Original Investigation

Nerve Growth Factor Gene Therapy
Activation of Neuronal Responses in Alzheimer Disease

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IMPORTANCE Alzheimer disease (AD) is the most common neurodegenerative disorder and lacks effective disease-modifying therapies. In 2001, we initiated a clinical trial of nerve growth factor (NGF) gene therapy in AD, the first effort at gene delivery in an adult neurodegenerative disorder. This program aimed to determine whether a nervous system growth factor prevents or reduces cholinergic neuronal degeneration in patients with AD. We present postmortem findings in 10 patients with survival times ranging from 1 to 10 years after treatment.

OBJECTIVE To determine whether degenerating neurons in AD retain an ability to respond to a nervous system growth factor delivered after disease onset.

DESIGN, SETTING, AND PARTICIPANTS Patients in this anatomicopathological study were enrolled in clinical trials from March 2001 to October 2012 at the University of California, San Diego, Medical Center in La Jolla. Ten patients with early AD underwent NGF gene therapy using ex vivo or in vivo gene transfer. The brains of all 8 patients in the first phase 1 ex vivo trial and of 2 patients in a subsequent phase 1 in vivo trial were examined.

MAIN OUTCOMES AND MEASURES Brains were immunolabeled to evaluate in vivo gene expression, cholinergic neuronal responses to NGF, and activation of NGF-related cell signaling. In 2 patients, NGF protein levels were measured by enzyme-linked immunosorbent assay.

RESULTS Among 10 patients, degenerating neurons in the AD brain responded to NGF. All patients exhibited a trophic response to NGF in the form of axonal sprouting toward the NGF source. Comparing treated and nontreated sides of the brain in 3 patients who underwent unilateral gene transfer, cholinergic neuronal hypertrophy occurred on the NGF-treated side ($P < .05$). Activation of cellular signaling and functional markers was present in 2 patients who underwent adeno-associated viral vectors (serotype 2)–mediated NGF gene transfer. Neurons exhibiting tau pathology and neurons free of tau expressed NGF, indicating that degenerating cells can be infected with therapeutic genes, with resultant activation of cell signaling. No adverse pathological effects related to NGF were observed.

CONCLUSIONS AND RELEVANCE These findings indicate that neurons of the degenerating brain retain the ability to respond to growth factors with axonal sprouting, cell hypertrophy, and activation of functional markers. Sprouting induced by NGF persists for 10 years after gene transfer. Growth factor therapy appears safe over extended periods and merits continued testing as a means of treating neurodegenerative disorders.

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Alzheimer disease (AD) is the most common neurodegenerative disorder, affecting approximately 50 million people worldwide. Therapies to slow the course of the disease do not exist and constitute an objective of great medical importance. Amyloid-modifying approaches have substantial mechanistic appeal for slowing disease progression. However, early clinical trial results with amyloid-depleting drugs have been disappointing, leading to the initiation of clinical trials in which amyloid-modifying treatment is initiated in presymptomatic patients or those at a very early stage. There remains a great unmet need to identify therapies with the potential to slow disease progression and improve cognitive function in AD.

Nervous system growth factors prevent neuronal death in various correlative animal models of AD, including amyloid-overexpressing mice, aged rats and primates, and rats and primates with lesions.[1-5] Of the approximately 50 identified nervous system growth factors, 2 are of particular relevance to AD, namely, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Specifically, NGF prevents the death of and stimulates the function of basal forebrain cholinergic neurons that undergo early and prominent degeneration in AD. It influences the nervous system in several species, including primates, and is present in the human brain.[4,6,7] Indeed, NGF levels in the basal forebrain region decline in AD.[8] Representing a second candidate growth factor treatment for AD, BDNF prevents the death of and stimulates the function of cortical neurons.[1,9] Work with NGF in models of AD was initiated approximately a decade earlier than work with BDNF, and it accordingly transitioned to human clinical trials in AD first[6] based on an extensive set of preclinical efficacy and safety studies.

In 2001, we initiated the first human clinical trial of gene delivery in an adult neurodegenerative disorder, administering the NGF gene to patients with early-stage AD.[6] We used gene delivery of NGF for 2 reasons. First, NGF is a large and polar protein; therefore, it does not penetrate the blood-brain barrier after peripheral administration and requires central administration to exert its effects on degenerating neurons. Second, if NGF broadly circulates throughout the brain, it elicits intolerable adverse effects, including pain and weight loss, by stimulating nociceptive and hypothalamic neurons, respectively.[10,11] Moreover, NGF induces Schwann cell migration into the brain.[11] Ironically, these effects of broad NGF availability in the nervous system reflect its biological potency on mature neurons. Accordingly, growth factor testing in humans requires a long-term delivery method that achieves central delivery and restricted distribution only to regions of degenerating basal forebrain cholinergic neurons. Gene therapy is one of the few means of achieving this goal.

We herein report biological responses to NGF in a large series of brain samples of humans who have undergone gene therapy. The brains were studied of 10 patients with AD having survival times ranging from 1 to 10 years after treatment.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

Patients in this anatomicopathological study were enrolled in clinical trials from March 2001 to October 2012 at the University of California, San Diego, Medical Center in La Jolla. Eight patients were enrolled in the first phase 1 clinical trial, which used methods of ex vivo gene therapy to deliver NGF. Briefly, autologous fibroblasts were expanded from skin biopsy specimens, transduced to express human NGF using Moloney leukemia viral (MLV) vectors, and implanted into the basal forebrain region to act as biological "minipumps" for NGF secretion.[6] The basal forebrain region contains cholinergic cell bodies that send their projections throughout the cortex and hippocampus, which are necessary for maintenance of cognitive function, and undergo atrophy in early AD.[12-14] The clinical findings of the phase 1 ex vivo trial have been reported,[6] suggesting increases in cortical positron emission tomographic metabolism and possible reductions in cognitive decline over a 2-year observation period compared with pretreatment rates of cognitive decline. Ten patients were enrolled in a second phase 1 clinical trial from January 2005 to October 2007 in which the method of gene delivery was changed to in vivo gene therapy. Adeno-associated viral vectors (serotype 2) (AAV2) were injected into the basal forebrain region, genetically modifying cells of the brain itself, rather than using grafts of autologous cells as in the phase 1 ex vivo trial. The AAV2-NGF vectors represent a simpler and less costly method of gene delivery and result in long-term gene expression in the nonhuman primate brain of at least 7 years' duration.[15] Vectors were prepared by Ceregene, Inc as indicated in public filing documents (recombinant advisory committee filings) using good manufacturing process practices. Patients 1 through 3 received a total vector dose of 1.2×10^{10} vector particles, patients 4 through 6 received a total vector dose of 5.8×10^{10} vector particles, and patients 7 through 10 received a total vector dose of 1.2×10^{11} vector particles.[16]

Study enrollment criteria have been reported.[6,16] In brief, patients had a clinical diagnosis of probable AD based on National Institute of Neurological Disorders and Stroke criteria, were 50 to 80 years old, and had baseline Mini-Mental State Examination scores in the range of 20 to 30 for the ex vivo trial and 16 to 26 for the in vivo trial.

All aspects of the study were approved by the institutional review boards for each of the participating centers. Consents for autopsy were obtained from all patients per approved Human Research Protection Program protocols. The clinical trial identifiers for studies described in the article are NCT00087789 and NCT00017940.

Details of the surgical methods for gene delivery have been summarized previously.[6] Briefly, magnetic resonance imaging (MRI) was performed with the patient in an MRI-compatible stereotaxic head frame to identify brain targets for cell or viral vector injection. In the ex vivo gene delivery trial, cells were stereotaxically implanted into 5 sites equally spaced over the rostral to caudal extent of the cholinergic nucleus basalis of

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Table. Patient Characteristics[a]

Patient No./Sex/Age at AD Diagnosis, y	Age at Gene Transfer, y	Time to Death After Gene Transfer	MMSE Score at Study Entry	Type of Gene Delivery
1/F/59	60	5 y	23	Ex vivo
2/F/69	70	7 y	20	Ex vivo
3/F/70	72	10 y	27	Ex vivo
4/F/71	72	7 y	21	Ex vivo
5/M/71	72	3 mo	20	Ex vivo
6/M/69	73	9 y	25	Ex vivo
7/F/63	64	7 y	18	Ex vivo
8/M/75	76	5 y	25	Ex vivo
9/F/53	56	3 y	17	In vivo
10/M/75	78	11 mo	21	In vivo

Abbreviations: AD, Alzheimer disease; MMSE, Mini-Mental State Examination.

[a] All patients had a Braak stage of V/VI.

Meynert (NBM), a total distance of approximately 12 mm (ie, injections were spaced at approximately 2.5-mm intervals). This spacing reflected the distance over which NGF spreads from gene delivery sites in nonhuman primates.[16] In the in vivo gene therapy trial, 3 vector injections were made targeting the caudal two-thirds of the NBM that constitute the majority cholinergic projection to the cortex, spaced at approximately 2.5-mm intervals.

Patient characteristics are summarized in the **Table**. In total, 4 men and 6 women were enrolled in the trial. The mean (SEM) age at AD diagnosis was 67.5 (2.2) years, and the mean (SEM) age at gene transfer was 69.3 (2.2) years. Patients survived a mean (SEM) of 5.4 (1.0) years after gene transfer. Three control AD brains were included in the analyses from 2 men and 1 woman with a mean (SEM) age at death of 79.3 (0.6) years (79, 79, and 80 years old, respectively) and a Braak stage of V, IV, and IV, respectively.

Histology and Analyses

Brains were harvested and placed in a solution of 4% paraformaldehyde for 72 hours and then switched to a 20% glycerin solution for 2 weeks. The brains were blocked into anterior, middle, and posterior sections, and blocks containing the NBM were sectioned on a freezing microtome set at 40-µm intervals. To evaluate effects of NGF delivery in the AD brain, we performed the following 5 sets of tissue labels: (1) Nissl stain for general cellular morphology, including visualization of fibroblast grafts in ex vivo patients; (2) NGF immunolabeling (NGF antibody, 1:500 dilution for fluorescence and 1:1000 dilution for light-level labeling)[17]; (3) p75 immunolabeling, which detects the low-affinity NGF receptor and is expressed by NGF-responsive cholinergic neurons of the basal forebrain,[2-4] to assess whether gene delivery altered the size or function of NGF-responsive cells (1:1000 dilution antihuman monoclonal antibody, clone NGFR5, produced by a hybridoma cell line from Mark Bothwell, PhD, University of Washington, Seattle); (4) c-fos, to assess activation of cell signaling classically related to NGF signaling (1:100 dilution, goat antihuman; Santa Cruz Biotechnology); and (5) phosphorylated tau immunolabeling to detect neurofibrillary degeneration (AT180, 1:50 dilution, mouse anti–PHF-1; Thermo). In addition, brain tissues from different regions (frontal cortex, superior temporal cortex occipital cortex, substantia nigra, basal ganglia, amygdala, and hippocampus/entorhinal cortex) were processed at the Human and Animal Tissue Technology Core at the University of California, San Diego, La Jolla, using an Aβ antibody (69D at 1:1500 dilution from Edward Koo), phosphorylated tau (PHF-1 at 1:300 dilution from Peter Davies), and phosphorylated synuclein antibody (81A at 1:15 000 dilution from Virginia Lee, PhD, University of Pennsylvania, Philadelphia). These sections were used to determine the Braak stage of the patients.

For light-level immunolabeling, sections were rinsed in 0.1M Tris-buffered saline (TBS), incubated in 0.1M sodium periodiate for 20 minutes, blocked in 3% donkey serum and 2% bovine serum albumin (BSA) in 0.05% Triton-X in TBS for 1 hour, and incubated in primary antibody (2.5 µg/mL for the 192 IgG) in 1% donkey serum plus 1% BSA in 0.05% Triton-TBS for 72 hours at 4°C. Bound antibodies were detected by incubating sections in 1.5 µg/mL of biotinylated IgG (goat antirabbit IgG [BA-1000] or horse antimouse IgG [BA-2000]; Vector Laboratories) for 2 hours and in an avidin-biotin peroxidase reagent (1:250 dilution ABC Elite; Vector Laboratories) for 90 minutes. Sections were then rinsed in imidazole acetate buffer (pH 7.4) and treated with a solution containing 0.05% diaminobenzidine tetrahydrachloride, 2.5% nickel ammonium sulfate, and 0.15% hydrogen peroxide in imidazole acetate solution. For NGF light-level immunolabeling, sections first were submitted to an antigen retrieval protocol and were postfixed with 2% paraformaldehyde and 0.2% parabenzoquinone.

For fluorescent immunolabeling, sections were postfixed in 2% paraformaldehyde and 0.2% parabenzoquinone. To block autofluorescence, sections were treated with 0.1M sodium borohydride for 30 minutes, followed by 30 minutes in 0.5% Sudan black. Sections then underwent blocking with 3% donkey serum and 2% BSA in TBS. Sections were incubated in primary antibodies for 72 hours at 4°C, and specific labeling was detected using Alexa Fluor (Life Technologies) secondary IgG antibodies and 4′,6-diamidino-2-phenylindole (1:1000) for nuclear labeling. Using a tyramide signal amplification protocol, c-fos labeling was enhanced.

Quantification of the size of p75 cell bodies was performed in 3 patients who received unilateral transplantation of the autologous fibroblasts expressing NGF. Using a stereological microscope system (StereoInvestigator; Microbrightfield Bioscience), the size of p75 cells was measured in the intermediate region of the NBM on the side of the graft and in

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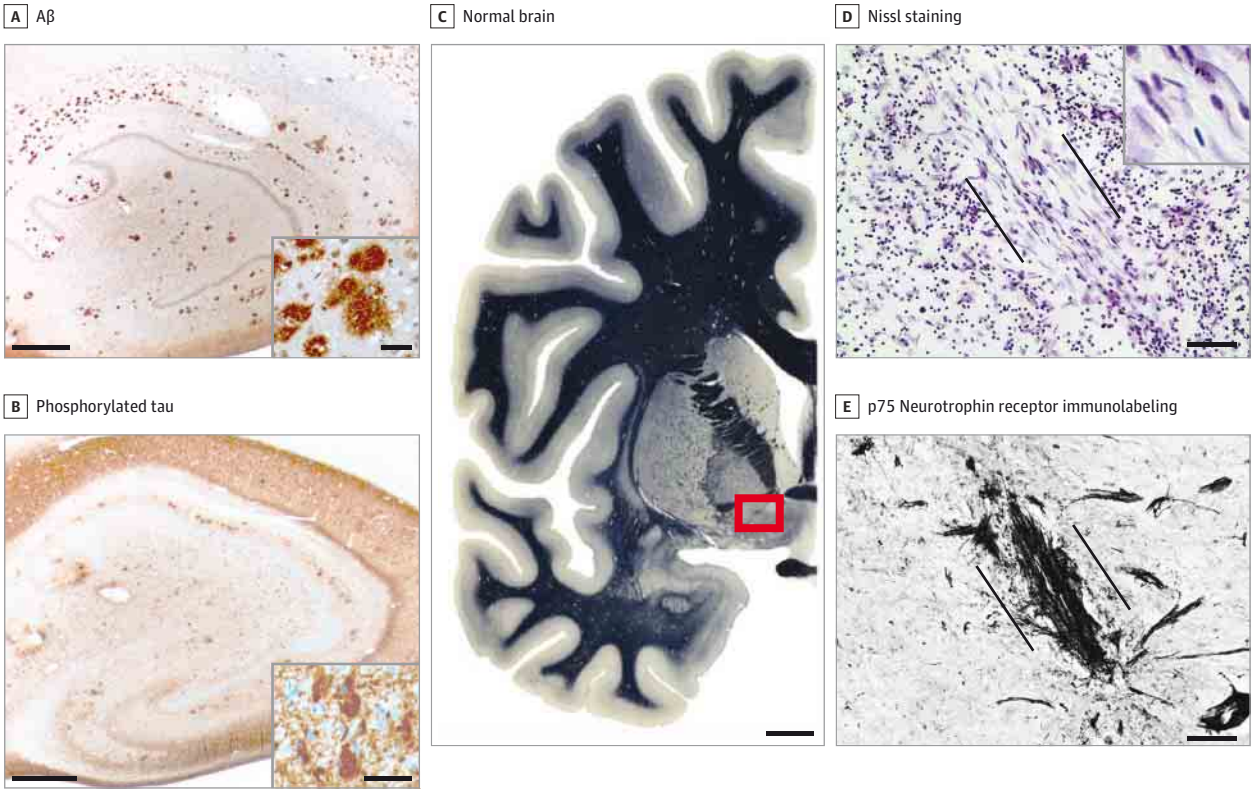
Figure 1. Alzheimer Disease Pathology and Site of Gene Delivery in a Patient 7 Years After Gene Transfer



All participants in this study exhibited Alzheimer pathology. A, The hippocampus shows numerous plaques. The inset shows diffuse and dense-core plaques. B, Tangles and dense neurofibrillary tau pathology are present (also evident in the inset), indicated by tau phosphorylation (PHF-1). C, Shown is an overview of the left half of a normal human brain, with the boxed region indicating a region containing neurons of the nucleus basalis of Meynert that are targeted by nerve growth factor gene delivery. Myelin staining is reproduced with permission from the *Atlas of the Human Brain*.[19] D, Shown is Nissl staining of a graft of autologous fibroblasts that were genetically modified to secrete human nerve growth factor and injected into the nucleus basalis of Meynert. Cells had been injected 7 years previously, and the graft is located between the parallel lines. Cells survived and exhibited typical fibroblast morphology (inset). E, In the adjacent section of D, p75 neurotrophin receptor immunolabeling shows basal forebrain cholinergic axons penetrating into the graft (between the parallel lines) in a linear fashion, and this concentration of p75 fibers is only present at graft sites (confirmed by Nissl staining). The bar represents 300 µm in A (inset 50 µm), 300 µm in B (inset 50 µm), 6 mm in C, 30 µm in D, and 25 µm in E.

the control hemisphere. A counting frame of 300 × 300 µm was used with a sampling grid of 600 × 600 µm (1:4 sample ratio) or 900 × 900 µm (1:9 sample ratio) to sample p75 neurons in the intermediate NBM. Cell body size was measured using a cross-sectional area of the soma at the level of the cell body based on the intersection of a 6-ray extension from the nucleus.

NGF Enzyme-linked Immunosorbent Assay

Using methods previously described,[18] levels of NGF were measured by enzyme-linked immunosorbent assay (ELISA) in patient 10 in the Table, who received AAV2-NGF and survived 11 months, and in patient 7 in the Table, who underwent ex vivo NGF gene transfer and survived 7 years. Briefly, the brains were blocked into 1-cm-thick coronal segments. The crossing of the anterior commissure across the midline demarcated the approximate first deposit of AAV2-NGF, while the presence of the mamillary bodies demarcated the approximate location of the third deposit. Brain punches were extracted from these regions, slightly dorsal to the known location of the cholinergic cell bodies using 2-mm-diameter skin biopsy punches. These specimens were snap frozen to −80°C. When all specimens were collected, NGF ELISA was performed as reported.[18]

Statistical Analysis

Within-patient comparisons were made using paired *t* test, with a significance criterion of $P < .05$. Results are reported as the mean (SEM).

Results

Among 10 patients, we found that NGF elicits classic "trophic" responses in the AD brain, including neuronal hypertrophy, axonal sprouting, and activation of cell signaling. These results provide evidence that growth factors consistently stimulate the functional state of degenerating neurons in chronic neurodegenerative disorders, constituting a rationale for continued testing of growth factors as neuroprotective agents in human degenerative nervous system disorders.

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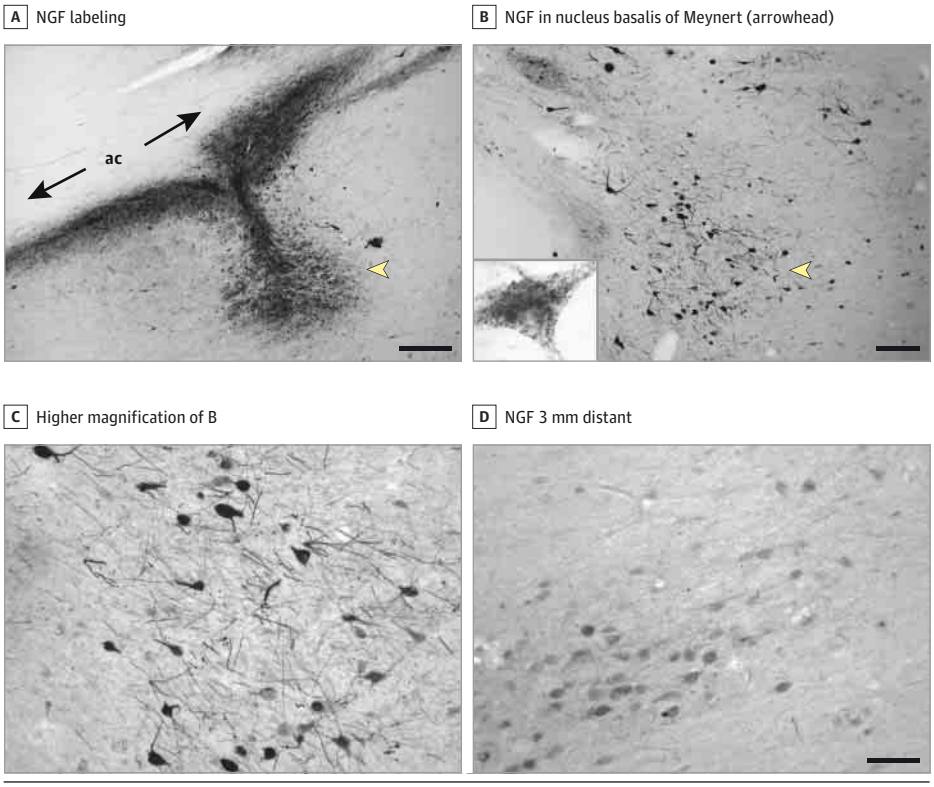
Figure 2. Adeno-Associated Viral Vectors (Serotype 2)–Nerve Growth Factor Gene Expression



A, Nerve growth factor labeling shows the site of nerve growth factor gene delivery in the nucleus basalis of Meynert (arrowhead) under the anterior commissure (ac). The patient had received the injection 3 years previously. B, Another site from same brain shows nerve growth factor uptake in neurons in the region of the nucleus basalis of Meynert (arrowhead). The inset shows a single neuron with granular intraneuronal labeling. C and D, Higher magnification of nerve growth factor–expressing neurons (C, from the region of the arrowhead in B) compared with less intense labeling in the nucleus basalis of Meynert neurons located 3 mm from the injection site (D). The bar represents 325 µm in A, 250 µm in B, and 100 µm in C and D.

Pathological Evaluation of Brains

A pathological diagnosis of AD was confirmed in all study participants (**Figure 1**), with late-stage disease (Braak stage V/VI) evident in each patient at the time of death (Table). Labeling for Aβ and tau showed classic signs of AD in all patients, including NGF-treated participants and control AD brains (Figure 1A and B). In patients treated with autologous fibroblasts genetically modified to secrete NGF (ex vivo gene therapy), surviving deposits of fibroblasts were observed within the region of the NBM up to 10 years after gene delivery (Figure 1C-E).

NGF Expression After NGF Gene Transfer

The 2 patients who underwent in vivo AAV2-NGF–mediated gene therapy exhibited targeting of the vector to the region of the NBM, as revealed by NGF immunolabeling, 1 year and 3 years after gene transfer, respectively (**Figure 2**). Neuronal NGF accumulation was distinctly more intense in neurons adjacent to sites of NGF delivery compared with neurons more distantly located (>3 mm) (Figure 2C and D). The presence of NGF labeling indicated that gene expression persisted throughout this period. Using ELISA, NGF protein levels were measured in one patient treated with AAV2-NGF who had undergone gene delivery 1 year earlier and detected 1230 pg of NGF per gram of tissue in the NBM. An adjoining control region of the NBM located 5 mm from the site of NGF delivery in the same patient contained only 22 pg of NGF per gram of tissue. Therefore, AAV2-NGF gene delivery had increased NGF protein by more than 50-fold at 1 year after gene delivery in this patient. In a patient who had undergone ex vivo gene transfer using grafts of autologous fibroblasts and died 7 years after treatment, NGF ELISA from the same region detected 50 pg of NGF per gram of tissue. This 2-fold increase compared with control regions is within the variance of the assay.

Trophic Responses to NGF in the AD Brain

Each graft in patients in the ex vivo gene therapy trial showed penetration by cholinergic axons labeled for p75, the low-affinity neurotrophin receptor that is exclusively expressed by cholinergic neurons and their axons in the basal forebrain region (Figure 1E and **Figure 3**). The growth of cholinergic axons toward and within an ectopic location reflects sprouting of these axons toward the growth factor produced by gene delivery, a classic trophic effect[4] that has been reported in animal studies.[4,20,21] Cholinergic axonal sprouting into sites of NGF gene delivery was evident in all brains, including that of the longest surviving ex vivo gene transfer patient (10 years after treatment), which showed p75 axons (Figure 3D and E).

Effects of NGF on Cell Size and Functional Markers

The p75-labeled cholinergic neurons located in regions of NGF delivery appeared to exhibit hypertrophy compared with neurons more remotely located (>3 mm) and compared with neurons in AD control brains (Figure 3A-C). To quantify this effect, cholinergic cell size in p75-labeled sections was measured in patients 1, 2, and 5 in the Table, who received unilateral delivery of NGF to the NBM. The mean somal area was measured on the treated side and the untreated side of the brain. Significant hypertrophy was present on the treated side. The

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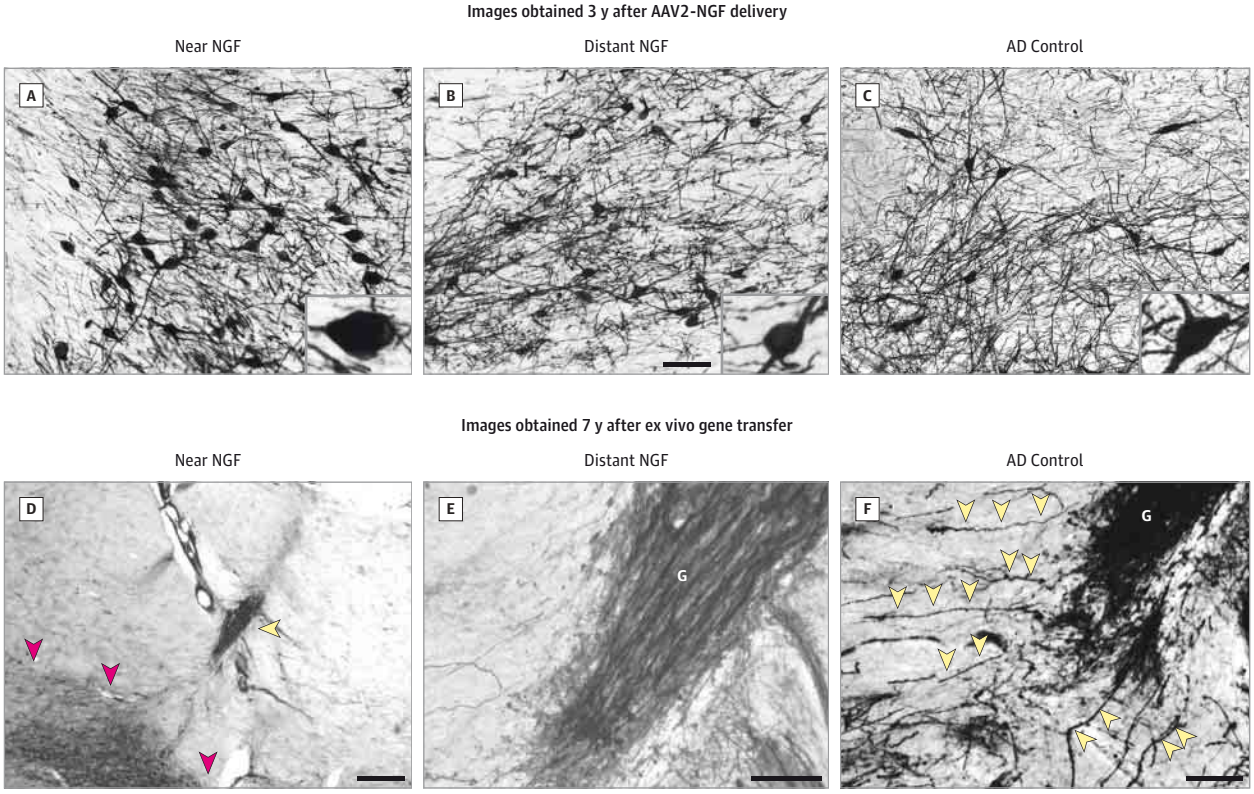
Figure 3. Cholinergic Neuronal Hypertrophy and Sprouting



Shown is labeling for p75, a neurotrophin receptor expressed on cholinergic neurons of the nucleus basalis of Meynert. Images were obtained 3 years after adeno-associated viral vectors (serotype 2)–nerve growth factor (AAV2-NGF) delivery (A-C) and 7 years after ex vivo gene transfer (D-F). A-C, Cholinergic neurons are labeled for p75 within the zone of transduction (A), 3 mm from the zone of transduction (B), and in a control Alzheimer disease (AD) brain of the same Braak stage (C). Cells near the NGF transduction region appear larger. The inset shows higher-magnification views of a typical neuron from each region. D, Shown is a graft of fibroblasts transduced to secrete NGF (yellow arrowhead) adjacent to the nucleus basalis of Meynert (red arrowheads). E, The graft (G) at higher magnification is densely penetrated by p75-labeled axons arising from the nucleus basalis of Meynert. These axons are sprouting toward the graft, a classic trophic response. F, Shown are p75-labeled axons from the nucleus basalis of Meynert sprouting toward the graft. Individual axons coursing toward the graft are evident (arrowheads). The bar represents 125 µm in A through C, 500 µm in D, and 100 µm in E and F.

mean (SEM) cholinergic cell soma areas were 948 (44) μm^2 on the treated side and 890 (28) μm^2 on the untreated side ($t_2 = 3.62$, $P < .05$, paired t test), a difference of 7%.

The AAV2-NGF vectors infected neurons with almost complete specificity. Double-labeling for NGF, glial fibrillary acid protein, and Olig-2 demonstrated that more than 99% of cells expressing a cell-specific marker were neurons. This result is consistent with findings in AAV2 gene transfer in the nonhuman primate brain.[22]

NGF gene transfer is predicted to stimulate the functional state of neurons based on canonical activation of CREB and c-fos signaling after trophic stimulation.[23] Phosphorylated CREB and c-fos expression was examined in the 2 patients who underwent AAV2-NGF gene transfer in the NBM. These patients were chosen because NGF expression was unequivocally detected by immunolabeling in both of them (Figure 2 and ELISA data above in the NGF Expression After NGF Gene Transfer subsection of the Results section). Compared with regions outside the zone of NGF delivery, expression of CREB and c-fos was elevated (**Figure 4**A-F). Moreover, neurons actively expressing tau pathology were infected by the AAV2-NGF vector, shown by co-labeling for NGF and tau (Figure 4G-I), indicating that actively degenerating neurons retain an ability to incorporate a therapeutic vector.

Safety Studies and the Absence of Off-Target Effects of NGF

In rat and nonhuman primate models, NGF distribution throughout the neuraxis after intraventricular or intrathecal infusions results in Schwann cell migration and hypertrophy in the subpial space of the brainstem and spinal cord.[11] This potential adverse effect is not observed after gene delivery to focal parenchymal targets in animal studies.[4,24,25] In all 10 human brains examined in this study, sectioning of the medulla failed to show evidence of Schwann cell hyperplasia (eFigure in the Supplement). Other than histological changes associated with AD, no tumor formation or evidence of neural toxicity was detected.

Discussion

Evidence from 10 patients with AD who underwent NGF gene transfer indicates that growth factors consistently promote

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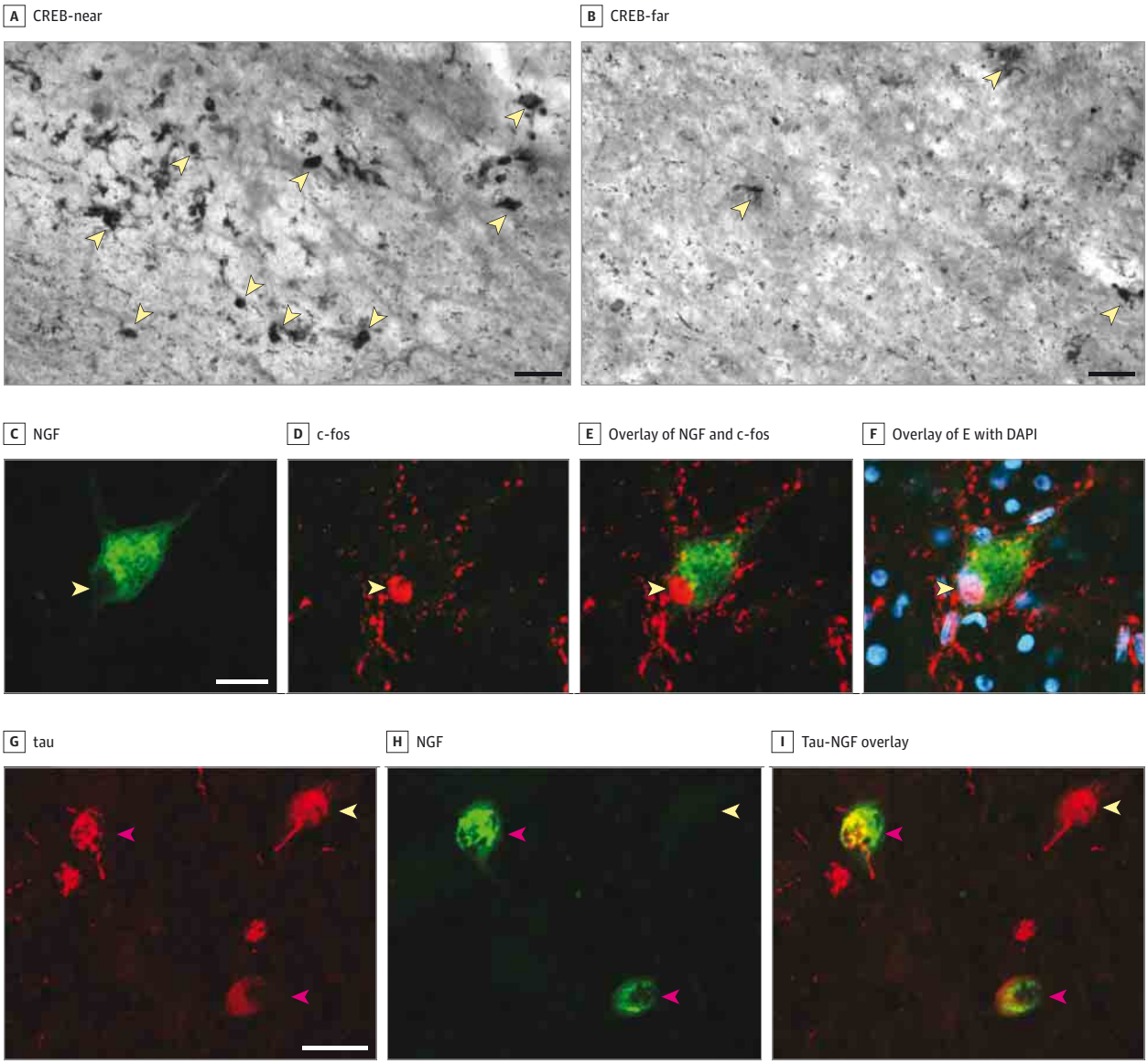
Figure 4. Cell Activation After Nerve Growth Factor (NGF) Gene Delivery



A and B, In regions of NGF expression (CREB-near), there is induction of phosphorylated CREB expression, a canonical mediator of downstream neurotrophin signaling and cell activation[23] compared with regions of the nucleus basalis of Meynert 3 mm from the site of NGF injection (CREB-far). Arrowheads indicate examples of individual nuclei labeled for CREB. The patient had received adeno-associated viral vectors (serotype 2)–NGF (AAV2-NGF) 3 years earlier. A similar response was evident in a patient who had received AAV2-NGF and survived 1 year. C-F, An individual neuron expressing NGF (green) also expressed c-fos, another canonical marker of neurotrophin-mediated activation of cell signaling.[23] As expected, c-fos labeling is nuclear (arrowheads). This image is from the brain of the patient who had undergone AAV2-NGF gene transfer 3 years earlier. F, Staining with DAPI for cell nuclei. G-I, Shown is NGF expression in degenerating neurons expressing tau pathology. G, Neurons in the nucleus basalis of Meynert exhibit characteristic tau pathology, indicated by tau phosphorylation (AT180 antibody). H, Two neurons from G also express NGF (red arrowheads). A third neuron in the field (yellow arrowhead) expressed tau but not NGF. I, The overlay shows that actively degenerating neurons can be infected by AAV2-NGF to express a potentially therapeutic gene product. DAPI indicates 4′,6-diamidino-2-phenylindole. The bar represents 50 µm in A and B, 35 µm in C through F, and 25 µm in G through I.

classic trophic responses in neurons of the degenerating human brain. Axons sprouted toward the local source of NGF in every brain examined, following concentration gradients of NGF.[4] Sprouting is a standard neuronal response to trophic factors applied to the brain.[4,26-30] Cell hypertrophy, another classic response to a trophic stimulus,[4,31,32] was also observed in the brains of 3 patients who underwent unilateral ex vivo NGF gene transfer. We measured cholinergic cell size in them because the contralateral, untreated side of the brain provided a within-patient control. Further evidence of activation of growth factor–induced canonical trophic signaling[23] was observed in 2 patients who had undergone AAV2-NGF in vivo gene transfer, with increased neuronal labeling for CREB in regions of gene delivery. Collectively, these anatomical findings support the rationale for clinical trials to test the hypothesis that sustained growth factor delivery over time can reduce

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cell degeneration and stimulate cell function in chronic neurodegenerative disorders, thereby slowing functional decline. A phase 2 multicenter, randomized, sham surgery-controlled trial of AAV2-NGF gene delivery is under way that is measuring cognitive outcomes in patients with AD.

To our knowledge, the present study presents anatomical findings from the largest cohort to date of patients in any gene transfer clinical trial. Previous case reports suggested that growth factors could influence trophic responsiveness in AD[6] and Parkinson disease,[33] but the consistency and extent of these responses across patient cohorts have not been examined previously. The findings herein demonstrate that every participant in an extended cohort of 10 patients exhibited trophic responses, reflecting the consistency and potency of the trophic response, despite the presence of an ongoing and severe neurodegenerative process. We show that cells exhibiting tau pathology can also express NGF, suggesting that individual neurons affected by neurodegeneration retain the ability to become infected with viral vectors and express a potentially therapeutic gene.

We also present an actual measure of growth factor protein levels after gene transfer in the human brain by ELISA and found that levels of the growth factor exceeded physiological levels by approximately 50-fold in one patient who had undergone AAV2-NGF gene transfer 1 year earlier. The AAV2 vector expressed NGF from the relatively potent CAG promoter, which has sustained in vivo gene expression for at least 7 years in a nonhuman primate brain model.[34] ELISA was also performed in one patient who had undergone ex vivo gene transfer 7 years earlier; in this patient, levels of NGF in the basal forebrain showed a small increase of 2-fold compared with a control region of the same brain, but this increase could be within the variance of the assay. As expected, the lower level of NGF at this extended 7-year time point suggests that gene expression after ex vivo gene transfer declines over time. In patients who had undergone ex vivo gene transfer, NGF was expressed by the MLV promoter, which is generally less potent than the CAG promoter.

Moreover, we demonstrate the safety of growth factor gene transfer for 10 years' duration. Over this protracted period, there remains persistent evidence of trophic responses to NGF, which raises the possibility that once-in-life treatment with growth factor gene transfer could enable protection for extended periods without retreatment.

Finally, we demonstrate long-term safety at the anatomical level after gene transfer in the nervous system. Growth factor gene expression in vivo did not lead to any of the pathological changes that have previously been reported after broad NGF distribution through the cerebrospinal fluid, including the migration and proliferation of Schwann cells in the subpial space of the medulla.[11] Moreover, at sites of NGF gene transfer in the NBM, there was no evidence of localized cell toxicity that resulted from gene transfer and no signs of broader cell toxicity or tumor formation.

These findings support the rationale for continued development of growth factor therapy to determine whether this potent class of biologically active molecules will slow neurodegeneration and activate the functional state of neurons in human disease. A phase 2 multicenter trial of NGF gene therapy in AD and a phase 2 trial of glial cell line–derived neurotrophic factor gene therapy in Parkinson disease are addressing this possibility. Earlier studies[21,35,36] indicate that the types of trophic responses elicited by NGF in the present study have been associated with amelioration of cognitive deficits in animal models of neurodegeneration. Moreover, several new neurological gene therapy indications are undergoing clinical translation, including trials in spinal muscular atrophy, retinitis pigmentosa, Leber congenital ameurosis, Parkinson disease, and other disorders.

Conclusions

The NGF gene therapy administered to patients with established AD resulted in classic trophic responses, characterized by axonal sprouting in all patients examined herein. Responses to NGF persist up to 10 years after gene transfer. Subsets of patients examined for neuronal hypertrophy and CREB activation provide additional evidence of trophic activity. No adverse pathological effects were observed over this period, supporting the safety of and the rationale for expanded clinical programs under way in AD, Parkinson disease, and other neurological indications.

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