Journal of Alzheimer's Disease 6 (2004) 231–242
IOS Press


# Alzheimer-associated neuronal thread protein mediated cell death is linked to impaired insulin signaling

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**Abstract**. Alzheimer-associated neuronal thread protein, AD7c-NTP, accumulates in cortical neurons and co-localizes with phospho-tau-containing cytoskeletal lesions in brains with AD. Over-expression of AD7c-NTP results in increased neuronal death mediated by apoptosis and mitochondrial dysfunction. Empirical studies demonstrating differential growth factor responses to AD7c-NTP led to us to further investigate the effects of insulin, insulin-like growth factor, type 1 (IGF-1), nerve growth factor (NGF), and platelet-derived growth factor (PDGF) stimulation on neuronal survival mechanisms in relation to AD7c-NTP expression. PNET2 human CNS-derived neuronal cells were stably transfected with a cDNA encoding AD7c-NTP or chloramphenicol acetyl transferase (CAT) whereby gene expression was regulated by an inducible promoter. In cells that expressed AD7c-NTP, insulin or IGF-1 stimulation was associated with reduced viability with increased levels of p53, p21/Waf-1, phospho-JNK, and phospho-*tau*, and reduced levels of Bcl-2 and phospho-Erk MAPK. In contrast, AD7c-NTP-transfected cells stimulated with NGF or PDGF, and CAT-transfected cells stimulated with any one of the four growth factors remained viable and had low levels of p53, p21/Waf-1, phospho-JNK, and phospho-*tau*, and abundant Bcl-2 and phospho-Erk expression. The results suggest that reduced survival in neurons that over-express AD7c-NTP may be mediated by impaired insulin/IGF-1 signaling, and that CNS neurons with abundant insulin or IGF-1 receptors may be particularly vulnerable to the adverse effects of AD7c-NTP.

Keywords: Neuronal thread protein, apoptosis, neuritic sprouting, growth factors, neuronal cytoskeleton

## 1. Introduction

AD7c-NTP is a member of a novel family of genes termed, "neuronal thread proteins" (NTPs). The ∼1.4 kB AD7c-NTP cDNA isolated from an AD brain library encodes a ∼41 kD protein [1]. Subsequence analysis predicted that AD7c-NTP has at least one trans-membrane domain, a hydrophobic leader sequence, a myristoylation motif, and probable cleavage site in the amino terminal region. The last three features suggest that AD7c-NTP may be secreted, and in this regard, elevated levels of AD7c-NTP are detectable in cerebrospinal fluid [1–3] and urine [3,4] of patients with early or moderately severe AD. The AD7c-NTP protein has 17 predicted phosphorylation motifs, the majority (14 of 17) of which correspond to glycogen synthase kinase $3_\beta$ sites [1]. GSK-$3_\beta$ is one of the major kinases responsible for tau phosphorylation in brains with AD [5]. In addition, AD7c-NTP has an insulin/IGF-1 chimeric receptor domain [1], which may be important for its functional regulation.

Analysis of postmortem brains revealed significantly increased levels of AD7c-NTP mRNA and protein expression in AD relative to age-matched control cases [1]. Immunohistochemical staining demonstrated increased AD7c-NTP immunoreactivity in degenerating neurons, neuropil threads, and dystrophic neurites, corresponding with dementia-associated structural and cytoskeletal lesions of AD [1, 6]. Double-label immunohistochemical staining showed that AD7c-NTP immunoreactivity was co-

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ISSN 1387-2877/04/$17.00 

localized with phospho-*tau* in degenerating neurons and dystrophic neurites [6]. However, there appeared to be a reciprocal relationship between AD7c-NTP and phospho-tau such that, neurons with normal or only slightly altered cyto-morphologies exhibited intense degrees of AD7c-NTP immunoreactivity and low levels of phospho-*tau*, whereas the presence of well-delineated intra-neuronal phospho-tau-immunoreactive neurofibrillary tangles was associated with low levels of AD7c-NTP [6]. These findings suggest that accumulation of AD7c-NTP in neurons may precede the formation of neurofibrillary tangles.

*In vitro* experiments demonstrated that over-expression of AD7c-NTP in transfected human neuronal cell lines produced a dimorphic phenotype characterized by reduced viability and increased neuritic sprouting [1]. Further investigations showed that AD7c-NTP-induced neuronal death was mediated by apoptosis and mitochondrial dysfunction [7]. However, the mechanisms by which AD7c-NTP promotes neuronal death are not known. In general, apoptosis of neuronal cells is mediated by alterations in intracellular signaling that result in inhibition of survival mechanisms or activation of pro-apoptosis pathways [8–17]. Phosphoinositol-3 kinase (PI3K)-Akt kinase mediated neuronal survival can be stimulated by growth factors or activity dependent signaling, whereas inhibition of growth factor signaling through PI3K-Akt results in neuronal apoptosis [13,18–21] and mitochondrial dysfunction [16,22,23]. Alternatively, neuronal apoptosis can occur with increased activation of stress kinase signaling [11,15,16,24]. In the present study, we demonstrate growth factor-dependent modulation of neuronal responses to AD7c-NTP and show that neuronal death in the context of AD7c-NTP over-expression is associated with impaired insulin stimulated survival signaling due to increased activation of stress kinases.

## 2. Methods

### *2.1. Establishment of an inducible expression system to study AD7c-NTP over-expression*

An inducible system using the LacSwitch II mammalian expression vector (Stratagene, La Jolla, CA) was used to regulate AD7c-NTP or chloramphenicol acetyltransferase (CAT) (negative control) gene expression in PNET2 human CNS neuronal cells [25–27], as previously reported [7]. AD7c-NTP expression or CAT activity was tightly regulated by isopropyl-1-$\beta$-D-thiogalactopyranoside (IPTG) such that increased gene expression or CAT activity was detected within 8 hours of adding IPTG, persisted for 48–96 hours, and was rapidly inhibited upon withdrawal of IPTG. Previous experiments established that 3 mM IPTG was sufficient for inducing gene expression [7]. The present experiments utilized AD7c-NTP- and CAT-expressing clones. To examine the effects of different growth factors in relation to AD7c-NTP expression, the cells were serum-starved for 16 hours, after which they were treated with 3 mM IPTG plus insulin (50 nM), IGF-1 (25 nM), nerve growth factor (NGF; 5 ng/ml), or platelet-derived growth factor (PDGF; 25 ng/ml) for 24–48 hours. Viability was measured using the crystal violet (CV) assay [28]. CV absorbances were measured with a Spectracount (Packard Instrument Co, Meriden, CT).

CV labels only viable cells, and in previous studies, a linear correlation between CV absorbance and viable cell density was demonstrated [15,28]. The CV assay is simple and reproducible. Other popular assays such as the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) or ATPLite actually measure mitochondrial function or ATP production, which are generally correlated with viable cell density. However, results generated with such assays could be confounded by impaired mitochondrial function or energy metabolism produced by oxidative stress conditions. The CV assay was specifically chosen to measure cell viability because previous studies demonstrated that over-expression of the AD7c-NTP gene impairs mitochondrial function in neuronal cells [7].

### *2.2. Immunoblotting methods*

Western blot analysis was used to measure expression of p53, Bcl-2, phospho-tau, tau, c-fos, NTP, and both activated (phosphorylated) and total Erk mitogen activated protein (MAP) kinase, amino-terminal c-jun-activated kinase (pJNK), and p38/HOG1. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA) containing protease and phosphatase inhibitors (1 mM NaF, 1 mM $Na_4P_2O_7$, 2 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu$g/ml each of aprotinin, pepstatin A, and leupeptin) [29] and the supernatant fractions obtained after centrifuging the samples at 14,000 $\times$ g for 15 minutes at 4 °C to pellet debris were used in the studies. Protein concentrations were measured using the bicinchoninic acid (BCA) assay (Pierce,

Rockford, IL). Samples containing 60 $\mu$g protein were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF membranes. Non-specific sites were blocked with SuperBlock (Pierce, Rockford, IL). Blots were incubated over night at 4°C with 0.5–1.0 $\mu$g/ml antibody diluted in TBS plus 0.05% Tween-20 and 0.5% bovine serum albumin (TBST-BSA). Immunoreactivity was detected with horseradish peroxidase conjugated secondary antibody, SuperSignal enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, IL), and either film autoradiography or the Kodak Digital Science Imaging Station (NEN Life Sciences, Boston, MA).

### 2.3. *Measurement of phosphatidylinositol-3 kinase* (*PI3K*) *activity*

Cells were homogenized in Triton lysis buffer (50 mM Tris-HCI, pH 7.5, 10 mM EDTA, 1% Triton) containing protease and phosphatase inhibitors (see above). Antibodies were incubated with 500 $\mu$g protein aliquots, and immune complexes captured with Protein A sepharose were suspended in 50 $\mu$l TNE (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) buffer. Reactions were initiated by sequentially adding 20 $\mu$g of sonicated phosphatidylinositol (10 $\mu$l), 10 $\mu$l of 100 mM $MgCl_2$, and 5 $\mu$l of [$\gamma^{32}$P]ATP working solution composed of 0.88 mM [$\gamma^{32}$P]ATP (30$_\mu$Ci [$\gamma^{32}$P]ATP/3000 Ci/mmol), 20 mM $MgCl_2$, and 150 mM cold ATP. After 10 minutes incubation at 30°C with constant agitation (300 rpm), reactions were stopped by adding HCl to a final concentration 1.2 N. Phospholipids extracted with chloroform/methanol were analyzed by thin layer chromatography using plates pre-coated with 1% potassium oxalate (Merck, White House Station, NJ). PI3K activity was quantified with a phosphorimager.

### 2.4. *Microtiter immunocytochemical ELISA* (*MICE*) *assay*

The MICE assay is a rapid and sensitive method for quantifying immunoreactivity in 96-well microcultures [30]. Briefly, the cells were fixed in Histochoice (Amresco, Solon, Ohio), permeabilized with 0.05% saponin in Tris-buffered saline (50 mM Tris, pH 7.5, 0.9% NaCl; TBS), and blocked with SuperBlock-TBS (Pierce, Rockford, IL). Cells were then incubated overnight at 4°C with 0.5–1.0 $\mu$g/ml primary antibody diluted in TBST-BSA. Immunoreactivity was detected with horseradish peroxidase conjugated secondary antibody (Pierce, Rockford, IL) and the TMB soluble peroxidase substrate (Pierce, Rockford, IL). Absorbances were measured at 450 nm using a Spectracount microplate reader. To normalize immunoreactivity with respect to cell density, the plates were subsequently washed in TBS and the cells were stained 0.1% Coomassie blue dye in 40% methanol/10% acetic acid. After extensive washing in water and drying the plates, the dye was eluted with 1% SDS in PBS (200 $\mu$l/well). Absorbances were measured at 560 nm using a Spectracount (Packard Instrument Company, Meriden, CT). The MICE index was calculated from the ratio of the absorbances measured for immunoreactivity and cell density. Eight or16 replicate culture wells were analyzed in each experiment. All experiments were repeated at least 3 times.

### 2.5. *Source of antibodies and chemicals*

Monoclonal antibodies to p53 and Bcl-2 were purchased from Oncogene Research Products (Cambridge, MA). Rabbit polyclonal antibodies to the p85 subunit of PI3K, and total and phospho-specific JNK, Erk MAPK, and p38/HOG1were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-tau was purchased from Dako Corp. (Carpenteria, CA), and the Alz-50 monoclonal antibody was used to detect phospho-tau (generously provided by Dr. Peter Davies). Protein A sepharose was purchased from Amersham-Pharmacia Biotechnology (Arlington Heights, IL). Recombinant human NGF, PDGF, and IGF-1 were purchased from Sigma Co (St. Louis, MO). Human insulin (Novolin) was purchased from Nova Nordisk Pharmaceuticals (Princeton, NJ).

### 2.6. *Statistical analysis*

Data depicted in the graphs and table represent the means $\pm$ S.D.'s of results obtained from 3 to 6 experiments. Inter-group comparisons were made using Student t-tests or analysis of variance (ANOVA) with the Fisher least significant difference (LSD) post-hoc test. Statistical analysis was performed using the Number Cruncher Statistical Systems (Dr. Jerry L. Hintze, Kaysville, UT).

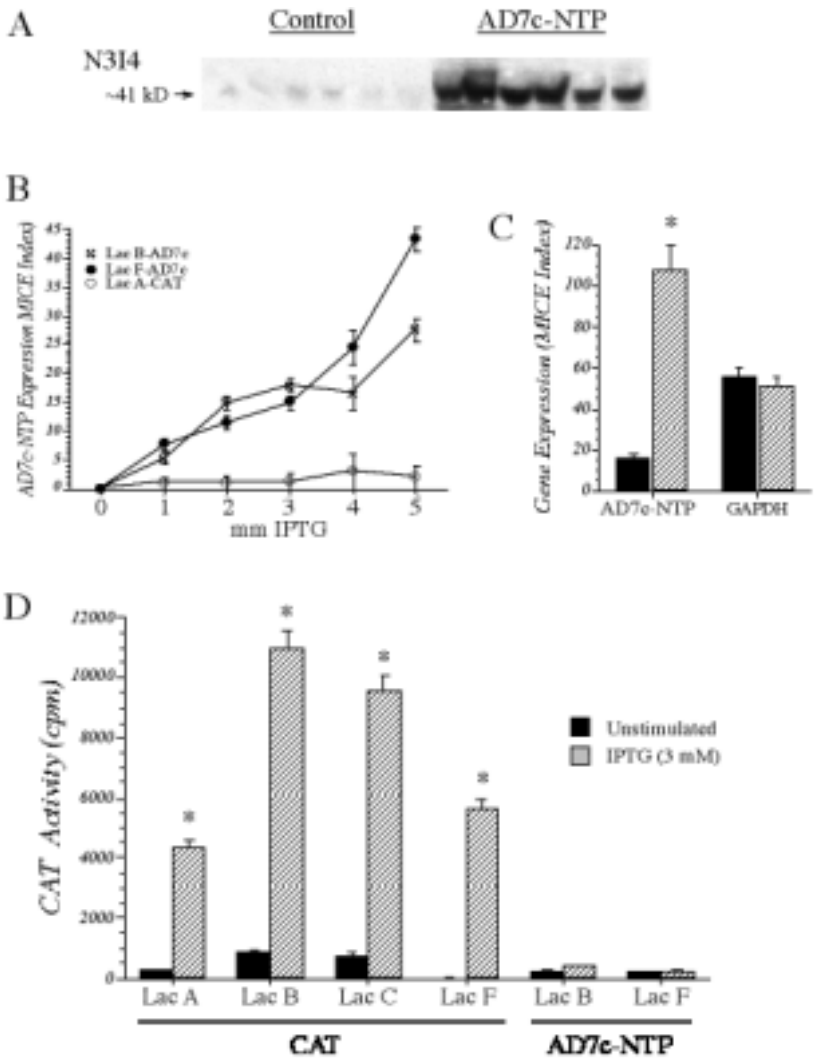


Fig. 1. IPTG induction of AD7c-NTP expression or CAT activity in PNET2 neuronal cells that were stably transfected with recombinant plasmid DNA in which gene expression was under the control of a LacZ promoter and induced with 1–5 mM IPTG. Six clones from each group (Lac A-Lac F-AD7c and Lac A-Lac F-CAT) were studied. (A) Western blot analysis demonstrating higher levels of NTP in cells transfected with the AD7c-NTP cDNA relative to control CAT-transfected cells. NTP immunoreactivity was detected with the N3I4 monoclonal antibody that was raised against the recombinant protein. Gene expression was induced with 3 mM IPTG. (B) Dose-dependent IPTG-induction of AD7c-NTP demonstrated using the Microtiter Immunocytochemical ELISA (MICE) assay. (C) Comparison of NTP and GAPDH expression in Lac B-AD7c cells that were treated with vehicle (solid black) or 3 mM IPTG (hatched). Immunoreactivity was measured using the MICE assay. The MICE index reflects levels of immunoreactivity corrected for cell density. (D) IPTG (3 mM)-induction of CAT activity in cells transfected with the CAT cDNA, and not in cells transfected with AD7c-NTP. The graphs in Panels B-d depict the mean $\pm$ S.D. of immunoreactivity or CAT activity (* $P < 0.001$ by Student T-test).

## 3. Results

### 3.1. AD7c-NTP over-expression in transfected PNET2 cells

The 6 AD7c-NTP- and 6 CAT-transfected clones selected for study exhibited tight regulation of gene expression (Fig. 1). After 24 hours of IPTG treatment (3 mM), cells transfected with the AD7c-NTP cDNA exhibited prominently increased levels of NTP expression, as demonstrated by Western blot analysis. In contrast, similarly treated CAT-transfected cells had low levels (endogenous) of NTP (Fig. 1A). Similar responses to IPTG were observed in all clones selected for study. Using the MICE assay, IPTG dose-dependent increases in the levels of NTP were demon-

strated in cells transfected with AD7c-NTP (Fig. 1B). In addition, studies showed that IPTG treatment selectively increased NTP expression, and had no effect on the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in cells transfected with AD7c-NTP (Fig. 1C). IPTG treatment (3 mM) induced CAT activity in cells transfected with the CAT cDNA, but not in cells transfected with AD7c-NTP (Fig. 1D). The low levels of CAT activity or NTP immunoreactivity observed in the absence of IPTG stimulation indicates that the promoter regulating gene expression exhibited modest degrees of leakiness which, for the purposes of these experiments, did not interfere with the data interpretation.

### 3.2. *Effects of specific growth factors on neuronal viability in relation to AD7c-NTP expression*

In previous studies, we obtained evidence that endogenous NTP expression and function may be regulated by growth factor stimulated signaling or agents that promote neuritic sprouting [26,31,32]. In the present studies, Western blot analysis demonstrated similar levels of NTP in AD7c-NTP-transfected PNET2 cells that were treated with 3 mM IPTG and stimulated with insulin, IGF-1, PDGF, or NGF (Fig. 2A), indicating that the levels of AD7c-NTP expression were regulated by IPTG activation of the LacZ promoter, and not by growth factor stimulation. Corresponding with the results shown in Fig. 1, in the absence of IPTG, the AD7c-NTP-transfected cells expressed relatively low levels of NTP, independent of growth factor stimulation. In these studies, growth factor modulation of endogenously expressed NTP was masked by the high levels of AD7c-NTP generated from the transgene.

Despite similar levels of AD7c-NTP expression, viability was significantly reduced in cultures that were stimulated with insulin or IGF-1 ($P < 0.001$) relative to NGF- or PDGF-stimulated cultures (Fig. 2B). In contrast, cells induced to express the CAT gene remained viable independent of the growth factor employed (Fig. 2C). In the absence of IPTG, cells transfected with the AD7c-NTP or CAT cDNA exhibited similarly high levels of viability. The reduced cell densities observed in cultures that were induced to express AD7c-NTP and also stimulated with insulin or IGF-1 were not due to differences in DNA synthesis since proliferating cell nuclear antigen (PCNA) expression and [3H]-thymidine incorporation levels were comparable in control and AD7c-NTP-transfected cultures, as previously reported [1].

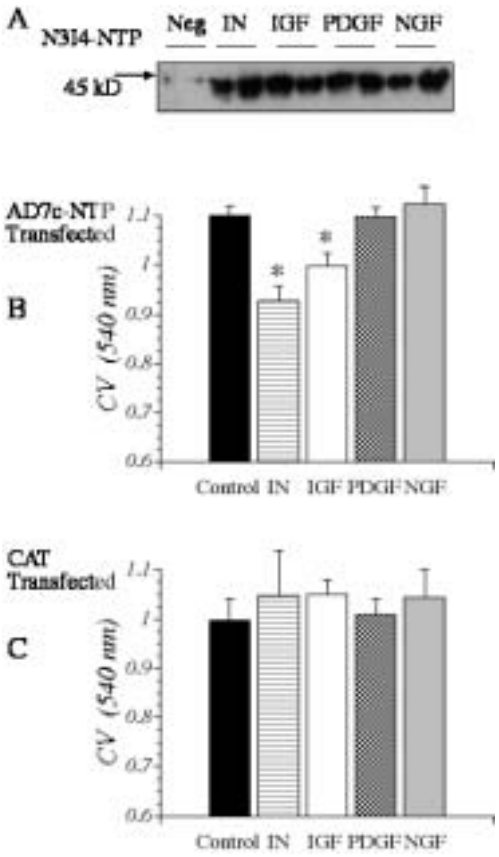


Fig. 2. Differential effects of growth factor stimulation on viability in cells induced to express AD7c-NTP. (A) Western blot analysis demonstrating similar levels of NTP expression in cultures stimulated with insulin (IN: 50 nM), IGF-1 (IGF: 25 nM), PDGF (PDGF: 25 ng/ml), or NGF (NGF: 5 ng/ml) and treated with 3 mM IPTG for 48 hours. Un-induced cultures (Neg/control) were simultaneously studied. (B, C) Crystal violet (CV) assays were used to measure viability in cultures that were induced to express AD7c-NTP (B) or CAT (C). Viability assays were performed with cells seeded into 96-well plates. CV absorbances were measured in a Spectracount. Asterisks indicate significant differences from un-induced controls as demonstrated by ANOVA ($P < 0.05$).

### 3.3. *Effects of specific growth factors on neuronal morphology in relation to AD7c-NTP expression*

Cells induced to express the CAT gene, and un-induced AD7c-NTP- or CAT-transfected cells that were stimulated with insulin exhibited polygonal morphology with short cell processes and only scattered cell rounding (Fig. 3A). In contrast, the same cells stimulated with IGF-1, PDGF, or NGF, exhibited neurite out-growth with formation of interconnecting processes (Fig. 3B). Cells induced to express AD7c-NTP and stimulated with insulin exhibited marked retraction of neuritic processes, increased rounding, and re-

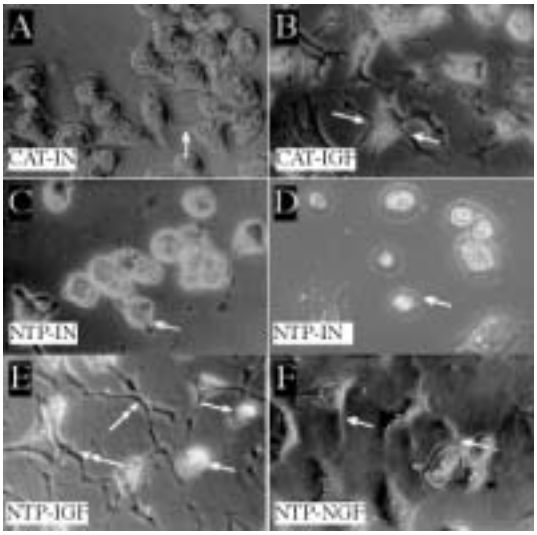


Fig. 3. Differential effects growth factor stimulation on cell morphology. PNET2 cells were induced to express CAT or AD7c-NTP (labeled NTP) by treatment with 3 mM IPTG for 24 or 48 hours, and simultaneously stimulated with insulin (IN: 50 nM), IGF-1 (IGF:25 nM), or NGF (NGF: 5 ng/ml). The cells were examined by phase contrast microscopy. (A) CAT-transfected cells stimulated with insulin for 48 hours. (B) CAT-transfected cells stimulated with IGF-1 for 48 hours. Arrows in Panels A and B show short neuritic processes. (C, D) AD7c-NTP-transfected cells stimulated with insulin for 24 (C) or 48 (D) hours. Arrows show prominent cell rounding (C) or apoptosis (D). (E) AD7c-NTP-transfected cells stimulated with IGF-1 for 48 hours. Note the dimorphic population of rounded cells (arrows) and neuritic process-bearing neuronal cells (double-headed arrows). (F) AD7c-NTP-transfected cells stimulated with NGF for 48 hours. Arrows indicate prominent neuritic processes. Similar effects of NGF stimulation were observed in CAT-transfected control cells (data not shown).

duced viability (floating and refractile) (Figs 3C–3D), while the same cells stimulated with NGF or PDGF remained flat and adherent, and developed long, prominent, branched and multi-polar, inter-connected neuritic processes (Fig. 3F). Cultures induced to express AD7c-NTP and stimulated with IGF-1 had two cell populations consisting of rounded, dying cells and sprouting cells (Fig. 3E), similar to the findings in a previous study in which the cells were stimulated with FCS [1], which contains IGF-1.

### *3.4. Analysis of pro-apoptosis and survival gene expression*

Previous studies demonstrated that apoptosis of AD7c-NTP-transfected neuronal cells was mediated by increased levels of p53 and CD95 (Fas-R) [7], and that survival of PNET2 cells was associated with high levels of Bcl-2 [15,16]. To determine if the cell death associated with AD7c-NTP expression and insulin or IGF-1 stimulation was mediated by pro-apoptosis/anti-survival mechanisms, Western blot analysis (Fig. 4) and the MICE assay (Fig. 5) were used to measure p53 and Bcl-2 expression. The levels of p21/Waf-1 were also examined because p53 signals through p21 to promote apoptosis [33]. The studies demonstrated high levels of p53 and p21, and low levels of Bcl-2 in insulin- and IGF-1-stimulated cultures, and low levels of p53 and p21 with high levels of Bcl-2 in cultures stimulated with NGF (Figs 4 and 5) or PDGF (data not shown). As a negative control, the samples were evaluated for c-fos expression. Western blot analysis demonstrated similar levels of c-fos protein in all cultures, irrespective of growth factor stimulation (Fig. 4G). In contrast to the findings in AD7c-NTP-transfected cultures, in control cells that were induced to express CAT, the levels of p53 and p21/Waf1 were not increased, and Bcl-2 was not reduced by insulin or IGF-1 stimulation (Figs 6A–6C).

### *3.5. Growth Factor Modulation of Phospho-tau in Neuronal Cells Expressing AD7c-NTP*

In AD, AD7c-NTP immunoreactivity co-localizes with phospho-tau [6], and in a previous report, we demonstrated increased levels of phospho-tau in neuronal cells that over-expressed AD7c-NTP [7]. Therefore, it was of interest to determine if phospho-tau accumulation was modulated by growth factor stimulation. Western blot analysis and MICE assay studies demonstrated significantly increased levels of phospho-tau in the insulin and IGF-1-stimulated, AD7c-NTP-expressing cells, and relatively low levels of phospho-tau in parallel cultures stimulated with NGF (Figs 4D and 5E) or PDGF (data not shown). Although tau protein levels were slightly higher in insulin- and IGF-1-stimulated relative to NGF-stimulated cells (Fig. 4H), the differences with respect to phospho-tau were substantially greater (see Figs 4D, 5E, and 5F) than could be explained on the basis of the small difference in tau protein expression. In cells induced to express the CAT gene, insulin and IGF-1 stimulation also resulted in increased levels of phospho-tau, but the responses were modest compared with those observed in AD7c-NTP-transfected cells (Fig. 6D). In the CAT-transfected control cells, c-fos and tau protein levels were similar in insulin, IGF-1, and NGF-stimulated cultures (Figs 6G and 6H).

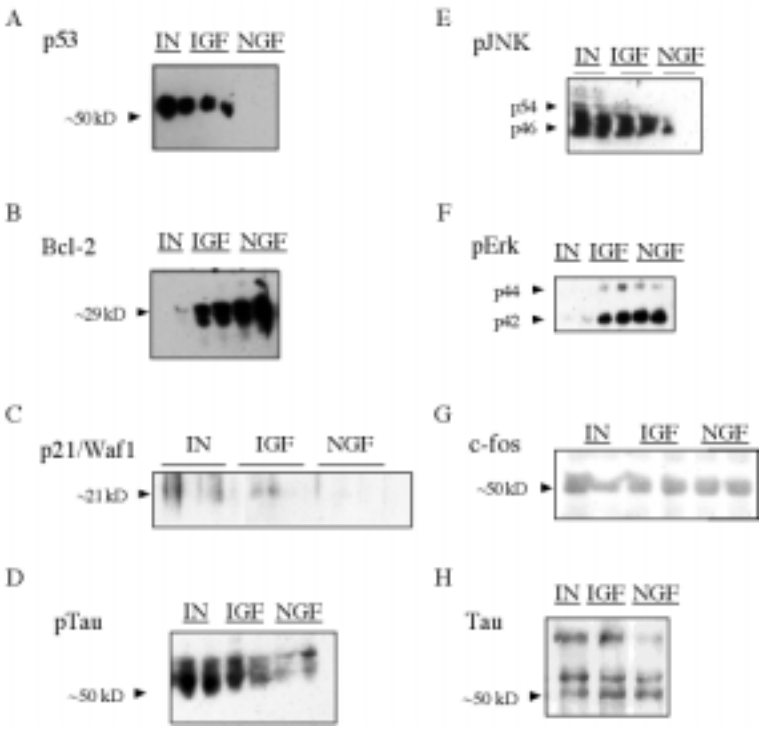


Fig. 4. Differential effects of growth factor stimulation on (A) p53, (B) Bcl-2, (C) p21/Waf-1, (D) phospho (p)-tau, (E) pJNK, (F) pErk MAPK (pErk), (G) c-fos, and (H) tau protein levels in cells induced to express AD7c-NTP by treatment with 3 mM IPTG for 24 hours. Cell homogenates were subjected to Western blot analysis with equivalent protein loading in all lanes. Activated (phospho) Erk and JNK were detected with phospho-specific antibodies. Positions of molecular weight standards are shown at the left in panels A-D, G, H. Note the unchanging levels of c-fos in relation to growth factor stimulation (Panel G). In Panels E and F, the two isoforms of pJNK and pErk are marked with arrowheads. Additional studies revealed absent modulation of total Erk, total JNK, activated p38 MAPK, and total p38 MAPK protein with growth factor stimulation and IPTG-induction of AD7c-NTP (data not shown).

### *3.6. Analysis of growth factor stimulated phospho-Erk MAPK and phospho-JNK expression*

The potential mechanisms by which the differential effects of insulin, IGF-1, NGF and PDGF occur were explored by examining signal transduction pathways that were previously demonstrated to mediate PNET2 neuronal survival and neuritic sprouting [15,16,26,34]. Apoptosis of neuronal cells can be mediated by activation of JNK (increased levels of phospho-JNK) [11, 15,16,24] or inhibition of PI3 kinase signaling [13,15, 16,18,19,34], whereas neuritic sprouting is associated with increased signaling through Erk MAPK or PI3 kinase [26,34–38]. In cells induced to express AD7c-NTP, stimulation with insulin was associated with high levels of phospho-JNK and low levels of phospho-Erk MAPK, while IGF-1 stimulation was associated with high levels of both phospho-JNK and phospho-Erk MAPK, and NGF or PDGF stimulation was associated with low levels of phospho-JNK and high levels of phospho-Erk MAPK (Figs 4E–4F). In contrast, total Erk, total JNK, phospho-p38/HOG, and total p38 protein levels were not modulated by growth factor stimulation (data not shown). Cells induced to express CAT had similar levels of phospho-JNK and phospho-Erk MAPK, irrespective of the type of growth factor stimulation (Figs 6E–6F).

### *3.7. No effect of AD7c-NTP on growth factor stimulated PI3 kinase*

PI3 kinase is an important mediator of neuronal survival [13,18,21]. PI3 kinase promotes survival by activating Akt (protein kinase B) and inhibiting both glycogen synthase kinase 3 (GSK-3) and BAD [13,18,21, 38,39]. Since previous studies demonstrated PNET2 survival mediated by growth factor activation of PI3 kinase [15,16,40], it was important to determine if the adverse effects of AD7c-NTP on insulin-stimulated neuronal survival were mediated by inhibition of PI3 kinase.

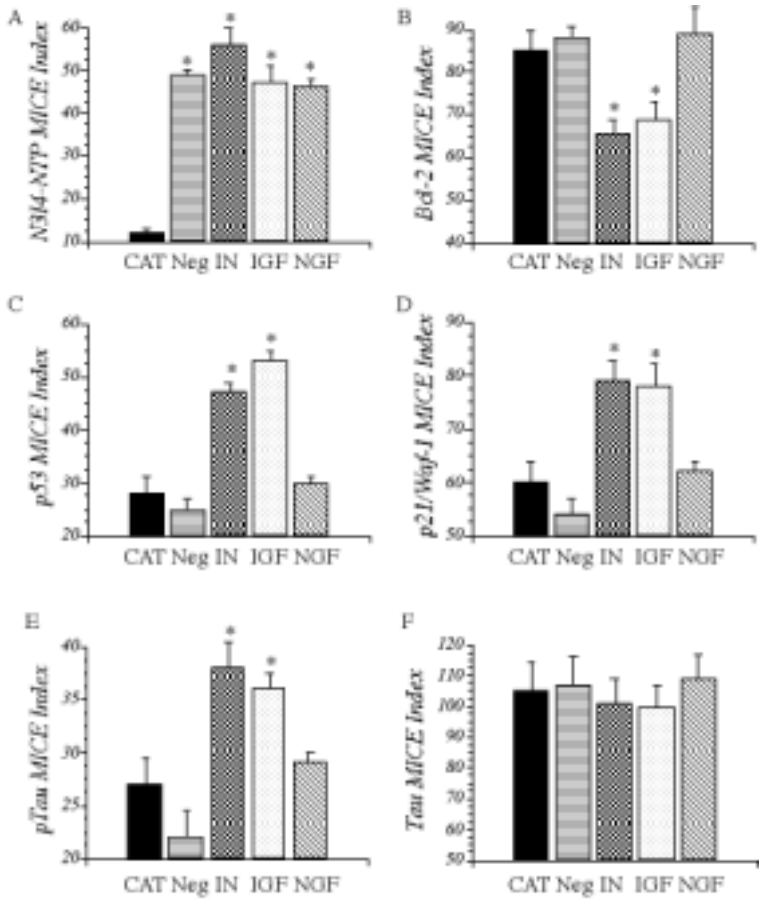


Fig. 5. Microtiter immunocytochemical ELISA (MICE) assays demonstrating differential effects of growth factor stimulation on (A) NTP (B) Bcl-2, (C) p53, (D) p21/Waf-1, (E) pTau, and (F) Tau protein levels. The cells treated with 3 mM IPTG to induce AD7c-NTP expression, and stimulated with insulin (IN; 50 nM), IGF-1 (IGF; 25 nM), or NGF (NGF; 5 ng/ml) for 24 hours. As controls, CAT-transfected cells that were stimulated with insulin and treated with 3 mM IPTG, and AD7c-NTP-transfected cells that were stimulated with insulin and treated with vehicle (Neg) were simultaneously studied. Immunoreactivity was detected using the MICE assay and 16 replicate cultures were used in the data analysis. Inter-group comparisons were made using ANOVA with Fisher's Least Significant Difference post-hoc test (*$P < 0.001$ relative to the corresponding negative control values).

Insulin and IGF-1 activate PI3 kinase by transmitting signals through insulin-receptor substrate (IRS) molecules [41–43], of which there are at least four known subtypes. Our studies were focused on IRS-1-transmitted signals because previous studies demonstrated IRS-1 expression in PNET2 cells [26,34]. Binding of tyrosyl phosphorylated (PY) IRS-1 to p85 activates PI3 kinase signaling [44]. The degree to which PY-IRS-1 associates with p85 correlates with the levels of IRS-1-associated PI3 kinase activity. Therefore, we assessed p85 interactions with PY-IRS-1 by Western blot analysis of IRS-1 immunoprecipitates. In addition, we measured total PI3 kinase activity in p85 immunoprecipitates since IRS-1-independent signaling also occurs, and PDGF and NGF activate PI3 kinase via IRS-independent mechanisms.

Un-stimulated cells had no detectable p85 binding to PY-IRS-1 or other tyrosyl phosphorylated signaling molecules, and correspondingly no detectable PI3 kinase activity. Insulin stimulated both IRS-1-associated and total PI3 kinase activities, and correspondingly increased the binding of p85 to PY-IRS-1 and other PY-associated signaling molecules (Fig. 7). As expected, PDGF stimulated total PI3 kinase activity and p85 binding to PY-associated signaling molecules, but had minimal effect on IRS-1-associated PI3 kinase since its actions are not mediated through IRS-1. The insulin- and PDGF-activated PI3 kinase activity remained detectable for 48 hours (Figs 7B and 7D), during which time cell loss was occurring. Cells transfected with the CAT gene exhibited similar patterns and degrees of PI3

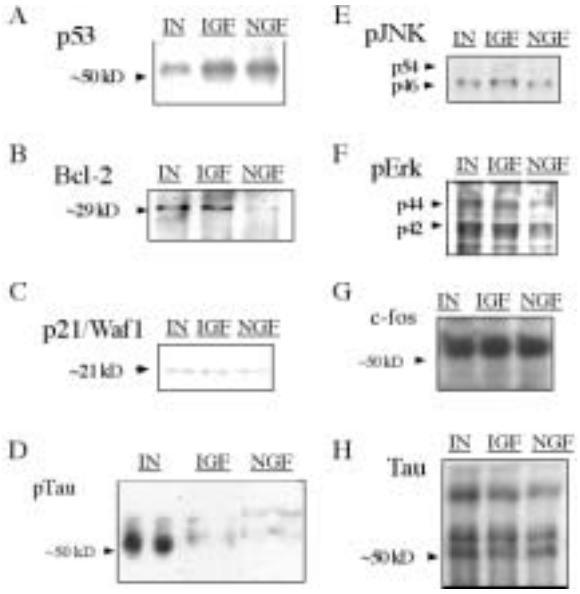


Fig. 6. Minimal growth factor modulation of (A) p53, (B), Bcl-2, (C) p21/Waf1, (D) pTau, (E) pJNK, (F) pErk, (G) c-fos and (H) Tau protein in cells induced to express CAT (3 mM IPTG) and stimulated with insulin (IN; 50 nM), IGF-1 (IGF; 25 nM), or NGF (NGF; 5 ng/ml) for 24 hours. Cell homogenates were subjected to Western blot analysis with equivalent amounts of protein loaded into each lane. Activated (phospho) Erk and JNK were detected with phospho-specific antibodies. Positions of molecular weight standards are shown at the left in Panels A-C and F. In Panels E and F, the two isoforms of pJNK and pErk MAPK detected are marked with arrowheads.

kinase activation in response to insulin and PDGF stimulation (data not shown), indicating that growth factor activation of the PI3 kinase survival pathway was intact in cells induced to express AD7c-NTP.

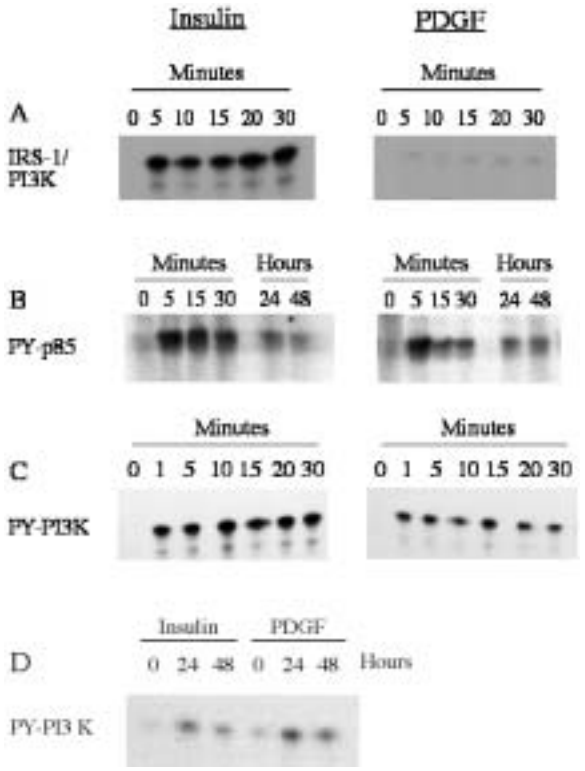


Fig. 7. Intact growth factor activation of phosphoinositol-3-kinase (PI3K) activity in PNET2 neuronal cells induced to express AD7c-NTP and stimulated with insulin (50 nM) or PDGF (25 ng/ml) for 0–30 minutes, and 0, 24, or 48 hours. (A) PI3K activity was measured in insulin receptor substrate, type 1 (IRS-1) immunoprecipitates. (B) Immunoprecipitation followed by Western blot analysis was used to assess interactions between the p85 subunit of PI3K and phosphotyrosine (PY) as a measure of total PI3K activity. (C and D) Total PI3K activity was measured in anti-PY immunoprecipitates after (C) short-term and (D) long-term growth factor stimulation to confirm results obtained by immunoprecipitation/Western blot analysis.

## 4. Discussion

The AD7c-NTP gene is over-expressed in brains with early to intermediate stages of AD [1,45,46]. AD7c-NTP immunoreactivity co-localizes with phospho-tau immunoreactive neuronal cytoskeletal lesions [6], and increased levels of AD7c-NTP and phospho-tau in CSF correlate with severity of dementia in AD [1,47]. To investigate the functional role of AD7c-NTP in relation to neuronal death, we established an inducible system in human CNS-derived PNET2 cells in which gene expression was regulated by IPTG. Using this model, we previously showed that AD7c-NTP over-expression causes neuronal death mediated by apoptosis and mitochondrial dysfunction [7]. The present study was designed to examine a novel mechanism by which AD7c-NTP may impair neuronal viability. The experiments were focused on the potential role of aberrant neuronal responses to growth factor stimulation, particularly insulin and IGF-1, because AD7c-NTP is predicted to have an insulin/IGF-1 chimeric receptor domain that may have functional significance [1].

The present study shows that AD7c-NTP-induced neuronal cell death occurs in the context of insulin and IGF-1 stimulation, but not with other growth factors. This suggests that over-expression of AD7c-NTP may interfere with insulin and IGF-1 stimulated survival mechanisms in CNS neurons. To explore this hypothesis, we examined the expression of pro-apoptosis and survival genes and signaling molecules in cells that were induced to express AD7c-NTP and stimulated with different growth factors. We observed that with in-

sulin or IGF-1, and not NGF or PDGF stimulation, the levels of pro-apoptosis gene products (p53, p21/Waf-1) were increased while survival molecules (Bcl-2) were reduced, whereas non-relevant gene products, e.g. c-fos were not modulated by growth factor stimulation. These effects were not associated with inhibition of growth factor-stimulated PI3 kinase activity, which has a major role in mediating survival in many cell types, including neurons. Instead, AD7c-NTP over-expression in insulin and IGF-1-stimulated cells was associated with increased levels of phospho-JNK and phospho-*tau*, and reduced levels of phospho-Erk MAPK, effects that mimic neuronal responses to oxidative injury [16, 22]. These results suggest that the findings in our previous study in which AD7c-NTP over-expression was demonstrated to increase oxidative stress and inhibit neuronal survival [7] were likely mediated by impaired neuronal responses to the IGF-1 present in FCS.

The present studies demonstrate that the adverse effects of AD7c-NTP expression may be linked to impaired survival signaling mediated by increased levels of activated stress kinase (phospho-JNK) and inhibition of growth factor signaling through Erk MAPK. How these effects occur is still unknown, but an important clue may reside in the co-accumulation of phospho-*tau*. Under physiological condition, *tau* can be phosphorylated in response to insulin or IGF-1 stimulation [48]. However, pathological states that result in increased oxidative stress and attendant activation of stress kinases [16,49–51] or GSK-3$\beta$ [52,53] can also promote *tau* phosphorylation, in which case, the cellular accumulation of phospho-*tau* may be sufficient to cause neuronal death [49]. In this regard, the cell loss that occurs with neurodegeneration, including AD, appears to be mediated in part by this type of mechanism [16, 50,51,54–58]. One potential explanation for the findings herein is that insulin and IGF-1 played critical roles in initiating *tau* phosphorylation, and AD7c-NTP-mediated impairment of mitochondrial function caused oxidative stress which then lead to the activation of stress kinases and further phosphorylation of *tau*, followed by its polymerization and cellular accumulation. In addition, recent studies demonstrating physical interactions between NTP and microtubule-associated proteins, including *tau*, provide an additional mechanism by which AD7c-NTP over-expression could contribute to phospho-*tau* accumulation in neurons [32]. Both of these scenarios could account for the co-accumulation of NTP and phospho-*tau* that occurs in brains with AD [6]. Moreover, one would predict that CNS neurons with abundant insulin or IGF-1 receptors might be more susceptible to the adverse effects of AD7c-NTP.

In the present study, we observed very prominent neuritic sprouting in cells that were induced to express AD7c-NTP and stimulated with IGF-1, NGF, or PDGF. Although control cells also exhibited neurite outgrowth, the responses appeared to be less elaborate, as was manifested by the presence of mainly short, unbranched processes compared with the relatively well-arborized neuritic processes in AD7c-NTP-transfected cells. Neuritic sprouting is positively regulated by signaling through MAP kinases [24,35–37,59], and correspondingly, the AD7c-NTP transfected cells that were stimulated with IGF-1, NGF, or PDGF had high levels of activated Erk MAPK. In contrast, the insulin-stimulated cells had relatively low levels of activated Erk MAPK, and correspondingly, they exhibited extensive cell rounding and neurite retraction. Insulin, IGF-1, NGF, and PDGF can support growth and promote neuritic sprouting (as observed herein), but with AD7c-NTP over-expression, the insulin-stimulated signaling mechanisms that promote neurite outgrowth were found to be impaired. Since neuritic growth is required for synaptic plasticity, the finding that AD7c-NTP inhibits this function in insulin-responsive CNS neuronal cells implies an additional mechanism by which AD7c-NTP may contribute to dementia in AD. Altogether, the results suggest that AD7c-NTP over-expression produces a state of relative insulin resistance in that insulin-stimulated survival and neuritic growth mechanisms are impaired. From the standpoint of therapeutic intervention, it may prove beneficial to restore insulin signaling mechanisms in neuronal cells that over-express AD7c-NTP by treatment with insulin sensitizer drugs [60]. Alternatively, since the NGF- and PDGF-stimulated cultures remained viable and exhibited prominent neuritic sprouting, exogenous supply of neurotrophic factors that function through insulin-independent pathways may prove to be effective for abrogating the adverse effects of AD7c-NTP in patients with early AD.

## Acknowledgements

Supported by Grants AA12458, AA02666, and AA11931 from the National Institute of Alcoholism and Alcohol Abuse, and COBRE Award P20RR15578 from the National Institutes of Health. The authors are also supported by Nymox Pharmaceutical Corporation.

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