

BDNF mRNA Is Decreased in the Hippocampus of Individuals with Alzheimer's Disease

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Summary

In recent years, nerve growth factor (NGF) has gained attention as a potential therapeutic agent for Alzheimer's disease (AD). To study the expression of NGF and its homologs, brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3), postmortem samples of hippocampus from AD and control donors were examined by *in situ* hybridization. Hybridization signal for BDNF, but not NGF or NT-3, was decreased in samples of hippocampus from donors with AD. Decreased transcript abundance of BDNF mRNA in hippocampi of individuals with AD was verified by an RNAase protection assay. These results suggest the possibility that decreased expression of BDNF may contribute to the progression of cell death in AD.

Introduction

Increasing evidence indicates that nerve growth factor (NGF) plays a role in trophic support of basal forebrain cholinergic neurons (Hefti et al., 1989; Perry, 1990). The association between loss of basal forebrain cholinergic neurons and memory loss in Alzheimer's disease (AD) has been taken to suggest that agents which maintain the function of these cells might be used to slow the deterioration of cognitive function that occurs in this disease (Hefti et al., 1989; Perry, 1990; Marx, 1990). Although NGF has been proposed as a potential therapeutic agent for AD (Hefti et al., 1989; Perry, 1990; Marx, 1990), there is no evidence to indicate that this disease is associated with a significant deficiency in NGF (Goedert et al., 1986). Reports concerning NGF receptor expression in AD have been somewhat conflicting, but suggest that a deficiency in NGF receptor expression is unlikely to be involved in the etiology of the disease (Higgins and Mufson, 1989; Goedert et al., 1989; Hefti and Mash, 1989; Kordower et al., 1989).

Recent observations suggest the possibility that an NGF homolog, brain-derived neurotrophic factor (BDNF), may also serve as a trophic factor for basal forebrain cholinergic cells; a third member of the NGF family, neurotrophin 3 (NT-3), is less likely to play a major role in support of these neurons (Alderson et al., 1990; Ernfors et al., 1990; Hofer et al., 1990; Knusel

et al., 1991; Phillips et al., 1990; Wetmore et al., 1990). To compare expression of NGF, BDNF, and NT-3 in AD and control individuals, samples of human postmortem hippocampus were examined by *in situ* hybridization.

Results

In Situ Hybridization Signal for BDNF, but Not NGF or NT-3, Is Reduced in AD

Hybridizations for BDNF, NGF, and NT-3 mRNAs were compared in hippocampi of 9 AD and 6 control samples (Table 1). Specificity of our hybridization protocol for localization of neurotrophins in rat and mouse brain has been previously demonstrated (Phillips et al., 1990). Furthermore, slot blots of synthetic human neurotrophin RNAs probed either with rat BDNF cRNA probe under conditions identical to those employed for *in situ* hybridization or with human BDNF DNA probe under less stringent conditions exhibit no cross-reactivity of the BDNF probe with NGF or NT-3 (data not shown).

Consistent with our earlier observations (Phillips et al., 1990), control specimens hybridized with BDNF antisense probe displayed intense label throughout the granule cell layer of the dentate gyrus, with a more dispersed signal across the pyramidal layer of CA1-CA4 (Figure 1A). In marked contrast, the majority of samples from the AD group displayed a weak signal over the granule cell layer of the dentate gyrus and a barely detectable signal in Ammon's horn (Figure 1C). As previously observed (Phillips et al., 1990), sections hybridized with sense-strand control probe demonstrated low levels of homogeneously distributed radioactivity. NGF hybridization signals were notably weaker than those for BDNF, but appeared equivalent in AD and control samples (Figures 1B and 1D). No consistent difference between NT-3 signals in control and AD samples was apparent (data not shown).

For quantification of hybridization intensities, the granule cell layer of the dentate gyrus was chosen because intensity and uniformity of labeling were greatest with the probes in this region and because minimal cell loss occurs there in AD (Coleman and Flood, 1987). BDNF hybridization intensities were also analyzed in the pyramidal layer of the hippocampus proper. The low signal to noise ratios for NGF and NT-3 did not allow quantitative analysis of hybridization for these mRNAs in this region.

A two-way analysis of variance on hybridization intensities in the dentate gyrus indicated that BDNF, NGF, and NT-3 mRNA signals are differentially affected in AD ($F = 5.55$, 2 and 9 d.f., $p < 0.01$). The hybridization signal for BDNF in the dentate gyrus was nearly 4-fold lower for the group of AD samples than for the nondemented control group (Figure 2; 0.05 ± 0.015 versus 0.192 ± 0.034 , $t = 4.34$, 13 d.f.

Table 1. Samples Examined by In Situ Hybridization

Donor #	Neuropathology	Sex	Age	Autolysis Time	Cell Density
1	None	M	64	16	39
2	None	M	49	9	33
3	None	M	79	19	42
4	PD	M	63	23	37
5	PD	M	62	13	39
6	PD	M	78	5	44
Mean (SE)			65.8 (4.6)	14.2 (2.7)	39 (1.5)
7	AD	F	63	19	42
8	AD	M	64	11	32
9	AD	F	82	16	37
10	AD	M	79	18	43
11	AD	F	78	6	49
12	AD	M	72	10	37
13	AD	M	67	21	40
14	AD	M	65	20	36
15	AD	M	73	10	39
Mean (SE)			71.4 (2.4)	15.2 (1.8)	39 (1.6)

Sample information included neuropathology report (AD, Alzheimer's disease; PD, Parkinson's disease), sex and age (years) of donor, autolysis times (hours), and cell density (per 100 μm^2). No significant differences are seen in age, autolysis time, or cell density of the samples in the AD versus control groups ($p > 0.05$, t test for all comparisons).

$p < 0.001$). BDNF hybridization intensities were also significantly reduced in the pyramidal layer of the hippocampus proper (0.010 ± 0.005 versus 0.035 ± 0.008 , $t = 2.64$, $p < 0.05$). In contrast to BDNF hybridization signals, NGF and NT-3 hybridization intensities did not differ in the dentate gyrus between the AD and control groups (Figure 2; for NGF, 0.102 ± 0.012 versus 0.125 ± 0.013 , $t = 1.33$, 13 d.f., $p > 0.10$; for NT-3, 0.046 ± 0.015 versus 0.094 ± 0.025 , $t = 1.72$, 13 d.f., $p > 0.10$).

The reduced BDNF hybridization signal in our group of AD samples cannot be explained on the basis of differences in donor age or sex (Table 1). There was no significant difference between the age of donors in our 2 samples. Furthermore, when our sample groups were matched more closely for age by eliminating samples from the youngest control donor and the 2 oldest AD donors, the values for BDNF hybridization remained significantly lower in the AD sample. (After matching sample groups, the age of the AD group = 68.9 ± 2.1 ; the age of the control group = 68.6 ± 3.5 ; BDNF optical density [OD] of the AD group = 0.053 ± 0.018 ; BDNF OD of the control group = 0.162 ± 0.020 , $t = 4.03$, 10 d.f., $p < 0.005$.) Similarly, matching of the groups for sex by the deletion of the 3 samples from female donors reduces the mean BDNF signal for the AD group and does not affect the significance value of the difference observed between AD and control groups. (Mean of AD samples = 0.050 [$n = 9$, female samples included] versus 0.032 [$n = 6$, female samples excluded].)

The decreased BDNF signal in our AD samples cannot be accounted for by differences in autolysis times or by loss of cell density in the granule cell layer of the dentate gyrus. Autolysis times did not differ between our AD and control samples (Table 1; $t = -0.13$, 13 d.f., $p = 0.90$). Furthermore, BDNF hybridization

intensity showed no reduction with longer autolysis times ($R = 0.03$). Analysis of cell density in the granule cell layer of the dentate gyrus indicated no difference between our AD and control samples (Table 1).

Hybridization for BDNF, but Not NGF or NT-3, Is Decreased in AD mRNA Relative to an Unrelated mRNA of Similar Abundance

To control for variability in hybridization intensity resulting from differences in RNA quality or a general reduction in mRNA that might occur with AD, we normalized hybridization signals in the dentate gyrus for BDNF, NGF, and NT-3 to signals measured in the same region following hybridization with a probe to the human neuronal cell adhesion molecule (N-CAM). N-CAM values were 0.173 ± 0.026 for control and 0.092 ± 0.017 for AD (Figure 2; $t = 2.77$, 13 d.f., $p < 0.05$). Correlation analysis revealed that, in general, N-CAM hybridization values for individual samples correlated as well with values for the neurotrophins as did values between members of the neurotrophin family (R values for N-CAM versus BDNF, N-CAM versus NGF, and N-CAM versus NT-3 = 0.82 , 0.56 , and 0.78 , respectively; R values for BDNF versus NGF, BDNF versus NT-3, and NGF versus NT-3 = 0.60 , 0.64 , 0.77 , respectively). These results are consistent with a lack of cross-hybridization of our neurotrophin probes with heterologous members of the family.

Two-way analysis of variance revealed that BDNF/N-CAM, NGF/N-CAM, and NT-3/N-CAM ratios in the dentate gyrus were differentially affected in AD ($F = 11.86$, 2 and 38 d.f., $p < 0.0005$). BDNF/N-CAM ratios in the dentate gyrus were significantly reduced in AD versus control samples (Table 2; $t = 4.75$, 13 d.f., $p < 0.0005$). Analysis of the BDNF/N-CAM ratios in the pyramidal cell layer of the hippocampus revealed

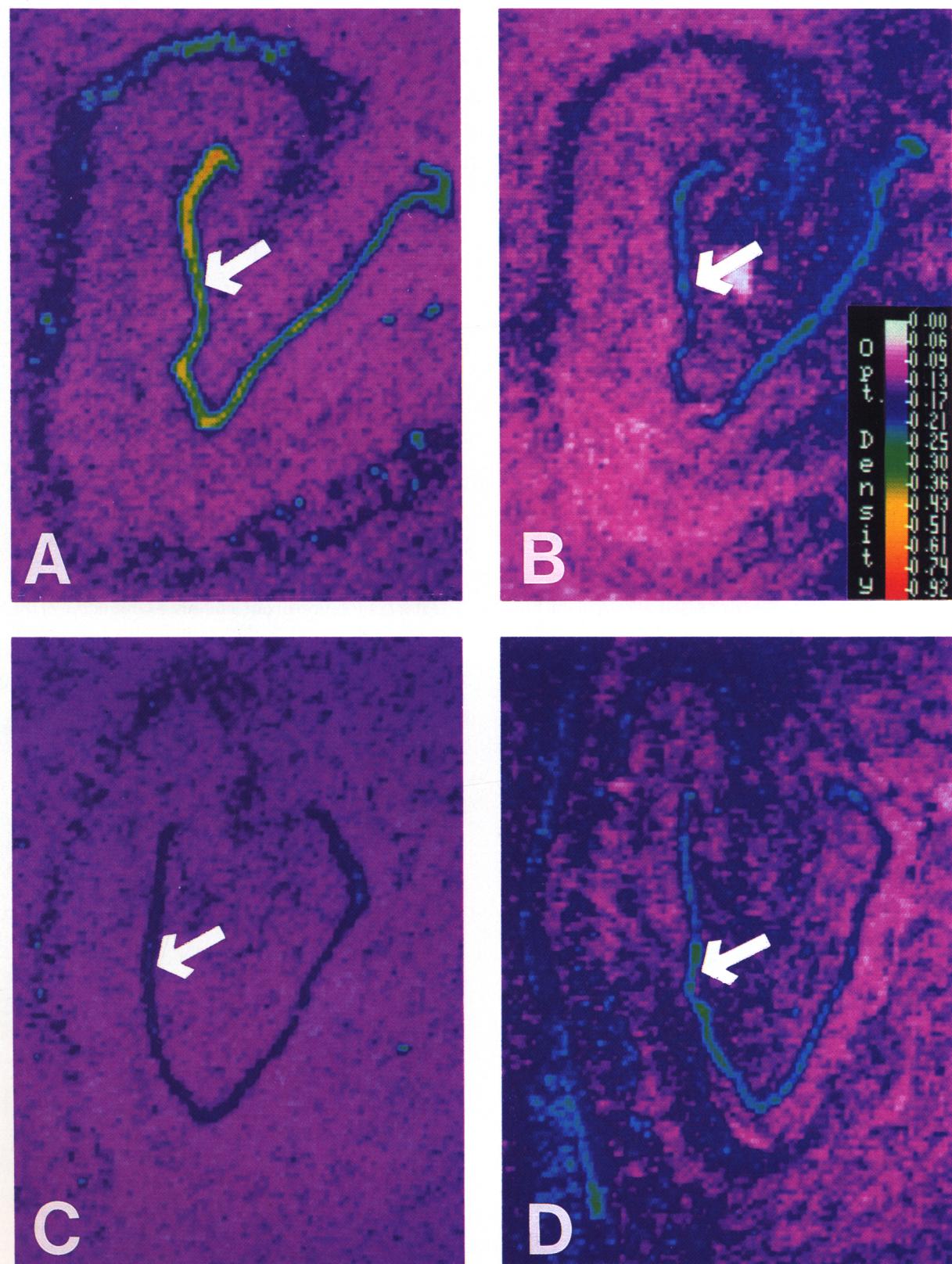


Figure 1. Computer Digitized Autoradiographs of Sections Hybridized to BDNF Antisense or NGF Antisense Probes (A and C) BDNF probe; (B and D) NGF probe. (A and B) Control donor; (C and D) AD donor. Color bar in (B) applies to all panels. Arrows indicate the granule cell layer of the dentate gyrus. BDNF hybridization signal is markedly lower in the AD sample (C) as compared with control sample (A). NGF signals are comparable in the two specimens (B and D).

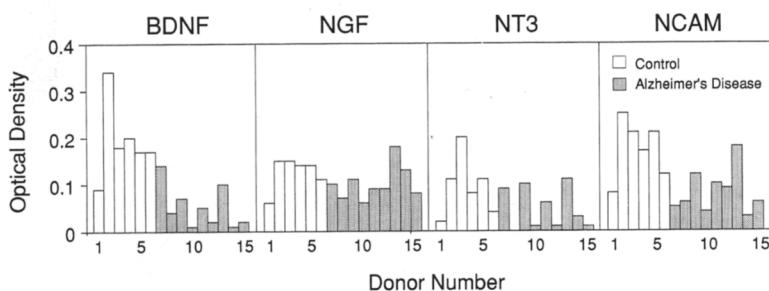


Figure 2. Comparison of BDNF, NGF, NT-3, and N-CAM Hybridization Signals in the Hippocampal Formation of AD and Control Donors

Each bar represents the specific hybridization signal from an individual sample. Donor numbers correspond to Table 1. Exposure times of autoradiographs were 1 day for N-CAM, 3 days for BDNF, 8 days for NGF, and 14 days for NT-3.

significant reduction in this region in AD versus control samples (0.09 ± 0.04 versus 0.26 ± 0.03 , $t = 2.91$, 13 d.f., $p < 0.02$). In marked contrast, neither NGF/N-CAM nor NT-3/N-CAM ratios in the dentate gyrus were decreased in AD (Table 2). NT-3/N-CAM ratios displayed no difference in AD and control samples ($t = 0.33$, 13 d.f., $p = 0.747$), whereas NGF/N-CAM ratios revealed a trend indicative of a relative sparing of NGF mRNA in AD. (In two separate experiments $t = -2.69$, 12 d.f., $p = 0.02$; see Table 2; control = 0.33 ± 0.05 ; AD = 0.54 ± 0.09 , $t = -1.86$, 12 d.f., $p = 0.083$.)

BDNF mRNA Abundance Is Reduced in the Hippocampus of Individuals with AD

To quantify the abundance of BDNF mRNA in the hippocampus, experiments were performed utilizing an RNAase protection assay. Examination of RNA from 17 samples of human hippocampus revealed a roughly 2-fold reduction in abundance of BDNF transcript in samples from AD donors compared with non-AD controls (Figure 3; 0.29 ± 0.04 pg per 10 μ g of RNA for AD; 0.57 ± 0.06 pg per 10 μ g of RNA for control, $t = 3.93$, 15 d.f., $p = 0.001$). Mean autolysis times did not differ between control and AD samples (control = 11.6 ± 3.2 hr; AD = 7.3 ± 2.3 hr, $p = 0.279$). Although the mean age for the AD samples was greater than that for the control donors (75.7 ± 2.2 years for AD

donors; 67.1 ± 2.0 years for control donors, $t = 2.69$, 15 d.f., $p < 0.05$), this difference is not likely to account for the observed difference in BDNF values, since BDNF values showed a trend (nonsignificant) toward increasing with age in both AD and control groups.

Although the majority of the samples analyzed by RNAase protection differ from those utilized in the in situ hybridization studies, there was some overlap in the sample sets. When only samples obtained from tissue banks not contributing tissue for the in situ hybridization analysis were included in the RNAase protection analysis, a significant reduction in BDNF mRNA in AD was still observed (AD = 0.36 ± 0.05 , $n = 7$; control = 0.66 ± 0.08 , $n = 4$, $t = -3.36$, $p < 0.01$).

After completion of our analysis, examination of the records of the control donors indicates a history of

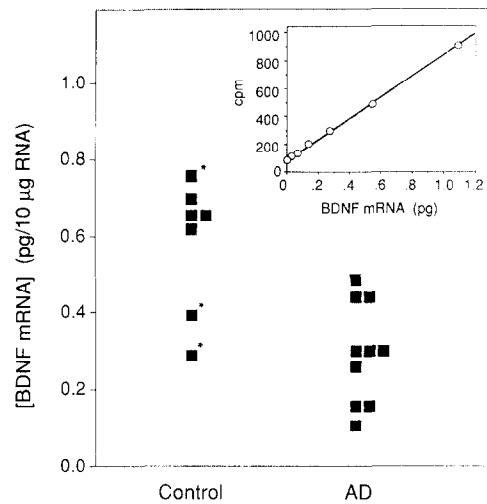


Figure 3. Quantification of BDNF mRNA in Hippocampi from AD and Control Donors by RNAase Protection Assay

Values displayed represent the mean of three determinations of the amount of BDNF mRNA sequence encoding mature protein present in 10 μ g of total RNA. The standard error of the mean for triplicate determinations averaged 8.6% of the mean value determined. Asterisks indicate control donors with clinical history of dementia.

(Inset) Standard curve generated by the hybridization of increasing amounts of synthetic sense-strand BDNF RNA to antisense probe. Values for BDNF indicate picograms of the 740 base coding sequence of mature BDNF. Values on the y-axis indicate the number of counts recovered by filtration following degradation of single-stranded RNA and precipitation.

Table 2. Neurotrophin Hybridization Signals Normalized to N-CAM

	BDNF/N-CAM	NGF/N-CAM	NT-3/N-CAM
Control	1.13 ± 0.12 (6)	0.75 ± 0.05 (6)	0.50 ± 0.09 (6)
AD	0.49 ± 0.08^b (9)	1.08 ± 0.10^a (8) ^c	0.45 ± 0.12 (9)

Hybridization signals for BDNF, NGF, and NT-3 in the dentate gyrus normalized to N-CAM signal in control and AD samples. The specific NGF, BDNF, and NT-3 signals for each sample were normalized by dividing by the N-CAM signal. Values shown represent the mean \pm SEM of the ratios determined for individual samples within a group. The number of samples per group is indicated in parentheses. Values were not corrected for exposure time of autoradiographs (N-CAM 1 day, BDNF 3 days, NT-3 8 days, NGF 21 days).

^a $P < 0.05$ versus control; t test.

^b $P < 0.0005$ versus control; t test.

^c One outlying point (>4 standard deviations above mean) has been eliminated from the analysis of NGF/N-CAM ratios.

dementia in 3 individuals. Curiously, the BDNF values for 2 of these individuals (1 with no neuropathology, but a clinical history of memory loss and 1 with a combination of Parkinson's disease and multi-infarct dementia) were the lowest values observed for control samples and fell well within the range observed for AD samples. The third individual, diagnosed with multi-infarct dementia, displayed normal levels of BDNF mRNA.

Discussion

Degenerative changes in the basal forebrain cholinergic system have been suggested to play a critical role in the development of dementia in AD (see Perry, 1990). While increasing evidence indicates that NGF may serve as a trophic agent for the basal forebrain cholinergic system (see Hefti et al., 1989), there is no evidence to support the suggestion that a deficiency in NGF occurs in AD. Recent findings indicate that BDNF, one of two known NGF homologs, is widely expressed by targets of basal forebrain cholinergic neurons and is capable of exerting trophic actions on these cells *in vitro* (Alderson et al., 1990; Ernfors et al., 1990; Hofer et al., 1990; Knusel et al., 1991; Phillips et al., 1990). In the present study, we report a reduction in BDNF mRNA abundance in hippocampi of individuals with AD. These findings suggest the possibility that a deficiency in BDNF expression may contribute to the loss of cholinergic neurons in the basal forebrain in AD.

Utilizing *in situ* hybridization, we have examined expression of three members of the NGF family, NGF, BDNF, and NT-3, in hippocampi of individuals with AD and of control donors. This analysis revealed a 4-fold reduction in BDNF hybridization intensity in the dentate gyrus of AD versus control samples. A reduction in BDNF hybridization intensity was also apparent in the pyramidal layer of Ammon's horn. In marked contrast to BDNF, no significant differences in hybridization signals for NGF or NT-3 were observed in the dentate gyrus. The lack of change in NGF hybridization intensity is consistent with earlier observations on the cortex (Goedert et al., 1986).

The differences in BDNF hybridization intensity in hippocampi of control and AD donors cannot be explained on the basis of donor age or sex, autolysis times of the samples, or loss of cell density in the region examined. Predeath agony is unlikely to account for the observed changes, as BDNF signals in the 3 nondemented controls that were victims of Parkinson's disease were quite similar to those of the 3 neurologically normal donors. Furthermore, the lack of change in NGF hybridization intensity between AD and control samples argues that the decrease in BDNF hybridization cannot be accounted for by a general reduction in mRNA within the granule cell layer of the dentate gyrus or by differences in RNA quality between our AD and control samples.

In spite of the lack of difference in NGF hybridiza-

tion intensity between our AD and control samples, the trend ($p > 0.10$) toward reduction in NT-3 signal in our AD samples brought to bear concerns over the specificity of reduction in BDNF hybridization signal. To address these concerns, we performed hybridizations with a probe to an unrelated mRNA comparable in abundance to BDNF, N-CAM. Normalization of the hybridization signals for BDNF, NGF, and NT-3 to those obtained with the N-CAM probe revealed that only BDNF/N-CAM ratios were decreased in AD.

It is not clear whether the roughly 2-fold reduction in N-CAM ($p < 0.05$) hybridization signal in our AD samples reflects reduction in this mRNA in AD. Given that most (if not all) of our AD samples represent end stages of the disease, it would not be surprising if expression of a variety of neuronal mRNAs is affected to some extent. Alternatively, it is possible that variations in quality of RNA may have contributed to the observed difference in N-CAM hybridization. It is not within the realm of this study to distinguish between these alternatives. It is, however, clear that while NGF/N-CAM and NT-3/N-CAM ratios showed no tendency toward reduction in AD, BDNF/N-CAM ratios showed a highly significant ($p < 0.0005$) reduction in the AD samples as compared with control samples.

The reduction in BDNF/N-CAM hybridization ratio in AD observed with *in situ* hybridization (57%) agrees nicely with the reduction in BDNF mRNA abundance observed in a second set of samples with RNAase protection assay (49%). Thus, in two sets of tissue samples assayed by different techniques, we have observed evidence for a reduction in BDNF mRNA abundance in hippocampi of individuals with AD. It is not clear whether the reduction in BDNF mRNA abundance is associated with decreased levels of protein, but previous studies on NGF have indicated a close correlation in regional brain content of NGF mRNA and protein (Korschning et al., 1985; Shelton and Reichardt, 1986). Although a physiological role for BDNF in support of basal forebrain cholinergic cells has not been established, if synthesis of BDNF is limiting for function of these neurons, then a reduction in BDNF mRNA abundance of the magnitude we observed might be expected to have profound consequences on cholinergic function. Choline acetyltransferase activity has been reported by several investigators to decrease 50%–60% in the hippocampus of individuals with AD (Araujo et al., 1988; Sakurada et al., 1990; see Perry, 1990; see Palmer and Gershon, 1990). Given the cholinergic deficiency in the cortex and profound cell loss in the nucleus basalis that occur in AD (see Palmer and Gershon, 1990; see Perry, 1990; Whitehouse et al., 1982), it will be extremely interesting to determine whether BDNF mRNA is reduced in the cortex with AD.

From our data, it is apparent that BDNF mRNA abundance is decreased in postmortem samples of hippocampus from individuals afflicted with AD. As our samples reflect end-stage disease, it is not possible to determine at what point in the disease BDNF mRNA is affected. Given the numerous pathological changes

that occur in AD, it is unlikely that decreased BDNF production is the primary event in the disease. The possibility that BDNF deficiency contributes to the progression of atrophy in the basal forebrain cholinergic system does, however, deserve consideration. Intriguingly, in the few cases of Parkinson's disease that we examined, those with accompanying dementia displayed BDNF hybridization values as low as those observed in individuals with AD (OD of 2 cases of Parkinson's disease with dementia = 0.01 and 0.07; OD of 3 cases of Parkinson's disease without dementia = 0.20, 0.17, and 0.17). These limited findings suggest the possibility that some non-AD forms of dementia might also be associated with decreased BDNF mRNA in the hippocampus. Additional studies are needed to determine the complete distribution of changes in BDNF expression in AD and to establish a correlation between levels of BDNF expression and progression of degenerative events that occur in this disease.

Experimental Procedures

In Situ Hybridization

Frozen, unfixed samples were sectioned, processed for *in situ* hybridization with ^{35}S -labeled RNA probes to human BDNF, NGF, NT-3, or N-CAM, and subjected to sheet film autoradiography.

For BDNF, probe synthesis and hybridizations were conducted as previously reported (Phillips et al., 1990) using an $[^{35}\text{S}]$ UTP-labeled, 1 kb probe of the human BDNF sequence. Utilizing our hybridization protocol, good signals for BDNF were observed on sheet film (Hyperfilm, β -max, Amersham) after a 3-day exposure at room temperature. Two pilot experiments employing a subset of samples and one complete experiment with all 18 samples were performed with BDNF sense and antisense probes. Data presented in Figure 2 come from the complete experiment and are confirmed by the findings of the two pilot experiments. For most of the samples, 4 sections were hybridized to antisense probe and 2 to sense control probe. As material from the specimens that had been used in pilot experiments was limited, 2 slides were hybridized to antisense probe and 1 to sense control probe.

An $[^{35}\text{S}]$ UTP-labeled RNA probe to NT-3 was produced utilizing a 0.9 kb (BglII-HindIII) fragment of the human NT-3 sequence (Rosenthal et al., 1990) subcloned into pGEM-3Z. Probe synthesis, hybridizations, autoradiography, and OD readings of the resulting autoradiographs were performed as described for BDNF. A single NT-3 experiment was conducted. For each sample, in some cases 2 slides were hybridized to antisense probe and 1 to sense control probe.

Two separate experiments of NGF hybridizations were performed employing sections from all 18 samples. Data from both experiments yielded similar results. In both cases, the conditions for hybridization and washing were identical to those employed in the BDNF experiments. In the first experiment, an $[^{35}\text{S}]$ UTP-labeled RNA probe was utilized (1 slide each was hybridized to sense and antisense probe); in the second experiment, probe was labeled with both $[^{35}\text{S}]$ UTP and $[^{35}\text{S}]$ CTP (2 slides each were used for sense and antisense probe). The template for probe synthesis in both cases was a 1 kb (BamHI-HindIII) fragment of human NGF subcloned into pGEM-3Z. Hybridizations conducted using the single-labeled nucleotide probe required long exposure times. (A 21-day exposure yielded a signal significantly weaker than that observed for BDNF after 3 days.) As for the single-labeled nucleotide probe, the double-labeled nucleotide probe was added to tissue sections at a final concentration of 8×10^6 cpm/ml. Use of the higher specific activity NGF probe at this reduced molar concentration resulted in a stronger hybridization signal and allowed shorter exposure times to be utilized.

Results with the higher specific activity probe are presented in Figure 2 and Table 2.

Probe to N-CAM was generated using a 1.1 kb template of the human N-CAM sequence cloned into pGEM-3Z. Template sequence represents residues 1571-2704 of the neural form of human N-CAM (Hemperly et al., 1990). Two slides each were used for sense and antisense probes. Hybridizations and quantification of resulting signals were performed as described for NGF homologs.

To quantify hybridization signals, the autoradiographs were digitized and numerical readings of OD were obtained with the aid of an image analysis system. Films were digitized, and optical density readings were obtained using a RAS-3000 image analysis system (Amersham). All autoradiographs from a given experiment were digitized under identical conditions. For each autoradiograph, the mean of 5 OD readings at points within the granule cell layer of the dentate gyrus was determined. By subtracting the mean background hybridization signal obtained on the control (sense probe) slides from the mean signal observed on the antisense probe slides, we obtained a measure of the specific hybridization signal for each sample. The standard error of BDNF hybridization signals from samples with 4 replicate slides averaged 6.5% of the OD value. For analysis of BDNF hybridization intensities in pyramidal layer, 5 OD readings were obtained from the region of most intense signal (CA3 in most cases).

RNAase Protection Assay

RNA was prepared from frozen pieces of human hippocampus as previously described (Chirgwin et al., 1979), or by following the RNAzol (CINNA/BIOTEX Labs. Int., Inc.) procedure as described by the manufacturer. Following ethanol precipitation, the RNA pellet was resuspended in sterile 10 mM Tris-HCl (pH 7.6), 1 mM EDTA buffer. The concentration of the RNA was determined by the absorbance at 260 nm. A_{260}/A_{280} ratios for human samples prepared by this method are typically in the range of 1.7-1.9 and do not differ for AD and control samples. The final RNA concentration was adjusted to 2.5 $\mu\text{g}/\text{ml}$.

The RNAase protection assay was carried out by a modification of the method of Lee and Costlow (1987). BDNF antisense probe was synthesized *in vitro* by T7 RNA polymerase utilizing a 740 bp DNA template that represents the coding sequence for mature human BDNF. The double-stranded DNA template was generated by polymerase chain reaction amplification of sequence from the cDNA-containing vector used for *in situ* hybridization. The sense and antisense primers each encoded 24 bases of DNA complementary to BDNF as well as 24 bases complementary to either SP6 or T7 RNA polymerase promoter. Primers are as follows: sense, 5'-TACGATTAGGTGACACTATAGAACAT-GACCATCTTTCTTACTATG-3'; antisense, 5'-TCTAAATACG-ACTCACTATAGGGAGACATCTCCCTTTAATGGTCAATGT-3'. The resulting template consisted of 740 bp encoding the mature human BDNF sequence flanked by T7 and SP6 promoters orientated so that T7 polymerase could be utilized to generate antisense probe. Antisense probe was generated *in vitro* utilizing [α - ^{32}P]UTP and [α - ^{32}P]CTP (800 Ci/mmol; Amersham) by a method similar to that employed for synthesis of the *in situ* hybridization probes. The probe resulting from *in vitro* transcription was analyzed by gel electrophoresis and judged to be essentially all full-length product. Specific activity of the probe was approximately 330 Ci/ μmol .

To generate a BDNF RNA standard, unlabeled sense-strand BDNF RNA was synthesized by SP6 polymerase from the linearized vector template utilized in the *in situ* hybridization experiments. The amount of RNA generated in this reaction was estimated by monitoring the incorporation and recovery of a trace amount of $[^{35}\text{S}]$ UTP added to a parallel reaction. As the length of standard generated from the linearized vector template was 1 kb, values were corrected by a factor of 0.74 to express all data as picograms of coding sequence of BDNF RNA.

For assay, 10 μg of sample human RNA or synthetic BDNF standard with 10 μg of tRNA carrier was added to 100,000 cpm of probe (synthesized within 24 hr of use) in 20 μl containing 50% formamide, 0.4 M NaCl, 25 mM HEPES, and 1 mM EDTA. The

mixture was overlaid with mineral oil, heated to 85°C for 5 min, and incubated overnight at 50°C. To each tube, 100 µl of solution containing 50 µg of salmon sperm DNA, 25 µg of RNAase A, and 1.5 µg of RNAase T1 (in 0.4 M NaCl, 25 mM HEPES, 1 mM EDTA) was added, and the mixture was allowed to incubate for 1–2 hr at 37°C. Each sample was then precipitated by the addition of 0.8 ml of ice-cold 1 M HCl, 0.1 M sodium pyrophosphate, filtered with a cell harvester onto receptor-binding filter mats (Skatron; Sterling, VA), and counted in a scintillation counter. The linear equation describing the standard curve ($y = 90 + 750x$, $R^2 = 0.999$) was used to determine the picograms of BDNF mRNA coding sequence present in the unknown samples.

Specificity of this method for detection of BDNF mRNA was confirmed by the observation that 10 pg of synthetic RNA encoding BDNF, but not NT-3 or NGF, protected a band visible by autoradiography following urea-acrylamide gel electrophoresis.

Tissue Samples

For *in situ* hybridization experiments, 18 samples of postmortem human hippocampus were obtained from the National Neurological Research Bank for study. The medical records including clinical histories and neuropathology report were obtained for each donor. All 9 samples included in our AD sample came from donors with confirmed clinical and neuropathological diagnoses of AD and showed no evidence of other neurological disease. Nine samples were obtained for inclusion in the non-AD control group. Of these 9 samples, 3 were from donors with no clinical or pathological evidence of neurological dysfunction, 5 were classified by the neuropathology reports as Parkinson's disease, and 1 was from a donor who had suffered a stroke. The medical records of 2 of the Parkinson's donors indicated a history of progressive memory loss of several years duration. *In situ* hybridization data obtained from these 2 samples were not included as controls in our analysis of control versus AD donors. The tissue specimen from the stroke victim was deleted from the study as it displayed obvious signs of infarction within the hippocampus. Cell counts were performed on the granule cell layer of the dentate gyrus in photographs of 12 µm, cresyl violet-stained sections.

For RNAase protection assays, hippocampal RNA was obtained from 4 donors included in the *in situ* hybridization analysis and 13 additional donors. Each sample included the entire extent of the hippocampal formation present in a coronal slice of brain approximately 1 cm thick. Care was taken to remove the temporal cortex and amygdala. Samples were obtained from the National Neurological Research Bank (3 control, 3 AD), the autopsy core of the ADRC of Southern California (3 control, 7 AD), and the Institute of Biogerontology (1 control). All 10 AD samples were from individuals with clinical and neuropathological diagnosis of AD. The 7 control samples were assigned on the basis of the neuropathology reports as normal ($n = 4$), multi-infarct dementia ($n = 1$), Parkinson's disease ($n = 1$), and Parkinson's disease with multi-infarct dementia ($n = 1$). As we did not have access to the detailed clinical histories for every patient, we did not eliminate any of the samples from the control group in spite of evidence for the presence of dementia in 3 cases.

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References

- Alderson, R. F., Alterman, A. L., Barde, Y.-A., and Lindsay, R. M. (1990). Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. *Neuron* 5, 297–306.
- Araujo, D. M., Lapchak, P. A., Robitaille, Y., Gauthier, S., and Quirion, R. (1988). Differential alteration of various cholinergic markers in cortical and subcortical regions of human brain in Alzheimer's disease. *J. Neurochem.* 50, 1914–1923.
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18, 5294.
- Coleman, P., and Flood, D. (1987). The value of alternative morphological approaches to Alzheimer's disease. *Neurobiol. Aging* 8, 521.
- Ernfors, P., Wetmore, C., Olson, L., and Persson, H. (1990). Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. *Neuron* 5, 511–526.
- Goedert, M., Fine, A., Hunt, S. P., and Ullrich, A. (1986). Nerve growth factor mRNA in peripheral and central rat tissues in the human central nervous system: lesion effects in the rat brain and levels in Alzheimer's disease. *Brain Res.* 387, 85–92.
- Goedert, M., Fine, A., Dawburn, D., Wilcock, G. K., and Chao, M. V. (1989). Nerve growth factor receptor mRNA distribution in human brain: normal levels in basal forebrain in Alzheimer's disease. *Mol. Brain Res.* 5, 1–7.
- Hefti, F., and Mash, D. C. (1989). Localization of nerve growth factor receptors in the normal human brain and in Alzheimer's disease. *Neurobiol. Aging* 10, 75–87.
- Hefti, F., Hartikka, J., and Knusel, B. (1989). Function of neurotrophic factors in the adult and aging brain and their possible use in the treatment of neurodegenerative diseases. *Neurobiol. Aging* 10, 515–533.
- Hemperly, J. J., Deguglielmo, J. K., and Reid, R. A. (1990). Characterization of cDNA clones defining variant forms of human neural cell adhesion molecule (NCAM). *J. Mol. Neurosci.* 2, 71–78.
- Higgins, G. A., and Mufson, E. J. (1989). NGF receptor gene expression is decreased in the nucleus basalis in Alzheimer's disease. *Exp. Neurol.* 106, 222–236.
- Hofer, M., Pagliusi, S. R., Hohn, A., Leibrock, J., and Barde, Y.-A. (1990). Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *EMBO J.* 9, 2459–2464.
- Knusel, B., Winslow, J. W., Rosenthal, A., Burton, L. E., Seid, D. P., Nikolics, K., and Hefti, F. (1991). Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. *Proc. Natl. Acad. Sci. USA* 88, 961–965.
- Kordower, J. H., Gash, D. M., Bothwell, M., Hersh, L., and Mufson, E. J. (1989). Nerve growth factor receptor and choline acetyltransferase remain colocalized in the nucleus basalis (Ch4) of Alzheimer's patients. *Neurobiol. Aging* 10, 67–74.
- Korschig, S., Auburger, G., Heumann, R., Scott, J., and Thoenen, H. (1985). Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. *EMBO J.* 4, 1389–1393.

- Lee, J., and Costlow, N. A. (1987). A molecular titration assay to measure transcript prevalence levels. *Meth. Enzymol.* 152, 633-648.
- Marx, J. (1990). NGF and Alzheimer's: hopes and fears. *Science* 247, 408.
- Palmer, A. M., and Gershon, S. (1990). Is the neuronal basis of Alzheimer's disease cholinergic or glutamatergic? *FASEB* 4, 2745-2752.
- Perry, E. K. (1990). Nerve growth factor and the basal forebrain cholinergic system: a link in the etiopathology of neurodegenerative dementias? *Alzheimer's Dis. Associated Disorders* 4, 1-13.
- Phillips, H. S., Hains, J. M., Laramee, G., Rosenthal, A., and Winslow, J. W. (1990). Widespread expression of BDNF but not NT3 by target areas of basal forebrain cholinergic neurons. *Science* 250, 290-294.
- Rosenthal, A., Goeddel, D. V., Nguyen, T., Lewis, M., Shih, A., Laramee, G. R., Nikolics, K., and Winslow, J. W. (1990). Primary structure and biological activity of a novel human neurotrophic factor. *Neuron* 4, 767-773.
- Sakurada, T., Alufuzoff, I., Winblad, B., and Nordberg, A. (1990). Substance P-like immunoreactivity, choline acetyltransferase activity and cholinergic muscarinic receptors in Alzheimer's disease and multi-infarct dementia. *Brain Res.* 521, 329-332.
- Shelton, D. L., and Reichardt, L. F. (1986). Studies on the expression of the β nerve growth factor (NGF) gene in the central nervous system: level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several distinct populations of neurons. *Proc. Natl. Acad. Sci. USA* 83, 2714-2718.
- Wetmore, C., Ernfors, P., Persson, H., and Olson, L. (1990). Localization of brain-derived neurotrophic factor mRNA to neurons in the brain by *in situ* hybridization. *Exp. Neurol.* 109, 141-152.
- Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W., Coyle, J. T., and DeLong, M. R. (1982). Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. *Science* 215, 1237-1239.