ (ANOVA and Student-Newman-Keuls test).

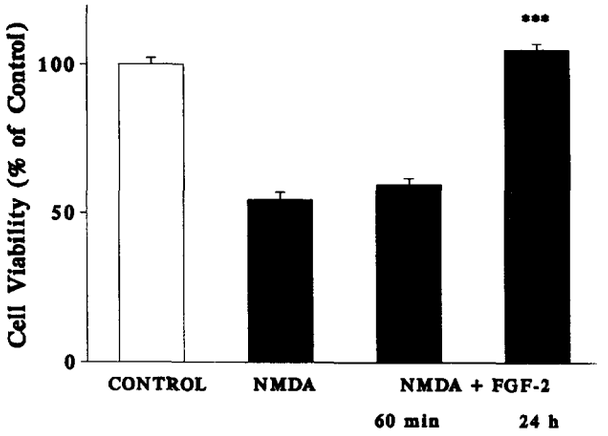

Fig. 3. Long-term pre-treatment with FGF-2 protected cultured rat hippocampal neurons against NMDA-induced degeneration. Cultured rat hippocampal neurons were treated with FGF-2 (50 ng/ml) for periods of either 60 min or 24 h. Afterwards, cells were exposed to 100 μM NMDA and stained with Trypan blue as described. Data are means $\pm$ S.E.M. from 4 cultures. Different from NMDA-exposed controls: *** $P < 0.001$ (ANOVA and Student-Newman-Keuls test). Experiment was performed in duplicate with similar results.

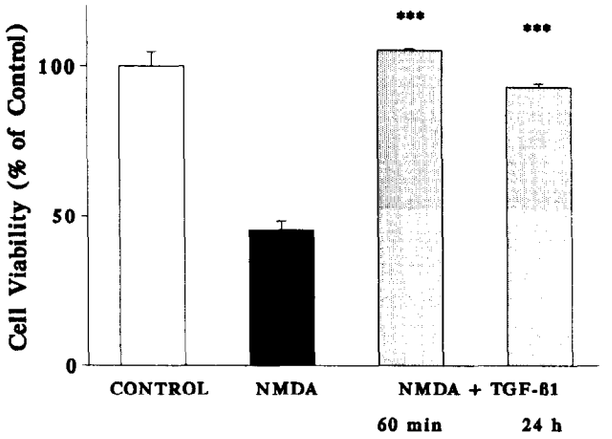

Fig. 4. Both short- and long-term pre-treatments with TGF-β1 blocked NMDA neurotoxicity in cultured rat hippocampal neurons. Cultured rat hippocampal neurons were treated with TGF-β1 (10 ng/ml) for either 60 min or 24 h and were then exposed to NMDA (100 μM) for 20 min (see legend of Fig. 1). Cell viability was determined 24 h afterwards by Trypan blue exclusion. Data are means ± S.E.M. from 4–5 cultures. Different from NMDA-exposed controls: *** $P < 0.001$ (ANOVA and Student-Newman-Keuls test). Experiment was performed in triplicate with similar results.

combined 24-h treatment with interleukin-1β (100 ng/ml) and a neutralizing monoclonal antibody to NGF (0.5 μg/ml) also significantly inhibited the neuroprotective activity of interleukin-1β, albeit to a lesser extent (Table 3, see also Strijbos and Rothwell, 1995). In contrast, co-administration of interleukin-1β (100 ng/ml) and a neutralizing antibody to CNTF (5 μg/ml) did not influence the protective action of interleukin-1β (Table 3).

3.5. BDNF potentiated NMDA neurotoxicity

Both prolonged and short-term pre-treatments with the neurotrophin BDNF failed to reduce the degree of NMDA-induced excitotoxic injury over a wide range of concentrations (0.5–50 ng/ml; Table 4). I observed a significant potentiation of NMDA neurotoxicity when the factor was applied 60 min before the exposure (Fig. 5).

Treatments with EGF (50 ng/ml) did not protect against NMDA-induced neuronal degeneration of the hippocampal cultures (data not shown).

Table 2
Dose-response relationship for the protective effect of a short-term TGF-β1 pre-treatment on NMDA-induced neuronal degeneration. Cultured rat hippocampal neurons were pre-treated for 60 min with TGF-β1 (0.3–10 ng/ml) and were then exposed to 100 μM NMDA as described in the legend of Table 1. Cell viability was determined 24 h later by Trypan blue exclusion. Data are means ± S.E.M. from n cultures

Treatment	TGF-β1 (ng/ml)	Cell viability (% of control)	n
Control	0	100.0 ± 4.1	4
	3	94.9 ± 3.6	3
	10	105.3 ± 5.7	3
NMDA	0	54.3 ± 1.9	4
	0.3	59.5 ± 4.5	4
	1	68.6 ± 2.2 [a]	4
	3	81.9 ± 1.7 [b]	4
	10	96.5 ± 1.4 [b]	4

[a] Different from NMDA-exposed controls: $P < 0.05$; [b] $P < 0.001$ (ANOVA and Student-Newman-Keuls test).

Table 3
Effect of neutralizing antibodies to NGF and CNTF on the neuroprotective activity of interleukin-1β. Cultured rat hippocampal neurons were pre-treated for 24 h with interleukin-1β (100 ng/ml) alone or in combination with a neutralizing mouse monoclonal antibody to NGF (0.5 μg/ml) or a neutralizing mouse monoclonal antibody to CNTF (5 μg/ml). Afterwards, cultures were exposed to 100 μM NMDA and stained with Trypan blue as described in the legend of Table 1. The antibodies themselves had no effect on NMDA neurotoxicity (not shown). Data are means ± S.E.M. from n cultures

Treatment	Cell viability (% of control)	n
Control	100.0 ± 1.3	4
NMDA	61.3 ± 1.1	4
NMDA + interleukin-1β	89.0 ± 1.6 [a]	4
NMDA + interleukin-1β + anti-NGF	78.7 ± 2.5 [b]	4
NMDA + interleukin-1β + anti-CNTF	85.8 ± 1.3	4

[a] Different from NMDA-exposed controls: $P < 0.001$. [b] Different from interleukin-1β-treated cultures: $P < 0.05$. ANOVA and Student-Newman-Keuls test were employed for statistical comparisons.

4. Discussion

NMDA-induced degeneration of cultured rat hippocampal neurons produced morphological changes characteristic

Table 4
Dose-response relationship for the effect of short- and long-term pre-treatment with BDNF on NMDA neurotoxicity of hippocampal neurons. Cultured rat hippocampal neurons were treated with BDNF (0.5–50 ng/ml) for periods of either 24 h or 60 min. Afterwards, cells were exposed to NMDA (100 μM) for 20 min and stained for the evaluation of neuronal viability as described in the legend of Table 1. Data are means ± S.E.M. from n cultures

Treatment	BDNF (ng/ml)	Cell viability (% of control)	n
Experiment 1: 24-h pre-treatment			
Control	0	100.0 ± 1.6	4
	5	103.0 ± 1.2	3
	50	100.6 ± 2.2	3
NMDA	0	64.3 ± 0.8	4
	0.5	67.2 ± 1.3	4
	5	62.5 ± 4.1	4
	50	60.5 ± 2.7	4
Experiment 2: 60 min pre-treatment			
Control	0	100.0 ± 1.1	4
NMDA	0	66.3 ± 1.5	5
	0.5	66.0 ± 1.4	4
	5	58.9 ± 0.8 [a]	4
	50	44.6 ± 2.3 [b]	4

[a] Different from respective NMDA-exposed controls: $P < 0.05$. [b] $P < 0.001$ (ANOVA and Student-Newman-Keuls test).

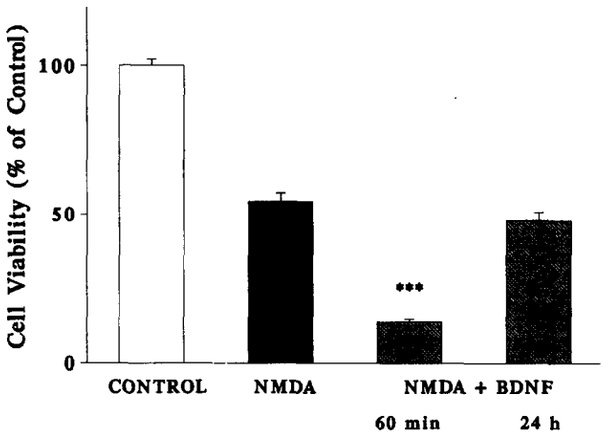

Fig. 5. BDNF potentiated NMDA-induced neuronal degeneration. Cultured rat hippocampal neurons were treated with BDNF (50 ng/ml) for either 60 min or 24 h. Afterwards, cells were exposed to NMDA (100 μM) and stained with Trypan blue as described in the legend of Fig. 1. Data are means + S.E.M. from 4 cultures. Different from NMDA-exposed controls: *** $P < 0.001$ (ANOVA and Student-Newman-Keuls test). Experiment was performed in duplicate with similar results.

of neuronal necrosis (Wyllie et al., 1980; Fig. 1). These changes included swelling and darkening of the neuronal soma upon exposure to NMDA, swellings on neurites ('blebbing') and an early loss of membrane integrity. On the other hand, certain morphological and biochemical changes characteristic of neuronal apoptosis became evident subsequent to the NMDA exposure. These latter changes included cell shrinkage, nuclear pyknosis and the formation of double-stranded DNA breaks, a hallmark of apoptosis (Gavrieli et al., 1992). Thus, NMDA neurotoxicity appeared to involve both necrotic and apoptotic processes. In agreement with previous reports, the present study demonstrated that neuronal growth factors and cytokines have the capacity to protect central neurons against this NMDA-induced excitotoxic injury. I now provide evidence that there are, nevertheless, marked differences in the anti-excitotoxic action of these factors.

Among the different factors tested, only TGF-β1 and TNF-α reduced NMDA-induced excitotoxic injury when administered briefly before the excitotoxic insult (e.g. Fig. 4). Interestingly, both factors are minimally expressed in the unlesioned nervous system, but are strongly induced in response to lesions, such as cerebral ischemia, infections, trauma and Alzheimer's disease (Hopkins and Rothwell, 1995). My data suggest that these injury-related factors might have the biological function of 'fast-acting' neuroprotectants. Both factors have been shown to stabilize neuronal Ca^{2+} homeostasis after an excitotoxic insult (Cheng et al., 1994; Prehn et al., 1994), an effect which may significantly contribute to their neuroprotective activity. Moreover, both factors may influence pathophysiological events occurring downstream of NMDA-induced Ca^{2+} overloading, such as NMDA-induced oxidative stress (Bondy and LeBel, 1993). For example, TNFs are known to rapidly induce the anti-oxidative mitochondrial enzyme, manganese superoxide dismutase (Wong and Goeddel, 1988), and TGF-β1 also has anti-oxidative activity (Prehn et al., 1994). Moreover, both TNFs and TGF-βs induce the expression of the cytoprotective, 'anti-apoptotic' Bcl-2 oncoprotein (Prehn et al., 1994; Genestier et al., 1995). It should be mentioned that human TNF-α, used in the present study only acts on one of the two known rodent TNF receptors (Smith et al., 1994).

Both interleukin-1β and FGF-2 protected the hippocampal neurons against NMDA-induced degeneration, but only after a longer period of pre-treatment (Fig. 2A Fig. 3; see also Mattson et al., 1989; Fernandez-Sanchez and Novelli, 1993; Louis et al., 1993; Strijbos and Rothwell, 1995). The neuroprotective effect of FGF-2 has been suggested to be related to the down-regulation of a functional 71 kDa protein associated with the NMDA receptor (Mattson et al., 1993), but other effects, such as enhanced synthesis and secretion of growth factors or inhibition of nitric oxide toxicity, may also be involved (Yoshida and Gage, 1991; Maiese et al., 1993).

Interleukin-1β has recently been demonstrated to protect neurons against NMDA-induced injury by increasing the synthesis or release of nerve growth factor (NGF) (Strijbos and Rothwell, 1995). Indeed, interleukin-1β is the strongest inducer of NGF described so far (Lindholm et al., 1987). In the present study, the neuroprotective activity of interleukin-1β could only be blocked partially by a neutralizing antibody to NGF (Table 3). The high-affinity receptor for NGF (*trk*A) appears not to be expressed in hippocampal cultures (Ip et al., 1993; Marsh et al., 1993). Several groups have also reported the absence of the low-affinity receptor for NGF ($gp75^{LNGFR}$) in hippocampal neuronal cultures (Marsh et al., 1993). I thus investigated the possibility that the neuroprotective effect of interleukin-1β could also be mediated by other factors. Previous reports have demonstrated that treatment of cultured cortical astrocytes with interleukin-1β strongly induced TGF-β1 expression (Da Cunha et al., 1993). My data show that this effect may well be associated with interleukin-1β's neuroprotective activity, since I could significantly inhibit its neuroprotective effect by co-administration of a neutralizing antibody to TGF-β (Fig. 2B).

In contrast to the above-mentioned factors, BDNF did not rescue the hippocampal neurons from NMDA-induced neurodegeneration (Fig. 5). It is well known that BDNF and related neurotrophins regulate developmental aspects of neurons, but evidence is growing that these factors also play an important role in the stabilization of synaptic transmission (Patterson and Nawa, 1993). BDNF in particular has been shown to be strongly induced after electrical or pharmacological stimulation of central neurons (Zafra et al., 1990; Patterson et al., 1992), to rapidly increase neurotransmitter release (Lohof et al., 1993) and to enhance excitatory synaptic transmission (Kang and Schuman, 1994). Accordingly, BDNF could also potentiate, rather

than ameliorate, neuronal death caused by intense activation of glutamate receptors.

Indeed, in the present study, BDNF significantly potentiated NMDA toxicity if applied 60 min before the NMDA exposure (e.g. Fig. 5). Similar potentiating effects of BDNF on excitotoxic neuronal injury have recently been observed by Fernandez-Sanchez and Novelli (1993), as well as by Koh and co-workers (1995). Other groups, on the contrary, observed a protective effect of a BDNF pre-treatment against excitotoxic neuronal injury (Lindholm et al., 1993; Shimohama et al., 1993), suggesting that BDNF may also induce some protective activity against this type of cell death. Interestingly, I did not observe the potentiating effect of BDNF if the factor was administered 24 h prior to the NMDA exposure. It is therefore reasonable to assume that BDNF induced some protective mechanisms during the long-term pre-treatment that counteracted the potentiating effects of BDNF observed with the short-term pre-treatment schedule. Clearly, with respect to ongoing clinical trials, the role of BDNF in excitotoxic neuronal degeneration requires further careful investigation.

Acknowledgements

The author is grateful to Elke Preis for technical assistance and Professors Josef Krieglstein and Richard J. Miller for their support. These experiments were supported by grants from the Deutsche Forschungsgemeinschaft (Pr 338/2-1 and Forschergruppe 'Neuroprotektion').

References

Bondy, S.C. and C.P. LeBel, 1993, The relationship between excitotoxicity and oxidative stress in the central nervous system, Free Rad. Biol. Med. 14, 633.

Carswell, S., 1993, The potential for treating neurodegenerative disorders with NGF-inducing compounds, Exp. Neurol. 124, 36.

Cheng, B. and M.P. Mattson, 1994, NT-3 and BDNF protect CNS neurons against metabolic/excitotoxic insults, Brain Res. 640, 56.

Cheng, B., S. Christakos and M.P. Mattson, 1994, Tumor necrosis factors protect neurons against metabolic excitotoxic insults and promote maintenance of calcium homeostasis, Neuron 12, 139.

Choi, D.W., 1988, Glutamate neurotoxicity and diseases of the nervous system, Neuron 1, 623.

Da Cunha, A., J.A. Jefferson, R.W. Jackson and L. Vitkovic, 1993, Glial cell-specific mechanisms of TGF-β1 induction by IL-1 in cerebral cortex, J. Neuroimmunol. 42, 71.

Eide, F.F., D.H. Lowenstein and L.F. Reichardt, 1993, Neurotrophins and their receptors – current concepts and implications for neurological diseases, Exp. Neurol. 121, 200.

Fernandez-Sanchez, M.T. and A. Novelli, 1993, Basic fibroblast growth factor protects cerebellar neurons in primary culture from NMDA and non-NMDA receptor mediated neurotoxicity, FEBS Lett. 335, 124.

Gavrieli, Y., Y. Sherman and S.A. Ben-Sasson, 1992, Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation, J. Cell Biol. 119, 493.

Genestier, L., N. Bonnefoy-Berard, J.-P. Rouault, M. Flacher and J.-P. Revillard, 1995, Tumor necrosis factor-α up-regulates Bcl-2 expression and decreases calcium-dependent apoptosis in human B cell lines, Int. Immunol. 7, 533.

Hartley, D.M., M.C. Kurth, L. Bjerkness, J.H. Weiss and D.W. Choi, 1993, Glutamate receptor-induced Ca^{2+} accumulation in cortical cell culture correlates with subsequent neuronal degeneration, J. Neurosci. 13, 1993.

Hopkins, S.J. and N.J. Rothwell, 1995, Cytokines and the nervous system I: expression and recognition, Trends Neurosci. 18, 83.

Ip, N.Y., Y. Li, G.D. Yancopoulos and R.M. Lindsay, 1993, Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF, J. Neurosci. 13, 3394.

Kang, H. and E.M. Schuman, 1994, BDNF and NT-3 enhance excitatory synaptic transmission in area CA1 of the rat hippocampus, Soc. Neurosci. Abstr. 20, 264.

Koh, J.-Y., B.J. Gwag, D. Lobner and D.W. Choi, 1995, Potentiated necrosis of cultured cortical neurons by neurotrophins, Science 268, 573.

Lindholm, D., R. Heumann, B. Hengerer and H. Thoenen, 1987, Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve, Nature 330, 658.

Lindholm, D., G. Dechant, C.-P. Heisenberg and H. Thoenen, 1993, Brain-derived neurotrophic factor is a survival factor for cultured rat cerebellar granule neurons and protects them against glutamate-induced neurotoxicity, Eur. J. Neurosci. 5, 1455.

Lindsay, R.M., C.A. Altar, J.M. Cedarbaum, C. Hyman and S.J. Wiegand, 1993, The therapeutic potential of neurotrophic factors in the treatment of Parkinson's disease, Exp. Neurol. 124, 103.

Lohof, A.M., N.Y. Ip and M.-m. Poo, 1993, Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF, Science 363, 350.

Louis, J.-C., E. Magal, W. Gerdes and W. Seifert, 1993, Survival-promoting and protein kinase C-regulating roles of basic FGF for hippocampal neurons exposed to phorbol ester, glutamate and ischemia-like conditions, Eur. J. Neurosci. 5, 1610.

MacDermott, A.B., M.L. Mayer, G.L. Westbrook, S.J. Smith and J.L. Barker, 1986, NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal neurones, Nature 321, 519.

Maiese, K., I. Boniece, D. DeMeo and J.A. Wagner, 1993, Peptide growth factors protect against ischemia in culture by preventing nitric oxide toxicity, J. Neurosci. 13, 3034.

Marsh, H.N., W.K. Scholz, F. Lamballe, R. Klein, V. Nanduri, M. Barbacid and H.C. Palfrey, 1993, Signal transduction events mediated by the BDNF receptor $gp145^{trkB}$ in primary hippocampal pyramidal cell culture, J. Neurosci. 13, 4281.

Mattson, M.P., M. Murrain, P.B. Guthrie and S.B. Kater, 1989, Fibroblast growth factor and glutamate: opposing roles in the generation and degeneration of hippocampal neuroarchitecture, J. Neurosci. 9, 3728.

Mattson, M.P., K.N. Kumar, H. Wang, B. Cheng and E.K. Michaelis, 1993, Basic FGF regulates the expression of a functional 71 kDa NMDA receptor protein that mediates calcium influx and neurotoxicity in hippocampal neurons, J. Neurosci. 13, 4575.

Nozaki, K., S.P. Finklestein and M.F. Beal, 1993, Basic fibroblast growth factor protects against hypoxia-ischemia and NMDA neurotoxicity in neonatal rats, J. Cereb. Blood Flow Metab. 13, 221.

Patterson, P.H. and H. Nawa, 1993, Neuronal differentiation factors/cytokines and synaptic plasticity, Cell 72, 123.

Patterson, S.L., L.M. Grover, P.A. Schwartzkroin and M. Bothwell, 1992, Neurotrophin expression in rat hippocampal slices: a stimulus paradigm inducing LTP in CA1 evokes increases in BDNF and NT-3 mRNAs, Neuron 9, 1081.

Pauwels, P.J., H.P. van Assouw and J.E. Leysen, 1989, Attenuation of neurotoxicity following anoxia or glutamate receptor activation in EGF- and hippocampal extract-treated neuronal cultures, Cell. Signal. 1, 45.

Prehn, J.H.M., C. Backhauss and J. Krieglstein, 1993a, Transforming growth factor-β1 prevents glutamate neurotoxicity in rat neocortical

cultures and protects mouse neocortex from ischemic injury, J. Cereb. Blood Flow Metab. 13, 521.

Prehn, J.H.M., M. Welsch, C. Backhauss, J. Nuglisch, F. Ausmeier, C. Karkoutly and J. Krieglstein, 1993b, Effects of serotonergic drugs in experimental brain ischemia: evidence for a protective role of serotonin in cerebral ischemia, Brain Res. 630, 10.

Prehn, J.H.M., V.P. Bindokas, C.J. Marcuccilli, S. Krajewski, J.C. Reed and R.J. Miller, 1994, Regulation of neuronal Bcl2 protein expression and calcium homeostasis by transforming growth factor type β confers wide-ranging protection on rat hippocampal neurons, Proc. Natl. Acad. Sci. USA 91, 12599.

Rothman, S.M. and J.W. Olney, 1987, Excitotoxicity and the NMDA receptor, Trends Neurosci. 10, 299.

Shimohama, S., Y. Tamura, A. Akaike, T. Tsukahara, O. Ohara, S. Watanabe and J. Kimura, 1993, Brain-derived neurotrophic factor pretreatment exerts a partially protective effect against glutamate-induced neurotoxicity in cultured rat cortical neurons, Neurosci. Lett. 164, 55.

Smith, C.A., T. Farrah and R.G. Goodwin, 1994, The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death, Cell 76, 959.

Strijbos, P.J.L.M. and N.J. Rothwell, 1995, Interleukin-1β attenuates excitatory amino acid-induced neurodegeneration in vitro: involvement of nerve growth factor, J. Neurosci. 15, 3468.

Thoenen, H., R.A. Hughes and M. Sendtner, 1993, Trophic support of motoneurons: physiological, pathophysiological, and therapeutic implications, Exp. Neurol. 124, 47.

Ventimiglia, R., P.E. Mather, B.E. Jones and R.M. Lindsay, 1995, The neurotrophins BDNF, NT-3 and NT-4/5 promote survival and morphological and biochemical differentiation of striatal neurons in vitro, Eur. J. Neurosci. 7, 213.

Wong, G.H.W. and D.V. Goeddel, 1988, Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism, Science 242, 941.

Wyllie, A.H., J.F.F.R. Kerr and A.R. Currie, 1980, Cell death: the significance of apoptosis, Int. Rev. Cytol. 68, 251.

Yoshida, K. and F.H. Gage, 1991, Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes, Brain Res. 538, 118.

Zafra, F., B. Hengerer, J. Leibrock, H. Thoenen and D. Lindholm, 1990, Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors, EMBO J. 9, 3545.