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Detection of NGF-Like Activity in Human Brain Tissue: Increased Levels in Alzheimer's Disease

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A two-site ELISA and a bioassay were used to detect NGF-like activity in human brain tissue. Both assays detected mouse NGF and recombinant human NGF with approximately equal sensitivity, whereas the antibodies showed little cross-reactivity with the recombinant human proteins NT-3 and brain-derived neurotrophic factor. NGF-like activity was detected in fresh human cortical samples obtained from epileptic patients, with the highest activity observed in the right hemisphere of men. NGF-like activity was subsequently measured in autopsy samples of frontal and occipital cortex from patients with Alzheimer's disease (AD) and from individuals with no history or pathological evidence of AD. Based on both the ELISA and the bioassay measurements, NGF-like activity was significantly elevated in both brain regions in AD. These results demonstrate the feasibility of detecting NGF-like activity in both fresh and postmortem human brain tissue and further suggest that AD is characterized by increased, rather than decreased, levels of cortical β-NGF. The AD-related increase in NGF may be a consequence of degenerative changes in the basal forebrain cholinergic system.

[Key words: NGF, neurotrophin, ELISA, Alzheimer's disease, bioassay, human]

NGF was the first discovered member of what is now thought to be a family of nerve growth factors (neurotrophins) that exhibit effects on the survival and neurite outgrowth of specific classes of neurons (Levi-Montalcini and Angeletti, 1968). Although early studies indicated that NGF was not present in the mammalian brain, there is now a large body of evidence demonstrating the presence, as well as regional distribution, of NGF-like protein and NGF mRNA in the brains of rodents and nonhuman primates (Crutcher and Collins, 1982; Korsching et al., 1985; Large et al., 1986; Shelton and Reichardt, 1986; Whittemore et al., 1986; Whittemore and Seiger, 1987; Hayashi et al., 1990). The demonstration that NGF exhibits pharmacological effects on basal forebrain cholinergic neurons both *in vitro* (Gähwiler and Hefti, 1987; Hsiang et al., 1989) and *in vivo* (Hefti, 1986; Mobley et al., 1986; Williams et al., 1986; Kromer, 1987; Gage et al., 1988; Koliatsos et al., 1991; Tuszynski et al., 1991) has led to the suggestion that NGF normally serves to maintain these cells and that decreased NGF could contribute to the loss of basal forebrain neurons in Alzheimer's disease (AD; Hefti, 1983; Hefti and Weiner, 1986). However, levels of NGF mRNA do not show declines in AD (Goedert et al., 1986; Ernfors et al., 1990b; Phillips et al., 1991). Using an ELISA, Allen et al. (1991) recently found no decline in NGF-like protein in AD cortex compared with age-matched control tissue, although no information was provided regarding the specificity of the antibodies and no bioassay measurements were reported.

The availability of sensitive immunological and biological assays to detect low levels of NGF and related factors (Furukawa et al., 1986; Korsching and Thoenen, 1987; Lärkfors and Ebendal, 1987; Weskamp and Otten, 1987; Heinrich and Meyer, 1988; Söderström et al., 1990) now makes it possible to detect NGF-like activity in human brain tissue samples. It is particularly important to employ both types of assays in order to avoid erroneous conclusions (Suda et al., 1978). Furthermore, the identification and purification of other neurotrophins such as brain-derived neurotrophic factor (BDNF), NT-3, and NT-4 (or NT-5) (Ernfors et al., 1990a,c; Maisonpierre et al., 1990a,b; Berkemeier et al., 1991; Lindsay et al., 1991) afford the opportunity to determine whether these factors can be detected with assays developed for measuring NGF (Acheson et al., 1991). In the present study, we characterized the specificity of a two-site ELISA and a sensitive biological assay in detecting the recombinant human proteins NGF (rhNGF), rhBDNF, and rhNT-3. We found that both assays reliably detect mouse NGF and rhNGF with approximately equal sensitivity. The ELISA failed to detect either rhBDNF or rhNT-3, whereas weak cross-reactivity was detected with the bioassay and with immunoblots. We then used these assays to measure NGF-like activity in cortical biopsy tissue removed from epileptic patients and in postmortem tissue samples from patients with or without AD. In the latter case, both assays revealed an increase in NGF-like activity in AD compared with control tissue.

Materials and Methods

Fresh human brain tissue. Fresh tissue samples were obtained from the right ($n = 9$) or left ($n = 12$) cortex of patients being treated for intractable epilepsy (samples were obtained through the assistance of Dr. Hwa-Shain Yeh, Department of Neurosurgery, and Dr. Michael Privitera, Department of Neurology, University of Cincinnati Medical Center). Immediately following surgical removal, the specimens were frozen on dry ice and stored at −70°C until the time of assay. Specimens were obtained from 21 patients (10 women and 11 men). Most were being

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treated for temporal lobe epilepsy, although five were being treated for multifocal epilepsy and underwent partial resections of the frontal lobe as well. Mean patient age was 31 years (range, 17–46), and the average history of seizures was 21 years prior to surgery. Samples were primarily taken from the temporal lobe, but in five patients samples were also obtained from the frontal lobe, and for these patients it was impossible to determine whether the samples were from the temporal or frontal lobe.

Postmortem tissue. All of the control tissue and six of the AD brains were obtained through the Alzheimer's Disease Research Center at the University of Cincinnati. Five additional AD brains were obtained from the AD Research Center at Case Western Reserve University. The diagnosis of AD was initially made on clinical criteria (McKhann et al., 1984) and was subsequently confirmed using neuropathological criteria (Khatchaturian, 1985). Control tissue samples were obtained from patients without clinical or neuropathological evidence of dementia. Tissue was frozen on dry ice at the time of removal and stored at −70°C until assayed (ranging from a few months to 8 years).

Immunological assay. The ELISA procedure was modified slightly from that described earlier (Saffran et al., 1989). The monoclonal antibody was raised against mouse NGF and kindly provided by Dr. William Mobley (University of California at San Francisco). The polyclonal antiserum was raised in a goat by Hazelton Research Products, Inc. (Denver, PA) using purified mouse NGF according to the procedure of Mobley et al. (1976). Brain tissue samples were dissected in a partially thawed state to facilitate removal of white matter and then homogenized at a 1:10 dilution in buffer containing 1% or 0.1% Tween. The amount of NGF-like activity in each tissue sample was estimated using standard curves generated on the same 96-well plates (NUNC, Irvine Scientific, Santa Ana, CA) using known concentrations (1.56, 3.12, 6.25, 12.5, 25, 50, and 100 pg/well) of purified mouse or rhNGF (provided by Genentech, Inc.). Most of the epilepsy tissue values were generated from plates using mouse NGF as the standard (rhNGF was not available at the time), whereas values from the remaining tissue samples were calculated based on rhNGF. Recoveries were determined by adding 25 pg/well of mouse (epilepsy tissue) or rhNGF (postmortem tissue) to parallel samples of homogenate. When correcting for recovery, the value obtained for each sample was used to calculate recovery for that sample. Optical densities were determined using either *ortho*-phenylenediamine (Sigma, St. Louis, MO) or tetramethyl blue (Transgenic Sciences Co., Worcester, MA) as chromagen. In some cases, portions of the same tissue block or homogenate were assayed at separate times in order to determine the extent of variability between assays. Because no consistent differences were found, the resulting values were averaged with the original measurements. Recombinant human BDNF and NT-3 (generously provided by Amgen, Inc.) were also tested in the ELISA at concentrations ranging from 0.5 ng/ml to 100 ng/ml.

Bioassay. Lumbar sympathetic or dorsal root ganglia were isolated from embryonic day 9 (E9) chick embryos (Spafas Inc., Roanoke, IL) under sterile conditions in unsupplemented Ham's F12 medium. After dissection into small pieces (~0.01 mm²), explants were transferred to 30 mm polyornithine-coated plastic dishes (20–40 explants per dish) containing F12 supplemented with 100 μM putrescine, 20 nM progesterone, 100 μg/ml human transferrin, 30 nM selenium, and 2% streptomycin. Dishes were then incubated at 37°C for 2–3 hr to allow explant attachment, after which known amounts of either rhNGF, rhBDNF, rhNT-3, or human brain extract were added, with or without anti-NGF antibodies. Human brain extract was prepared by homogenizing freshly thawed tissue with an equal volume of F12 and centrifuging at 15,000 × *g*, followed by sterilization of the supernatant using a 0.22 μm filter. Extract was added to the dish at 5% of final volume (1.5 ml). Cultures were incubated for an additional 16–18 hr and then fixed and stained with silver nitrate (Crutcher, 1989).

Assessment of explant outgrowth was performed using a Nikon Microphot-FX microscope linked to a Macintosh IIfx computer running IMAGE 1.43 morphometric software (National Institutes of Health). Areal measurements of neurite outgrowth per explant (halo area) as well as of the explant proper were taken from calibrated screen images and subsequently transferred to a statistical program. The validity of employing areal measurements was initially determined by comparing the number of neurites per explant with the "growth ratio" of the explant (halo area divided by explant area) across a series of increasing rhNGF concentrations (S. A. Scott and K. A. Crutcher, unpublished observations). The results indicated that areal growth was highly predictive of neurite number ($r = 0.92$), in particular at relatively low NGF concentrations (i.e., those calculated to be present in tissue extracts based on ELISA).

Immunoblotting. Mouse NGF and the three recombinant human neurotrophins (NGF, BDNF, and NT-3) were diluted at least 1:1 with SDS-sample buffer (2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 250 mM Tris-Cl, pH 6.8, 20% glycerol, 0.02% bromphenol blue) prior to electrophoresis and boiled for 5 min. Samples were separated by electrophoresis on minigels in a Daiichi apparatus (1 mm × 10 cm × 10 cm) prepared with an acrylamide gradient of 5 to ≈30% in Tris/HCl, pH 8.8, overlaid by a discontinuous stacking gel of 5% acrylamide, pH 6.8. Following electrophoresis, proteins were electrophoretically transferred to nylon membrane (Magna NT, Magnagraph Sciences, Inc.) in transfer buffer (Tris-glycine, pH 8.3, containing 20% methanol) in a Hoeffer transfer apparatus at 0.3 A for 2 hr. After transfer, filters were (1) blocked in Tris buffer (TBS) containing 0.3% Tween 20, 50 mM Tris/HCl, pH 7.5, 500 mM NaCl (TTBS), 5% nonfat dry milk powder, 5% gelatin from cold water fish skin (Sigma) and 1% BSA for 1 hr; (2) washed in TBS for 3 × 2 min; (3) incubated at room temperature in TBS containin g primary antibody [goat anti-mouse (GAM) at 1:5000 dilution] 16–20 hr; (4) washed in TTBS (3 × 15 min); (5) incubated in TBS containing goat anti-rabbit alkaline phosphatase–conjugated antibody at 1:10,000 dilution (Promega Corporation) for 2 hr; (6) washed in TTBS (2 × 20 min); and (7) washed in TBS for 15 min. Following this series, bands were visualized by addition of substrate (nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate) in buffer (100 mM Tris-HCl, pH 10.0, 100 mM NaCl, 10 mM $MgCl_2$) and incubated at room temperature until bands were apparent (15 min to 24 hr).

Statistical analysis. Multifactorial analyses of variance and covariance were used to identify overall condition effects, while Student's *t* test served for selective comparison of individual data groups, unless otherwise indicated. Pearson product–moment correlations and regression analyses were used to identify relationships between variables within a group.

Results

Sensitivity and specificity of the two-site ELISA for NGF

This two-site ELISA is generally adapted from the procedure of Weskamp and Otten (1987) and has previously been shown to detect mouse NGF reliably (Saffran et al., 1989). The first antibody is a polyclonal antiserum (GAM) raised in a goat immunized with purified mouse NGF (prepared and kindly provided by Dr. William Mobley, UCSF). The second is a monoclonal antibody (1G3) also raised against mouse NGF (provided by Dr. Mobley). The ELISA was initially tested for its ability to detect mouse NGF and rhNGF (Genentech, Inc.). Figure 1 shows that although both proteins were reliably detected, the human protein was detected with slightly less sensitivity than the mouse protein. The lower limit of sensitivity for both proteins was ~10 pg/ml.

Both rhBDNF and rhNT-3 (Amgen, Inc.) were tested in the ELISA as well, given their extensive sequence homology to NGF (Maisonpierre et al., 1990) as well as overlapping affinities for neurotrophin receptors (Rodrigueztebar et al., 1992). Neither protein was detected with the ELISA even when run at a concentration of 100 ng/ml (10,000 times the lowest concentration of NGF detectable in this assay). Therefore, any proteins detected in human tissue samples using this assay are closely related to NGF.

Bioassay detection of NGF and related neurotrophins

Although immunological assays are extremely sensitive and specific, they provide no information regarding the biological activity of the detected proteins. NGF potently stimulates neurite outgrowth from embryonic sympathetic and dorsal root ganglion neurons (Levi-Montalcini and Angeletti, 1968). NT-3 is active on both neuronal populations, whereas BDNF stimulates dorsal root ganglion neurons but not sympathetic neurons (Lind-

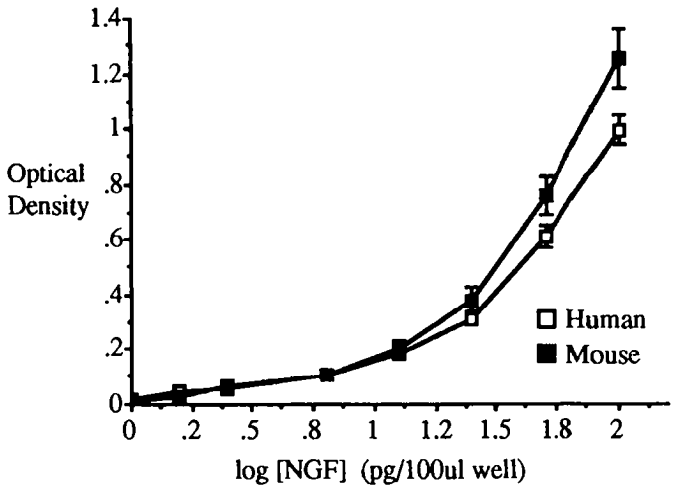

Figure 1. This figure plots two average curves obtained when using NGF purified from mouse submandibular gland or rhNGF in the present two-site ELISA. The curves represent the average (±SEM) obtained from experiments in which both human and mouse NGF standards were run on the same plates (17 curves for human and 7 for mouse) and are expressed as optical density versus the log of NGF concentration (pg/well). This ELISA procedure results in detection of purified mouse NGF and rhNGF, with slightly greater sensitivity for the mouse protein. The lower limit of detection is approximately 1 pg/well (10 pg/ml). The recombinant human proteins NT-3 and BDNF were undetectable at any concentration tested (up to 100 ng/ml).

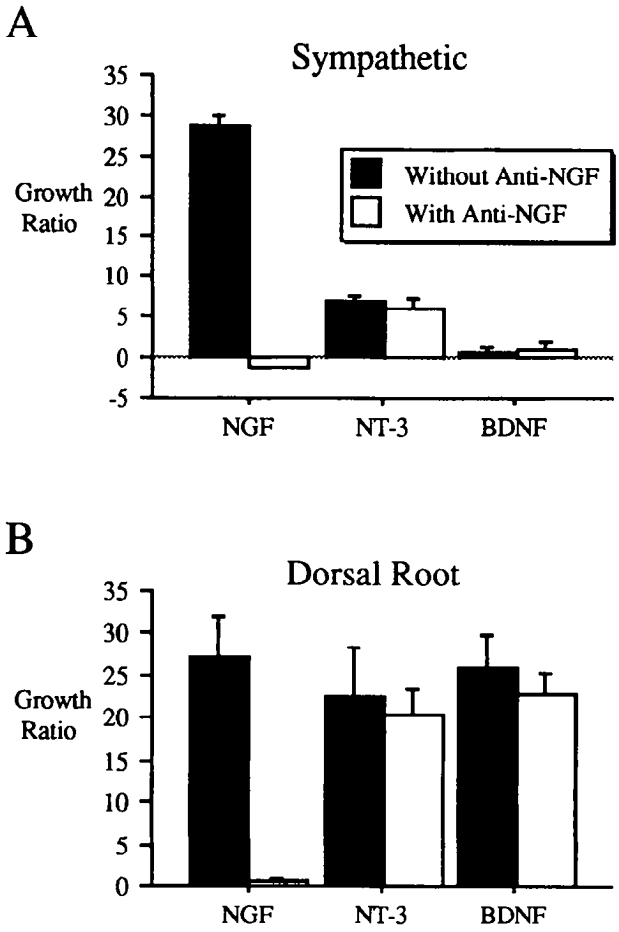

Figure 2. Neurite outgrowth, expressed as "growth ratio" (see Materials and Methods), from E9 chick sympathetic (*A*) and dorsal root explants (*B*) exposed to rhNGF, rhNT-3, or rhBDNF (100 pg/ml). *Bars* indicate the mean ± SEM per dish (20–40 explants) in the presence and absence of polyclonal anti-NGF (GAM, 20 μg/ml). Average growth ratio elicited by culture medium alone was subtracted from all dishes, resulting in values that presumably reflect only the condition in question. *t* tests indicated that the anti-NGF antibodies significantly blocked neurite outgrowth only in the dishes containing NGF ($p < 0.0001$).

say et al., 1991). We used explants from both tissues to assess the bioactivities of all three factors and to examine the ability of GAM to inhibit their action. All three neurotrophins stimulated dorsal root ganglion neurite outgrowth, whereas only NGF and NT-3 stimulated sympathetic neurite outgrowth (Fig. 2). GAM completely blocked the effects of NGF on both embryonic tissues, but was ineffective in antagonizing the actions of NT-3 and BDNF at the same concentration of antibody (Fig. 2). Similar results were obtained using the monoclonal antibody (data not shown).

Immunoblot characterization of antibody specificity

The ability of GAM to detect each of the neurotrophins was further tested using Western blots. GAM reliably detected both mouse and rhNGF proteins, although it appeared to react more strongly with mouse NGF than with the human protein (Fig. 3). While GAM showed little cross-reactivity with NT-3, a distinct band was observed with BDNF, but only when BDNF was run at relatively high concentrations (Fig. 3). These results support the findings of the bioassay and indicate that our anti-NGF antibodies selectively recognize NGF.

ELISA detection of NGF-like activity in human biopsy tissue

The feasibility of reliably detecting NGF-like activity in human brain tissue was first tested using fresh tissue samples obtained from patients undergoing surgical resection for the treatment of epilepsy to avoid postmortem changes. The ELISA detected NGF-like protein in all samples and the average value was approximately 600 pg/gm wet weight. This value represents the mean after correcting each sample for recovery, which ranged from 36% to 90%. A two-way ANOVA revealed significant overall effects of gender [$F(1,19) = 9.5$, $p = 0.0068$] and side of brain [$F(1,19) = 6.6$, $p = 0.0197$] on NGF-like activity, as well as a significant interaction between these variables [$F(1,19) = 6.9$, $p = 0.0179$]. The overall increase in males was confirmed using a *t* test [$t(1,19) = 2.24$, $p = 0.0374$] and was due primarily to higher levels in the right hemisphere (Fig. 4*A*). This increase could not be accounted for by seizure history or patient age at the time of resection, or by differences in recovery, which averaged 62% in tissue samples from women and 56% in tissue samples from men. No correlation was observed between patient age and NGF-like activity either within or across gender groups (Fig. 4*B*).

Bioassay of human biopsy tissue extracts

Cortical tissue extracts from three of the epilepsy patients were also run in the bioassay. These samples contained variable levels of NGF-like activity as measured using the ELISA (80, 400, and 630 pg/gm, uncorrected for recovery). Each of the samples stimulated neurite outgrowth from sympathetic explants beyond the effect of medium alone and a significant portion of this outgrowth could be inhibited by GAM, whereas preimmune serum showed little effect on the outgrowth (Fig. 5). Furthermore, the degree of outgrowth that could be inhibited by GAM

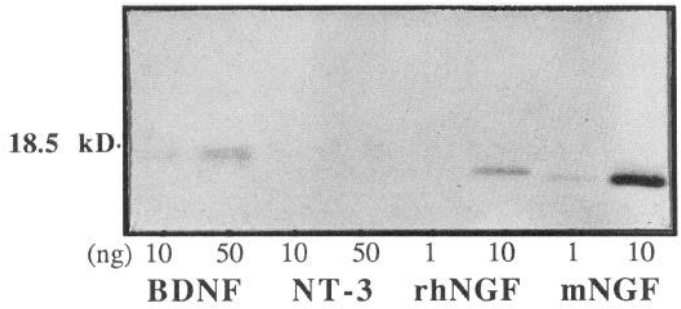

Figure 3. Western blot of anti-NGF antiserum (GAM) tested against two different concentrations of four neurotrophins: rhBDNF, rhNT-3, rhNGF, and mouse NGF (*mNGF*). The polyclonal antiserum was used at a concentration of 1:5000. Distinct bands are present in lanes containing mouse NGF and the 10 ng concentration of rhNGF. There is also slight immunopositive staining in lanes containing 10 ng and 50 ng of rhBDNF, but no staining of rhNT-3 was observed under these conditions.

varied directly with the ELISA value generated from the same tissue block (Fig. 5). These results demonstrate that broad differences in NGF-like activity, as indicated by ELISA, can be confirmed using this bioassay.

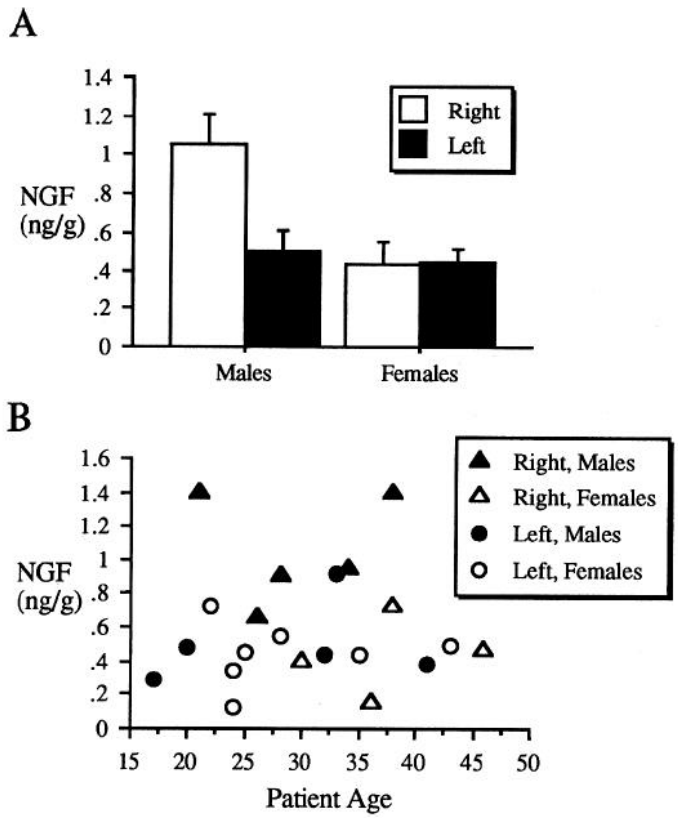

Figure 4. *A*, Bar graph showing recovery-corrected NGF-like activity levels (mean ± SEM) in epileptic human temporal cortical tissue. *t* tests indicated that more NGF-like activity was present in the right hemisphere of males versus any other subgroup ($p < 0.05$). *B*, Scattergram plotting individual NGF-like activity values versus patient age, split by gender and brain side. There is no obvious effect of age on NGF-like activity within any subgroup. When the data were split by either gender or brain side (increasing sample size), regression analyses indicated no effect of age on NGF-like activity.

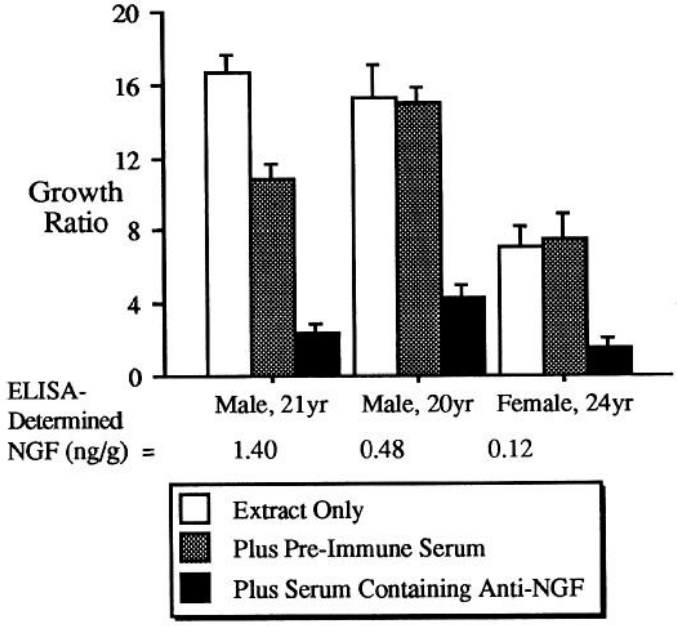

Figure 5. E9 chick sympathetic ganglia incubated in temporal cortical biopsy tissue extract containing either (1) extract diluted in medium at a 1:20 v/v ratio, (2) extract plus anti-NGF (5 μg/ml), or (3) extract plus preimmune serum (5 μg/ml). Each condition was represented by duplicate dishes (20–40 explants per dish) and the values (mean ± SEM) were corrected for medium-only outgrowth as in Figure 2. Anti-NGF (GAM) significantly inhibited extract-induced outgrowth in each case (unpaired *t* tests, $p < 0.001$), whereas preimmune serum showed a slight inhibition of growth only for the 21 year old male. Moreover, recovery-corrected NGF-like activity levels previously determined for each of these samples (shown at bottom) correlated with the difference between the extract-only bar and the extract plus anti-NGF bar (i.e., the outgrowth that could be blocked by anti-NGF). The specificity of anti-NGF was confirmed in parallel dishes containing rhNGF at various concentrations with either anti-NGF or preimmune serum. The first two samples were taken from the right side of the brain, whereas the third was taken from the left.

ELISA measurement of NGF-like activity in postmortem tissue

Once the feasibility of detecting NGF-like activity in fresh cortical tissue was determined, we studied postmortem tissue samples obtained from patients with clinically and pathologically confirmed AD ($n = 11$) as well as neurological controls ($n = 14$) (Table 1). The samples were taken from the frontal and occipital poles of the cortex, and in 17 of 25 cases, both areas were studied from the same brain. Mean postmortem delay was not significantly different between control donors (15 hr) and AD donors (13 hr), and there were no within-group correlations between postmortem delay and NGF-like activity levels. In order to correct for the presence of NGF binding proteins or other components that can mask endogenous NGF, each sample was run in the presence of 25 pg of rhNGF. There was no difference in the recovery of exogenous NGF from AD and control samples (averaging 50% in each group).

A two-way ANOVA revealed a significant effect of AD [$F(1,40) = 30.1, p < 0.0001$] but not brain region ($p > 0.5$) on the amount of recovery-corrected NGF-like activity in the tissue samples. Subsequent *t* tests indicated that in both brain regions, NGF-like activity levels were significantly greater (approximately 250%) in AD versus control samples (Fig. 6). A similar effect of AD was obtained when based on the NGF values uncorrected

Table 1. Subject information

Control subjects			Alzheimer subjects		
Sex	PMI[a]	Age at death (yr)	Sex	PMI[a]	Age at death (yr)
F	23	67	M	5	69
M	12	49	M	6	81
F	6	27	M	6	88
F	14	65	M	16	76
F	20	73	M	5	89
F	11	57	M	55	80
F	20	75	F	3	72
M	11	59	M	16	72
M	15	40	F	3	84
M	10	53	M	13	78
M	40	64	M	12	63
F	6	69			
M	13	71			
M	5	58			
7 M, 7 F	15 ± 2.4[b]	59 ± 3.6	9 M, 2 F	13 ± 4.5[b,c]	77 ± 2.4*

[a] Interval (hr) between death and freezing of the tissue.
[b] Mean ± SEM.
[c] Not significantly different from control ($p > 0.05$, unpaired t test), with or without the 55 hr postmortem interval case.
* Significantly different from control ($p < 0.05$, unpaired t test).

for recovery [$F(1,40) = 19.91$, $p < 0.0001$], with similar magnitudes of increases. The AD-related increases persisted when the values were expressed as ng/mg total protein, instead of per gram of tissue wet weight ($p < 0.05$). Comparing only the values obtained from male patients also showed significantly greater activity in AD versus control ($p < 0.05$).

Although AD subjects were significantly older than controls (Table 1), regression analyses failed to demonstrate a significant relationship between patient age and NGF-like activity in either the control or AD group (Table 2). Furthermore, since there was no significant difference between the slopes of the regression lines obtained from the correlational analyses (Table 2), an additional test of the effect of age was carried out using the least-squares means procedure of the covariance analysis. The differences between AD and control tissue remained significant in both cortical regions ($p < 0.05$), and were of the same magnitude as without correction for age. Finally, the individual t tests were recomputed after omitting the oldest AD patients ($n = 8$) and the youngest control patients ($n = 5$) in order to obtain samples that were age matched (AD mean = 71 years; control mean = 67 years; $p > 0.26$). The differences between AD and control samples were still significant ($p < 0.05$), and of similar magnitude (two- to threefold increases), in both brain regions. Taken together, these results demonstrate that the increased NGF-like activity in AD patients did not result from differential patient age.

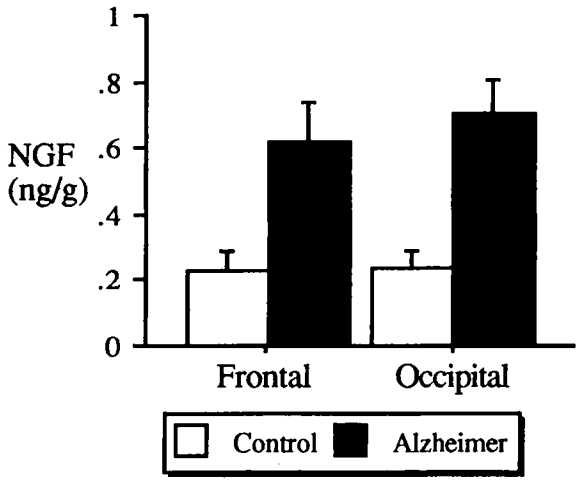

Figure 6. Recovery-corrected ELISA measurements of NGF-like activity from frontal and occipital cortex samples from AD (*solid bars*) and non-AD brains (*open bars*). In both brain regions there was more NGF-like activity in AD samples versus control (unpaired two-tailed t test; frontal, $p = 0.003$; occipital, $p = 0.0002$).

Bioassay of control and AD postmortem tissue

Bioassays were subsequently performed on a subset of the above samples to determine whether the AD-related increase in NGF-like immunoreactivity coincided with increased biological activity. Frontal cortical samples from five AD and five age- and postmortem-equivalent control brains were tested in separate experiments (one control was paired with one AD sample in each experiment). All extracts were found to stimulate neurite outgrowth from E9 chick sympathetic ganglia (Fig. 7). The percentage of the extract-induced outgrowth that could be blocked by anti-NGF was subsequently calculated for each extract in each experiment (Fig. 7). By paired t test analysis this percentage was significantly greater in AD [$t(1,4) = 5.49$, $p = 0.0054$]. An overall comparison of the raw difference in outgrowth between extract and extract-plus-antibody dishes (i.e., the outgrowth blocked by anti-NGF) also revealed higher levels of NGF-like outgrowth in AD. While the significance of this difference was marginal according to a paired t test ($p = 0.0598$), a Mann–Whitney U test indicated statistical significance at $p = 0.0283$

Table 2. Effect of patient age on cortical NGF-like activity

Patient group	Correlation (r) between age, NGF-like activity	p value (ANOVA)	Test of common slope
Control, frontal cortex (n = 12)	−0.07	0.83 (NS)	No difference between slopes[a]
AD, frontal cortex (n = 8)	+0.26	0.54 (NS)	
Control, occipital cortex (n = 14)	+0.10	0.73 (NS)	No difference between slopes
AD, occipital cortex (n = 10)	+0.52	0.12 (NS)	

Data are based on recovery-corrected NGF-like activity values. Significant correlations were also lacking (all less than r = 0.24) when based on the uncorrected values.
[a] Based on ANOVA (frontal, p = 0.4128; occipital, p = 0.0948).

(df = 8). Thus, results of the bioassay underscore the AD-related increase in NGF-like activity as determined by the ELISA.

Discussion

Specificity of assays for detecting NGF

Although NGF was not originally detected in CNS tissue (Levi-Montalcini and Angeletti, 1968), subsequent studies demonstrated the presence of NGF-like activity in the brain with highest levels in the hippocampal formation (Crutcher and Collins, 1982; Korsching et al., 1985; Shelton and Reichardt, 1986; Auburger et al., 1987; Whittemore et al., 1987). The development of sensitive immunological assays (Furukawa et al., 1983; Korsching and Thoenen, 1987; Lärkfors and Ebendal, 1987; Weskamp and Otten, 1987) has permitted more detailed studies of the distribution and regulation of NGF in the brain. However, immunological assays are limited in that no information is provided regarding the biological activity of the proteins that are detected. For this reason, the use of both immunological and biological assays is desirable (Suda et al., 1978; Söderström et al., 1990).

NGF has never been purified from brain, so its precise molecular identity in the CNS is unknown. For example, a recent study demonstrated the presence of pro-NGF-like molecules in rat hippocampal tissue (Dicou, 1992). For this reason we refer to the activity detected with both immunological and biological assays as "NGF-like." The protein(s) responsible for this activity is presumably a human homolog to the male mouse submandibular gland protein, against which most available antibodies have been raised. It is also likely that NGF-like proteins from different species are homologous since the genomic sequences are highly conserved (Ullrich et al., 1983). In the present study, mouse NGF and rhNGF were detected with an ELISA using two different antibodies raised against mouse NGF. The fact that the rhNGF was detected with slightly lower sensitivity is probably due to a lower affinity of the polyclonal antibody for the human protein, as revealed by the immunoblot analysis (Fig. 3).

With the recent discovery of other putative neuronal growth factors that show homology to NGF, the interpretation of both ELISA and bioassay results must consider the possibility that antibodies raised against NGF will cross-react with other neurotrophins. Such cross-reactivity has been demonstrated for some polyclonal antibodies raised against NGF (Acheson et al., 1991). However, neither BDNF nor NT-3 was detected with the present ELISA, even at concentrations 10,000 times greater than the detection limit for NGF. This is not surprising since our ELISA employs two different anti-mouse NGF antibodies, a monoclonal and a polyclonal. Failure of either antibody to cross-react with the antigen prevents detection of the protein. We are therefore reasonably confident that this ELISA does not detect known neurotrophins other than NGF.

It is equally important to establish the specificity of the bioassay for detecting NGF and related factors. We found, as have others (Lindsay et al., 1991), that BNDF had no effect on sympathetic ganglia but did stimulate neurite outgrowth from sensory ganglia. NT-3 stimulated neurite outgrowth from both types of explants but was much less effective than NGF on sympathetic explants. The results obtained from adding anti-NGF antiserum to the cultures indicate that the polyclonal antibody (GAM) is quite selective for NGF under the conditions used in our bioassay (Fig. 2). The neurite-promoting activity of NGF was completely blocked by GAM as well as the monoclonal antibody (data not shown) but the activities of BDNF and NT-3 were not inhibited. Thus, the results of the bioassay indicate that both antibodies specifically block NGF but not NT-3 or BDNF.

Further evaluation of antibody cross-reactivity was carried out using immunoblotting techniques. The monoclonal antibody failed to detect either BDNF or NT-3 but did detect the NGF proteins when run on Western blots (data not shown). The polyclonal antibody showed slight cross-reactivity with BDNF

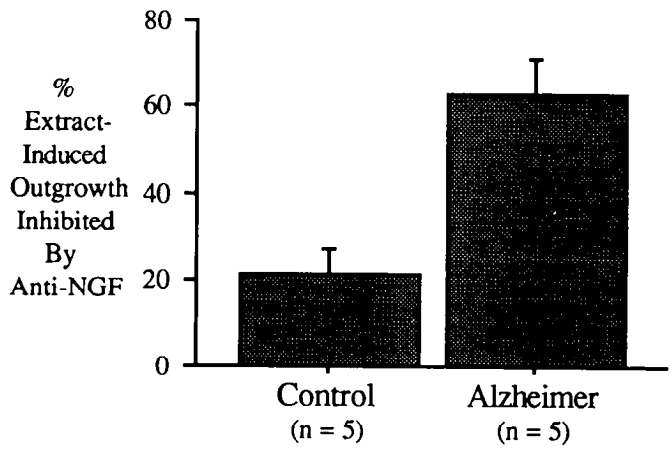

Figure 7. Detection of NGF-like bioactivity in control and AD frontal cortical tissue extracts. This figure summarizes five experiments involving 10 control and AD extracts. One control extract was run in parallel with one AD extract in a single experiment. All conditions were run in duplicate. The amount of outgrowth inhibited by anti-NGF (GAM) was calculated for each extract as follows: the difference in growth ratio between (1) extract-only and (2) extract-plus-antibody conditions was divided by the former and multiplied by 100. *Bars* represent the mean percentage reduction (±SEM) across experiments. The difference in this value between control and AD groups was statistically significant (paired t test, p = 0.0054).

(Fig. 3) but no cross-reactivity with NT-3 above background. In addition, GAM immunoreactivity against the mouse NGF was stronger than against rhNGF. This difference in detection may be due to species differences in the proteins, conformational changes in the recombinant protein, or loss of epitopes on SDS-PAGE. All three detection methods (ELISA, bioassay, and immunoblotting) generally confirm that GAM is selective in binding to NGF-like molecules. The activity detected in our human brain tissue samples by two-site ELISA is therefore likely to be from human NGF, or a closely related protein.

Detection of NGF-like activity in fresh human brain tissue

Our results demonstrate the feasibility of detecting NGF-like proteins in fresh human brain tissue using an ELISA and a bioassay that were developed for detecting mouse NGF. Both assays reliably detect rhNGF as well as NGF-like activity in fresh cortical tissue taken from patients undergoing surgery for the treatment of epilepsy. The range of activity detected in these cortical samples (0.12–1.4 ng/gm) encompasses the values reported for the macaque cortex (0.3–0.6 ng/gm; Hayashi et al., 1990) and for postmortem human AD and control tissue (0.2–0.5 ng/gm; Allen et al., 1991). Furthermore, the average value for our samples (0.6 ng/gm), most of which were obtained from temporal cortex, is close to that reported for temporal cortex from macaque (0.5 ng/gm; Hayashi et al., 1990) and postmortem human brains (0.4 ng/gm; Allen et al., 1991). The extent to which these levels reflect the normal concentration of NGF in human cortex is unknown. All of the tissue used in the present study was obtained from patients with a history of epilepsy (average duration of 21 years) and was removed from sites exhibiting abnormal excitability, usually representing seizure foci. Seizures might affect NGF levels, as documented in rodents, where epileptogenic activity or injury results in elevated NGF mRNA in the hippocampus (Gall and Isackson, 1989; Bakhit et al., 1991; Ballarin et al., 1991; Gall et al., 1991; Lindefors et al., 1992; Rocamora et al., 1992). Therefore, the levels of NGF-like activity detected in these samples may not reflect levels present in nonepileptic tissue. The values obtained from the epileptic tissue are not directly comparable to values obtained from our postmortem tissue because (1) they represent different brain regions; (2) the AD tissue was acquired after death, whereas the epilepsy samples were obtained from surgical resections; (3) the epilepsy patients were much younger than the AD patients; and (4) the epilepsy samples were run using mouse NGF as the standard (due to the unavailability of human NGF at the time these samples were run).

Although the quantitative ELISA findings are supported by the bioassay results, it should be emphasized that tissue extracts include a complex mixture of factors that may affect the overall growth potential of the cultured tissue. In addition, the response of chick sympathetic neurons to NGF is not a linear function of dose. As a result, it is difficult to estimate the absolute amount of NGF-like protein in tissue extracts using bioassay data alone. However, the bioassay allows one to assess the *relative* NGF-like activity present within tissue extracts run under similar conditions. Our results demonstrate that extracts of human cortical tissue are capable of stimulating neurite outgrowth. Up to 80% of this stimulation was specifically blocked by anti-NGF antibodies. Thus, some of the NGF-like protein detected in the ELISA is biologically active. Furthermore, in three epilepsy tissue samples that we tested, the relative potency of NGF-like bioactivity paralleled the concentration determined by ELISA from the same tissue blocks (Fig. 5). This strengthens the conclusion that the bioassay detects the same activity detected with the ELISA and that relative differences between tissue samples can be detected with both methods.

Asymmetrical distribution of NGF-like activity in epileptic cortical tissue

A surprising result was the finding of greater NGF-like activity in cortical samples taken from the right hemisphere of men when compared with the NGF-like activity in samples from the left hemisphere of men or from either hemisphere in women. It is unclear whether this asymmetry reflects the normal distribution of cortical NGF levels in men or if it is unique to the presently sampled patient population. Because the asymmetry was not observed in females, however, the finding does not appear simply related to the presence of epilepsy. Determining whether this sexually dimorphic asymmetry is present in normal subjects will require additional studies. The main value of the results obtained in this study is the demonstration that NGF-like activity can be reliably detected in biopsy samples of human brain. Somewhat surprisingly, these values are not substantially different from values obtained from postmortem tissue samples.

Increased NGF-like activity in AD

AD is characterized by specific neuropathological changes including a reduction in cortical cholinergic markers (Davies and Maloney, 1976; Araujo et al., 1988) and loss and atrophy of basal forebrain cholinergic neurons (Whitehouse et al., 1981; Mufson et al., 1989). The suggestion has been made that these changes are secondary to loss of trophic support in the cortex (Appel, 1981), the most likely candidate being NGF (Hefti, 1986). However, a recent study found stability of hippocampal and cortical β-NGF levels in AD using a two-site ELISA (Allen et al., 1991), and the present results suggest that AD is accompanied by an increase in neocortical β-NGF. Our findings cannot be compared directly with those of Allen et al. (1991) because different cortical regions were sampled and different antibodies were used in the ELISAs. However, in the Allen et al. (1991) study, mean values for each neocortical region were higher in AD than in control samples and the prefrontal cortex values, while not statistically significant, were 50% greater on average.

NGF is thought to upregulate its own receptor (Higgins et al., 1989; Lindsay et al., 1990; Fusco et al., 1991). Therefore, our finding of increased NGF-like activity in AD may explain the recent finding of a novel population of NGF receptor–positive neurons in AD (Mufson and Kordower, 1992). NGF mRNA levels appear to be normal in AD brain tissue (Goedert et al., 1986) while reports of mRNA for the low-affinity NGF receptor in AD tissue are conflicting (Goedert et al., 1989; Higgins et al., 1989; Ernfors et al., 1990b). However, the percentage of basal forebrain cholinergic neurons that continue to express NGF receptors in AD appears to be stable (Hefti and Mash, 1989; Kordower et al., 1989; Mufson et al., 1989). These data suggest that residual basal forebrain neurons in AD are responsive to NGF (Mufson et al., 1989). Moreover, the region of the basal forebrain that projects to the hippocampal formation, which contains the highest brain levels of NGF, is relatively spared in AD (Kordower et al., 1989; Mufson et al., 1989). Thus, increased NGF-like activity in AD may represent a response to degenerative changes occurring within the cholinergic basal forebrain and may serve to ameliorate these changes. Additional studies will be needed to determine whether increases in NGF-like ac-

tivity also occur in other neurodegenerative diseases, since the present results are based only on Alzheimer's and nondemented control tissue.

The cellular source of the increased NGF-like activity is unknown. Results from immunohistochemical studies are inconsistent (Ayer-LeLievre et al., 1983; Conner et al., 1992), but there is evidence for NGF production by neurons (Rennert and Heinrich, 1986; Ayer-LeLievre et al., 1988; Ceccatelli et al., 1991; Lu et al., 1991b) as well as by glial cells (Lindsay, 1979; Furukawa et al., 1986; Assouline et al., 1987; Lu et al., 1991a). NGF synthesis has been shown to increase in response to injury or increased activity (Gall and Isackson, 1989; Bakhit et al., 1991; Ballarin et al., 1991; Gall et al., 1991; Lindefors et al., 1992; Rocamora et al., 1992). Thus, the neuropathological changes occurring in the cortex in AD may lead to increased glial production of NGF. Alternatively, increased NGF-like activity could be due to loss of retrograde transport due to the death of NGF-responsive basal forebrain neurons (Whitehouse et al., 1982; Mufson et al., 1989). For example, fimbrial transection or medial septal lesion in the rat results in elevation of NGF-like activity in the hippocampal formation (Collins and Crutcher, 1985, 1989; Gasser et al., 1986; Weskamp et al., 1986a,b; Lärkfors et al., 1987a) with no change in NGF mRNA (Goedert et al., 1986; Korsching et al., 1986). If basal forebrain cholinergic neurons are degenerating in AD through a mechanism independent of NGF, secondary accumulation of NGF in the cortex might be expected due to loss of retrograde transport.

Regardless of the underlying cause of the increase in NGF-like activity in AD brain, the present results go contrary to the hypothesis that AD-related changes in the basal forebrain are due to reduced cortical target NGF levels. Mechanisms involving retrograde transport, utilization of available NGF-like activity, or reductions in other putative neurotrophic factors, such as BDNF, may contribute to basal forebrain cell loss and atrophy. A recent study (Phillips et al., 1991) demonstrated decreased BDNF mRNA in the hippocampus from AD patients with no change in NGF mRNA, although NGF protein levels and biological activity were not examined.

If the increase in NGF-like activity reflects an active response to atrophic changes in the basal forebrain, then the rationale for the clinical use of exogenous NGF may be strengthened (Phelps et al., 1989). However, intracerebral infusion of NGF has been shown to increase the sympathetic innervation of extracerebral blood vessels in rats (Saffran et al., 1989; Isaacson et al., 1990, 1992) and similar effects might be obtained in AD patients. A recent report (Olson et al., 1992) demonstrating increased cerebral blood flow in an AD patient receiving intracerebral administration of NGF might reflect alterations in the sympathetic innervation of cerebral blood vessels. Other consequences of using NGF as a possible therapeutic agent, such as the documented NGF-stimulated increase in the expression of the amyloid precursor protein (APP; Mobley et al., 1988) and the recent demonstration of APP as a mediator of NGF-stimulated neurite outgrowth (Milward et al., 1992), must be assessed before engaging in clinical trials.

Increased growth-promoting activity in AD

The present findings are consistent with other reports of increased neurite growth-promoting activity in extracts of AD brain tissue. For example, Uchida et al. (1988) and Uchida and Tomonaga (1989) have reported elevations in neurotrophic support in AD tissue, a result that appears to be due to decreased levels of a growth inhibitory factor (Uchida et al., 1991). In addition, a recent study (Marshak et al., 1991) found increased S100β activity in AD tissue as detected with both radioimmunoassay and bioassay measurements. Thus, considerable evidence supports the conclusion that AD is characterized by increases in putative growth factors. Such findings raise the question of whether growth factors might contribute to the neuropathological changes in this disease (Butcher and Woolf, 1989). Aberrant neurite outgrowth (Scheibel and Tomiyasu, 1978; Arendt et al., 1986; Ihara, 1988), perhaps stimulated by components associated with senile plaques (Probst et al., 1983; Geddes et al., 1986; Roher et al., 1991), appears to be characteristic of AD. However, when used as a substrate for neurite growth in tissue culture, senile plaques do not stimulate neurite growth from sympathetic or hippocampal neurons (Crutcher et al., 1991; Carpenter et al., 1993). It is possible that growth-stimulating as well as growth-inhibiting factors are active in AD. Destruction of basal forebrain neurons in rodents results in elevated NGF-like activity in cortical target tissues as well as aberrant sympathetic axonal sprouting (Crutcher, 1987), so it is possible that this, or other, sprouting occurs in AD (Geddes et al., 1985). Further experiments are required to determine whether alterations in neurotrophic factors in neurological diseases represent compensatory mechanisms or whether they contribute to the neuropathological changes and behavioral deficits.

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