Binding of β-Amyloid to the p75 Neurotrophin Receptor Induces Apoptosis

A Possible Mechanism for Alzheimer's Disease

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Abstract

Alzheimer's disease is a neurodegenerative disorder characterized by the extracellular deposition in the brain of aggregated β-amyloid peptide, presumed to play a pathogenic role, and by preferential loss of neurons that express the 75-kD neurotrophin receptor ($p75^{NTR}$). Using rat cortical neurons and NIH-3T3 cell line engineered to stably express $p75^{NTR}$, we find that the β-amyloid peptide specifically binds the $p75^{NTR}$. Furthermore, 3T3 cells expressing $p75^{NTR}$, but not wild-type control cells lacking the receptor, undergo apoptosis in the presence of aggregated β-amyloid. Normal neural crest–derived melanocytes that express physiologic levels of $p75^{NTR}$ undergo apoptosis in the presence of aggregated β-amyloid, but not in the presence of control peptide synthesized in reverse. These data imply that neuronal death in Alzheimer's disease is mediated, at least in part, by the interaction of β-amyloid with $p75^{NTR}$, and suggest new targets for therapeutic intervention. (*J. Clin. Invest.* 1997. 100:2333–2340.) Key words: melanocytes • neurons • nerve growth factor • receptor binding • neurodegenerative

Introduction

Alzheimer's disease is a neurodegenerative disorder affecting 20 million people worldwide (1–3). It is characterized clinically by progressive dementia and neuropathologically by deposits in the brain parenchyma and in the walls of meningeal and cortical blood vessels that consist primarily of the so-called β-amyloid protein, a 4.2-kD polypeptide (4, 5). Cholinergic neurons of the basal nuclear complex that express the highest levels of the p75 neurotrophin receptor ($p75^{NTR}$) in the brain are preferentially affected (6), but the disease affects cortical neurons as well (7).

The structure of $p75^{NTR}$ is similar to that of p55 TNF receptor and Fas, and all three proteins are known to have a conserved sequence in their cytoplasmic domain, the death domain motif, which generates a cell death signal upon activation (8, 9). Furthermore, when $p75^{NTR}$ is activated by nerve growth factor (NGF)[1] in cells that do not also express the NGF-specific receptor $p140^{trkA}$, $p75^{NTR}$ activation leads to cell death (10, 11). Also, PC12 cells, like neurons, undergo cell death when exposed to β-amyloid; the expression of $p75^{NTR}$ is required for β-amyloid to kill these cells (12). In a mutant PC12 cell line that lacks $p75^{NTR}$, β-amyloid has hardly any effect on cell viability (12). Taken together, these data suggest an interaction between β-amyloid and $p75^{NTR}$ that predisposes neuronal death. Here we demonstrate that this interaction constitutes direct binding of β-amyloid to $p75^{NTR}$.

Methods

Cell culture

$p75^{NTR}$-NIH 3T3 and wild-type (WT) NIH-3T3 cells (kindly provided by Dr. M.V. Chao, Cornell University Medical College, New York) were maintained in DMEM supplemented with 10% FBS in the presence of penicillin (45 ng/ml), streptomycin (68 ng/ml), and hygromycin B (17.5 ng/ml) (only $p75^{NTR}$-NIH 3T3 cells). For definitive experiments cells were provided with serum-free DMEM containing transferrin (5 μg/ml) and insulin (5 μg/ml). Melanocytes were established from neonatal foreskins, and were maintained as described (13). Homogenous cultures consisting of neural crest–derived melanocytes, known to express both $p75^{NTR}$ (14) and $p140^{trkA}$ (13), were serially passaged at confluence and used at second to fourth passage. In definitive experiments cells were maintained in serum-free Medium 199 (GIBCO BRL, Gaithersburg, MD) supplemented with epidermal growth factor (10 ng/ml) (GIBCO BRL), insulin (10 μg/ml), (Sigma Chemical Co., St. Louis, MO), triiodothyronine (10^{-9} M) (Sigma Chemical Co.), transferrin (10 μg/ml) (Sigma Chemical Co.), hydrocortisone (1.4×10^{-6} M) (Calbiochem Corp., La Jolla, CA), cholera-gen (10^{-9} M) (List Biological Laboratories, Inc., Campbell, CA), and basic fibroblast growth factor (10 ng/ml) (Collaborative Biomedical Products, Bedford, MA). Neuronal cultures were established from the cerebral cortex of E17 fetal rats (Long Evans; Charles River Lab., Portage, MI) as described (15), with minor modifications. In brief, the cerebral cortices were removed, resuspended in 0.025% trypsin in HBSS buffered with 10 mM Hepes, and incubated at 37°C. After 10 min, serum-containing medium supplemented with 0.05% DNAse was added, and the content was centrifuged and suspended in serum-containing medium supplemented with 0.02% DNAse. Cells were then dissociated by gentle trituration, filtered through 73.7-μm and 35-μm screens, and the dissociated cells were resuspended in serum-free neurobasal medium (GIBCO BRL). Cells were plated in T75 tissue culture flasks precoated overnight with 2 $\mu g/cm^2$ poly D-lysine in PBS (30,000–70,000, pH 7.5; Sigma Chemical Co.). Cultures were used 2 d after plating.

In definitive experiments, cultures were examined daily for gross differences in cell morphology using an inverted phase contrast microscope (Nikon, Japan) without reference to culture identity.

Toxicity assays

β-amyloid toxicity was determined by cell yields and Trypan blue exclusion.

Cell yield. After removing the medium and rinsing once with PBS to remove unattached cells, cultures were incubated either in 0.25% trypsin or in EDTA at 37°C, and cell yields were determined using a particle counter (Coulter Corp., Hialeah, FL).

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1. *Abbreviations used in this paper:* NGF, nerve growth factor; WT, wild-type.

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Trypan blue exclusion assay. $p75^{NTR}$-NIH 3T3 cells were incubated in EDTA. Suspended cells were incubated for 5 min with 0.5% Trypan blue at room temperature. Trypan blue–positive and –negative cells were determined using a hemocytometer.

β-Amyloid preparations

Lyophilized HPLC-purified β-amyloid, containing the first 40 amino acids of the β-amyloid peptide (1–40), or an identically handled peptide containing the first 40 amino acids synthesized in reverse order (40–1) used as a negative control (Bachem California, Torrance, CA), were dissolved in ddH_2O at a concentration of 5 mg/ml, aliquoted, and frozen at −20°C. Before use, preparations were thawed once, diluted in DMEM, and were either added directly to medium (monomeric, soluble), or incubated at 37°C for 16 h before addition to medium (aggregated).

To confirm that the latter preparation contained aggregated β-amyloid, presumptive soluble and aggregated β-amyloid preparations were dissolved in Laemmli sample buffer and subjected to electrophoresis on Tris-tricine SDS/10–20% PAGE under reducing conditions, and were stained with Coomassie Blue to reveal bands of different electrophoretic mobilities. Gels were scanned into a computer (Macintosh IIsi). The intensity of the bands at 4.5-kD electrophoretic mobility in the soluble and the aggregated preparation was determined using Scan Analysis (68000) program, and the percent of aggregated β-amyloid was calculated. [^{125}I]β-amyloid was always used in its soluble, monomeric form.

Immunoprecipitation

80% confluent $p75^{NTR}$-NIH 3T3 cells or rat cortical neurons were lifted from the dish with EDTA and incubated in suspension with monomeric soluble [^{125}I]β-amyloid (Peninsula Laboratories, Inc., Belmont, CA) with or without 1,000-fold excess unlabeled aggregated β-amyloid at 4°C. After incubation, 1 mM of disuccinimidyl suberate was added for 30 min. After centrifugation, cells were lysed with RIPA buffer (50 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 0.5% sodium deoxycholate, 1% Triton X-100) in the presence of 1 μg/ml aprotinin and 75 μg/ml PMSF, sonicated for 1–3 s, and immunoprecipitated with anti–human $p75^{NTR}$ antibodies (mouse monoclonal IgG1; Cedarlane Laboratories Ltd., Ontario, Canada, $p75^{NTR}$-NIH 3T3 cells), anti–rat $p75^{NTR}$ antibodies (mouse monoclonal IgG1; Boehringer Mannheim Biochemicals, Indianapolis, IN, rat cortical neurons) or with mouse IgG as control for 16 h at 4°C in the presence of 15 μl of protein G plus protein A agarose and 1 M NaCl adjusted to pH 8.0. After several washes with 20 mM Tris HCl, pH 8.0, 1 M NaCl, 5 mM $MgCl_2$, 0.2% Triton X-100, and 1 mM PMSF, immunoprecipitates were separated over 8% PAGE and subjected to autoradiography.

Binding assays

$p75^{NTR}$-NIH 3T3 cells were incubated in binding medium (DMEM, 10 mM Hepes, 0.1 mg/ml cytochrome C, 0.01% Tween 80, 1 mg/ml BSA) with 0.2 nM [^{125}I]β-amyloid 1–40 and increasing concentrations of cold aggregated β-amyloid for 4 h at 15°C; or 1 nM [^{125}I]β-amyloid 1–40 and increasing concentrations of cold soluble β-amyloid for 4 h at 4°C. In NGF displacement studies, cells were incubated with 1 nM [^{125}I]β-amyloid 1–40 and increasing concentrations of cold human NGF (Boehringer Mannheim Biochemicals) at 4°C. At the end of the incubation period, cells were rinsed in PBS, lysed in 1 N NaOH, and lysates were subjected to γ counting. For each point of the Scatchard analysis, nonspecific binding, determined with a 400-fold excess of unlabeled competitor, was subtracted from total binding. Each point was the average of at least two separate determinations.

Apoptosis analysis

In situ labeling of DNA strand breaks. Detached as well as adherent cells were collected and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature as described (16). Cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate (2 min at 4°C), then incubated for 60 min at 37°C with fluorescein-tagged dUTP in the presence of terminal deoxynucleotidyl transferase (Boehringer Mannheim Biochemicals), and cellular fluorescence was analyzed by flow cytometry (Becton Dickinson, San Jose, CA).

DNA extraction. Cells were washed with cold PBS and disrupted in lysis buffer, pH 8.0 (10 mM Tris, 150 mM NaCl, 0.1 mM EDTA, 1% SDS, 200 μg/ml proteinase K). After a 15-h incubation at 37°C, samples were extracted twice with phenol plus chloroform (1:1 vol/vol) and precipitated overnight with ethanol (2.5× vol) and 3 M sodium acetate (1/10× vol). The DNA was then digested with DNAse-free ribonuclease (10 μg/ml) for 1 h at 37°C and separated on 1% agarose gel.

Results

β-amyloid binds $p75^{NTR}$. To determine if β-amyloid interacts directly via receptor binding, soluble monomeric [^{125}I]1–40 β-amyloid peptide was added to NIH-3T3 cells engineered to stably express $p75^{NTR}$ ($p75^{NTR}$-NIH 3T3 cells) in the presence of disuccinimidyl suberate, a widely used reagent for cross-linking ligand–receptor complexes (17). Cells were then immunoprecipitated with anti-$p75^{NTR}$ antibodies or an irrelevant mouse IgG. Autoradiograms revealed a protein band of 75–80 kD only in lysates immunoprecipitated with anti $p75^{NTR}$ antibodies (Fig. 1 *A*). Excess cold aggregated β-amyloid competitively inhibited the binding of [^{125}I]β-amyloid (Fig. 1 *B*). Scatchard analysis of aggregated β-amyloid binding to $p75^{NTR}$-NIH 3T3 cells demonstrated a K_d of 23 nM (Fig. 1 *C*), indicating that in its aggregated state, β-amyloid has a lower receptor affinity than NGF, for which the reported $p75^{NTR}$ dissociation constant is 4–7 nM (18, 19). Compared to the aggregated β-amyloid, soluble β-amyloid showed a higher affinity for $p75^{NTR}$ (displacement of 50% of radiolabeled tracer at ∼ 12 nM, data not shown). To determine if β-amyloid similarly binds $p75^{NTR}$ expressed on normal neurons, the cross-linking and immunoprecipitation experiments were repeated using rat cortical neurons. [^{125}I]β-amyloid again specifically bound $p75^{NTR}$ (Fig. 1 *D*). Furthermore, increasing concentrations of unlabeled human NGF competitively displaced the [^{125}I]β-amyloid binding in $p75^{NTR}$-NIH 3T3 cells with 50% displacement at a concentration of ∼ 6 nM (Fig. 1 *E*). SDS-PAGE/Coomassie blue staining showed that some of the β-amyloid preparation that had been maintained at 37°C overnight was either retained at the top of the gel or migrated at a slower electrophoretic mobility (Fig. 1 *F*). These results are similar to those shown in previous studies (20). Densitometric analysis showed that ∼ 20% of the preparation was present in the aggregated form.

β-amyloid is toxic to $p75^{NTR}$-NIH 3T3 cells. To determine the effect of $p75^{NTR}$-bound β-amyloid on cells, $p75^{NTR}$-NIH 3T3 cells were supplemented with HPLC-purified aggregated β-amyloid corresponding to amino acids 1–40. In Alzheimer's disease and related disorders, β-amyloid protein is found in an aggregated, poorly soluble form (21), and in vitro β-amyloid–induced neurotoxicity is markedly enhanced when the peptide is preaggregated (22). We initially chose to examine the effect of 25 μM aggregated β-amyloid, a concentration much higher than the calculated K_d, but one that is routinely used in other in vitro studies to induce neurotoxicity (12, 23). The parental WT NIH-3T3 cells that lack $p75^{NTR}$ were used as a control. Within 72 h there was a significant decline in cell yields of $p75^{NTR}$-NIH 3T3 cells, but not WT NIH-3T3 cells (Fig. 2 *A*). While WT NIH-3T3 cells appeared healthy (Fig. 2 *B*), $p75^{NTR}$-NIH 3T3 cells were rounded and detached from the dish surface (Fig. 2 *C*). In contrast to aggregated β-amyloid, mono-

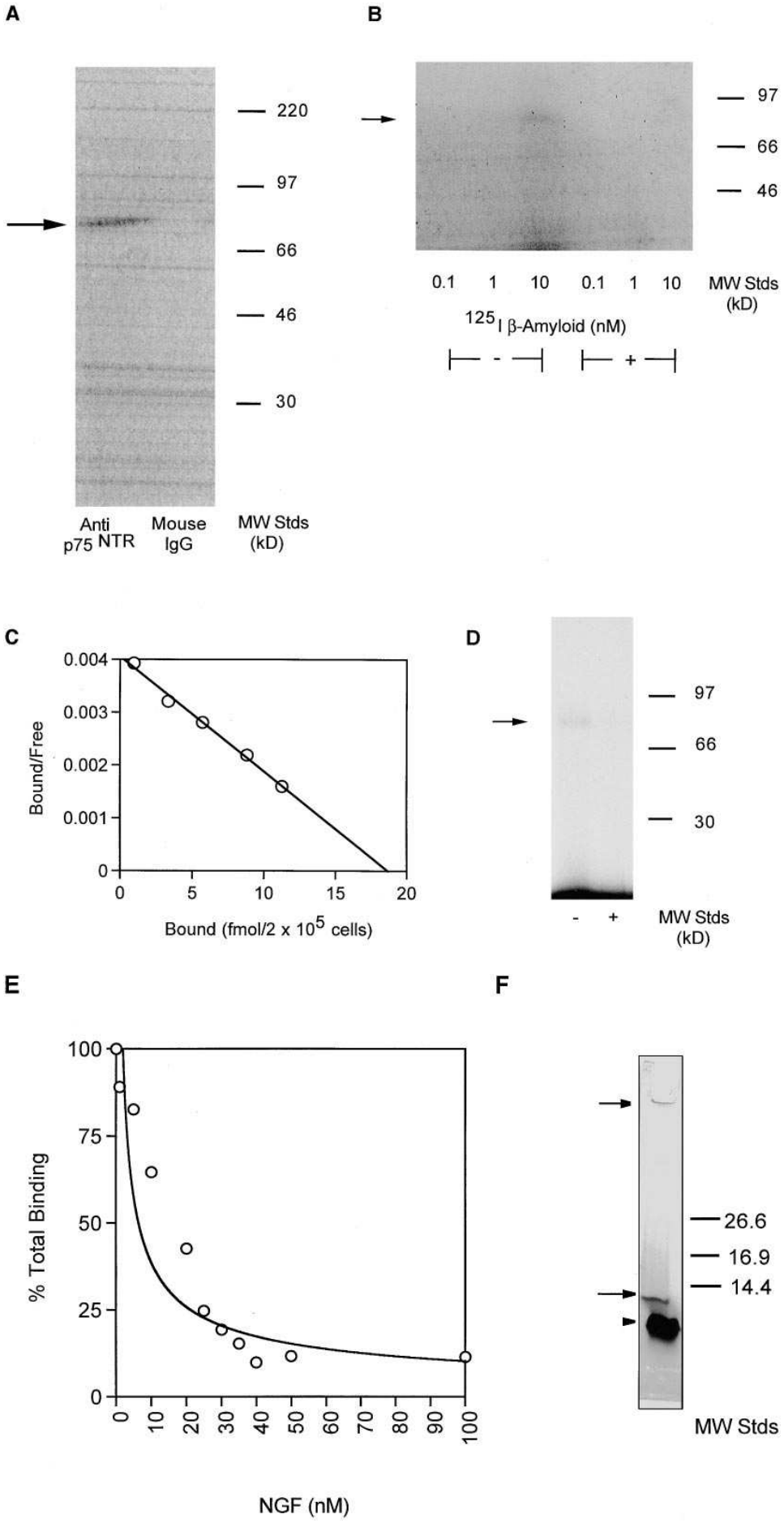

Figure 1. β-amyloid binds the $p75^{NTR}$. (*A*) $p75^{NTR}$-NIH 3T3 cells were incubated in suspension with 5 μCi (5 nM) soluble [^{125}I]β-amyloid 1–40 (specific activity 1615 Ci/mmole) at 4°C. Cell lysates were immunoprecipitated with anti-$p75^{NTR}$ antibodies (mouse monoclonal IgG1; Cedarlane Laboratories Ltd., Ontario, Canada) or with mouse IgG as control, and separated over 8% PAGE. Autoradiography shows a band of 75–80 kD only in lysates immunoprecipitated with anti-$p75^{NTR}$ antibodies (*arrow*) and not in lysates immunoprecipitated with irrelevant mouse IgG. (*B*) $p75^{NTR}$-NIH 3T3 cells were incubated in suspension with 0.1 nM, 1 nM, or 10 nM of soluble [^{125}I]β-amyloid 1–40 (specific activity 1926 Ci/mmole) in the presence (+) or absence (−) of 10 μM cold aggregated β-amyloid and immunoprecipitated as above with anti-$p75^{NTR}$ antibodies. The expected band of 75–80 kD is present only in lysates incubated with 10 nM [^{125}I]β-amyloid. The presence of 1,000-fold excess cold aggregated β-amyloid blocked [^{125}I]β-amyloid binding. (*C*) Scatchard analysis of aggregated β-amyloid binding to $p75^{NTR}$-NIH 3T3 cells. 0.2 nM [^{125}I]β-amyloid was used as tracer, and increasing concentrations of aggregated β-amyloid was used as competitor. The derived K_d is 23 nM, and B_{max} is ∼ 60,000 sites per cell, consistent with the published 90,000 sites per cell (18) and well within the range of $p75^{NTR}$ in non-transfected cells (i.e., PC12 cells ∼ 60,000 sites/cell [58]; A875 melanoma, 100,000 sites/cell [18]). Similar K_d results of ∼ 25 nM were obtained in each of four different displacement curves. (*D*) Rat cortical neurons were incubated in suspension with 10 nM [^{125}I]β-amyloid 1–40 (specific activity 1058 Ci/mmole) in the presence (+) or absence (−) of 10 μM cold aggregated β-amyloid and immunoprecipitated with anti–rat $p75^{NTR}$ antibodies. The expected band of 75–80 kD is present only in lysates incubated with 10 nM [^{125}I]β-amyloid. The presence of excess cold aggregated competitor blocked [^{125}I]β-amyloid binding to neurons. (*E*) $p75^{NTR}$-NIH 3T3 cells were incubated for 4 h in binding medium with 1 nM [^{125}I]β-amyloid 1–40 and increasing concentrations of human NGF (0–100 nM). A concentration-dependent inhibition of [^{125}I]β-amyloid binding by NGF was observed with 50% inhibition at ∼ 6 nM NGF concentration, consistent with the reported K_d for NGF of 4–7

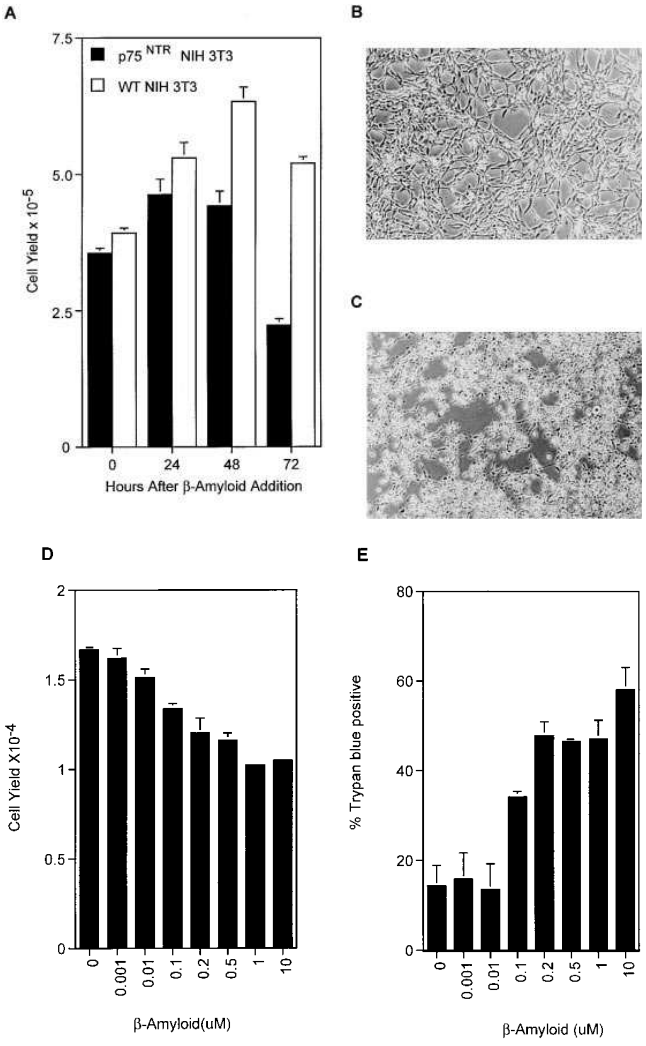

Figure 2. β-amyloid induces death of p75^{NTR}-NIH 3T3 cells. (*A*) p75^{NTR}-NIH 3T3 cells and WT NIH-3T3 cells were maintained in serum free medium, and yields 72 h after addition of β-amyloid between p75^{NTR}-expressing cells and control cells were highly statistically significant ($P < 0.007$, ANOVA. Bonferroni post-hoc comparisons demonstrated a difference at a significance level of $P = 0.001$ between p75^{NTR}-NIH 3T3 and WT NIH-3T3 cells at 72 h, and a difference at a significance level of $P = 0.05$ between p75^{NTR}-NIH 3T3 and WT NIH-3T3 cells at 24 and 48 h). Error bars represent standard deviations of two different dishes. (*B*) The majority of the WT NIH-3T3 cells have a typical bipolar morphology, and are spread on the dish surface. (*C*) The majority of p75^{NTR}-NIH 3T3 cells are rounded and detached from the dish surface. (*D* and *E*) p75^{NTR}-NIH 3T3 cells were maintained as above and supplemented with increasing concentrations (0–10 μM) of aggregated β-amyloid 1–40 peptide. (*D*) 72 h after addition of aggregated β-amyloid, there is a dose-dependent decrease of cell yield. (*E*) The same cultures processed for Trypan blue exclusion assay show a dose-dependent increase in Trypan blue–positive cells.

meric soluble β-amyloid did not affect cell yield (data not shown).

Furthermore, increasing concentrations (0–10 μM) of aggregated 1–40 β-amyloid decreased p75^{NTR}-NIH 3T3 cell yield and viability in a dose-dependent manner (Fig. 2, *D* and *E*). Regression analysis showed significant dose-dependent decreases in cell yield ($r^2 = 0.9070$, $P < 0.000001$) and Trypan blue exclusion ($r^2 = 0.9189$, $P < 0.000001$).

At least one pathway of β-amyloid–induced cellular toxicity involves the formation of free radicals (24). To rule out the possibility that expression of p75^{NTR} simply renders the cells more sensitive to oxidative stress generally, p75^{NTR}-NIH 3T3 cells and WT NIH-3T3 cells were supplemented with 10–300 μM hydrogen peroxide (H_2O_2). At the lower doses, no effect was observed. At 100 and 300 μM H_2O_2 there were progressive decreases in cell yields, determined daily for 4 d up to ~ 50%, but no difference between the cell lines (data not shown).

β-amyloid induces apoptosis of p75^{NTR}-NIH 3T3 cells. Recent in vivo and in vitro data suggest that β-amyloid–induced cell death exhibits classical characteristics of programmed cell death (25, 26). To determine if p75^{NTR}-NIH 3T3 cells undergo apoptosis after exposure to aggregated β-amyloid, DNA fragmentation was assessed. Total cellular DNA isolated from cultures 72 h after β-amyloid addition displayed the characteristic endonuclease-induced DNA fragmentation into multimers (Fig. 3 *A*). The DNA showed a random degradation pattern in addition to the characteristic fragmentation into multimers, suggesting that β-amyloid induces cell death via both necrosis and apoptosis as has been reported (27, 28). In situ labeling of DNA strand breaks was performed as well. After 72 h, 50.5±6.5% of β-amyloid–treated p75^{NTR}-NIH 3T3 cells as compared to 6.9±0.3% of WT NIH-3T3 cells ($P < 0.0001$, nonpaired *t* test, four separate determinations) showed label incorporation, indicating apoptosis (Fig. 3, *B* and *C*).

β-amyloid is toxic to normal human neural crest–derived melanocytes. To determine the effect of β-amyloid on normal human cells that express physiologic numbers of p75^{NTR} (14), homogenous cultures consisting of neural crest–derived melanocytes were supplemented with increasing concentrations (up to 50 μM) of aggregated 1–40 β-amyloid fragment, or 40–1 as a negative control (Fig. 4 *A*). Regression analysis showed significant dose-dependent decreases in cell yield with β-amyloid 1–40 ($R^2 = 0.8475$, $P < 0.00001$), but no significant effect on cell yield with β-amyloid 40–1 ($R^2 = 0.06$, $P = 0.44$). Phase contrast microscopy showed that the majority of melanocytes maintained in the presence of the 1–40 peptide were dying and detaching from the dish surface (Fig. 4 *B*). In contrast, cells maintained with the same concentration of the control peptide appeared healthy (Fig. 4 *C*). Similar but less prominent effects were observed when lower doses of β-amyloid were used (Fig. 4 *D*). Within 48 and 72 h, 100 nM of aggregated β-amyloid 1–40 decreased melanocyte yields by 26±1% and 28±4%, respectively, compared to cultures maintained with the same concentration of the control 40–1 peptide that showed no sig-

Figure 1 legend (Continued)

nm (18, 19). (*F*) SDS-PAGE analysis of aggregated β-amyloid. Aggregated β-amyloid was run over an SDS/10–20% PAGE. Soluble fraction is migrating predominantly as a single band at ~ 4.5 kD (*arrowhead*). In contrast, with soluble β-amyloid that runs as a single band at ~ 4.5 kD (data not shown), a prominent proportion of the preparation is either retained at the top of the gel (*upper arrow*) or migrates at slower electrophoretic mobility (*lower arrow*). One representative experiment is shown.

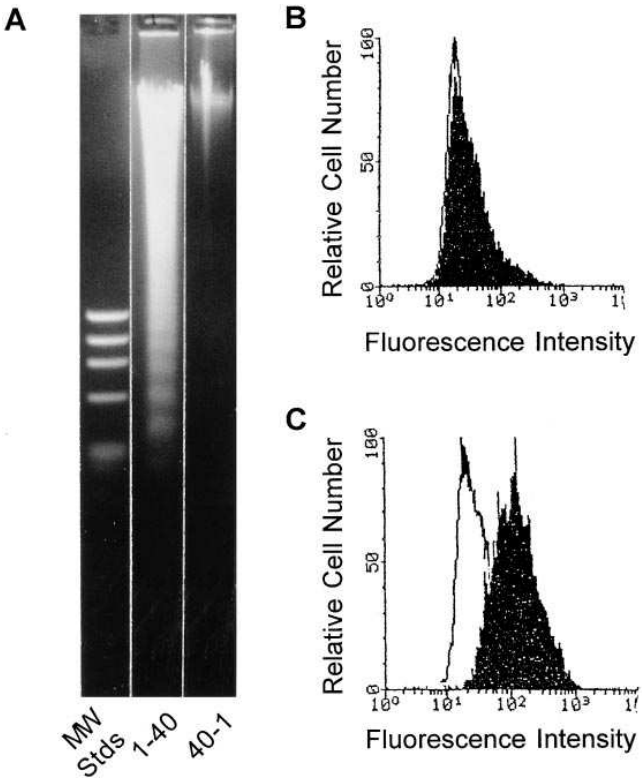

Figure 3. β-amyloid induces apoptosis of $p75^{NTR}$-NIH 3T3 cells. (*A*) Within 72 h after addition of aggregated β-amyloid to $p75^{NTR}$-NIH 3T3 cells, DNA fragmentation was observed in cultures supplemented with 1–40 peptide, but not in cultures supplemented with 40–1 peptide. The DNA also shows a random degradation pattern suggesting necrosis in addition to apoptosis. (*B* and *C*) Flow cytometric analysis of NIH-3T3 cell apoptosis. Detached as well as adherent β-amyloid treated WT NIH-3T3 cells (*B*, *black curve*) and $p75^{NTR}$-NIH 3T3 cells (*C*, *black curve*) were collected and processed for DNA strand breaks. Background (*white curve*) represents fluorescence intensity obtained when cells were incubated without terminal deoxynucleotidyl transferase.

nificant change in cell yields after supplementation. ANOVA indicated significance of $P < 0.003$ at 48 h and $P < 0.007$ at 72 h. Bonferroni post-hoc comparisons demonstrated a significant difference between the 100 nM β-amyloid–treated group and all other groups ($P < 0.0001$). After 48 h, in situ labeling of DNA strand breaks showed 20.6±7.2% of apoptotic cells in cultures provided 100 nM 1–40 as compared to 2% in cultures provided control peptide (Fig. 4, *E* and *F*).

Discussion

Our studies document that β-amyloid–induced apoptosis can be mediated through its binding to $p75^{NTR}$. This binding occurs in cells that normally express no neurotrophin receptors, but that were engineered to stably express $p75^{NTR}$. β-amyloid also binds $p75^{NTR}$ in primary rat cortical neurons and human epidermal melanocytes, both known to express physiologic levels of $p75^{NTR}$ in addition to a neurotrophin-specific receptor of the trk family. Although classically considered to mediate survival in cells that express its specific $p140^{trkA}$ receptor (29, 30), NGF binding to $p75^{NTR}$ in cells that lack $p140^{trkA}$ may lead to cell death (10, 11). Our data confirm and expand the recent observation that, in such cells, expression of $p75^{NTR}$ is required for β-amyloid–induced cell death (12).

Our studies also demonstrate that both the soluble and the aggregated form of β-amyloid bind $p75^{NTR}$, but only the aggregated form is cytotoxic. The toxicity data correlate well with the calculated K_d for the aggregated molecule. In both $p75^{NTR}$-NIH 3T3 cells and melanocytes, there is hardly any toxic effect on cells treated with doses ≤ 10 nM, but there is a significant cytotoxicity with doses of 100 nM or higher. In vitro studies show that β-amyloid toxicity for neurons, astrocytes, microglia, and endothelial cells require aggregation of the molecule (31). Also, the in vivo association between aggregated, fibrillar β-amyloid, and local neuronal toxicity has been confirmed by light and electron microscopic studies of Alzheimer's disease brains (31). It is possible that signaling by β-amyloid through $p75^{NTR}$ requires cross-linking of $p75^{NTR}$ rather than one-on-one ligand–receptor binding. Such a model could explain why aggregated β-amyloid, but not the soluble form, is toxic to cells. Indeed, it was shown for Fas, another member of the death domain receptor family that includes $p75^{NTR}$, that cross-linking of receptors is required for signaling (32). Such a mechanism is strongly suggested by our data, but further experiments are required to delineate this point.

Two parallel signaling pathways are coupled to $p75^{NTR}$: one that leads to apoptosis and one that leads to survival. It was shown that in mature oligodendrocytes (10) and early developing retinal neurons (11), NGF binding to $p75^{NTR}$ results in a sustained increase of intracellular ceramide, activation of the c-Jun amino-terminal kinase, and apoptotic cell death. Apoptosis through $p75^{NTR}$ activation, however, appears to depend on the cell type and its differentiation state. For example, in developing avian sympathetic ganglia, NGF activation of $p75^{NTR}$ induces primarily proliferation (33), and NGF binding to $p75^{NTR}$-expressing immortalized neuronal cells (34), or neuroblastoma cells that do not express $p140^{trkA}$, enhances their survival.

NGF, the best-studied ligand for $p75^{NTR}$, is known to bind in addition the $p140^{trkA}$, a tyrosine kinase receptor. It is understood that when both $p75^{NTR}$ and $p140^{trkA}$ are expressed on the cell surface, NGF receptor binding affinity is in the picomolar range (35), and NGF signals through $p140^{trkA}$ leading to cell survival (36). Indeed, in the adult organism, presumptively through coordinated binding to both $p75^{NTR}$ and $p140^{trkA}$, NGF can augment survival of aged neurons (37–39) as well as the survival of neurons after various injuries like hypoglycemia (40, 41), axotomy (42–44), or exposure to neurotoxic substances (45). Furthermore, in some studies NGF has been reported to improve cognitive function and attenuate loss of cholinergic neurons in animal models or in patients with Alzheimer's disease (46–48). In melanocytes that express both $p75^{NTR}$ and $p140^{trkA}$, β-amyloid induces cell death, while NGF induces their survival (49). Therefore, our studies are consistent with β-amyloid interacting exclusively with $p75^{NTR}$ in contrast with NGF that interacts with $p75^{NTR}$ and $p140^{trkA}$.

β-amyloid–containing senile plaques are known to exist in the brain parenchyma years before dystrophic neuritic alterations occur, and before signs of dementia appear (21). Furthermore, diffuse amyloid plaques occur in many clinically normal aged humans, suggesting that β-amyloid deposition is not sufficient by itself to produce the clinical manifestations of Alzheimer's disease. Instead, it appears that additional local changes in the brain tissue render neurons susceptible to the toxic effects of β-amyloid. Of note, the presumptive survival

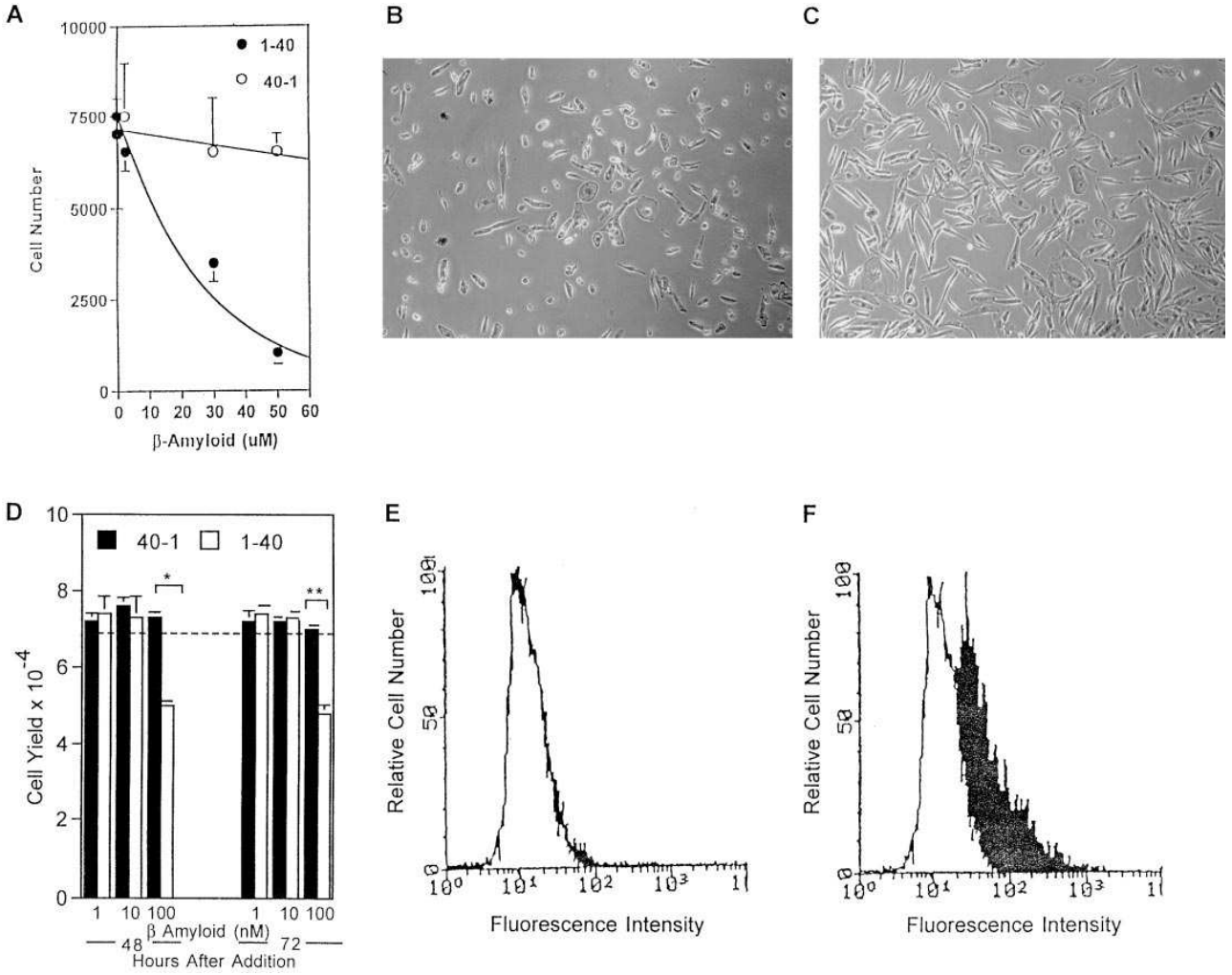

Figure 4. Effect of β-amyloid on melanocyte apoptosis. 2 d after plating, melanocytes were supplemented with increasing concentrations of aggregated β-amyloid 1–40 or the control peptide 40–1 (0–50 μM). (*A*) Cell yields determined 72 h after addition of β-amyloid show a dose-dependent decrease of cell yield in cultures maintained with the 1–40 peptide. No effect on cell yield was observed in cultures maintained with the control 40–1 peptide. One representative experiment with mean and STDEV of two separate counts is shown. (*B*) The majority of melanocytes maintained in the presence of 25 μM 1–40 peptide are rounded and detached from the dish surface. (*C*) Melanocytes maintained in the presence of 25 μM 40–1 control peptide have a typical bipolar to polygonal morphology. (*D*) 2 d after plating, melanocytes were supplemented with 1–100 nM of aggregated β-amyloid or the same concentrations of the control peptide 40–1. Cell yields determined 48 and 72 h after addition of β-amyloid show a significant decrease of cell yield in cultures maintained with 100 nM 1–40 peptide as compared to cultures maintained with the control peptide. (*E* and *F*) Flow cytometric analysis of melanocyte apoptosis 48 h after addition of aggregated β-amyloid. β-amyloid 40–1 peptide–treated cultures (*E*, *black curve*) and 1–40 peptide-treated cultures (*F*, *black curve*) were processed for DNA strand breaks. Background (*white curve*) represents fluorescence intensity obtained when cells were incubated without terminal deoxynucleotidyl transferase.

factor NGF is locally produced in the brain (50, 51), and its abundance has been reported to decrease with aging (52), suggesting that a relative lack of NGF may predispose the elderly to neuronal loss.

Recently, the cell surface neuronal receptor for advanced glycation endproducts has been reported to bind aggregated β-amyloid (53, 54), leading to the generation of oxidative stress and induction of macrophage–colony stimulating factor, which in turn enhances microglia cell proliferation and migration. In addition, microglia express scavenger receptors that promote their adhesion to β-amyloid, leading to β-amyloid uptake by the receptors and release of cytokines and reactive nitrogen and oxygen species that damage neurons (55, 56). These behaviors of neurons and microglial cells are postulated to contribute to the pathophysiology of Alzheimer's disease.

Based on our findings, we propose an alternative/additional mechanism for the pathophysiology of Alzheimer's disease. We propose that in neurons expressing both $p75^{NTR}$ and $p140^{trkA}$, NGF binds $p75^{NTR}/p140^{trkA}$ with an affinity in the picomolar range and enhances neuronal survival (29, 30, 35), but that under conditions characterized by increased surface expression of $p75^{NTR}$ (57) and/or greatly increased levels of β-amyloid in the extracellular space in the absence of saturating amounts of NGF, cell death may result from binding of β-amyloid to the $p75^{NTR}$ alone, with subsequent activation of the apoptotic pathway. Such a situation might occur in older individu-

als, particularly those with a genetically determined excessive production of β-amyloid fragments (21). This model for the pathophysiology of Alzheimer's disease may suggest new therapeutic interventions to slow or arrest its progression.

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References

1. Yamazaki, T., D.J. Selkoe, and E.H. Koo. 1995. Trafficking of cell surface β-amyloid precursor protein: retrograde and transcytotic transport in cultured neurons. *J. Cell Biol.* 129:431–442.

2. Barinaga, M. 1995. Missing Alzheimer's gene found. *Science (Wash. DC).* 269:917–918.

3. Levy-Lahad, E., E.M. Wijsman, E. Nemens, L. Anderson, K.A.B. Goddard, J.L. Weber, T.D. Bird, and G.D. Schellenberg. 1995. A familial Alzheimer's disease locus on Chromosome I. *Science (Wash. DC).* 269:970–972.

4. Selkoe, D.J. 1991. The molecular pathology of Alzheimer's disease. *Neuron.* 6:487–498.

5. Glenner, G.G., and C.W. Wong. 1993. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* 120:885–890.

6. Woolf, N.J., E. Gould, and L.L. Butcher. 1989. Nerve growth factor receptor is associated with cholinergic neurons of the basal forebrain but not the pontomesencephalon. *Neuroscience.* 30:143–152.

7. Bauer, J., M. Hull, and M. Berger. 1995. Pathogenetic factors of Alzheimer disease. *Z. Gerontol. Geriatr.* 28:155.

8. Feinstein, E., A. Kimchi, D. Wallach, M. Boldin, and E. Varfolomeev. 1995. The death domain: a module shared by proteins with diverse cellular functions [letter]. *Trends Biochem. Sci.* 20:342–344.

9. Boldin, M.P., E.E. Varfolomeev, Z. Pancer, I.L. Mett, J.H. Camonis, and D. Wallach. 1995. A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J. Biol. Chem.* 270:7795–7798.

10. Casaccia-Bonnefil, P., B.D. Carter, R.T. Dobrowsky, and M.V. Chao. 1996. Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. *Nature (Lond.).* 383:716–719.

11. Frade, J.M., A. Rodríguez-Tébar, and Y.A. Barde. 1996. Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature (Lond.).* 383:166–168.

12. Rabizadeh, S., C.M. Bitler, L.L. Butcher, and D.E. Bredesen. 1994. Expression of the low affinity nerve growth factor receptor enhances β-amyloid peptide toxicity. *Proc. Natl. Acad. Sci. USA.* 91:10703–10706.

13. Yaar, M., M.S. Eller, P. DiBenedetto, W.R. Reenstra, S. Zhai, T. McQuaid, M. Archambault, and B.A. Gilchrest. 1994. The trk family of receptors mediates nerve growth factor and neurotrophin-3 effects in melanocytes. *J. Clin. Invest.* 94:1550–1562.

14. Peacocke, M., M. Yaar, C.P. Mansur, M.V. Chao, and B.A. Gilchrest. 1988. Induction of nerve growth factor receptors on cultured human melanocytes. *Proc. Natl. Acad. Sci. USA.* 85:5282–5286.

15. Gray, C.W., and A.J. Patel. 1995. Neurodegeneration mediated by glutamate and beta-amyloid peptide: a comparison and possible interaction. *Brain Res.* 691:169–179.

16. 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.* 119:493–501.

17. Waugh, S.M., E.E. DiBella, and P.F. Pilch. 1989. Isolation of a proteolytically derived domain of the insulin receptor containing the major site of cross-linking/binding. *Biochemistry.* 28:3448–3455.

18. Baldwin, A.N., and E.M. Shooter. 1995. Zone mapping of the binding domain of the rat low affinity nerve growth factor receptor by the introduction of novel N-glycosylation sites. *J. Biol. Chem.* 270:4594–4602.

19. Mahadeo, D., L. Kaplan, M.V. Chao, and B.L. Hempstead. 1994. High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors. *J. Biol. Chem.* 269:6884–6891.

20. Boland, K., M. Behrens, D. Choi, K. Manias, and D.H. Perlmutter. 1996. The serpin-enzyme complex receptor recognizes soluble, nontoxic amyloid-β peptide but not aggregated, cytotoxic amyloid-β peptide. *J. Biol. Chem.* 271:18032–18044.

21. Selkoe, D.J. 1994. Normal and abnormal biology of the beta-amyloid precursor protein. *Ann. Rev. Neurosci.* 17:489–517.

22. Davis-Salinas, J., and W.E. Van Nostrand. 1995. Amyloid beta-protein aggregation nullifies its pathologic properties in cultured cerebrovascular smooth muscle cells. *J. Biol. Chem.* 270:20887–20890.

23. Pike, C.J., D. Burdick, A.J. Walencewicz, C.G. Glabe, and C.W. Cotman. 1993. Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. *J. Neurosci.* 13:1676–1687.

24. Behl, C., J.B. Davis, R. Lesley, and D. Schubert. 1994. Hydrogen peroxide mediates amyloid beta protein toxicity. *Cell.* 77:817–827.

25. Cotman, C.W., and A.J. Andersen. 1995. A potential role for apoptosis in neurodegeneration and Alzheimer's disease. *Mol. Neurobiol.* 10:19–45.

26. Su, J.H., A.J. Andersen, B.J. Cummings, and C.W. Cotman. 1994. Immunohistochemical evidence for apoptosis in Alzheimer's disease. *Neuroreport.* 5:2529–2533.

27. Behl, C., J.B. Davis, P.G. Klier, and D. Shubert. 1994. Amyloid beta peptide induces necrosis rather than apoptosis. *Brain Res.* 645:253–264.

28. Gschwind, M., J.R. Martin, J.L. Moreau, and G. Huber. 1996. Beta-APP cognitive function versus beta-amyloid-induced cell death. *Ann. NY Acad. Sci.* 77:293–296.

29. Crowley, C., S.D. Spencer, M.C. Nishimura, K.S. Chen, S. Pitts-Meek, M.P. Armanini, L.H. Ling, S.B. MacMahon, D.L. Shelton, A.D. Levinson, and H.S. Phillips. 1994. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell.* 76:1001–1011.

30. Deckwerth, T.L., and E.M. Johnson, Jr. 1993. Temporal analysis of events associated with programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor. *J. Cell Biol.* 123:1207–1222.

31. Selkoe, D.J. 1996. Amyloid β-protein and the genetics of Alzheimer's disease. *J. Biol. Chem.* 271:18295–18298.

32. Nagata, S., and P. Goldstein. 1995. The fas death factor. *Science (Wash. DC).* 267:1449–1456.

33. Goldstein, R.S., C. Avivi, and R. Geffen. 1997. In vivo NGF treatment increases proliferation on the primary sympathetic ganglia of chick embryos. *Dev. Biol.* 181:116–120.

34. Cortazzo, M.H., E.S. Kassis, K.A. Sproul, and N.F. Schor. 1996. Nerve growth factor (NGF)-mediated protection of neural crest cells from antimitotic agent-induced apoptosis: the role of the low-affinity NGF receptor. *J. Neurosci.* 16:3895–3899.

35. Battleman, D.S., A.I. Geller, and M.V. Chao. 1993. HSV-1 vector-mediated gene transfer of the human nerve growth factor receptor p75hNGFR defines high-affinity NGF binding. *J. Neurosci.* 13:941–951.

36. Chao, M.V., and B.L. Hempstead. 1995. p75 and Trk: a two-receptor system. *Trends Neurosci.* 19:321–326.

37. Ruit, K.G., P.A. Osborne, R.E. Schmidt, E.M. Johnson, Jr., and W.D. Snider. 1990. Nerve growth factor regulates sympathetic ganglion cell morphology and survival in the adult mouse. *J. Neurosci.* 10:2412–2419.

38. Manfridi, A., G.L. Forloni, E. Arrigoni-Martelli, and M. Mancia. 1992. Culture of dorsal root ganglion neurons from aged rats: effects of acetyl-L-carnitine and NGF. *Inter. J. Develop. Neurosci.* 10:321–329.

39. Jiang, Z.G., and R.A. Smith. 1993. Effects of nerve growth factor on the survival of primary cultured adult and aged mouse sensory neurons. *J. Neurosci. Res.* 35:29–37.

40. Cheng, B., and M.P. Mattson. 1992. Glucose deprivation elicits neurofibrillary tangle-like antigenic changes in hippocampal neurons: prevention by NGF and bFGF. *Exp. Neurol.* 117:114–123.

41. Cheng, B., and M.P. Mattson. 1991. NGF and bFGF protect rat hippocampal and human cortical neurons against hypoglycemic damage by stabilizing calcium homeostasis. *Neuron.* 7:1031–1041.

42. Kawaja, M.D., M.B. Rosenberg, K. Yoshida, and F.H. Gage. 1992. Somatic gene transfer of nerve growth factor promotes the survival of axotomized septal neurons and the regeneration of their axons in adult rats. *J. Neurosci.* 12: 2849–2864.

43. Henderson, T.A., R.W. Rhoades, C.A. Bennett-Clarke, P.A. Osborne, E.M. Johnson, and M.F. Jacquin. 1993. NGF augmentation rescues trigeminal ganglion and principalis neurons, but not brainstem or cortical whisker patterns, after infraorbital nerve injury at birth. *J. Comparative Neurol.* 336:243–260.

44. Tuszynski, M.H., H.S. U, D.G. Amaral, and F.H. Gage. 1990. Nerve growth factor infusion in the primate brain reduces lesion-induced cholinergic neuronal degeneration. *J. Neurosci.* 10:3604–3614.

45. Shimohama, S., N. Ogawa, Y. Tamura, A. Akaike, T. Tsukahara, H. Iwata, and J. Kimura. 1993. Protective effect of nerve growth factor against glutamate-induced neurotoxicity in cultured cortical neurons. *Brain Res.* 632: 296–302.

46. Seiger, A., A. Nordberg, H. von Holst, L. Backman, T. Ebendal, I. Alafuzoff, K. Amberla, P. Hartvig, A. Herlitz, A. Lilija, et al. 1993. Intracranial infusion of purified nerve growth factor to an Alzheimer patient: the first attempt of a possible future treatment strategy. *Behav. Brain Res.* 57:255–261.

47. Lapchak, P.A. 1993. Nerve growth factor pharmacology: application to

the treatment of cholinergic neurodegeneration in Alzheimer's disease. *Exp. Neurol.* 124:16–20.

48. Olson, L., A. Nordberg, H. von Holst, L. Backman, T. Ebendal, I. Alafuzoff, K. Amberla, P. Hartvig, A. Herlitz, A. Lilija, et al. 1992. Nerve growth factor affects 11C-nicotine binding, blood flow, EEG, and verbal episodic memory in an Alzheimer patient (case report). *J. Neural. Transm. Parkinson's Dis. Dementia. Sect.* 4:79–95.

49. Zhai, S., M. Yaar, S. Doyle, and B.A. Gilchrest. 1996. Nerve growth factor rescues pigment cells from ultraviolet-induced apoptosis by up-regulating BCL-2 levels. *Exp. Cell Res.* 224:335–343.

50. Takahashi, Y. 1992. Gene expression in cells of the central nervous system. *Prog. Neurobiol. (NY).* 38:523–569.

51. Gall, C.M., and P.J. Isackson. 1989. Limbic seizures increase neuronal production of messenger RNA for nerve growth factor. *Science (Wash. DC).* 245:758–761.

52. Larkfors, L., T. Ebendal, S.R. Whittemore, H. Persson, B. Hoffer, and L. Olson. 1987. Decreased level of nerve growth factor (NGF) and its messenger RNA in the aged rat brain. *Brain Res.* 427:55–60.

53. Shi, D.Y., Z. Huaijie, J. Fu, S.F. Yan, A. Roher, W.W. Tourtellotte, T. Rajavashisth, X. Chen, G.C. Godman, D. Stern, and A.M. Schmidt. 1997. Amyloid-β peptide-receptor for advanced glycation endproduct interaction elicits neuronal expression of macrophage-colony stimulating factor: a proinflammatory pathway in Alzheimer disease. *Proc. Natl. Acad. Sci. USA.* 94:5296–5301.

54. Yan, S.D., X. Chen, J. Fu, M. Chen, H. Zhu, A. Roher, T. Slattery, L. Zhao, M. Nagashima, J. Morser, et al. 1996. RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. *Nature (Lond.).* 382:685–691.

55. Khoury, J.E., S.E. Hickman, C.A. Thomas, L. Cao, S.C. Silverstein, and J.D. Loike. 1996. Scavenger receptor-mediated adhesion of microglia to β-amyloid fibrils. *Nature (Lond.).* 382:716–719.

56. Paresce, D.M., R.N. Ghosh, and F.R. Maxfield. 1996. Microglial cells internalize aggregates of the Alzheimer's disease amyloid beta-protein via a scavenger receptor. *Neuron.* 17:553–565.

57. Yankner, B.A., A. Caceres, and L.K. Duffy. 1990. Nerve growth factor potentiates the neurotoxicity of beta amyloid. *Proc. Natl. Acad. Sci. USA.* 87: 9020–9023.

58. Benedetti, M., A. Levi, and M.V. Chao. 1993. Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. *Proc. Natl. Acad. Sci. USA.* 90:7859–7863.