Role of p75 Neurotrophin Receptor in the Neurotoxicity by β-amyloid Peptides and Synergistic Effect of Inflammatory Cytokines

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Abstract

The neurodegenerative changes in Alzheimer’s disease (AD) are elicited by the accumulation of β-amyloid peptides (Aβ), which damage neurons either directly by interacting with components of the cell surface to trigger cell death signaling or indirectly by activating astrocytes and microglia to produce inflammatory mediators. It has been recently proposed that the p75 neurotrophin receptor (p75NTR) is responsible for neuronal damage by interacting with Aβ. By using neuroblastoma cell clones lacking the expression of all neurotrophin receptors or engineered to express full-length or various truncated forms of p75NTR, we could show that p75NTR is involved in the direct signaling of cell death by Aβ via the function of its death domain. This signaling leads to the activation of caspases-8 and -3, the production of reactive oxygen intermediates and the induction of an oxidative stress. We also found that the direct and indirect (inflammatory) mechanisms of neuronal damage by Aβ could act synergistically. In fact, TNF-α and IL-1β, cytokines produced by Aβ-activated microglia, could potentiate the neurotoxic action of Aβ mediated by p75NTR signaling. Together, our results indicate that neurons expressing p75NTR, mostly if expressing also proinflammatory cytokine receptors, might be preferential targets of the cytotoxic action of Aβ in AD.

Key words: p75NTR • cell death • human neuroblastoma cells • cytokines • Alzheimer’s disease

Introduction

Alzheimer’s disease (AD)* is characterized by progressive loss of neurons, formation of fibrillary tangles within neurons, and numerous plaques in affected brain regions. According to the “β-amyloid cascade hypothesis,” the key pathogenetic event responsible for the degenerative changes in neurons is the excessive formation and/or accumulation of fibrillar β-amyloid peptides (Aβ), a set of 39–43 amino acid (aa) peptides derived from the cleavage by β- and γ-secretases of a membrane glycoprotein, named β-amyloid precursor protein (APP) (1–3). Aβ are neurotoxic in vitro, and this cytotoxicity correlates with their β-sheet structure and fibrillar state (4–6). However, recent findings have shown that not only fibrils, but even protofibrils and small soluble oligomers of Aβ can be neurotoxic (7).

Two main mechanisms have been postulated to be responsible for the neurotoxicity by Aβ: (i) Aβ may interact with components of cell membranes and thus injure neurons directly (4–8) and/or enhance the vulnerability of neurons by a variety of common insults, such as excitotoxicity, hypoglycemia, or peroxidative damage (9); (ii) Aβ may damage neurons indirectly by activating microglia and astrocytes to produce toxic and inflammatory mediators, such as nitric oxide (NO), cytokines, and reactive oxygen intermediates (ROI) (10–16).

*Abbreviations used in this paper: α7NACHr, α-7-nicotinic acetylcholine receptor; aa, amino acid; Aβ, β-amyloid peptide; AD, Alzheimer’s disease; AO, acridine orange; APP, β-amyloid precursor protein; BBP, β-amyloid-binding protein; DD, death domain; DPI, diphenyleneiodonium; EB, ethidium bromide; JICD, juxtamembrane domain; NGF, nerve growth factor; NO, nitric oxide; p75NTR, neurotrophin receptor p75; RAGE, advanced glycation endproducts receptors; ROI, reactive oxygen intermediates; Trk, tropomyosin-related kinase.
The mechanisms by which A\beta interact with the cell surface remain to be clarified. Besides interacting with phospholipids of cellular plasmamembrane and forming selective cation channels and/or disrupting membrane integrity by virtue of their lipophilic nature (17–19), A\beta bind to a variety of cell surface receptors, such as scavenger receptors (13) and NH\textsubscript{2}-formylpeptide receptor 2 in microglia (20), advanced glycation end products receptors (RAGE) in neurons and microglia (21), serpin-enzyme complex receptor (22), α-7-nicotinic acetylcholine receptor (\alpha\textsubscript{7}nAChR) (23), neurotrophin receptor p75 (p75\textsubscript{NTR}) (24–25), amyloid precursor protein (APP) (26) and a β-amylloid binding protein (BBP) containing a G protein-coupling module (27) in neurons. Some of these binding interactions (21, 23–27) have been correlated with the direct neurotoxicity of A\beta. The multiplicity of the receptors involved raises the problem of the specificity of their interactions with A\beta and active roles in signaling cell death.

p75\textsubscript{NTR} binds NGF and the other neurotrophins (28) and belongs to the family of death receptors (29, 30). In recent years, several groups have shown that p75\textsubscript{NTR} mediates both ligand-dependent and ligand-independent apoptosis (31–37) including that by A\beta (24, 25). Furthermore, the cholinergic neurons of the basal forebrain, which are A\beta-sensitive (13) and NH\textsubscript{2}-formylpeptide receptor 2 in microglia (20), advanced glycation end products receptors (RAGE) in neurons and microglia (21), serpin-enzyme complex receptor (22), α-7-nicotinic acetylcholine receptor (\alpha\textsubscript{7}nAChR) (23), neurotrophin receptor p75 (p75\textsubscript{NTR}) (24–25), amyloid precursor protein (APP) (26) and a β-amylloid binding protein (BBP) containing a G protein-coupling module (27) in neurons. Some of these binding interactions (21, 23–27) have been correlated with the direct neurotoxicity of A\beta. The multiplicity of the receptors involved raises the problem of the specificity of their interactions with A\beta and active roles in signaling cell death.

Materials and Methods

A\beta-Peptides. A\beta(25–35), A\beta(1–40), A\beta(1–42), and A\beta(35–25) were from Bachem AG. A\beta(25–35) was dissolved at 1.5 mM in PBS, A\beta(1–40) at 1.5 mM in double-distilled water to be next diluted at 250 μM in PBS, and A\beta(1–42) at 500 μM in double-distilled water. Fibrillogenesis by A\beta(25–35) was rapid (minutes) at room temperature, whereas A\beta(1–40) and A\beta(1–42) required 5–6 d at 37°C. A\beta(35–25) was dissolved as A\beta(25–35), but did not form fibrils. Fibrillogenesis was monitored by thioflavine test (16) before the experiments. When A\beta were dissolved in DMSO they did not form fibrils and remain in solution.

p75\textsubscript{NTR} and Tropomyosin-related Kinase A Constructs. The construct encoding for the wild-type (wt) p75\textsubscript{NTR} (pCEP4\textsubscript{p75}) was generated by cloning the full-length human p75\textsubscript{NTR} cDNA into the PvuII site of the pCEP4\beta mammalian expression vector which carries the hygro resistance gene (see Fig 1 A; Invitrogen). The p75\textsubscript{DD} mutant, lacking aa from 352 to 427, was generated according to Hantzopoulos (53). The other deletion mutants p75\textsubscript{JICD}, p75\textsubscript{jadICD}, and p75\textsubscript{jadIC} were obtained by PCR using specific primers and cloning the respective products into the pCEP4\beta vector. Tropomyosin-related kinase (Trk)A expression plasmid was obtained by inserting the full-length cDNA encoding for the human TrkA receptor (54) into the episomal expression vector pCEP4\beta which carries the neo resistance gene.

Cell Clones. The human neuroblastoma SK-N-BE cell line, which expresses neither p75\textsubscript{NTR} nor TrkA (BENTR-free) (34) was grown in RPMI 1640 medium (BioWhittaker) containing FBS (15% vol/vol; Life Technologies, Inc.), glutamine (2.0 mM), and gentamycin (50 μg/ml) and transfected by the liposome technique (Lipofectin Reagent; GIBCO BRL) (55) with 10 μg of each of the p75 constructs or with the TrkA codifying plasmid. As control BENTR-free cells were also transfected with the two empty vectors. Transfected cells were selected in complete medium containing either hygromycin (150 μg/ml) or G418 (300 μg/ml) (Roche Molecular Biochemicals). The antibiotic-resistant clones were characterized for expression of wt and mutated p75\textsubscript{NTR} proteins or the wt TrkA protein. The SK-N-BE-derived cell clones generated were (see Fig 1): (i) BEp75 expressing the full-length p75\textsubscript{NTR}; (ii) BEp75\textsubscript{jadICD} lacking the fourth cysteine-rich repeat of the extracellular domain (aa 36–230); (iii) BEp75\textsubscript{jadICD} lacking the whole intracellular region (aa 280–427); (iv) BEp75\textsubscript{jadDD}, lacking the DD (aa 352–427); (v) BEp75\textsubscript{jadICD} missing the intracellular JICD (aa 275–340); and (vi) BETrka expressing the full-length TrkA protein. BETrka was further transfected with the plasmid encoding the full-length p75\textsubscript{NTR} and derived cell clones (BEp75TrkA) were selected with both hygromycin and G418.

Western Immunoblot and Immunocytochemistry Analysis. Immunoblotting was used to test the cellular levels of the various forms of p75\textsubscript{NTR} and TrkA. Cells were lysed, fractioned by 8% SDS-PAGE and transferred onto nitrocellulose filters as described previously (34). Nitrocellulose filters were probed with one of the following antibodies: (i) anti-p75\textsubscript{NTR} 9992 polyclonal antiserum.
raised against the intracellular region (provided by M.V. Chao, New York University School of Medicine, New York, NY) (see Fig. 1 B); (ii) anti-TrkA rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.). The p75<sub>NTR</sub> and TrkA proteins were detected with a HRP-conjugated secondary antibody (Amersham Pharmacia Biotech) and revealed by the ECL method (Amersham Pharmacia Biotech). The expression level of p75<sub>NTR</sub> in BeP75 cell clones was 3–5-fold higher than in PC12 cells (34). The localization in the plasmamembrane of the various p75<sub>NTR</sub> and TrkA proteins was detected immunohistochemically using either the mAb ME20.4 (a gift from M.V. Chao) raised against the p75<sub>NTR</sub> extracellular domain or polyclonal antisem 9992 (see Fig. 1 C) or anti-TrkA rabbit polyclonal antibody as described previously (34).

**Experimental Protocol.** Cell clones were plated at 12,500 cells/cm<sup>2</sup> for microscopic analysis and at 30,000 cells/cm<sup>2</sup> for MTS assay. At the onset of the experimental treatments, the growth medium was replaced with a fresh complete RPMI 1640 medium containing 1% (vol/vol) PBS. Cultures were then exposed for various times to (i) Aβ peptides (1–42 or 1–40 or 25–35) in fibrillary state, (ii) human recombinant nerve growth factor-β (hrNGF-β) (Sigma-Aldrich), (iii) anti–human p75<sub>NTR</sub> mAb 8211 (Chemicon Int., Inc.) (56); or (iv) staurosporine (Calbiochem). In some instances, these treatments were also preceded by 2-h exposure to one of the following agents: Z-VD-FMK (100 μM; Calbiochem), a nonspecific inhibitor of caspases; Z-IETD-FMK (20 μM; Calbiochem), a specific inhibitor of caspase-8; human recombinant TNF-α (10 ng/ml hrTNF-α) or 20 ng/ml IL-1β (PeproTech EC Ltd.); or 100 nM diphenylethionediode (DPI) (Sigma-Aldrich). All the experiments throughout the work were performed by using 20 μM Aβ(25–35) or 5 μM Aβ(1–40) and Aβ(1–42) since, on the basis of preliminary experiments, these concentrations correspond to those giving the maximal cytotoxicity in our experimental conditions. However, the cytotoxic effect of Aβ started to be detectable at a rather low concentration of Aβ (~100 nM).

**Assessment of Cell Damage and Viability.** Cell damage was analyzed by means of epifluorescence microscopy after staining the cells with a solution 1:1 (vol/vol) of acridine orange (AO; filter setting for FITC) and ethidium bromide (EB; filter setting for rhodamine) (both at 0.1 mg/ml in PBS; Molecular Probes), a procedure that reveals both apoptosis and necrosis (57). Annexin V–FITC binding test (Roche Molecular Biochemicals) evaluated by epifluorescence microscopy was also used for the detection of V-FITC binding test (Roche Molecular Biochemicals) evaluated by epifluorescence microscopy after staining the suspension were first treated for 1 h at 4°C with primary mAb anti–TNFR55 H398 (donated by P. Scheurich, University of Stuttgart, Stuttgart Germany), mAb anti–TNFR75 utr-1 (Bachem; Peninsula Laboratories, Inc.) or mAb anti–IL-1R1 (a gift from A. Mantovani, Istituto Mario Negri, Milano, Italy). After cell washing, the secondary biotin-conjugated IgG (Sigma-Aldrich) was added for 30 min at 4°C, followed by several washings and addition of 10 μl of streptavidin–phycoerythrin (Sigma-Aldrich). Cytofluorographic analysis was performed on a FACSscan™ (Becton Dickinson) using CELLQuest™ software.

**Statistical Analysis.** Multiple data points were compared by one-way ANOVA test with posthoc Dunnett multiple comparison test. The interaction between Aβ and TNF-α or IL-1β was determined by two-way ANOVA. All statistical tests were performed by SPSS 10 statistical package (SPSS, Inc.).

**Results.**

**Expression of p75<sub>NTR</sub> and the Cytotoxicity of Aβ.** We first investigated the effect of these peptides on the BENTR-free cells (34), and on BeP75 (Fig. 1). Our results showed that Aβ(25–35), Aβ(1–40), and Aβ(1–42) were able to induce cell death in BeP75 cells, while being totally harmless for BENTR-free cells (Fig. 2 and Table I) or BENTR-free cells transfected with an empty pCEP4β vector (data not shown). The morphologic assessment (Fig. 2) of cell damage showed that, in our experimental conditions, Aβ induced cell death via both apoptosis and necrosis, as reported previously (24, 58, 59). Aβ were toxic only in a fibrillar state, as previously shown (4–6). Reverse order Aβ were harmless (not shown).

We also investigated the role of NGF receptor TrkA by examining the effect of Aβ on cell clones expressing TrkA only (BETrKA), or on cell clones expressing both TrkA and full-length p75<sub>NTR</sub> (BeP75TrkA). The results (Table I) show that BETrKa clones were insensitive to the toxic action by Aβ, whereas BeP75TrkA clones were sensitive to the action of Aβ to the same extent as were BeP75 clones.

The cytotoxic effect of Aβ was further verified using MTS reduction test. The data in Fig. 3 show that the MTS assay gave results similar to those obtained by using the double-staining epifluorescence method.

**Signaling for Cell Death Induced by Aβ via p75<sub>NTR</sub>.** Our results show that the toxicity by Aβ was associated with the activation of both caspase-8 and caspase-3 in BeP75 cells (Fig. 4 A). A role for these caspases in Aβ-induced, p75<sub>NTR</sub>-mediated cell death was further supported by the finding (Fig. 4 B) that Aβ neurotoxicity was prevented by Z-VD-FMK, a nonspecific inhibitor of caspases, and by Z-IETD-FMK, a specific inhibitor of caspase-8. In the same experiments, the cell death induced by staurosporine, a well known protein kinase inhibitor that induces apoptosis, was prevented by the unspecific inhibitor of caspases, but could not be suppressed by the inhibitor of caspase-8. The cytotoxic effect by Aβ was also prevented by DPI (Fig. 4 B), an inhibitor of oxygen-free radicals forming NADPH oxidase and of other flavoprotein dehydrogenases (16) indicating that p75<sub>NTR</sub>-mediated cell death induced by Aβ was associated with the activation of ROI sources and oxidative stress.

The Extracellular Region of p75<sub>NTR</sub> Is Necessary for the Cytotoxic Effect. Two mechanisms might be responsible for the role of p75<sub>NTR</sub> in the cytotoxicity by Aβ: (i) p75<sub>NTR</sub> is per-
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Figure 1. Expression of p75NTR in SK-N-BE neuroblastoma clones. (A) Schematic depiction of the full-length and truncated p75NTR proteins expressed in transfected SK-N-BE clones. Specifically, p75NTR, full-length receptor; p75ΔECD, p75 lacking the extracellular region (aa 36–230); p75ΔICD, p75 lacking the whole cytoplasmatic region (aa 280–427); p75ΔDD, p75 lacking the intracellular DD (aa 352–427); p75ΔJICD, p75 lacking the cytoplasmic JICD (aa 275–340). TM, transmembrane region. (B) p75NTR protein levels (Western blot analysis) in BENTR-free cell clones transfected with different constructs of p75NTR. (C) Localization of the p75NTR protein at the plasma membrane by immunostaining with 9992 antiserum in BENTR-free, BEp75, BEp75ΔECD, BEp75ΔDD, and BEp75ΔJICD cell clones, and with mAb ME20.4 in BEp75ΔICD cell clones; the detection was performed by Cy3-conjugated anti-rabbit IgG or anti-mouse IgG; nuclei are blue-stained with DAPI.

Figure 2. Epifluorescence microscopic analysis of cell damage by Aβ. (A1) and (A2) BENTR-free cells, untreated and treated for 24 h with Aβ(25–35) (20 μM), respectively. (B1) and (B2) BEp75 cells, untreated and treated for 24 h with Aβ(25–35) (20 μM), respectively. A pale green nuclear fluorescence by AO identifies still normal cells. A dazzling yellow nuclear fluorescence (arrowheads) reveals the progressive chromatin condensation, collapse, and marginalization proper of apoptosis. A vivid red fluorescence of chromatin remnants by EB (arrows) denotes cells, whose membrane integrity was lost as the death process shifted from apoptosis to necrosis. +, mitosis.
cell damage examined by means of the double staining with AO and EB were similar to those by Aβ/H9252 (Fig. 6 A). Furthermore the cells treated with NGF or mAb appeared Annexin V-positive indicating the presence of an apoptotic process (Fig. 6 B). The signals for cell death triggered by the binding of NGF or mAb to the extracellular region of p75NTR appear to be similar to those triggered by the binding of Aβ/H9252. In fact, also the cell death by NGF or mAb was inhibited by Z-VAD-FMK, a nonspecific inhibitor of caspases, by Z-IETD-FMK, the specific inhibitor of caspase-8, and by DPI, an inhibitor of ROI-forming NADPH oxidase and other flavin-dehydrogenases (Fig. 4 B).

To clarify the relations between the mechanisms of p75NTR activation by Aβ/H9252, NGF or mAb, we investigated the effect of Aβ/H9252 in BEp75 cells, whose p75 NTR receptor had been previously occupied by NGF or mAb. The results (Fig. 5) show that Aβ/H9252, NGF, or mAb exerted cytotoxic activities of comparable magnitude when added each by itself, but when given serially — mAb first and Aβ next — their cytotoxic effects were neither additive nor synergistic. These findings suggest that Aβ, NGF, and mAb act via a similar mechanism by binding the same or closely related sequences of the extracellular region of p75NTR and thereby triggering an alike activation of the receptor.

### Table I. Cell Death Induced by Aβ Peptides

|                     | BENTR-free | BEp75 | BEp75TrkA |
|---------------------|------------|-------|------------|
|                     | 24 h       | 48 h  | 24 h       | 48 h       | 24 h       | 24 h       |
| Controls            | 5.9 ± 2.6* (18) | 7.8 ± 2.6 (18) | 8.2 ± 2.2 (62) | 9.2 ± 2.4 (20) | 5.3 ± 2.5 (3) | 10.3 ± 2.1 (3) |
| Aβ(25–35)           | 5.2 ± 2.6 (12) | 6.2 ± 2.8 (12) | 29.7 ± 4.5* (65) | 34.0 ± 4.8* (14) | 7.1 ± 3.1 (5) | 28.5 ± 6.3* (5) |
| Aβ(1–40)            | 5.6 ± 2.3 (5) | 5.5 ± 2.8 (4) | 27.6 ± 2.3* (5) | 33.2 ± 6.2* (4) |
| Aβ(1–42)            | 5.6 ± 1.6 (5) | 7.3 ± 1.8 (4) | 30.0 ± 2.3* (3) | 30.9 ± 2.2* (4) |

Cells were treated with Aβ(25–35) (20 μM), Aβ(1–40) (5.0 μM), or Aβ(1–42) (5.0 μM) and cell death was assessed by epifluorescence microscopy after 24 and 48 h.

*The values express percentages of cell death and are means ±SD of the experiments indicated within brackets.

**P < 0.001 with respect to the controls of the corresponding time point.
Role of the Intracellular Region of p75NTR in the Cytotoxicity by Aβ, mAb 8211, and NGF. The results so far presented, showing that Aβ are cytotoxic by binding to the extracellular region of p75NTR, raise the problem whether Aβ–binding activates p75NTR and triggers cell death via the receptor’s intracellular region, or uses p75NTR as an anchor allowing the induction of cell damage via other mechanisms. To solve this problem, we investigated the effect of Aβ on BEp75ΔJICD cell clones expressing a truncated p75NTR devoid of the entire intracellular region (Fig. 1).

The results (Fig. 5) show that these cells were insensitive to the toxic effects of Aβ, NGF, or mAb 8211, indicating that p75NTR directly participates to the cell damage by these ligands via the signaling function of its intracellular region.

We next investigated the roles played in p75NTR-dependent cell death by the DD and the JICD domains. In spite of many studies (41, 46–52), the respective functions of these two domains remain unclear. We challenged with Aβ, mAb 8211, or NGF BEp75ΔDD cell clones expressing a truncated p75NTR devoid of the largest part of the DD (Fig. 1). The results (Fig. 5) show that these cells were insensitive to the toxic actions of Aβ, mAb 8211, or NGF, demonstrating that the ligand-induced p75NTR-mediated cell death does require the function of the DD. As a control, we found that staurosporine could induce cell death in all the cell clones expressing various truncated forms of p75NTR (Fig. 5), indicating that these clones remained susceptible to apoptogenic agents, whose activity is independent of p75NTR signaling. To understand the role of the juxtamembrane region, we treated BEp75ΔJICD cell clones expressing a truncated p75NTR lacking the whole JICD with Aβ, mAb 8211, or NGF (Fig. 1). The results (Fig. 5) show that these cells were sensitive to the cytotoxic effects of all three ligands, just as BEp75 cells were, indicating that the function of the JICD is not involved in the death signaling triggered by such agonists. Here it is worth noting that, in the absence of agonists, BEp75ΔJICD clones exhibited a far higher level of spontaneous mortality than did all the other clones we tested (Fig. 5).

TNF-α and IL-1β Synergize with the Aβ Neurotoxicity Mediated by p75NTR. Several studies have reported that in AD, besides a direct effect of Aβ on neurons, cell death is due also to an inflammatory reaction mainly correlated with the activation of microglia and astrocytes by Aβ to produce inflammatory mediators, including NO, ROI, IL-1β, IL-6, TNF-α, and monocyte chemoattractant protein 1 (10–16). The role of TNF in brain injury and neurodegenerative diseases is still controversial (62–65). Since neurons within AD plaques are attacked by Aβ, TNF-α, and other cytokines, we investigated the effects of TNF-α on the Aβ–induced p75NTR-mediated neurotoxicity. For this purpose we pretreated with this cytokine and then with Aβ(25–35) BEp75 cell clones, which express TNF receptors (Fig. 7 A). The results show that (i) TNF-α by itself exerted a slight cytotoxic action and could synergistically potentiate the toxic effect by Aβ (Fig. 7 B); (ii) the effects of both TNF-α by itself and TNF-α plus Aβ were inhibited by the inhibitor of caspase-8, Z-IETD-FMK (20 μM; Fig. 7 C).

To understand if the synergistic effect is specific of TNF-α we investigated the activity of IL-1β, another cy-
I. The rationale of the first problem is based on the findings that Aβ-induced cell damage associates with the presence of p75NTR on the cell surface (24–25). We first confirmed (Table I) these findings by using an experimental model consisting in the treatment with Aβ of BENTR-free cell clones devoid of all the neurotrophin receptors and clones expressing full-length p75NTR.

Once we confirmed that p75NTR is necessary for the toxic action of Aβ, we tried to clarify the mechanism by which p75NTR works. Three findings demonstrate that the first step of this mechanism is the interaction of Aβ with the external region of this receptor. (i) Consistently with the previous findings by others (24, 25) that Aβ bind to p75NTR, BEp75ΔECD cells, which are devoid of the four cysteine-rich repeats of the extracellular region of the receptor, were insensitive to the cytotoxic action of Aβ; (ii)
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NGF and mAb 8211, which interacts with the region including the binding site of NGF, mimicked the cytotoxic effect of Aβ in BEp75 cells expressing the full-length p75NTR, but were harmless for BEp75ΔECD cells, whose p75NTR lacks the four cysteine-rich repeats; and (iii) the pretreatment of BEp75 cell clones with NGF or mAb 8211 did not elicit any additive toxic effect by Aβ, likely due to an hindrance to the binding of Aβ, as suggested by previous results showing that NGF displaced bound Aβ from p75NTR (25, 26).

We do not know what is the precise domain of the extracellular region of p75NTR responsible for the binding of Aβ. The fact that Aβ bind different and structurally unrelated receptors (13, 20–27) might suggest that such interactions occur in nonspecific ways (66) and that the binding of Aβ to p75NTR takes place in a manner differing from the recently described interaction between NGF and p75NTR (67). This raises the problem of whether the binding between fibrillar Aβ and p75NTR might activate this receptor or only permit the tethering of Aβ to the cell membrane and its subsequent toxic activity independently of any activation of p75NTR. The finding that BEp75ΔICD cells, retaining the extracellular binding region but lacking the whole intracellular region, were insensitive to the action of Aβ clearly demonstrates that, in our experimental model, cell death by Aβ requires the activation of p75NTR and its signaling via the intracellular region. The two main portions of the intracellular region are the DD and the JICD but the functions of these domains are not yet understood. Recently, various factors have been identified that interact with different sequences of the intracellular region of p75NTR and are thus potentially involved in signal transduction, i.e., TRAF family proteins, of which TRAF-2 interacts with the helical COOH-terminal region corresponding to the DD, and TRAF-4 and TRAF-6 interact with the JICD region (46, 50); FAP-1, which binds to the intracellular region at a COOH-terminal Ser-Pro-Val residue (47); NRIF, which interacts with two discrete sequences, the JICD and the DD (49); SC-1, a zinc finger protein (51), and NRAGE (52), both of which bind to the JICD region; and NADE (48), RhoA (68) and RIP2 (69) which bind to the DD. Some data indicate that the JICD region, but not the DD, is required for neuronal death in an experimental model, in which a ligand-independent kind of apoptosis is induced by an overexpressed p75NTR (41). We have investigated whether the JICD region were involved in cell death by Aβ, NGF, or mAb 8211, and our results show (Fig. 5) that this is not the case, because all the three agonists were toxic for BEp75/JICD cells expressing a JICD-devoid p75 NTR. The reasons for the discrepancy between our results and those of others (41) remain to be investigated. The different experimental conditions, i.e., a ligand-independent apoptotic stimulus in p75NTR-overexpressing cells (41), and, as in our case, ligand-dependent apoptotic stimuli in cells overexpressing p75NTR along with the specific composition of p75NTR interactors within these cells, could be responsible for such a discrepancy. Interestingly, in 1% serum medium, BEp75/JICD cell clones, expressing a p75NTR devoid of most of the JICD, exhibited a spontaneous greater mortality than BEp75 cell clones. This indicates that, under our experimental conditions, the JICD is necessary for the optimal survival of BEp75 cells.
Regarding the role of the DD of p75NTR, our results show that this domain is necessary for the cell death induced by Aβ. In fact, BEp75ΔDD cell clones were insensitive to the cytotoxic effects by Aβ, NGF, or mAb 8211. These results agree with those showing that the DD of p75NTR is involved in ligand (NGF)-dependent p75NTR-mediated cell death via the binding (at 338–396) of the cell death executor protein NADE (48), and in cell death by serum withdrawal (70) or by p75NTR-induced expression (71).

In conclusion, on the basis of the previous results showing that Aβ bind to p75NTR (24, 25) and of those presented here, we propose that the mechanism of p75NTR-mediated cell death by Aβ occurs through a cascade of biochemical processes signaled by the receptor DD. Among these processes we have identified the oxidative stress (Fig. 4 B) and the activation of caspase-8 and -3 (Fig. 4 A), the former being the proteolytic enzyme mediating signal transduction downstream the death receptors family (72). Many studies have been performed on the role of caspases in neuronal cell death by Aβ and the results are not conclusive, because several caspases were found to be activated, i.e., caspase-2 (73), caspase-3 (74), caspase-8 (75, 76), caspase-12 (77), and caspases-2, -3, and -6 (78). Conversely, caspases-3, -6, and -9, but not caspase-8, were found to be activated during apoptosis by induction of p75NTR expression (71), and caspases-1, -2, -3, but not -8, in cell death by p75NTR-bound NGF (79). The reasons for such discrepancies remain unclear. In any case, the finding that the specific inhibition of caspase-8 prevented cell death by Aβ (Fig. 4 B) could indicate that in our experimental model caspase-8 acts upstream in the cell death signaling.

II. As previously mentioned, it has been suggested that in AD an inflammatory reaction, which does not involve the migration of blood cells, but only the local production of cytokines and other mediators by glial cells, contributes primarily to tissue damage and to Aβ formation (14, 16, 65). Herein we have shown that this inflammatory reaction can cooperate with the direct mechanism of cytotoxicity by Aβ. In fact, TNF-α and IL-1β can synergistically potentiate the ability of Aβ to induce death in neuronal cells expressing the full-length p75NTR (Fig. 7). The finding that inflammatory mediators, produced by Aβ-activated microglia and astrocytes, were able to synergize with the p75NTR-mediated toxicity by Aβ is of relevance for the pathogenesis of neuronal damage in AD. In fact, the exposure of p75NTR-expressing neurons to Aβ fibrils and to TNF-α and/or IL-1β mimics the condition occurring in the brain of AD, in which both the direct and indirect mechanisms of cell damage are present and work concurrently. Thus, the death signals triggered by p75NTR could be a unifying pathway upon which converge the effects of both Aβ and inflammatory cytokines. It will be of interest to investigate if other cytokines produced by glial cells activated by Aβ (10–16) have a synergistic effect similar to TNF-α and IL-1β.

III. The findings that p75NTR is involved in neurotoxicity by Aβ raise some problems worth to be investigated, such as the type of interaction between p75NTR and Aβ,
the structural changes of the receptor triggered by the bound Aβ corresponding to the assumption of an activated state, and the other mechanisms, besides those of the activation of caspases and oxidative stress, by which neuronal death is enacted (40, 80).

Another problem, relevant to the pathogenesis of neurodegeneration in AD, is the actual role of p75NTR in neuronal damage in vivo. The results of the in vitro experiments presented here and in other reports (24, 25), and the correlation between the expression of p75NTR and the vulnerability of the cholinergic neurons in the brain of AD patients (38, 39) are in keeping with an involvement of this receptor. However, one cannot underestimate the fact that Aβ can nonspecifically interact with several proteins (66), and that in vitro Aβ can induce cell death by interacting also with other receptors of neuronal surface, such as RAGE (21), α7NACHR (23), and APP (26), or with additional molecules, such as phospholipids and gangliosides (19). Furthermore, our finding that the activity of caspase-8 is stimulated by Aβ, supports the concept that Aβ activates a receptor-mediated, rather than a stress-mediated, cell death pathway. Thus, in AD, alongside with p75NTR, it is likely that also other receptors or interactors be involved, depending on their distribution and level of surface expression and on the types and functional states of the neurons characteristic of the brain regions where the extracellular formation of fibrillar aggregates can be favored. However, the results presented here indicate that neurons expressing p75NTR might be preferential targets of the toxic activity of Aβ, especially if they express also receptors of TNF or other cytokines.

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Due to a production error, the column heading of BETrkA was missing. The corrected table appears below.

Table I.  

|                   | BENTR-free | BEp75 | BETrkA | BEp75TrkA |
|-------------------|------------|-------|--------|------------|
|                   | 24 h       | 48 h  | 24 h   | 24 h       |
| Controls          | 5.9 ± 2.6  | 7.8 ± 2.6 | 8.2 ± 2.2 | 9.2 ± 2.4  | 5.3 ± 2.5 | 10.3 ± 2.1 |
| Aβ(25–35)         | 5.2 ± 2.6  | 6.2 ± 2.8 | 29.7 ± 4.5 | 34.0 ± 4.8  | 7.1 ± 3.1 | 28.5 ± 6.3  |
| Aβ(1–40)          | 5.6 ± 2.3  | 5.5 ± 2.8 | 27.6 ± 2.3 | 33.2 ± 6.2  |          |            |
| Aβ(1–42)          | 5.6 ± 1.6  | 7.3 ± 1.8 | 30.0 ± 2.5 | 30.9 ± 2.2  |          |            |

Cells were treated with Aβ(25–35) (20 μM), Aβ(1–40) (5.0 μM), or Aβ(1–42) (5.0 μM) and cell death was assessed by epifluorescence microscopy after 24 and 48 h.

The values express percentages of cell death and are means ± SD of the experiments indicated within brackets.

*P < 0.001 with respect to the controls of the corresponding time point.