Akt-dependent Expression of NAIP-1 Protects Neurons against Amyloid-β Toxicity*

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Neurotrophins are a family of growth factors that attenuate several forms of pathological neuronal cell death and may represent a putative therapeutic approach to neurodegenerative diseases. In Alzheimer disease, amyloid-β (Aβ) is thought to play a central role in the neuronal death occurring in brains of patients. In the present study, we evaluate the ability of neurotrophin-3 (NT-3) to protect neurons against the toxicity induced by aggregated Aβ. We showed that in primary cultures of cortical neurons, NT-3 reduces Aβ-induced apoptosis by limiting caspase-8, caspase-9, and caspase-3 cleavage. This neuroprotective effect of NT-3 was concomitant to an increased level of Akt phosphorylation and was abolished by an inhibitor of the phosphatidylinositol-3 kinase (PI-3K), LY294002. In parallel, NT-3 treatment reduced Aβ induced caspase-3 processing to control levels. In an attempt to link PI-3K/Akt to caspase inhibition, we evaluated the influence of the PI-3K/Akt axis on the expression of a member of the inhibitors of apoptosis proteins (IAPs), the neuronal apoptosis inhibitory protein-1. We demonstrated that NT-3 induces an up-regulation of neuronal apoptosis inhibitory protein-1 expression in neurons that promotes the inhibition of Aβ-induced neuronal apoptosis. Together, these findings demonstrate that NT-3 signaling counters Aβ-dependent neuronal cell death and may represent an innovative therapeutic intervention to limit neuronal death in Alzheimer disease.

Alzheimer disease (AD) is a neurodegenerative disease characterized by the accumulation of a 40–42-amino acid-long peptide termed amyloid-β (Aβ) into amyloid deposits and by the hyperphosphorylation of the protein tau, leading to neurofibrillary tangles (1–3). Aβ is thought to play a central role in the neuronal death occurring in brains of AD patients. The importance of Aβ is notably supported by the effects of genetic mutations that cause familial AD (4), all of which predispose to amyloid deposition. Moreover, fibrillar Aβ is specifically neurotoxic in primary cortical neurons, whereas soluble monomeric Aβ is not (5, 6). Neuronal death induced by fibrillar Aβ exposure displays characteristic features of apoptosis including plasma membrane blebbing, nuclear condensation, DNA fragmentation (7), and caspase activation. Caspases are cysteine aspartate proteases regulating the entry of the cell into programmed cell death by two principal pathways: the Fas/TNF receptor-mediated pathway associated with caspase-8 and the mitochondria-dependent pathway linked with caspase-9 (8). The caspases 8 and 9, also called initiator caspases, trigger the activation of the effector caspases. Following Aβ treatment, activation of several caspases has been identified in dying neurons (9–14), and caspase inhibitors block Aβ-induced cell death (11, 12, 14, 15), highlighting the contribution of apoptosis to Aβ-induced toxicity.

During brain development, where apoptosis plays a fundamental role in regulating cell fate (16), neurotrophins (NTs) antagonize naturally occurring programmed cell death and thus promote neuronal survival in many mammalian species (17). Four related members of the neurotrophin growth factor family have been identified: nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). They act on a set of high affinity tyrosine kinase receptors (18) (i.e. TrkA, TrkB, and TrkC). When activated, Trk receptors are autophosphorylated within tyrosine residues located at the cytoplasmic tail of Trk receptors, which serve as docking sites for kinases such as phosphatidylinositol-3-kinase (PI-3K). Because of their survival promoting properties during development, a widely held concept has emerged: neurotrophins may represent a potential candidate for therapeutic treatment of neurobiological disorders (19). Indeed, experimentally NTs have been shown to attenuate the neuronal death induced by various types of insults (20, 21). Recently, a clinical trial with nerve growth factor for the treatment of early onset Alzheimer disease began in 2001 at the University of California (22).

Among these growth factors, NT 3 has raised a particular interest as a potential therapeutic tool for limiting neuronal death during AD. Opposite to brain-derived neurotrophic factor, whose expression is up-regulated in the brains of AD patients, NT-3 expression decreases during adult life (16), and treatment with recombinant NT-3 might offer protection to neurons from the proapoptotic environment observed in AD. In the present study, we investigate how and by which mechanism recombinant NT-3 may protect neurons against Aβ-induced apoptotic cell death.

EXPERIMENTAL PROCEDURES

Dulbecco’s modified Eagle’s medium, poly-b-lysine, cytokine β-2-arabinoside (AraC), horse and fetal calf sera, propidium iodide, hydrochloride (2-(4-morpholinyl)-8-phenyl-1-(4H)-benzopyran-4-one hydrochloride) (LY294002), anti-MAP-2, and anti-actin antibodies were obtained from Sigma. N-Methyl-D-aspartate, α-ami-n03-hydroxy-5-methyl-

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† The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid-β; NT, neurotrophin; PI-3K, phosphatidylinositol-3-kinase; DAPI, 4′-6′-diamino-2-phenylindole dihydrochloride; ANOVA, analysis of variance; CHX, cycloheximide; ERK, extracellular signal-regulated kinase; IAP, inhibitor of apoptosis proteins.
4-isoxazoleproprionate, 6-cyano-7-nitroquinolxaline-2,3-dione, kainate, and (−)-5-methyl-10,11-dihydro-5H-dibenzo(a,b)cylohepten-5,10-imine maleate were from Tocris (Bristol, UK). Laminin was from Invitrogen. Mitogens-activated protein kinase/extracellular signal-regulated kinase inhibitor U0126 was from Promega France (Charbonnieres, France), and recombinant human neurotrophin-3 was obtained from R&D Systems, Inc. (Minneapolis, MN).

**Primary Cell Cultures**—Mouse cortical cultures of neurons were prepared from 14–15-day-old embryos as previously described (23), plated in 2-cm² dishes previously coated with poly-D-lysine and 0.5 mg/ml laminin. Cultures were maintained in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine and 5% fetal bovine serum and 5% horse serum. After 3 days in vitro, neurons were treated with 10 μM AraC to inhibit proliferation of nonneuronal cells. Experiments were realized on pure neuronal cultures (>98% microtubule-associated protein-2 immunoreactive cells) after 12–14 days in vitro.

**Aβ Exposures**—Amyloid-β peptides (Aβ-(25–35) and Aβ-(1–42)) (Sigma) were dissolved in sterile deionized water to obtain a stock solution of 2 mM. Aβs were kept at 37 °C for 48 h and stored at 4 °C until use. The presence of fibrillar Aβ was estimated by SDS-PAGE prior to application onto cultured neurons. Aβ-induced apoptosis was performed in the presence of (−)-5-methyl-10,11-dihydro-5H-dibenzo(a,b)cylohepten-5,10-imine maleate (10 μM) to prevent excitotoxic cell death.

**Estimation of Neurocellal Cell Death**—Toxicity was determined by estimating the activity of lactate dehydrogenase released in the media by dying neurons and by counting of apoptotic neurons eliciting either nuclear DNA condensation or DNA fragmentation by stained by 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) (1 μg/ml). Application of DAPI was realized onto paraformaldehyde-fixed neurons for 30 min, and then neuronal cultures were washed three times in phosphate-buffered saline. Apoptotic and total neurons were blind counted, and the ratio between neurons displaying morphological nuclear changes and total neurons stained was performed to assess apoptotic-induced toxicity.

**DNA-Agarose Electrophoresis**—Genomic DNA was harvested by using the Wizard® Genomic DNA kit (Promega, Charbonnieres, France) as described by the manufacturer. DNA amounts were estimated by spectrometry and loaded into 3% agarose electrophoresis gels containing 0.1% ethidium bromide. Gels were visualized by UV illumination and scanned with a CCD camera.

**Western Blotting**—Cells were harvested in a lysis solution containing 50 mM Tris-HCl (pH 7.6), 1% Nonidet P-40 (Sigma), 150 mM NaCl, 2 mM EDTA with 1 mM phenylmethylsulfonyl fluoride in the presence of a protease inhibitor mixture (Sigma). Cell lysates were centrifuged for 10 min at 12,000 rpm, supernatants were isolated, and the corresponding pellet was resuspended with the protease inhibitor-containing lysis buffer. Proteins were separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore). Western blots were stained with DAPI following a 24-h application of Aβ-(25–35)-induced apoptosis. Densitometric analysis was realized by ANOVA (n = 16, p < 0.001) followed by Bonferroni-Dunn’s test. *, p < 0.01, compared with controls; #, p < 0.001, compared with Aβ at the maximal doses. B, dose-response effect of NT-3 in the presence of Aβ-(25–35)-induced apoptosis. Dark bars, Aβ-(25–35)-treated neurons; light gray bars, co-application of CHX (at 1 μM); hatched bars, co-treatment with recombinant human NT-3 (0.1–10 ng/ml). Normalized number of neurons stained with DAPI displaying nuclear alterations characteristic features of apoptosis. Statistical analysis was realized by ANOVA (n = 16, p < 0.001) followed by Bonferroni-Dunn’s test. *, p < 0.001, compared with controls; #, p < 0.01, compared with Aβ at the maximal dose. C, microphotographs of neuronal nuclei stained with DAPI following a 24-h application of Aβ in the presence of NT-3 (at 10 ng/ml) or CHX (at 1 μM). Scale bar, 10 μm.

1% bovine serum albumin and 0.1% Tween 20. Cells were then washed and incubated for 1 h with the appropriate secondary biotin-conjugated antibody, secondary Alexa Fluor 488- or Alexa Fluor 555-conjugated antibodies (Molecular Probes Europe, Leiden, The Netherlands).

**Antibodies**—The following primary antibodies were used: Asp175 (1:500) against cleaved caspase-3 (Cell Signaling Technologies, Ozyme, Montigny Le Bretonneux, France), anti-caspase-8 (1:5000) (Calbiochem), anti-caspase-9 (1:1000) (Stressgen Biotechnologies, Victoria, Canada), phospho-Akt (Ser473) and Akt antibodies (1:1000 and 1:50) (Cell Signaling Technologies, sc-11067 (1:200) against NAIP-1, sc-7383 (1:200) and sc-93 (1:200) against caspase-8 and actin (1:250), respectively (Sigma).

**Densitometric Analysis**—Agarose gels or blots from three independent experiments were acquired by a CCD camera and saved in a resolution of 600 dpi for software analysis. PCR products and Western blot signals were quantified by two-dimensional densitometry analysis with OptiQuant® software (Packard Instrument Co.).

**Statistical Analysis**—Results are expressed as mean ± S.D.

NAIP-1 Protects Neurons against Aβ

**FIG. 1. NT-3 rescues neurons from Aβ-(25–35)-induced apoptotic cell death.** A, neuronal cell death was estimated by measuring the activity of lactate dehydrogenase in the bathing media and by counting apoptotic cells eliciting nuclear alterations revealed by a DAPI staining. Dark bars, lactate dehydrogenase values normalized to neuronal death induced by prolonged NMDA application (at 100 μM); empty bars, normalized number of neurons stained with DAPI displaying the characteristic nuclear alterations induced by apoptosis. Statistical analysis was realized by ANOVA (n = 16, p < 0.001) followed by Bonferroni-Dunn’s test. *, p < 0.01, compared with controls; #, p < 0.001, compared with Aβ at the maximal doses. B, dose-response effect of NT-3 in the presence of Aβ-(25–35)-induced apoptosis. Dark bars, Aβ-(25–35)-treated neurons; light gray bars, co-application of CHX (at 1 μM); hatched bars, co-treatment with recombinant human NT-3 (0.1–10 ng/ml). Normalized number of neurons stained with DAPI displaying nuclear alterations characteristic features of apoptosis. Statistical analysis was realized by ANOVA (n = 16, p < 0.001) followed by Bonferroni-Dunn’s test. *, p < 0.001, compared with controls; #, p < 0.01, compared with Aβ at the maximal dose. C, microphotographs of neuronal nuclei stained with DAPI following a 24-h application of Aβ in the presence of NT-3 (at 10 ng/ml) or CHX (at 1 μM). Scale bar, 10 μm.
RESULTS

**NT-3 Protects Neurons against Aβ-induced Toxicity**—To study the potential activity of NT-3 against neuronal death mediated by Aβ, we exposed primary cultures of mouse cortical neurons (14 days in vitro) to Aβ-(25–35) for 24 h. The Aβ-(25–35) peptide is a fragment of Aβ previously described to reproduce neurotoxicity of full-length Aβ peptides (5). Aβ-induced neuronal death was estimated by measurement of the lactate dehydrogenase activity in the extracellular media and by counting in a blind manner neurons stained by DAPI displaying nuclear condensation. A 24-h exposure to Aβ-(25–35) induced approximately 40% of neuronal death (Fig. 1) which displayed the characteristic feature of apoptosis (i.e., cell body shrinkage; nuclear condensation). This neuronal death was prevented by the co-application of the inhibitor of protein synthesis, cycloheximide (CHX), a compound that has been shown to protect against several apoptotic paradigms in neurons (20) (Fig. 1A).

To test the influence of NT-3 on Aβ-induced neurotoxicity, we applied NT-3 at 0.1, 1, and 10 ng/ml in the presence of Aβ-(25–35) for 24 h (Fig. 1B). We found that NT-3 elicits a neuroprotective activity in a dose-dependent manner with a maximum effect at 10 ng/ml.

Next, we studied the action of NT-3 (at 10 ng/ml) against Aβ-induced apoptosis at 3, 6, 12, and 24 h (Fig. 2A). Neuronal death could be observed at 6 h following application of aggregated Aβ-(25–35). NT-3 rescued ~50% (46.92 ± 9.47) and 33% (33.57 ± 11.21) of neurons after 12 or 24 h of incubation of aggregated Aβ-(25–35), respectively.

**NT-3 Attenuates Aβ-induced Caspase Cleavage**—As previously described (9–12, 14), Aβ exposure in neurons led to the processing of inactive procaspase-8 and -9 into their active forms. Similarly a 3–4-fold increase of activated caspase-3 (p20) was also detected following Aβ-(25–35) induced neurotoxicity at 3, 6, 12, and 24 h (Fig. 2A). Neuronal death was prevented by the co-application of the inhibitor of protein synthesis, cycloheximide (CHX), a compound that has been shown to protect against several apoptotic paradigms in neurons (20) (Fig. 1A).

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controls; #, 0.001) followed by Bonferroni-Dunn's test. *, triplicate. Statistical analysis was realized by ANOVA (n). Results are the mean of three independent experiments performed in triplicate. NT-3 in the presence of LY294002 or exposed to NT-3 alone for 24 h.

Akt and total Akt in cultured neurons exposed to A inhibits ERK5 activation (26). The co-treatment of NT-3 with NT-3/H9262, or exposed to NT-3 alone for 15 min, 1 h, 12 h, and 24 h in the presence of NT-3. As previously reported, NT-3 alone induced a transient phosphorylation of ERKs 15 min and 1 h after its incubation (Fig. 3, B and C). Aβ treatment alone led to an activation of ERKs, which was slightly enhanced by co-application of NT-3 without, however, being significant. Whereas phospho-ERKs were markedly detected during the first 1 h of treatment, they declined to basal levels at either 12 or 24 h (Fig. 3C).

NTs have also been described as a putative activator of the PI-3K/Akt pathway. This pathway has been the subject of emerging interest over the past years for its ability to reduce Aβ-induced apoptosis in PC12 cells or in neuroblastoma cell lines (28, 29). To determine whether NT-3 mediates its protective effect against Aβ-induced neurotoxicity in primary cultured cortical neurons through the activation of the PI-3K/Akt pathway, we first tested the influence of the PI-3K pathway inhibitor, LY294002. Because several studies have shown that the LY294002 alone induces apoptosis in primary neuronal culture, we chose a concentration of LY294002 that did not show any proapoptotic activity on its own (at 10 μM). The co-application of LY294002 reversed the neuroprotection promoted by NT-3, whereas LY294002 alone did not exhibit any detrimental effect (Fig. 3D). Accordingly, Western blotting revealed that Akt is rapidly phosphorylated and remained activated under NT-3 exposure alone or in the presence of Aβ for 12 or 24 h (Fig. 4A). The addition of LY294002 (pretreatment plus co-treatment) prevented NT-3-induced phosphorylation of Akt (Fig. 4B). Finally, neurons exposed to Aβ and LY294002 did not exhibit phospho-Akt levels different from controls.

Under the same experimental conditions, active caspase-3 levels (p20) were reduced in the conditions when phospho-Akt

Next we investigated which survival-promoting pathways activated by NT-3 are responsible for the observed neuroprotection. The Ras/mitogen-activated protein kinase pathway, including extracellular-signal regulated kinases 1/2 (ERK1/2) and ERK5, has been identified as one of the survival-promoting pathways used by NTs in the central nervous system (18, 24–27). To determine the contribution of the ERK family members to NT-3-induced neuroprotection against Aβ neurotoxicity, we used the ERK1/2 inhibitor U0126 (at 10 μM), which also inhibits ERK5 activation (26). The co-treatment of NT-3 with U0126 did not abolish the neuroprotective effect of the neurotrophin, indicating that ERKs do not contribute to this process (Fig. 3A).

To confirm these pharmacological data, we estimated ERK1/2 activation by performing a Western blotting of the proteins recovered from neurons exposed to Aβ at 15 min, 1 h, 12 h, and 24 h. Results are representative of three independent experiments performed in triplicate.

Results are representative of three independent experiments performed in triplicate. Densitometry analysis of phospho-AKT/AKT in cultured neurons exposed to Aβ-(25–35) (20 μM) in the presence of either CHX (1 μg/ml) or NT-3 (10 ng/ml) for 15 min, 1 h, 12 h, and 24 h. Results are the mean of three independent experiments performed in triplicate. Statistical analysis was realized by ANOVA (n = 12, p < 0.003) followed by Bonferroni-Dunn’s test. *p < 0.005, compared with controls; double asterisk, p < 0.001, compared with controls. No statistical differences were observed in neurons subjected to Aβ, Aβ + NT-3, or NT-3 alone. B, Western blot of phosphorylated Akt, total Akt, and activated caspase-3 (p20) in cultured neurons exposed to Aβ-(25–35) (20 μM for 24 h) in the presence of either CHX (1 μg/ml) or NT-3 (10 ng/ml). Results are representative of three independent experiments performed in triplicate. Densitometry analysis of cleaved caspase-3 levels in cultured neurons exposed to Aβ-(25–35) (20 μM for 24 h) in the absence of either CHX (25–35) with or without NT-3 in the presence of LY294002 or exposed to NT-3 alone for 24 h. Results are the mean of three independent experiments performed in triplicate. Statistical analysis was realized by ANOVA (n = 12, p < 0.001) followed by Bonferroni-Dunn’s test. *p < 0.005, compared with controls; #p < 0.001, compared with Aβ. C, Immunodetection of phospho-activated Akt (red fluorescence) in cultured neurons exposed to Aβ-(25–35) (20 μM for 24 h) in the absence or the presence of NT-3 (10 ng/ml). Neuronal nuclei were counterstained with DAPI (blue fluorescence). Overlaid images are presented in upper panels, and phospho-Akt immunoreactivity is displayed below to better appreciate its subcellular localization. Scale bar, 25 μm.

FIG. 4. NT-3-induced neuroprotection requires activation of the PI-3K/Akt pathway. A, Western blot analysis of phosphorylated Akt and total Akt in cultured neurons exposed to Aβ-(25–35) (20 μM) in the presence of either CHX (1 μg/ml) or NT-3 (10 ng/ml) for 15 min. Results are representative of three independent experiments performed in triplicate. Densitometry analysis of phospho-AKT/AKT in cultured neurons exposed to Aβ-(25–35) (dark bars), co-treated with NT-3 (hatched dark bars), or exposed to NT-3 alone (gray bars) for 15 min, 1 h, 12 h, and 24 h. Results are the mean of three independent experiments performed in triplicate. Statistical analysis was realized by ANOVA (n = 12, p < 0.003) followed by Bonferroni-Dunn’s test. *, p < 0.005, compared with controls; double asterisk, p < 0.001, compared with controls. No statistical differences were observed in neurons subjected to Aβ, Aβ + NT-3, or NT-3 alone. B, Western blot of phosphorylated Akt, total Akt, and activated caspase-3 (p20) in cultured neurons exposed to Aβ-(25–35) (20 μM for 24 h) in the presence of either CHX (1 μg/ml) or NT-3 (10 ng/ml). Results are representative of three independent experiments performed in triplicate. Densitometry analysis of cleaved caspase-3 levels in cultured neurons exposed to Aβ-(25–35) (20 μM for 24 h) in the absence of either CHX (25–35) with or without NT-3 in the presence of LY294002 or exposed to NT-3 alone for 24 h. Results are the mean of three independent experiments performed in triplicate. Statistical analysis was realized by ANOVA (n = 12, p < 0.001) followed by Bonferroni-Dunn’s test. *p < 0.005, compared with controls; #p < 0.001, compared with Aβ. C, Immunodetection of phospho-activated Akt (red fluorescence) in cultured neurons exposed to Aβ-(25–35) (20 μM for 24 h) in the absence or the presence of NT-3 (10 ng/ml). Neuronal nuclei were counterstained with DAPI (blue fluorescence). Overlaid images are presented in upper panels, and phospho-Akt immunoreactivity is displayed below to better appreciate its subcellular localization. Scale bar, 25 μm.

FIG. 5. NT-3-dependent activation of the PI-3K pathway reduces the cleavage of initiator caspase-8 and -9. Western blots of caspase-8 and caspase-9 (lower) derivatives in cultured neurons exposed to Aβ-(25–35) (20 μM for 24 h) in the presence of NT-3 (10 ng/ml) with PI-3K pathway inhibitor, LY294002 (1 μM). Actin levels were determined to confirm equal protein loadings (data not shown). Results are representative of three independent experiments performed in triplicate.
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Fig. 6. NAIP-1 immunoprecipitates with Smac under NT-3 exposure. A, fluorescent immunolabeling of NAIP-1 (green) and phosphorylated Akt (red) in cultured neurons exposed to Aβ(25–35) (20 μM for 24 h) in the presence of NT-3 (10 ng/ml). Overlaid pictures are presented in the right column. Scale bar, 50 μm. B, Western blot analysis of NAIP-1 and actin in neurons exposed to Aβ(25–35) (20 μM for 24 h) in the presence of NT-3 (10 ng/ml) with PI-3K pathway inhibitor LY294002 (1 μM). Results are representative of three independent experiments performed in triplicate. Densitometry analysis of NAIP-1/actin (p42) ratio in cultured neurons exposed to Aβ(25–35) (dark bars) co-treated with NT-3 (hatched bars) in the presence or not of LY294002 or exposed to NT-3 alone for 24 h (empty bar). Results are the mean of three independent experiments performed in triplicate. Statistical analysis was realized by ANOVA (n = 9, p < 0.004) followed by Bonferroni-Dunn’s test. *, p < 0.004, compared with Aβ; #, p < 0.002, compared with Aβ + NT-3. C, immunoprecipitation of NAIP-1 from the same protein extracts used in B revealed either with an antibody raised against SMAC or NAIP-1. The immunoprecipitation of NAIP-1 revealed with NAIP-1 confirmed results presented in B, showing an increased expression of NAIP-1 under NT-3 treatment with or without Aβ(25–35).

was detected (Fig. 4B). Conversely, immunocytochemical studies reveal that neurons eliciting phospho-Akt473 immunoreactivity did not exhibit characteristic apoptotic nuclear alterations (Fig. 4C), suggesting that NT-3-mediated Akt activation rescued cortical neurons from Aβ-induced apoptosis. In addition, we determined by immunoblotting the level of activated initiator caspases in neurons exposed to Aβ in the presence of NT-3 and LY294002 (Fig. 5). NT-3 treatment (at 10 ng/ml) lowered both levels of activated caspase-8 and -9, whereas co-application of LY294002 restored levels of the proapoptotic forms of caspases to those observed in neurons exposed to Aβ alone.

Activation of the PI-3K/Akt Pathway Induces NAIP-1 Expression—PI-3K/Akt signaling pathway has been described as a putative activator of proteins involved in regulating cell survival in various cell systems (30). Among these potential targets of the PI-3K/Akt pathway, members of the inhibitors of apoptosis proteins (IAPs), a family of proteins that directly inhibit caspase function (i.e. XIAP and HIAP (human IAP), and NAIP (neuronal apoptosis inhibitor protein)) have raised particular interest as a potential target for antiapoptotic drug treatment (31). Indeed, it has been shown in different cell systems that Akt activates the expression of survival IAP genes (32–36). Thus, we evaluated the influence of PI-3K/Akt activation on the expression of the neuronal member of IAPs, NAIP-1 (Fig. 6). First, we performed double immunochemistry experiments in primary cortical neurons exposed to Aβ that revealed a co-localization of NAIP-1 with phospho-Akt-immunoreactive neurons when treated NT-3 (Fig. 6A). To confirm this link between the PI-3K/Akt pathway and NAIP up-regulation, we estimated the level of expression of NAIP-1 by Western blotting (Fig. 6B). We observed a ~2–2.5-fold increase in NAIP-1 expression induced by NT-3 alone. This up-regulation of NAIP-1 expression induced by NT-3 was also observable when experiments were performed in the presence of Aβ. This effect was abolished by the co-application of LY294002, an inhibitor of the PI-3K pathway.

Because the antiapoptotic activity of IAP proteins is inhibited by a mitochondria-derived activator of caspase, the protein SMAC (also called Diablo), we performed immunoprecipitations with an antibody raised against NAIP-1, and we revealed the blots with an antibody raised against SMAC to determine whether NT-3 influences its interaction with SMAC (Fig. 6C). We found that Aβ application resulted in the formation of a Smac-NAIP-1 complex in the cytosol, whereas SMAC was nearly undetectable in controls. Co-treatment of NT-3 prevented formation of such a complex. Of note, NT-3-induced NAIP-1 up-regulation was confirmed in these conditions. In addition, co-incubation with LY294002 restored the formation of the deleterious assembly of NAIP-1-SMAC. To resume, these results suggest that the antiapoptotic effect of NT-3 on Aβ-
induced neurotoxicity could be due to both an increased expression of NAIP-1 and a reduced interaction between NAIP-1 and SMAC by a mechanism dependent on PI-3K/Akt activation.

**DISCUSSION**

AD is a neurodegenerative disease characterized by the abnormal accumulation of small aggregative peptides (Aβ) in brain tissue that leads to severe cortical dystrophy (4). To determine the biochemical pathways involved on the neurotoxic action of aggregated Aβ, studies on primary cell culture systems have been extensively used. Various deleterious mechanisms have been identified to support Aβ-induced cell toxicity, and there is now a large consensus in the scientific community that application of aggregated Aβ induces neuronal apoptosis in primary neuronal cultures (37–39). However, no valid therapeutic approaches have emerged from these studies. Here, we describe that a growth factor, NT-3, is able to reduce the proapoptotic action of aggregated Aβ on primary cortical neurons. This effect is mediated by the activation of PI-3K/Akt pathway and results in the reduction of Aβ-induced caspases activation. In addition, we identified the mechanism by which NT-3 limits caspase activation; NT-3 induces both an increased expression of NAIP-1, a neuronal form of IAPs that can directly inhibit caspases, and a reduced interaction between NAIP and its endogenous inhibitor SMAC. Because IAPs has been shown to reduce activation of caspase-3 and -9 in many systems, it is likely that the effect of NT-3 is downstream of caspase-8 activation.

Significant progress has been made in identifying the molecular mechanisms causing neuronal cell death in AD. Rare genetic cases of AD that develop an early onset have provided mechanistic insights relevant to most common sporadic cases. This has led to the hypothesis that familial and probably sporadic AD is initially caused by an abnormal accumulation of Aβ. Although the role of oligomeric forms of Aβ has been recently reappraised (40, 41), it is generally believed that aggregated forms of Aβ are responsible for the neurotoxic actions observed in AD. Here we showed that aggregated Aβ triggers caspase-dependent apoptosis in primary cortical neurons. Similar results were obtained by others in primary culture systems (9–11, 13, 14). More recently, analyses from post-mortem tissue from patient, transgenic animal and cell culture system experiments have confirmed the putative implication apoptosis in the pathophysiology of AD.

A possible therapeutic approach of AD is to develop strategies to block the apoptotic trigger or to develop antiapoptotic strategies. Because neurotrophins have been identified as one of the most powerful antiapoptotic agents in the CNS, it was tempting to test the influence of NT-3 against Aβ-induced apoptosis. We report that NT-3-induced neuroprotection against Aβ-driven apoptosis requires the activation of the PI-3K/Akt pathway, which induces an up-regulation of the expression of NAIP-1, a protein that directly inhibits caspase-3 and -7 (42). The PI-3K/Akt has already been identified as second messenger system providing antiapoptotic signal to various cell types (30). Upon activation of this signaling pathway, the induction of the IAP family members, including XIAP and HIAP (also called ITA) by PI-3K/Akt, has been reported in different cell types (32, 43, 44). Recently, Zhang et al. (45) have demonstrated that p75 TNFR-induced neuroprotection against extracellular Aβ toxicity was dependent on PI-3K but not Akt in primary human neurons. Their data and ours strengthen the importance of the PI-3K/Akt pathway in Aβ-induced neurotoxicity in primary neuronal cultures.

Finally, we reported that whereas Aβ application promotes the formation of a Smac-NAIP-1 complex enhancing caspase-dependent apoptotic pathways, NT-3 prevents this association. Thus, by enhancing NAIP-1 expression, NT-3 treatment decreases caspase activation and reduces Aβ-induced neurotoxicity. The NAIP gene encodes for a protein of 156 kDa with a
strong homology to the other inhibitor of apoptosis proteins (42). An antiapoptotic effect of NAIP and other members of the human IAP family has been shown in many cell culture systems. However, the mode of action of NAIP is not as well understood as its family homologues. MacKenzie’s group (46) recently pointed out that NAIP overexpressed in HeLa cells was able to bind to caspase-9 in the presence of ATP in an SMAC-independent manner. Although we do not know whether NT-3-induced up-regulation of NAIP-1 led to a binding with caspase-9, under our conditions, we report that endogenous NAIP-1 is able to complex with SMAC in primary cortical neurons subjected to Aβ fibrils. To summarize, our results provide a strong support for a model in which NAIP is playing a critical role in regulation of Aβ-induced neuronal apoptosis by directly inhibiting effector caspases.

In addition to the regulation of NAIP-1 expression by the PI-3K/Akt axis, this same signaling pathway can also affect other proteins that are able to inhibit apoptosis, including the FOXO family of Forkhead transcription factors (47, 48). In other proteins that are able to inhibit apoptosis, including the PI-3K/Akt axis, this same signaling pathway can also affect the extent of neuronal lesion occurring in the brain of an AD adult (51, 55–57). In the present study, we reduced neuronal vulnerability to aggregated Aβ fibrils. To summarize, our results may represent an innovative therapeutic strategy to limit the extent of neuronal lesion occurring in the brain of an AD patient.

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