Published in final edited form as:

Ann Neurol. 2009 March ; 65(3): 294–303. doi:10.1002/ana.21578.

Selective Vulnerability in Alzheimer's Disease: Amyloid Precursor Protein and p75^{NTR} Interaction

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Abstract

Objective—Selective neuronal vulnerability in neurodegenerative diseases is poorly understood. In Alzheimer's disease, the basal forebrain cholinergic neurons are selectively vulnerable, putatively because of their expression of the cell death mediator p75^{NTR} (the common neurotrophin receptor), and its interaction with proapoptotic ligands pro-nerve growth factor and amyloid- β peptide. However, the relation between amyloid precursor protein (APP) and p75^{NTR} has not been described previously.

Methods—APP and p75^{NTR} were assayed for interaction by coimmunoprecipitation in vitro and in vivo, yeast two-hybrid assay, bioluminescence resonance energy transfer, and confocal microscopy. Effects on APP processing and signaling were studied using immunoblotting, enzyme-linked immunosorbent assays, and luciferase reporter assays.

Results—The results of this study are as follows: (1) p75^{NTR} and APP interact directly; (2) this interaction is modified by ligands nerve growth factor and β -amyloid; (3) APP and p75^{NTR} colocalization in vivo is modified in Alzheimer's model transgenic mice; (4) APP processing is altered by p75^{NTR}, and to a lesser extent, p75^{NTR} processing is altered by the presence of APP; (5) APP-dependent transcription mediated by Fe65 is blocked by p75^{NTR}; and (6) coexpression of APP and p75^{NTR} triggers cell death.

Interpretation—These results provide new insight into the emerging signaling network that mediates the Alzheimer's phenotype and into the mechanism of basal forebrain cholinergic neuronal selective vulnerability. In addition, the results argue that the interaction between APP and p75^{NTR} may represent a therapeutic target in Alzheimer's disease.

The common neurotrophin receptor, p75^{NTR}, has been implicated as a potential mediator of Alzheimer's disease pathogenesis in several different ways. First, p75^{NTR} expression is highly restricted in the adult nervous system, and its main site of expression, the basal forebrain cholinergic neurons, represents a selectively vulnerable region in Alzheimer's disease.^{1–3} Second, p75^{NTR} mediates programmed cell death,^{4–7} and the expression of p75^{NTR} sensitizes cells to β -amyloid (A β) toxicity.⁸ Third, A β has been shown to interact directly with p75^{NTR},⁹ and this interaction may lead to apoptosis induction.^{9,10} Fourth,

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Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

p75^{NTR} has been shown to mediate apoptosis in response to pro-nerve growth factor (pro-NGF), which is increased in the brains of patients with Alzheimer's disease.¹¹ Fifth, small-molecule neurotrophin mimetics that bind p75^{NTR} have been suggested as potential therapeutics in Alzheimer's disease.^{12,13}

These studies suggest that one mechanism by which p75^{NTR} may participate in Alzheimer's disease pathogenesis is by binding A β peptide and triggering programmed cell death. However, recent studies suggest that A β peptide also binds to its parent, APP (β -amyloid precursor protein), multimerizing APP, inducing caspase cleavage of APP intracytoplasmically at Asp664, and inducing programmed cell death.^{14,15} Furthermore, the A β -induced cleavage of APP at Asp664 may play a key role in nonapoptotic features of the Alzheimer's phenotype, such as hippocampal synapse loss, dentate gyrus atrophy, and reduction in excitatory postsynaptic potentials at CA1 after stimulation of the Schaeffer collaterals of CA3 neurons, because the addition of an APP Asp664→Ala mutation to Alzheimer's model transgenic mice [PDAPP(D664A) mice] prevents all of these features, as well as memory loss and neophobia, even though A β accumulation is unaffected.^{16–19}

These results from the PDAPP(D664A) transgenic mice argue that, at least in this mouse model of AD, A β binding to p75^{NTR} is insufficient to reproduce the Alzheimer's disease phenotype, because the preventive mutation in this model, D664A, does not affect these two interactors. However, the D664A results do not exclude the possibility that p75^{NTR} may collude with A β and APP to affect APP signal transduction that requires the caspase cleavage at Asp664 and its resultant downstream signaling. In a more general sense, the relation between p75^{NTR} and APP, and its potential role in Alzheimer-relevant signal transduction, has not been explored. In particular, the well-described selective vulnerability of the p75^{NTR}-positive basal forebrain cholinergic neurons in Alzheimer's disease is incompletely understood (although, as noted earlier, p75^{NTR} may mediate proapoptotic signaling by both pro-NGF and A β), and indeed, the selective neuronal vulnerabilities observed in all of the major neurodegenerative diseases remain, for the most part, unexplained. Therefore, in this work, we examined the relation between p75^{NTR} and APP. We report here the following results: (1) p75^{NTR} and APP interact directly; (2) this interaction is modified by ligands NGF and A β ; (3) APP and p75^{NTR} colocalization in vivo is modified in Alzheimer's model transgenic mice; (4) APP processing is altered in the presence of p75^{NTR}; (5) transcriptional activity that is dependent on APP and mediated by Fe65 is blocked by p75^{NTR}; and (6) coexpression of APP and p75^{NTR} triggers cell death.

These results provide new insight into the emerging network of signaling molecules that mediates the Alzheimer's phenotype and into the underlying mechanism by which p75^{NTR} expression may confer selective vulnerability on the basal forebrain cholinergic neurons in Alzheimer's disease. In addition, the results argue that the interaction between APP and p75^{NTR} may represent a therapeutic target in Alzheimer's disease.

Materials and Methods

Cell Culture Conditions and Transfection Procedures

B103 rat neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Transient transfection of B103 cells was performed using either Lipofectamine Plus reagent (Invitrogen, Carlsberg, CA) for 24 hours (immunoprecipitation and caspase-3 activity assays) or 48 hours (enzyme-linked immunosorbent assay and ToxiLight assays), or Fugene6 (Roche, Alameda, CA) for 48 hours (luciferase assay) according to the manufacturer's instructions. Recombinant NGF and A β 1–42 were purchased from Sigma (St. Louis, MO) and Anaspec (San Jose, CA), respectively. Brefeldin A and tunicamycin were purchased from Sigma.

Plasmid Constructs

Full-length APP695 (pcDNA3-APP), APP-C99 (pcDNA3-C99), full-length p75^{NTR} (pcDNA3-p75^{NTR}), flag-p75^{NTR} (pcDNA3-flag-p75^{NTR}), Unc5h2 (pcDNA3-HA-Unc5h2), Fe65 (pCMV5-Fe65), Gal4-luciferase reporter (PG5E1B-luc), and APP-Gal4 (pMST-APP) were previously described.^{20–23} APP-C83 (APP613-695) was obtained by polymerase chain reaction (PCR) using pcDNA3-C99 as a PCR template and inserted into a pcDNA3.1TOPOd (Invitrogen). Gal4 transcriptional activating domain (AD)-APP-EC fusion and Gal4 DNA binding domain (DNA BD)-p75^{NTR}-EC fusion were obtained by PCR using pcDNA3-APP and pcDNA3-p75^{NTR}, respectively, as a PCR template and inserted into pGADT7 and pGBKT7 vectors (Clontech, Mountain View, CA) through *NdeI-XmaI* and *NdeI-SalI* insertion, respectively. YFP-p75^{NTR} and luciferase-APP constructs consist of the signal peptide sequence fused to YFP or luciferase protein, respectively, and to the full-length protein sequence. YFP-p75^{NTR} was made using the peYFP vector (Clontech) as PCR template. The amplified fragment and pcDNA3-p75^{NTR} were then digested with BspLU and ligated. A complementary DNA encoding firefly luciferase²⁴ was PCR amplified, and *ClaI* restriction endonuclease sites were introduced at the 5' and 3' ends via the PCR primers listed in the Table. A *ClaI* restriction enzyme site was introduced near the 5' end of the APP complementary DNA by site-directed mutagenesis using the primers listed in the Table. Luciferase-APP was generated by embedding the gene encoding firefly luciferase within the *ClaI* sites introduced into the APP complementary DNA downstream of the signal sequence. Sequences of PCR primers used are listed in the Table.

Antibodies

Monoclonal c-myc, polyclonal anti-APP (which recognizes a C-terminal epitope or N-terminal epitope), monoclonal anti-flagM2, and polyclonal anti-HA were purchased from Sigma. Polyclonal anti-p75^{NTR} was from Promega (San Luis Obispo, CA), monoclonal anti-p75^{NTR} from Abcam (Cambridge, MA), monoclonal anti-soluble APP α (APP α) from IBL (Minneapolis, MN), mixed monoclonal antibodies (5A3, 1G7) were kindly provided by Dr Ed Koo,²⁵ and monoclonal anti-Fe65 was purchased from Biotechnology (Lake Placid, NY).

Immunoblotting and Immunoprecipitation

B103 cells were lysed and sonicated in 50mM Hepes, pH 7.6, 125mM NaCl, 5mM EDTA, and 0.1% NP-40, in the presence of protease inhibitor cocktail (Roche). Mouse and rat adult brains were mechanically dissociated, frozen at -80°C , and lysed in the same buffer by 40 strokes in a Dounce homogenizer. Solubilized proteins were obtained in the supernatant after centrifugation at 13,000 rpm for 5 minutes.

Western blots were performed as described previously²⁶ using a polyclonal anti-p75^{NTR} (1:10,000 dilution), a poly-clonal anti-APP (C-terminal epitope: 1:10,000 dilution; N-terminal epitope: 1:10,000 dilution), a monoclonal anti-flagM2 (1:7,000 dilution), a monoclonal anti-Fe65 (1:3,000 dilution), or a polyclonal anti-HA tag (1:5,000 dilution) antibody. Coimmunoprecipitations were performed on pre-cleared solubilized proteins or supernatants using a monoclonal anti-APP (5A3,1G7) antibody kindly provided by Dr Ed Koo, a monoclonal anti-p75^{NTR}, monoclonal anti-flagM2, a polyclonal anti-APP C-terminal, or a monoclonal anti-sAPP α antibody. Coimmunoprecipitations from brains were performed using the microbead system developed by Miltenyi Biotech (Bergisch Gladbach, Germany). As negative control, normal IgGs were used. Coimmunoprecipitations and Western blots were repeated at least three times. The intensities of the bands were determined by densitometric scanning using BioRad Gel doc XR scanner and Quantity One software.

Two-Hybrid Analysis

The Matchmaker Gal4 two-hybrid system III, developed by Clontech, was used according to the manufacturer's instructions. The AH109 yeast strain was cotransformed with APP-EC (the extracellular domain of APP) fused to the AD of Gal4 and p75^{NTR}-EC fused to the DNA binding domain of Gal4. Cells were then allowed to grow in the absence of leucine, tryptophan, adenine, and histidine, and in the presence of X-alpha Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, (10mg/ml) and 3-amino-1,2,4-triazole (15mM). As a negative control, yeast cells were cotransformed with APP-EC and empty Gal4 DNA-BD vector, or with p75^{NTR}-EC and empty Gal4 AD vector. Total proteins were extracted from yeast, and protein expressions were detected by Western blot using a polyclonal anti-APP N-terminal (1:10,000 dilution) and a monoclonal anti-c-myc (1:4,000 dilution) antibody to detect the expression of APP-EC and p75^{NTR}-EC, respectively.

Luciferase Assay

To study bioluminescence resonance energy transfer, we cotransfected B103 cells in 12-well plates with different quantities of YFP-p75^{NTR} (0.2, 0.5, 0.75, 1μg) and 1μg luciferase-APP construct. As a transfection control, renilla luciferase vector was used for each condition (0.016μAg). As a negative control, cells were transfected with 1μg luciferase-APP and different quantities of either peYFP vector or p75^{NTR} (0.2, 0.5, 0.75, 1μg). Luminescence was measured using the Dual-luciferase reporter assay system (Promega), according to the manufacturer's instructions, and a Luminoskan Ascent luminometer (Labsystems, Ramsey, MN).

Confocal Analysis of Mouse Brains

Fifty-micrometer Vibratome brain sections from 2-month-old mice (C57BL/21 strain) nontransgenic or transgenic PDAPP as Galvan and colleagues¹⁶ described were saline perfused and immersion fixed 24 hours in 4% paraformaldehyde and were then stained with a monoclonal anti-APP (5A3,1G7) (10μAg/ml) and a polyclonal anti-p75 NTR (1: 250) followed by Alexa 555 donkey anti-mouse IgG (1:500; Vector Laboratories, Burlingame, CA) and Alexa 488 donkey anti-rabbit IgG (1:250; Vector Laboratories), respectively. Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI), and images were acquired using an LSM 510 NLO Confocal Scanning System (mounted on an Axiovert 200 Inverted Microscope) (Carl Zeiss, Thornwood, NY) equipped with a two-photon Chameleon laser (Coherent). Images were acquired using LSM 510 Imaging Software (Carl Zeiss, Santa Clara, CA). Images were scanned using Argon, 543 HeNe, and Chameleon (750–780nm for DAPI) lasers with a 40× objective and 2× digital zoom. Colocalization was determined by measuring correlation coefficients (r^2) between the two proteins using Imaris software (Bit-plane, Saint-Paul, MN).

β-Amyloid Enzyme-Linked Immunosorbent Assay

B103 cells were cotransfected in six-well plates with C99 + empty pcDNA3 constructs or with C99 + p75^{NTR}. Aβ 1-40 was quantitated by enzyme-linked immunosorbent assay (Biosource, Camarillo, CA) on total protein from B103 cells, according to the manufacturer's instructions. The fluorescence intensity was measured using a Victor station (Perkin-Elmer, Waltham, MA). The relative fluorescence determined using the standard curve for each sample was normalized by the protein quantity.

Cell Death Analysis and Caspase-3 Assay

Cell death was analyzed using the ToxiLight assay according to the manufacturer's instructions (Lonza, Allendale, NJ). In brief, B103 cells were seeded at 50,000 cells/well in 24-well plates. The following day, cells were transfected using Lipofectamine Plus

(Invitrogen) with 1 μ g total DNA per well (0.5 μ g p75^{NTR} and 0.5 μ g APP). Forty-eight hours after transfection, the release of adenylate kinase by cells was measured from the supernatant and normalized to total adenylate kinase from cell lysates by luminometer (Infinite-F500; Tecan, San Jose, CA).

Caspase-3 activity was measured 24 hours after B103 cell transfections using the caspase-3 fluorometric assay kit (BioVision, Mountain View). This assay uses the DEVD-AFC (Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin) substrate. The activity was measured every 5 minutes for 1 hour according to the manufacturer's instructions. For each condition, caspase-3 activation was calculated as follows: We first calculated the average of the slopes of the regression curves representing the number of fluorescent units for minutes; we then calculated the ratios between the caspase activity of each sample and that measured in B103 cells transfected with empty vector (control).

Data Analysis

Statistical significance of the differences observed between experimental groups was determined by the Mann–Whitney *U* test using the PRISM software computer program. For multiple comparisons, one-way analysis of variance followed by post hoc comparisons made with Bonferroni test was used. Differences were accepted as significant if $p < 0.05$ (designated “a”) or $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$.

Results

Amyloid Precursor Protein Interacts with p75^{NTR}

To assess APP interaction with p75^{NTR}, we used B103 cells that do not express APP or amyloid precursor-like proteins.²⁷ Cells were cotransfected with expression constructs for APP and p75^{NTR}. Immunoprecipitation of APP was accompanied by coimmunoprecipitation of p75^{NTR} (Fig 1A; see also Fig 4A), suggesting an interaction of p75^{NTR} with APP (both highly glycosylated and less glycosylated forms of p75^{NTR}; see Supplemental Fig 1). To analyze this putative interaction further, and given that A β binds the extracellular domain of p75^{NTR} receptor,⁹ we performed targeted yeast two-hybrid studies using the APP ectodomain (which includes part of the A β region) fused to the AD of Gal4 (APP-EC), and the p75^{NTR} ectodomain (p75^{NTR}-EC) fused to the DNA-binding domain of Gal4 (see Fig 1B). As shown in Figures 1C and D, coexpression of p75^{NTR} and APP ectodomains in yeast allowed Gal4-dependent transcription, visualized as blue colonies in the presence of X-alpha gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, 10mg/ml) in comparison with control (yeast transformed with APP-EC or p75^{NTR}-EC alone), in which no colonies were detected. The expression of both constructs in yeast was checked by Western blotting (see Fig 1D). Thus, APP and p75^{NTR} interact directly through their ectodomains. Moreover, as shown in Figure 1E, a bioluminescence resonance energy transfer study demonstrated that, in B103 cells, full-length p75^{NTR} interacts directly with full-length APP. Indeed, the decrease of luciferase measured in the presence of fusion protein, luciferase-APP, and YFP-p75^{NTR} demonstrates energy transfer, indicating a close proximity (less than 2Å) of these two proteins. In comparison, no reduction of luciferase activity was detected in control conditions in which YFP-p75^{NTR} was replaced by the fluorescent protein alone (peYFP) or untagged p75^{NTR}.

To investigate whether APP and p75^{NTR} interact not only when ectopically expressed in B103 cells or in yeast but also in more physiological settings, we analyzed the APP-p75^{NTR} interaction in mouse brain: Confocal analysis was performed on brain sections stained with anti-p75^{NTR} (green) and anti-APP (red). Yellow fluorescence (resulting from the overlap of green and red light) was detected in the corpus callosum-containing projections from basal

forebrain (Fig 2A, merge). These data show a colocalization of p75^{NTR} and APP. Moreover, immunoprecipitation of p75^{NTR} from both rat and mouse brain extract coimmunoprecipitated APP (see Fig 2B). Thus, these results show that APP and p75^{NTR} interact both in vitro and in vivo.

β-Amyloid Region of Amyloid Precursor Protein Participates in the Amyloid Precursor Protein-p75^{NTR} Interaction

To characterize the domain of APP required for APP-p75^{NTR} interaction further, and because the ectodo-main of APP was shown to be sufficient for this interaction (see Figs 1B, C), we analyzed whether the C-terminal 99-amino acid fragment of APP (C99 or β-CTF) or C-terminal 83-amino acid fragment of APP (C83 or α-CTF) was sufficient to support coimmunoprecipitation of APP and p75^{NTR}. The C99 fragment (β-CTF) derives from β-secretase cleavage of APP, whereas the C83 fragment (α-CTF) results from α-secretase cleavage (Fig 3A). As shown in Figure 3B, pull-down of p75^{NTR} was associated with immunoprecipitation of C99. However, the larger deletion of APP, leaving the C-terminal 83 amino acids, resulted in a loss of APP-p75^{NTR} coimmunoprecipitation (see Fig 3B). Thus, β-secretase cleavage of APP does not preclude the APP-p75^{NTR} interaction with the remaining C-terminal fragment (β-CTF), but α-secretase cleavage does.

Ligands Nerve Growth Factor and β-Amyloid Peptide Modulate the Amyloid Precursor Protein-p75^{NTR} Interaction

Because NGF and Aβ are both known ligands of p75^{NTR} and Aβ is a ligand of its precursor, APP,^{14,15} we assessed their effects on the interaction between APP and p75^{NTR}. As shown in Figure 4, the interaction between APP and p75^{NTR} is modulated by ligand-receptor interactions. NGF binds p75^{NTR}, as well as TrkA, and when NGF (10ng/ml) was added to B103 cells expressing APP and p75^{NTR}, the degree of coimmunoprecipitation was reduced (see Figs 4A, B). A similar effect was achieved with Aβ peptide (15μg/ml), even though Aβ peptide has been shown to interact with both APP and p75^{NTR}. To determine whether a similar effect may occur in vivo, we evaluated and quantitated immunohistochemically colocalization of APP and p75^{NTR} on brain sections of transgenic Alzheimer model mice (PDAPP, J20 line) and compared with results in littermate controls. As shown previously, brains of Alzheimer's model mice that overexpress APP bearing Swedish and Indiana mutations show greater concentrations of Aβ peptide in comparison with littermate controls.¹⁶ Confocal analysis of p75^{NTR} (in green) and APP staining (in red) showed low colocalization visualized by low yellow fluorescence detected in the corpus callosum (see Fig 4C) in comparison with control mice (see Fig 2A). Quantitation of the colocalization represented by the correlation coefficient (r^2) showed that the degree of this colocalization was significantly reduced in the Alzheimer's model mice (see Fig 4D). Together, these results suggest that the APP-p75^{NTR} interaction is capable of ligand modulation, both in vitro and in vivo.

Amyloid Precursor Protein Processing Is Modified by p75^{NTR} Expression

APP processing is critical in Alzheimer's disease pathogenesis: Cleavage of APP at the α-secretase site (chiefly by ADAM-10) prevents the production of Aβ peptide, whereas cleavage at the β-secretase and γ-secretase sites is required to produce the Aβ peptide. Moreover, mutations in APP that are associated with familial Alzheimer's disease map predominantly near the β-secretase and γ-secretase sites.^{28,29} Furthermore, as noted earlier, mutation of the caspase cleavage site of APP prevents the Alzheimer's phenotype in transgenic mice modeling Alzheimer's disease. Thus, the effect of the APP-p75^{NTR} interaction on APP processing is of interest. To detect extracellular sAPPα fragment, we performed an immunoprecipitation on supernatants of B103 cells using a specific antibody directed against the α-secretase-cleaved site of APP, followed by a Western blot using an

anti-APP N-terminal antibody. As shown in Figure 5A, the presence of p75^{NTR} led to a reduction in sAPP α production (and as shown in Supplementary Fig 2, a more minor effect was observed on p75^{NTR} α -secretase cleavage [which is conducted by ADAM-17/TACE], with an increase in processing). However, this effect was completely prevented in the presence of NGF (10ng/ml), and a similar prevention was observed in the presence of A β (15 μ g/ml). To ensure that an equal amount of APP was expressed in each experiment, we performed Western blots using an anti-APP C-terminal antibody on cell proteins corresponding to each condition.

Complementing the effect on sAPP α net production, the C99 fragment was used to analyze A β production in the presence of p75^{NTR}. As shown earlier, the C99 fragment interacts with p75^{NTR}. Moreover, it needs to be cleaved only by γ -secretase to produce A β peptide. Thus, A β production may be more readily observed using the C99 fragment than using full-length APP. p75^{NTR} in the presence of C99 led to a marked increase in A β production (see Fig 5B). Taken together, these results suggest that the p75^{NTR}-APP interaction modulates APP processing, reducing net sAPP α production and, at least for β -secretase-cleaved APP (C99, β -CTF), increasing net production of A β . However, the presence of ligand, either NGF or A β , blocks this effect of p75^{NTR} that would otherwise reduce net sAPP α production.

Fe65-Mediated Amyloid Precursor Protein–Dependent Transcriptional Activation Is Blocked by p75^{NTR} Expression

It has been shown that Fe65 interacts with APP and mediates an APP-dependent transcriptional effect via the APP intracytoplasmic domain that is derived from APP by γ -secretase cleavage.^{30,31} To examine the effect of p75^{NTR} on Fe65-mediated APP-dependent transcription, we performed a luciferase assay using a Gal4-luciferase reporter and an APP-Gal4 construct in which the intracellular domain of APP is fused to the DNA binding domain of yeast transcription factor Gal4.³⁰ As shown in Figure 6A, Fe65 induced APP intracytoplasmic domain–dependent transcription, visualized as an increase of luciferase. This transcriptional effect was almost completely suppressed by p75^{NTR}, whereas a control receptor, Unc5H2, had no effect. To assess the suppressive effect of p75^{NTR} on Fe65-mediated, APP-dependent transcription mechanistically, we analyzed the Fe65-APP interaction in the presence of p75^{NTR} by immunoprecipitation assay. Our data show that p75^{NTR} expression induced a marked reduction in the Fe65-APP coimmunoprecipitation in comparison with the control, Unc5H2 (see Fig 6B). These results demonstrate that the APP-p75^{NTR} interaction affects not only APP processing but also APP signaling mediated by its interactor, Fe65.

Coexpression of Amyloid Precursor Protein and p75^{NTR} Induces Cell Death

As noted earlier, both p75^{NTR} and APP have, under different circumstances, been implicated as mediators of programmed cell death, and as shown earlier, the presence of p75^{NTR} alters APP-mediated processing and signaling. Therefore, we asked what effect the coexpression of these two molecules would have, using B103 neuroblastoma cells. As shown in Figure 7A, the coexpression of p75^{NTR} and APP enhanced cell death significantly over that induced by either APP or p75^{NTR} alone. This increase of cell death was accompanied by caspase-3 activation (see Fig 7B).

Discussion

The mechanism(s) by which neurodegenerative diseases that are associated with widely expressed mutant or misfolded proteins display selective neuronal regional and subregional vulnerability remains poorly understood. Indeed, the major neurodegenerative diseases are associated with proteins whose expression is not limited to the selectively vulnerable

neurons of each disease. Understanding selective neuronal vulnerability may offer new therapeutic targets, not only by providing new insight into the pathogenetic mechanisms underlying such disease states but also, given that selective vulnerability implies complementary selective resistance in “unaffected” regions, by demonstrating the operative resistance mechanisms.

Alzheimer's disease is associated with degeneration of several different regions of the central nervous system; chief among these are the entorhinal cortex-hippocampal connections and the basal forebrain cholinergic neurons, the latter of which express p75^{NTR}. p75^{NTR}, the common neurotrophin receptor, has been implicated in Alzheimer's disease pathogenesis by virtue of its previously described mediation of programmed cell death, its interaction with A β peptide, its interaction with pro-NGF, and the potentially ameliorative effect of molecules that bind p75^{NTR}. Here we show that p75^{NTR} interacts directly with APP, that the β -secretase-derived C-terminal fragment (C99) is sufficient for this interaction (or alternatively, the extracellular domain), and that the interaction affects APP processing and downstream signal transduction. Furthermore, ligands that interact with these receptors, NGF and A β peptide, block this interaction and thus block its downstream effects. Considered in toto, these results suggest the possibility that the interaction between p75^{NTR} and APP shifts the effect of APP from a trophic one, with the production of sAPP α and the Fe65-dependent transcriptional effects, to an antitrophic one, with the reduction in sAPP α and the increase in A β peptide production. Reciprocally, APP may also affect p75^{NTR} processing and thus modulate its signaling as well.

Several questions remain unanswered: For example, what is the effect of pro-NGF on the p75^{NTR}-APP interaction? What is the effect of p75^{NTR} on the A β 1-42/A β 1-40 ratio? Is this effect modulated by ligand binding to p75^{NTR}? Does blocking the APP-p75^{NTR} interaction ameliorate the Alzheimer's disease phenotype? What is the effect of p75^{NTR} on APP interactions with molecules other than Fe65, such as X11 α , ABP (AMPA receptor Binding Protein, AMPA = α -amino-3-hydroxy-5-methyl-4-isoxazole propionate), and Jip-1b? Despite the fact that these and other questions remain to be addressed, this report offers new insight into APP-mediated signaling that is potentially relevant to Alzheimer's disease pathogenesis and provides a newly recognized mechanism by which the expression of p75^{NTR} may confer selective vulnerability on basal forebrain cholinergic neurons in Alzheimer's disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Ligue Contre le Cancer (to P.M.), the ANR (Agence Nationale pour la Recherche to P.M.), the NINDS (NS45093 to P.M. and D.E.B.), the NIA (AG12282 to D.E.B.), and the Joseph Drown Foundation (to D.E.B.).

We thank F. Calheiros and V. Corset for discussions, critiques, and technical assistance; C. Guix for technical assistance; and Prof E. Koo for anti-APP antibodies. We thank M. Susag for manuscript preparation.

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Fig 1.

Amyloid precursor protein (APP) immunoprecipitates with p75^{NTR}. (A) B103 cells were transfected with APP in the presence or absence of p75^{NTR} expression construct. Immunoprecipitation (IP) was performed using a monoclonal anti-APP antibody (5A3, 1G7) followed by a Western blot using anti-p75^{NTR}. The pulled-down band (arrow in top panel) is a 60kDa band. Bottom panel shows p75^{NTR} and APP C-terminal immunoblot from the same samples (before IP). (B) Schematic representation of the extracellular domain of APP (APP-EC) and p75^{NTR}-EC constructs used in (C) and (D) luciferase-APP and YFP-p75^{NTR} used in (E). The length of sequences is indicated by number of nucleotides. (C) Direct two-hybrid assay performed on AH109 yeast cotransformed with a Gal4 transcriptional activation domain (AD)-APP-EC fusion (pGADT7-APP-EC) and a Gal4 DNA binding domain (DNA BD)-p75^{NTR}-EC fusion (pGBKT7-p75^{NTR}-EC). Cells were allowed to grow on an agar plate in the absence of leucine, tryptophan, adenine, and histidine, and in the presence of X-alpha galactosidase (10mg/ml) and 3-amino-1,2,4-triazole (15mM). (D) Western blot from yeast using a polyclonal anti-APP N-terminal and a monoclonal c-myc antibody to detect the expression of APP-EC and p75^{NTR}-EC, respectively. The pGADT7-APP-EC lane shows a 117kDa band (arrow) corresponding to Gal4 AD-HA-APP-EC fusion protein. In the bottom panel, the 30kDa band detected in the pGBKT7 lane corresponds to Gal4 DNA BD protein in the empty vector and shifts to a 60kDa band (arrow) with p75^{NTR}-EC expression (lane pGBKT7-p75EC). (E) Luminescence measurement from B103 cells cotransfected for 48 hours with luciferase-APP and YFP-p75^{NTR}, peYFP, or p75^{NTR}.

Histograms represent the mean values standard error of the mean of firefly over renilla. Significance was determined by Mann–Whitney U test. *** p 0.001.

Fig 2.

Amyloid precursor protein (APP)-p75^{NTR} interaction occurs in vivo. (A) APP and p75^{NTR} colocalize in brain sections. Brain sections of 2-month-old mice were stained with p75^{NTR} (green fluorescence) and APP (red fluorescence) antibodies. Nuclei are stained with 4'-6-diamidino-2-phenylindole (DAPI). Analysis of images, acquired with an LSM 510 NLO Confocal Scanning System (Carl Zeiss, Thornwood, NY) equipped with a two-photon Chameleon laser (Coherent, Santa Clara, CA) and using LSM 510 Imaging Software (Carl Zeiss, Thornwood, NY), show a colocalization (yellow staining in “merge”) in the corpus callosum—containing projections from basal forebrain. (B) Pull-down (immunoprecipitation [IP]) was performed on total protein from adult rat and mouse brain using normal IgGs or a monoclonal anti-p75^{NTR} antibody followed by an immunoblot using a polyclonal anti-APP C-terminal antibody. A 110kDa band was demonstrated (*arrow*).

Fig 3.

A p75^{NTR} binding domain of amyloid precursor protein (APP) lies within the Aβ region of APP. (A) Schematic representation of APP and α-, β-, and γ-secretase cleavages. (B) B103 cells were transfected for 24 hours with a C99 (APP597-695)- or C83 (APP613-695)-expressing construct in the presence or in the absence of FLAG-p75^{NTR} expression construct. Pull-down was performed using a monoclonal anti-FLAGM2 antibody, and the immunoblot used an antibody raised against the C-terminal domain of APP, then an anti-FLAGM2 antibody. Pulled-down band is indicated by an *arrow* (lane C99+FLAG-p75^{NTR}). Western blot using an anti-APP C-terminal antibody was performed on total protein extracts from the same samples (before IP).

Fig 4.

Ligands modulate the amyloid precursor protein (APP)-p75^{NTR} interaction. (A) Pull-down was performed using a monoclonal anti-APP antibody (5A3, 1G7) from B103 cells transfected with APP expression construct or APP+p75^{NTR} in the absence of treatment or in the presence of nerve growth factor (NGF, 10ng/ml) or β -amyloid (A β , 15g/ml) and immunoblot used an antibody to p75^{NTR}. Bottom panels show p75^{NTR} and APP immunoblot from the same samples (before IP). (B) Quantitation of p75^{NTR} bands after APP pull-down followed by Western blot using p75^{NTR} antibody shown in (A). (C, D) Brain sections from 2-month-old nontransgenic (Non Tg) and transgenic PDAPP (Tg) mice were stained with anti-p75^{NTR} (green fluorescence) and anti-APP (red fluorescence) antibodies. (C) Representative images of p75^{NTR}, APP, or merged staining in the corpus callosum-containing projections from basal forebrain of PDAPP mice. (D) Histogram represents the mean values $\pm$ standard error of the correlation coefficient (r^2) obtained between both stainings. A Mann-Whitney U test was used to compare Tg ($n = 6$) versus Non Tg ($n = 5$). $**p < 0.01$.

Fig 5.

Amyloid precursor protein (APP) processing is modified by p75^{NTR} expression. (A) Pull-down was performed using a monoclonal anti-soluble APP α (sAPP α) antibody on the supernatant from B103 cells transfected for 24 hours with pcDNA3, APP expression construct, or APP+ p75^{NTR} in the absence of treatment or in the presence of nerve growth factor (NGF, 10ng/ml) or β -amyloid (A β , 15 μ g/ml) for 20 hours, and immunoblots used a polyclonal anti-APP N-terminal (APP N-t) antibody. As APP expression control, Western blot was performed on total protein extracts (bottom panel) from the corresponding cells using a polyclonal anti-APP C-terminal (APP C-t) antibody. Both immunoblots show 110kDa bands. (B) Quantitation of soluble A β 1-40 by enzyme-linked immunosorbent assay in total protein extracts from B103 cells over-expressing C99 expression construct or C99+p75^{NTR} for 48 hours. Histogram shows a normalization by A β 1-40 levels detected after C99 overexpression and corresponds to one representative experiment.

Fig 6.

Fe65-dependent amyloid precursor protein (APP) transcriptional activation is blocked by p75^{NTR} expression. (A) Luminescence measurement from B103 cells transfected for 48 hours with APP-Gal4 fusion protein and a Gal4-luciferase reporter plasmid in a 1:2 ratio, in the presence or in the absence of Fe65, p75^{NTR}, and Unc5h2 expression construct. Histograms represent the mean values $\pm$ standard error of the mean. ^a $p < 0.05$ (Gal4luc + APPGal4 + Fe65 + p75 vs Gal4luc + APPGal4 + Fe65); ^{**} $p < 0.01$ (all conditions vs Gal4luc + APPGal4). Significance was determined by one-way analysis of variance. Post hoc comparisons between all groups were made with the Bonferroni test. (B) Pull-down was performed using a polyclonal anti-APP C-terminal antibody (IP: APP) on total protein extracts from B103 cells transfected for 24 hours with Fe65 expression construct or Fe65+APP in the absence or in the presence of p75^{NTR} or Unc5h2-HA, and Western blot used a monoclonal anti-Fe65 antibody showing a 95kDa band (arrow). Bottom panels show immunoblots performed with anti-APP, anti-Fe65, anti-p75^{NTR}, and anti-HA antibodies on total protein extracts from the same samples (before immunoprecipitation [IP]), and the sizes of bands observed were 95, 110, 60 to 70, and 105kDa, respectively.

Fig 7.

Coexpression of amyloid precursor protein (APP) and p75^{NTR} triggers cell death. B103 cells were transfected with empty vector (Ctrl), p75^{NTR} expression vector (p75^{NTR}), APP expression vector (APP), or APP + p75^{NTR} (1μg DNA total). Cell death was analyzed by (A) ToxiLight assay 48 hours after transfection or (B) caspase-3 activity measurement 24 hours after transfection using DEVD-AFC substrate. *** $p < 0.001$.

Table
Polymerase Chain Reaction Primers Used for APP-C83, APP-EC, p75^{NTR}-EC, YFP-p75, and Amyloid Precursor Protein Luciferase Constructions

Constructs	Sequences (5'-3')	PCR Matrices	Size of Amplified Fragment (bp)
APP-C83	5'-caccatgttggtgttcttgcaga-3'	pcDNA3-APP-C100	249
	5'-ctagtctgcacatgctcaaa-3'		
APP-EC	5'-gaattccatatgatgctgcccgggttggcactgctcct-3'	pcDNA3-APP695	2,098
	5'-aattccccgggttgaaccacatcttctgcaaa-3'		
p75 ^{NTR} -EC	5'-gggaattccatatgatgaggaggcagggtgctgcctgca-3'	pcDNA3-p75 ^{NTR}	753
	5'-gaattcgtcgacgttgcgggtgccgggtcac-3'		
YFP-p75	5'-gaattcacatgtgtgagcaaggcaggagctgttcac-3'	peYFP	729
	5'-gaattcacatgtctccttctgtacagctgtccatgcc-3'		
APP- <i>Cla</i> I	5'-gctcgggcgctggaggtaccatcgcgatggaatgctggcctgctggct-3'	pcDNA3-APP695	N/A
	5'-agccagcagccagcattaccatcgcgatgggtacctccagcggccgagc-3'		
Luc- <i>Cla</i> I (-ATG)	5'-gaattcatcgatgaagacgcaaaaacataaagaaggccggcg-3'	κB-luc	1,653
	5'-gaattcatcgattacaatttgacattccgcccttcttggcctt-3'		

APP = amyloid precursor protein; APP-EC = extracellular domain of APP. p75^{NTR}-EC = extracellular domain of p75^{NTR}; YFP = yellow fluorescent protein; Luc = luciferase