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Nerve growth factor potentiates the neurotoxicity of β amyloid

(Alzheimer disease/neurotrophic factors/nerve growth factor receptor/hippocampal neurons)

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ABSTRACT **The role of growth factors in the pathogenesis of Alzheimer disease is unknown. The β-amyloid protein accumulates abnormally in the brain in Alzheimer disease and is neurotoxic to differentiated hippocampal neurons in culture. Nerve growth factor (NGF) increased the neurotoxic potency of a β-amyloid polypeptide by a factor of ≈100,000, which resulted in a reduction of the β-amyloid neurotoxic EC_{50} from 0.1 μM to 1 pM. This potentiating effect of NGF was reversed by a monoclonal antibody against NGF and was not observed for a variety of other neurotrophic growth factors. Exposure of hippocampal neurons to very low concentrations of β amyloid alone resulted in a marked induction of immunoreactive NGF receptors. Addition of NGF with β amyloid resulted in the appearance of neurodegenerative changes in NGF receptor-positive neurons. The early and profound degeneration of hippocampal and basal forebrain cholinergic neurons that occurs in Alzheimer disease may result from a neurotoxic interaction of β amyloid with NGF.**

A physiologic role for nerve growth factor (NGF) in the function of central nervous system cholinergic neurons has been suggested by the localization of NGF receptors in basal forebrain cholinergic neurons and by the synthesis of NGF in cholinergic target cells of the neocortex and hippocampus (1). Transection of the fimbria–fornix, a primary projection tract from basal forebrain to hippocampus, results in the degeneration of basal forebrain cholinergic neurons (2, 3). The ability of exogenous NGF to rescue degenerating cholinergic neurons has led to the suggestion that these neurons normally depend on NGF for survival (4–7). This idea is supported by the finding that NGF treatment improves age-related memory impairments in rats, with a concomitant increase in the number of basal forebrain cholinergic neurons (8). On the basis of these results, it has been suggested that NGF may also reverse the cholinergic neuronal degeneration characteristic of Alzheimer disease (AD).

Intraventricular administration of NGF in hamsters has been demonstrated to increase synthesis of the amyloid precursor protein in basal forebrain cholinergic neurons (9). The significance of this finding in the context of the pathogenesis of AD is unclear. The β-amyloid protein is the primary constituent of senile plaques and cerebrovascular deposits in AD and aged Down syndrome (10–14). Accumulation of β amyloid is one of the earliest detectable pathologic changes in the brains of patients with Down syndrome destined to develop Alzheimer-type dementia (15). In AD, the extent of cortical amyloid plaque development correlates with disease severity (16). These findings, although suggestive of an association of β amyloid with the development of AD, do not address the issue of whether β amyloid plays a causal role in the neurodegenerative process. Studies on the biological activity of β amyloid in rat PC-12 cells and primary hippocampal cultures have demonstrated both trophic and toxic effects (17–19). We have recently shown that β amyloid is trophic only for immature undifferentiated neurons and is toxic to older differentiated neurons in rat hippocampal cultures (19). In this report, we examine the effect of NGF on the neurotoxicity of β amyloid in differentiated hippocampal neurons. We find that NGF has a striking potentiating effect on the neurotoxic potency of β amyloid.

MATERIALS AND METHODS

Peptides, Growth Factors, and Antibodies. A polypeptide corresponding to the first 40 amino acids of β amyloid [β-(1–40)] was synthesized on a Milligen peptide synthesizer and purified by reverse-phase HPLC on C_{18} columns; the sequence was confirmed with an Applied Biosystems model 470 sequencer. The β-(1–40) peptide was solubilized and added to primary rat hippocampal cultures as described (19).

Highly purified mouse 2.5S NGF was obtained from Boehringer Mannheim. Insulin-like growth factor 1 and acidic and basic fibroblast growth factor were obtained from Boehringer Mannheim. Insulin was obtained from Sigma. Epidermal growth factor and human platelet-derived growth factor were obtained from Collaborative Research. Monoclonal antibodies against mouse 2.5S NGF, clone 27/21 (20), and against the rat NGF receptor, clone 192 (21, 22), were obtained from Boehringer Mannheim.

Cell Culture. Primary rat embryonic hippocampal cultures were established by a modification of the protocol previously described (23, 24). The hippocampus was dissected from the brains of embryonic day 18 rat embryos, incubated in 0.05% trypsin (30 min at 37°C), dissociated by gentle trituration, and cultured at a density of 2×10^4 cells per 16-mm polylysine-coated tissue culture well (Costar) in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) iron-supplemented calf serum (HyClone), 5% (wt/vol) Ham's F-12 nutrient mixture, 2 mM glutamine, 1 mM sodium pyruvate, 2.5% (wt/vol) Hepes, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Determination of Viable Neurons. For scoring and immunohistochemistry, cultures were fixed in 4% paraformaldehyde/0.12 M sucrose in phosphate-buffered saline for 30 min at 37°C. Pyramidal neurons were readily identified morphologically as cells that elaborated one primary axon and several dendrites during the first 5 days in culture as described (23, 24). This morphological identification of neurons was confirmed by immunohistochemical staining for neuron-specific markers including the class III β-tubulin isotype, microtubule-associated protein 2, and tau (19, 25–27). Identifiable neurons were judged to be viable by intactness of neurites [lack of beading or retraction (Fig. 4 *d* and *e*)] and intactness of soma (absence of vacuolar inclusions and ex-

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Abbreviations: NGF, nerve growth factor; AD, Alzheimer disease.

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clusion of 0.25% trypan blue). Determinations of viable pyramidal neurons were performed in triplicate 16-mm tissue culture wells; five 1.5-mm^2 fields were scored per well. At least 10 neurons per field or 50 neurons per well were counted in control cultures.

Immunohistochemistry. Hippocampal cultures grown on polylysine-coated glass coverslips were fixed, preincubated with 5% (vol/vol) bovine serum albumin in phosphate-buffered saline, and then incubated with the anti-NGF receptor monoclonal antibody (clone 192) (21, 22) at a 1:10 dilution for 12 hr at 4°C. Cells were then incubated with an affinity-purified biotinylated anti-mouse IgG (1:40) for 30 min, and reaction product was visualized by incubation with ExtrAvidin–peroxidase (Sigma) and then diaminobenzidine. There was no significant immunoreactivity in the absence of primary antibody.

RESULTS

Primary embryonic day 18 rat hippocampal cultures were used to determine the effects of β amyloid and NGF on neuronal viability. The advantages of this culture system are that it is >90% neuronal, neurons can be cultured at low density to facilitate quantitation, and neurons can be readily identified morphologically and by immunohistochemical markers (19, 23–27). We have previously shown that a synthetic polypeptide corresponding to the first 40 amino acids of β amyloid [β-(1–40)] is neurotoxic in 4-day-old cultures of differentiated hippocampal neurons (19). Treatment of 4-day-old hippocampal cultures with β-(1–40) resulted in a 40–50% decrease in the number of viable pyramidal neurons relative to untreated control cultures after 24 hr (Fig. 1). Treatment with a 1000-fold lower concentration of β-(1–40) (20 nM) had no detectable effect on neuronal viability. Addition of 2.5S NGF at 10 ng/ml together with the higher concentration of β-(1–40) (20 μM) resulted in slightly increased neurotoxicity relative to β-(1–40) alone. When NGF was added together with the lower inactive concentration of β-(1–40) (20 nM), there now appeared a significant neurotoxic response not observed with low concentrations of β-(1–40) alone (Fig. 1). A monoclonal antibody against NGF completely reversed this neurotoxic potentiation by NGF at a low dose of β-(1–40) (Fig. 1). NGF and the NGF antibody alone had no detectable effects on neuronal viability. A peptide corresponding to the first 16 amino acids of β amyloid, which is not neurotoxic to hippocampal neurons (19), was also not neurotoxic when added together with NGF (data not shown).

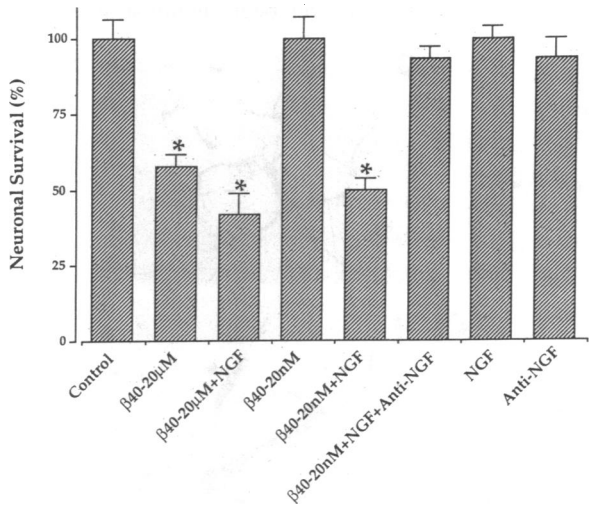

FIG. 1. NGF potentiates the neurotoxicity of β-(1–40). Four-day-old hippocampal neurons were treated as designated, and neuronal survival was determined 24 hr later. NGF (10 ng/ml) significantly potentiated the neurotoxicity of low doses of β-(1–40) (20 nM), and this potentiation was reversed by an NGF monoclonal antibody (clone 27/21; final concentration = 0.5 μg/ml) (20). Neuronal survival (ordinate) is expressed as the percentage of surviving neurons relative to the number of neurons in untreated control cultures and represents the mean ± SEM (n = 10–15). *$P < 0.001$ by analysis of variance. β40, β-(1–40).

The β-(1–40) concentration dependence of the neurotoxic response in the absence and presence of NGF is shown in Fig. 2. The neurotoxic response to β-(1–40) alone was observed in the 10^{-8}–10^{-7} M concentration range, and the EC_{50} was about 1×10^{-7} M β-(1–40) as reported (19). The neurotoxic response to β-(1–40) in the presence of NGF was observed in the 10^{-13}–10^{-12} M concentration range, and the EC_{50} was 6×10^{-13}–1×10^{-12} M. Thus, NGF increases neuronal sensitivity to the neurotoxic effect of β-(1–40) by a factor of ≈100,000.

A variety of other known growth factors that have been demonstrated to act on neuronal cells (1) were tested for their ability to potentiate β-(1–40) neurotoxicity. The combination of a low dose of β-(1–40) with either insulin, epidermal growth factor, platelet-derived growth factor, insulin-like growth factor 1, acidic fibroblast growth factor, or basic fibroblast growth factor did not significantly affect neuronal survival (Table 1). These growth factors had no significant effect even at much higher concentrations than NGF. The NGF concentration dependence for potentiation of β-(1–40) neurotoxicity is shown in Fig. 3. NGF was active at 0.1–1 ng/ml, with a half-maximal potentiating concentration of 0.2–0.3 ng/ml (≈0.01 nM), which is similar to the concentration range required for the neurite outgrowth response to NGF and for occupancy of the high-affinity NGF receptor (28–30).

To determine if neurons that degenerated in response to β-(1–40) possess NGF receptors, immunohistochemical analysis was performed with a monoclonal antibody to the rat NGF receptor (clone 192) (21, 22). In control untreated hippocampal cultures, only slight immunoreactivity was detected (Fig. 4 *a* and *b*). Neurons treated with NGF alone were not significantly different. However, in cultures treated with β-(1–40), >80% of the neurons showed high levels of immunoreactivity with the NGF receptor monoclonal antibody after 24 hr (Fig. 4 *c*, *d*, and *e*). NGF receptor induction appeared to be maximal at a concentration of 0.05 nM β-(1–40). When NGF was added together with a low dose of β-(1–40), many of the NGF receptor-positive neurons developed neurodegenerative changes, which included retraction

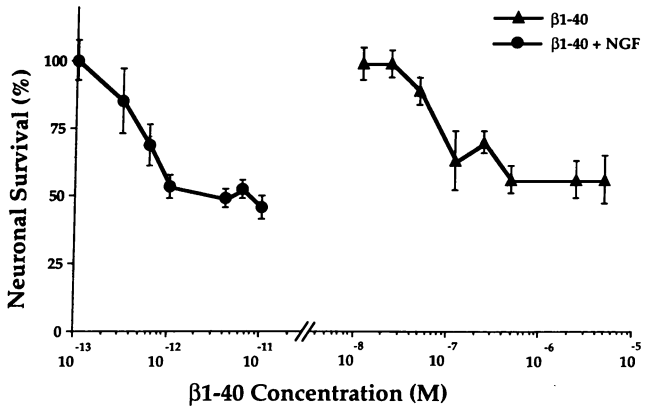

FIG. 2. NGF increases neuronal sensitivity to β-(1–40). Four-day-old hippocampal neurons were treated with the indicated concentrations of β-(1–40) in the absence or presence of NGF (10 ng/ml). Note that in the presence of NGF neurotoxicity is observed at about 10^5-fold lower concentrations of β-(1–40). Values represent the mean ± SEM (n = 10–15).

Table 1. Potentiation of β-(1–40) neurotoxicity is specific for NGF

Treatment	Neuronal survival, %
β-(1–40)	100 ± 7
β-(1–40) + NGF (0.01 nM)	47 ± 3
β-(1–40) + basic FGF (1 μM)	88 ± 6
β-(1–40) + acidic FGF (1 μM)	86 ± 5
β-(1–40) + IGF-1 (1 μM)	97 ± 7
β-(1–40) + insulin (0.2 μM)	105 ± 7
β-(1–40) + EGF (0.2 μM)	97 ± 6
β-(1–40) + PDGF (20 units/ml)	87 ± 7

β-(1–40) (20 nM) alone and in combination with each of the indicated growth factors was added to 4-day-old hippocampal neurons, and neuronal survival was determined 1 day later. Values are given as the percentage of surviving neurons relative to the number of neurons in control cultures and represent the mean ± SEM (n = 10–15). The only growth factor that significantly potentiated the low dose of β-(1–40) was 2.5S NGF ($P < 0.001$ compared to the control, two-tailed t test). The slight decreases in neuronal survival observed for platelet-derived growth factor (PDGF) and acidic and basic fibroblast growth factor (FGF) were not statistically significant ($P > 0.2$). IGF-1, insulin-like growth factor 1.

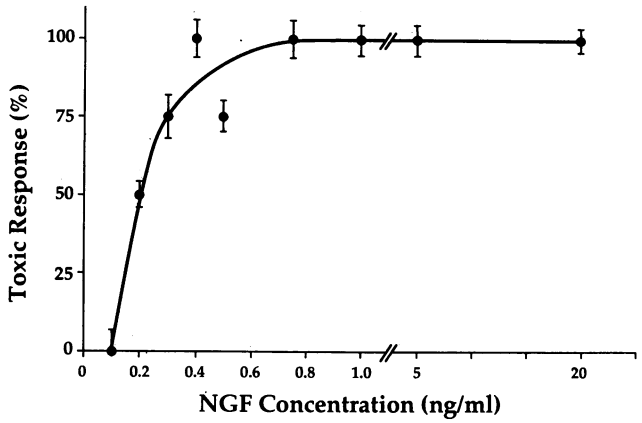

FIG. 3. NGF concentration dependence for potentiation of β-(1–40) neurotoxicity. Hippocampal neurons were treated with the indicated concentrations of 2.5S NGF in the presence of β-(1–40) (20 nM), and neuronal survival was determined 24 hr later. Values are normalized to the maximal decrease in neuronal survival (100% toxic response) observed for the combination of NGF with β-(1–40). Each value represents the mean ± SEM (n = 10).

and disruption of neurites and the development of vacuolar inclusions (Fig. 4*f*). Some of the neurons exhibiting increased NGF receptor immunoreactivity did not show morphologic evidence of degenerative changes. Exposure of hippocampal neurons to a neurotoxic concentration of glutamate (400 μM), which resulted in widespread neuronal degeneration, was not accompanied by increased NGF receptor immunoreactivity, suggesting that the β-(1–40) effect is not just a consequence of neuronal degeneration (data not shown).

DISCUSSION

In a previous report, we demonstrated that the β-(1–40) amyloid peptide is neurotrophic to immature hippocampal neurons at low concentrations and neurotoxic to older differentiated neurons at higher concentrations (19). In this report, we show that NGF potentiates the neurotoxicity of β amyloid on differentiated hippocampal neurons in culture. Although the maximal extent of β-(1–40)-induced neuronal cell death was not substantially changed in the presence of NGF, the sensitivity of neurons to β-(1–40) toxicity was increased by a factor of ≈100,000. The NGF concentration required for this potentiating effect was similar to that observed for the neurite outgrowth response and for occupancy of the high-affinity NGF receptor (28–30).

This is, to our knowledge, the first report of a neurotrophic factor potentiating a neurotoxic response. The role of NGF in the maintenance and viability of responsive neurons in the peripheral nervous system has been well documented (30). In the central nervous system, NGF has been reported to stimulate choline acetyltransferase activity in rat basal forebrain neurons, both *in vivo* and *in vitro*, and to promote the survival of cholinergic projection neurons after fimbria–fornix transection *in vivo* (1–7). Although rat hippocampal pyramidal neurons have been found to synthesize NGF (31–33) and possess NGF receptors (34), a physiological response to NGF has not yet been demonstrated in non-

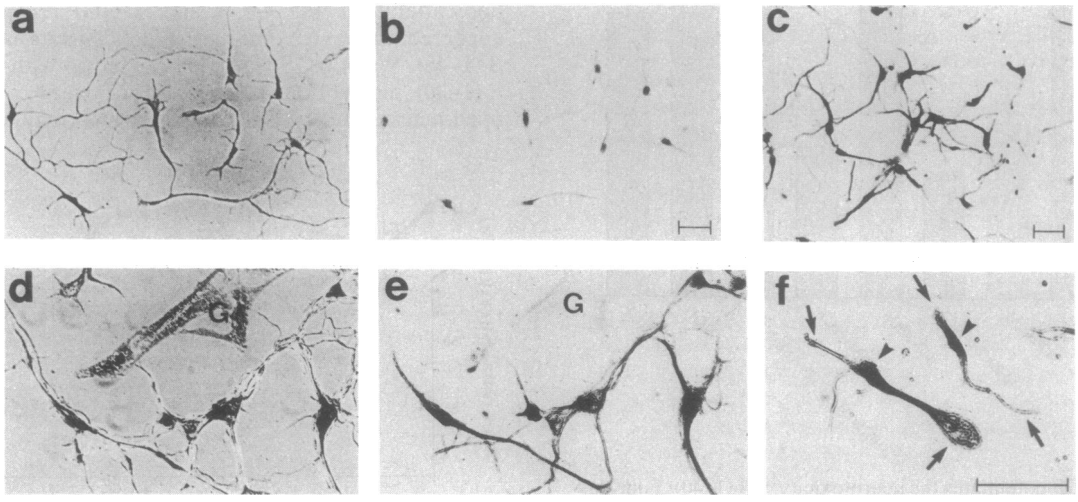

FIG. 4. NGF receptor immunoreactivity in cultured hippocampal neurons. Four-day-old hippocampal cultures were untreated (*a* and *b*), treated with 0.05 nM β-(1–40) (*c–e*), or treated with 0.05 nM β-(1–40) plus NGF (10 ng/ml) (*f*). (*a* and *d*) Phase contrast. (*b*, *c*, *e*, and *f*) Bright field. (*d–f*) High magnification. Treatment with β-(1–40) induced cell surface immunoreactive NGF receptors (*b* compared to *c*). When NGF was added together with β-(1–40) (*f*), receptor-positive neurons showed degenerative changes in the neurites (arrows) and cell soma (arrowheads). Glial cells (G) did not show significant NGF receptor immunoreactivity. Four-day-old hippocampal cultures were treated as indicated for 24 hr, fixed, incubated with the NGF receptor monoclonal antibody, and visualized with a biotinylated secondary antibody by the ExtrAvidin–peroxidase method. Cells were not permeabilized prior to treatment so that only cell surface receptor immunoreactivity was detected. (Bars = 30 μm.)

cholinergic central nervous system neurons. In primary hippocampal cultures, treatment with NGF or NGF antibody alone did not have detectable effects on neuronal viability or process outgrowth (Fig. 1). However, the combination of NGF and β amyloid had significant deleterious effects on neuronal viability.

Treatment of hippocampal neurons with low concentrations of β amyloid alone resulted in a significant induction of cell surface receptors for NGF. NGF receptor-positive neurons appeared intact in the presence of low concentrations of β amyloid alone, but when NGF was added, receptor-positive neurons developed neurodegenerative changes. Induction of the NGF receptor has been demonstrated following tissue damage in the adult rat striatum (35) and in response to NGF itself in PC-12 cells (36). In cultured rat hippocampal neurons, induction of the NGF receptor was not observed in response to glutamate-mediated neuronal damage or after addition of NGF, fibroblast growth factor, or insulin alone (B.A.Y. and A.C., unpublished data). Our results raise the possibility that in AD β-amyloid deposits may cause induction of the NGF receptor in neuronal cell types typically unresponsive to NGF. Such an induction may be difficult to detect if it is transient or results in the degeneration of affected cells.

Neurons of the hippocampus and basal forebrain cholinergic nuclei are among the earliest and most severely affected neuronal populations in AD (37). The data presented here suggests that one possible explanation for the vulnerability of NGF-responsive neurons in AD may be a neurotoxic interaction of β amyloid with NGF. Although β amyloid alone exhibits neurotoxic effects in culture (19), it is not known whether the 10^{-8}–10^{-7} M concentration range required for neurotoxicity is effectively achieved in the AD brain. However, in the presence of physiological levels of NGF, subpicomolar concentrations of β amyloid become neurotoxic. The interaction of β amyloid with NGF and possibly other as yet unidentified neurotrophic factors may be necessary for the progression of benign amyloidosis to a neurodegenerative process.

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