Neurobiology

Cell Death and Learning Impairment in Mice Caused by *in Vitro* Modified Pro-NGF Can Be Related to Its Increased Oxidative Modifications in Alzheimer Disease

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Pro-nerve growth factor (pro-NGF) is expressed at increased levels in Alzheimer's disease (AD)-affected brains and is able to induce cell death in cultures; however, the reasons for these phenomena remain elusive. Here we show that pro-NGF in human AD-affected hippocampus and entorhinal cortex is modified by advanced glycation and lipoxidation end-products in a stage-dependent manner. These modifications block pro-NGF processing to mature NGF, thus making the proneurotrophin especially effective in inducing apoptosis of PC12 cells in culture through the p75 neurotrophin receptor. The processing of advanced glycation and lipoxidation end-products *in vitro* modified recombinant human pro-NGF is severely impaired, as evidenced by Western blot and by examining its physiological functionality in cell cultures. We also report that modified recombinant human pro-NGF, as well as pro-NGF isolated from human brain affected by AD, cause impairment of learning tasks when administered intracerebroventricularly in mice, which correlates with AD-associated learning impairment. Taken together, the data we present here offer a novel pathway of ethiopathogenesis in AD caused by advanced glycation and lipoxidation end-products modification of pro-NGF. (Am J Pathol 2009, 175:2574–2585; DOI: 10.2353/ajpath.2009.090018)

Oxidative stress occurs early in the progression of Alzheimer's disease (AD) even before the development of the pathological hallmarks, neurofibrillary tangles and senile plaques, depending on the stage of the disease and cerebral region. This is accompanied by degeneration of synapses and dendrites, and by cell death and neuronal loss.^{1–7}

All classes of macromolecules are affected by oxidative stress and it is one of the mechanisms leading to neuronal dysfunction.

Oxidative protein damage arises from direct exposure of susceptible amino acid residues to reactive oxygen species, generating oxidative products such as glutamic and amino-adipic semi-aldehydes.³ These chemical and nonenzymatic modifications may also arise from reaction with low-molecular-weight, reactive carbonyl compounds such as glyoxal (GO), methylglyoxal (MGO), and malondialdehyde (MDA), resulting from damaged carbohydrates or unsaturated fatty acids. These carbonyl compounds could react primarily with Lys, Arg, and Cys residues in proteins, leading to the formation of both adducts and cross-links denominated advanced glycation/lipoxidation end products (AGE/ALEs). N ϵ -(carboxyethyl)-lysine (CEL), N ϵ -(carboxymethyl)-lysine (CML), and N ϵ -(malondialdehyde)-lysine (MDAL) are three of these adducts, derived from the reaction of MGO, GO, and MDA, respectively, with the free amino groups of lysine residues on protein. Mass spectrometry analysis of

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human brain homogenates has demonstrated a significant increase in CEL, CML, and MDAL in AD.³ It is also known that the oxidative nonenzymatic modifications increase protein crosslinking, which could affect protein function.^{8,9}

Neuroketals (NKTs) are isoprostane-like derivatives specifically produced by free radical-induced peroxidation of docosahexaenoic acid, which is highly enriched in the brain.^{10,11} NKTs were found to be formed in abundance *in vitro* during oxidation of docosahexaenoic acid, and were shown to rapidly adduct to Lys, forming Schiff base adducts. The fact that polyunsaturated fatty acids are prone to free radical attack and free radicals have been implicated in a number of neurodegenerative diseases makes NKTs a unique and valuable marker of oxidative injury in the brain.

Recent studies have shown that AD brain levels of pro-nerve growth factor (pro-NGF) are increased in a stage-dependent manner.^{12–14} Some evidence supports the idea that pro-NGF binding to a pair of p75 neurotrophin receptor (p75NTR) and Sortilin can mediate cell death in different neuronal models.^{15,16}

Synthesis of precursors and processing by proteolysis is a common feature for most neurotrophins. Pro-NGF is characterized by its non-trophic support action and ability to induce cell death and has been shown to be the predominant form of nerve growth factor (NGF) in human brain.^{13,14,17} Several pro-NGF forms with apparent molecular weights ranging from 16 to 60 kDa have been described.^{13,18–21} These pro-NGF forms that can vary from one tissue to another are provided by the combinations of two different possible transcript products,^{21,22} together with the existence of several potential targets for convertase cleavage and glycosylation.

Isolated by chromatography from AD-affected human brains, pro-NGF (ADhbi-pro-NGF) induces apoptotic cell death in neuronal cell cultures through its interaction with the p75NTR receptor.^{13,14,17} ADhbi-pro-NGF stimulates the processing of p75NTR by α - and γ -secretases, yielding a 20-kDa intracellular domain (p75^{ICD}), which translocates to the nuclei. This process is accompanied by apoptosis.¹⁴ Pro-NGF isolated from AD-affected brains differs functionally from pro-NGF isolated from control brains at comparable ages, with the latter being susceptible to processing to NGF when added to cell cultures.¹⁴

In the present work, we show that pro-NGF in human AD-affected hippocampus and entorhinal cortex is oxidatively modified at least by AGE/ALEs in a stage-dependent manner. We also show that these modifications produced *in vitro* lead to an increased resistance of the protein to processing and decreased maturation to NGF, thus making the proneurotrophin especially effective in inducing apoptosis through its interaction with p75NTR. Further, we demonstrate that intracerebroventricular administration of AGE/ALEs modified pro-NGF to mice impairs learning tasks, thus reinforcing the idea that pro-NGF could have a relevant role in the ethiopathogenesis of the disease.

Materials and Methods

Human Samples

Postmortem human brain samples from patients with AD and controls were obtained from the Institute of Neuropathology Brain Bank following informed consent and in accord with the guidelines of the local ethics committee. At autopsy, half of each brain was fixed in formalin, while the other half was cut in coronal sections 1-cm thick, frozen on dry ice, and stored at -80°C until use. For diagnostic morphological studies, the brains were fixed by immersion in 4% buffered formalin for 2 or 3 weeks. Neuropathological diagnosis followed the nomenclature of Braak and Braak,²³ adapted for paraffin sections.²⁴ Stage I/II are characterized by NFTs and dystrophic neurites restricted to the transentorhinal and entorhinal cortices. No clinical symptoms are noted at these stages. Stages III/IV are characterized by the additional involvement of the hippocampus and inner regions of the temporal cortex. Mild cognitive impairment may occur in association with AD Braak stages III/IV. Finally, stages V/VI involve the neocortex, and they are clinically manifested as Alzheimer's dementia. Regarding amyloid plaque burden, lesions were categorized as 0: no amyloid; A: amyloid plaques in the orbitary and temporal cortices; B: moderate numbers of plaques in the cerebral convexity; and C: large numbers of plaques in the whole cerebral cortex including primary motor and somatosensory areas. Cases with additional pathology (either tau: ie, grains; α -synuclein: Lewy bodies in other brain regions; or vascular in any area) were excluded. Cases with no neuropathological lesions (including vascular, hypoxic, inflammatory, and degenerative) were considered as controls.

The brains of nine patients with AD, including three AD stage I-II/A-B, three AD III-IV/A-B, three AD V/C, and three controls, were obtained post-mortem, and were immediately prepared for morphological and biochemical studies. For biochemical studies, the entorhinal cortex and the hippocampus were separately processed in every case. Control and diseased cases were processed in parallel. Special care was taken to reduce the post-mortem delay and the temperature of the storage of the sample to ensure protein preservation.²⁵ Since previous studies have shown that post-mortem delays between 12 and 18 hours were associated with modifications in the profile of oxidation markers,²⁶ for this study we used samples with post-mortem delay between 3 and 8 hours in control and diseased cases. The pH of the brain tissue varied from 6.7 to 6.9. A summary of control and AD cases is shown in Table 1.

Antibodies

The antibody against pro-NGF pro-domain was made as described previously by Beattie et al (2002).²⁷ In brief, glutathione-S-transferase-fusion protein containing the 23 to 81 (asp23–arg81) peptide from human pro-NGF was used to immunize New Zealand rabbits (Charles River Laboratories). The antibody was purified first by

Table 1. Summary of Cases Examined in the Present Study

No	AD stage	Age (years)	Sex
1	ADI/A	57	F
2	ADI/A	65	M
3	ADII/A	66	M
4	ADIV/A	80	F
5	ADIV/B	82	M
6	ADIII/A	66	M
7	ADV/C	90	F
8	ADV/C	79	M
9	ADV/C	74	F
10	No lesions	39	M
11	No lesions	56	M
12	No lesions	47	M

F, female; M, male.

immuno-absorption in a GST column to remove total GST immunoreactive fraction, and after that by absorption and elution from the glutathione column in which GST-pro-NGF was immobilized. Antibody against mature NGF was purchased from Santa Cruz. Secondary antibodies (anti-mouse IgG-horseradish peroxidase and anti-rabbit IgG-horseradish peroxidase) were obtained from Amersham Biosciences (Piscataway, NJ). Blocking pro-NGF in cell culture was performed by 2 hours pre-incubation with 200 ng/ml of anti-pro-NGF.¹³ p75NTR was blocked by adding 200 ng/ml anti-p75NTR antibody directed against the extracellular domain of p75NTR (kindly provided by L.F. Reichardt, Howard Hughes Medical Institute, University of California) to the cell culture media 2 hours before treatments. Monoclonal antibodies against CEL, CML, and AGEs were purchased from TransGenic Inc. (Kobe). Goat polyclonal antibodies to MDAL and NKTLs were from Academy Bio-Medical Company (Houston) and Chemicon (Temecula), respectively. For immunodetection, anti-pro-NGF and mature NGF were used diluted at 1:2500 in Tris-buffered saline/Tween 20 (TBS-T). Anti-CEL, anti-CML, and anti-AGEs antibodies were used at 1:2000, anti-MDAL antibody at 1:1000, and anti-NKTLs antibody at 1:5000 in TBS-T.

Protein Immunodetection

After the transfer, the membranes were blocked for 1 hour at room temperature in TBS-T (50 mmol/L Tris, pH 8.0, 133 mmol/L NaCl, 0.2% Tween 20) with blocking reagent (Tropix, MA). For immunodetection the membranes were incubated overnight at 4°C with agitation using the appropriate dilution for each antibody. After washing three times in TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000 in TBS-T) at room temperature for 1 hour. For detection, after washing three times in TBS-T, an enhanced chemiluminescence reagent (ECL plus, Amersham Biosciences, Piscataway, NJ) was used in accordance with the manufacturer's instructions.

Pro-NGF Immunoprecipitation

Samples of 200 to 300 mg tissue from hippocampus and entorhinal cortex, obtained from different control individ-

uals and different individuals affected by AD of the same stage (see human samples), were homogenized and pooled. For immunoprecipitation, the human samples were homogenized in radioimmunoprecipitation assay buffer (150 mmol/L NaCl; 0.1% SDS; 50 mmol/L Tris, pH 8.0; 1% NP-40; 0.5% deoxycholic acid) by sonication, and centrifuged at $12,000 \times g$ for 10 minutes.

The suspension was left on ice for 30 minutes and centrifuged at $16,000 \times g$ at 4°C for 15 minutes. The total amount of proteins was determined and normalized among the samples. Then the supernatant was transferred to a fresh microcentrifuge tube. The antibody-conjugated beads were prepared by mixing 30 μ l of 50% protein A-Sepharose bead slurry, 0.5 ml ice-cold PBS, and 1 μ g of anti-mature NGF, and then tumbled end over end for the mixture for ≥ 1 hour at 4°C in a tube. The mixture was centrifuged for 2 seconds at $16,000 \times g$ and 4°C, the supernatant (containing unbound antibodies) was removed, and the beads were washed 3 times with 1 ml non-denaturing buffer. The lysate was precleared by mixing 1 ml of lysate with 30 μ l of 50% protein A-Sepharose bead slurry and tumbled end over end for 30 minutes at 4°C in a tube rotator. It was then centrifuged for 5 minutes at $16,000 \times g$. The immunoprecipitation was performed by mixing 10 μ l of 10% bovine serum albumin to the tube containing anti-mature NGF antibody bound to protein A-Sepharose beads, and the entire volume of cleared lysate was incubated for 2 hours at 4°C while mixing end over end in a tube rotator. The mixture was centrifuged for 5 seconds at $16,000 \times g$, the supernatant containing unbound proteins was removed, and the beads were washed three times with 1 ml ice-cold washing buffer (0.1% [w/v] Triton X-100; 50 mmol/L Tris Cl, pH 7.4; 300 mmol/L NaCl; 5 mmol/L EDTA; 0.02% [w/v] sodium azide) and once with PBS. Finally, the supernatant was removed and the remaining sepharose beads containing bound antigen was boiled in loading buffer (50% glycerol; 10% SDS; 25% β -mercaptoethanol; 0.005% bromophenol blue; 375 mmol/L Tris, pH 6.8) for 5 minutes and centrifuged for 5 seconds at $16,000 \times g$. Supernatant was divided into six equal parts and each part was loaded in separate gel and analyzed by electrophoresis and immunoblotting. Every membrane was immunoblotted with a different antibody: Anti-CEL, anti-CML, anti-AGE, anti-MDAL, and anti-NKTLs, and one of the membranes with H₂O. This membrane blotted with H₂O was used as a loading control for all five Western blots for different protein modifications. As a control for immunoprecipitation specificity was performed immunoblotting with anti-pro-NGF antibody.

Expression, Purification, and Refolding of Pro-NGF from *Escheria coli*

The procedure is a modification of the method previously described by Rattenholl et al,²⁸ wherein *E. coli* strain BL21 (DE3) was used for the expression of genes under the T7 promoter control. Human pro-NGF gene was subcloned from cDNA of SHSY-5Y human neuroblastoma cells into a pET11a (Novagene) expression vector. The

resulting vector, pET11a-Pro-NGF, was co-transformed with plasmid pUBS520, which contains the *dnaY* gene, encoding the tRNA that recognizes the rare arginine-codons, in *E. coli*. pUBS520 contains the gene for kanamycin resistance and bears the p15A origin of replication, which is compatible with ColE1-based pET vectors. BL21 (DE3) transformants were grown at 37°C in 2× LB medium (17g tryptone, 10g yeast extract, and 5g NaCl per 1L, supplemented with the appropriate antibiotics) to an optical density 600 of 0.5 to 0.8. Gene expression was induced by the addition of 3 mmol/L isopropyl thio- β -galactoside and subsequent cultivation for 4 hours. Cells were harvested by centrifugation for 15 minutes at $6,000 \times g$. Inclusion bodies were isolated by resuspending the pellet in 5 ml of buffer (0.1 M/L Tris-Cl pH 7; 1 mmol/L EDTA) for every 1g of bacterial pellet. One mg lysozyme was added, and kept on ice for 30 minutes. Then, $MgCl_2$ up to 3 mmol/L and DNase I up to 10 μ g/ml were added, and the suspension was well sonicated. After sonication the same amount of DNase I was added and kept for 30 minutes at room temperature. After 30 minutes, half of the volume of (60 mmol/L EDTA, 6% Triton X100, 1.5 M/L NaCl, pH 7.0) was added and the suspension was incubated for 30 minutes at room temperature with a magnetic stirrer. The suspension was spun down and resuspended in 5 ml (60 mmol/L EDTA, 6% Triton X100, 1.5 M/L NaCl, pH 7.0; 0.1 M/L Tris-Cl; 20 mmol/L EDTA), and then spun down and resuspended in 5 ml (0.1 M/L Tris-Cl; 20 mmol/L EDTA). Finally, it was spun down yet again (repeated five times). The pellets were dissolved in 1 ml (6 M/L guanidine; 0.1 M/L Tris-Cl pH 8.0; 1 mmol/L EDTA; 100 mmol/L dithiothreitol added *ex tempore*) for every 0.2g pellet, by shaking vigorously for more than 2 hours at 37°C. The solution was dialyzed against dialysis buffer (4M/L guanidine; 5 mmol/L EDTA, pH 3 to 4) to completely remove dithiothreitol. The protein concentration was adjusted to 50 mg/ml with dialysis buffer and refolded by adding it stepwise ($\times 100 \mu$) to 100 ml (0.1 M/L Tris-Cl; 1 M/L arginine; 5 mmol/L EDTA; 1 mmol/L glutathione Ox; 5 mmol/L glutathione Red; pH 9.5) with slow mixing at 4°C. The proteins were concentrated after refolding using an ultra filtration device (Millipore).

For purification of renatured rh-pro-NGF, the renaturation solution was dialyzed against 50 mmol/L sodium phosphate, pH 7.0, 1 mmol/L EDTA, and then applied onto a DEAE Sepharose FF (GE Health care) column (4.6 $\times$ 100 mm). Refolded species were eluted in a single peak at 980 mmol/L NaCl after application of a linear gradient from 0 to 2 M/L NaCl. Purity was checked with SDS-polyacrylamide gel electrophoresis and biological activity by application to cell cultures, as previously described.¹⁴

Modifications of rh-Pro-NGF

To test the effects of nonenzymatic protein modifications in increasing the resistance to proteolysis of recombinant human pro-NGF, we used the reactive carbonyl species GO and MGO, which react with free amino groups of Lys

residues on proteins, as well as Arg and Cys residues, leading to the formation of CML and CEL adducts and intermolecular crosslinks. The reactions were performed by mixing 500 μ g recombinant protein with 250 μ l 50 μ mol/L GO or MGO and 250 μ l 100 mmol/L sodium phosphate, and then incubating at 37°C for 24 hours. The mixtures was passed three times through Amicon Ultra-4 Centrifugal Filter Units (Millipore) to replace the buffer containing excess of GO and MGO with PBS.

The effects of lipoxidation reactions on recombinant pro-NGF were also tested. The lipoxidation was induced by mixing 500 μ g recombinant pro-NGF with OML solution (70 μ l 10 mmol/L methyl linoleate, 70 μ l 25 mmol/L ascorbate, 70 μ l 100 μ mol/L $FeCl_3$, and 70 μ l acid H_2O [159 μ N CH_3COOH]) and incubating for 6 hours at 37°C. The mixture was passed three times through Amicon Ultra-4 Centrifugal Filter Units (Millipore) to replace the buffer containing excess OML solution with PBS. The efficiency of the modifications was detected with specific antibodies for each modification.

Furin cleavage of modified and non-modified rh-pro-NGF was performed by incubation for 30 minutes in the presence of Furin following the manufacturer's instructions (R&D Systems).

Isolation of Pro-NGF from Human Brain

The methodology followed is essentially as described previously.¹⁴ Briefly stated: frozen human brain tissue from AD-affected or control frontal cortex (8 to 10 g) was homogenized in 20 ml sterile water using a Potter on ice. After centrifugation of homogenates ($2500 \times g$ for 1 hour at 4°C), supernatants were dialyzed against 20 mmol/L Na_2HPO_4/NaH_2PO_4 (pH 6.8) overnight using a 10kD molecular weight cut-off membrane (Sigma). The samples were loaded on a DEAE-sepharose FF column (Amersham) pre-equilibrated in the same buffer. Eluted fractions having absorbance $A_{280} > 0.5$ were equilibrated by a second dialysis against 20 mmol/L Na_2HPO_4/NaH_2PO_4 (pH 6.8) overnight. Salt concentration was adjusted to 0.4 mol/L NaCl in 50 mmol/L CH_3COONa (pH 4.0). The sample was centrifuged at $2500 \times g$ for 30 minutes and the supernatant was loaded on a DEAE-Sepharose FF column previously equilibrated with the same buffer. All of the procedures were performed at 4°C. Eluted fractions with absorbance $A_{280} > 0.1$ were collected, concentrated and analyzed by Western blot using antibodies against either NGF (H20, Santa Cruz) or pro-NGF antibody. NGF protein was undetectable in all of the fractions obtained using H20 anti-NGF antibody. Absence of β -amyloid peptide or derived aggregates in the eluted fractions was ruled out by Western blot using rabbit polyclonal anti- $A\beta_{1-40}$ and $A\beta_{1-42}$ antibodies (Dr. M. Sarasa, Zaragoza), or anti- $A\beta_1$ (Boehringer) used at a dilution of 1:50.

Cell Cultures and Treatments

Rat pheochromocytoma cell line, PC12 cells, were grown in 24-well plates in Dulbecco's Modified Eagle Medium

supplemented with 6% fetal bovine serum, 6% horse serum, 2 mmol/L HEPES, and antibiotics. Before treatment, cells were washed twice with serum-free medium and treatments were performed in the absence of fetal bovine serum.

The PC12 differentiation was evaluated through the stage of dendrite elongations. Positively differentiated cells were considered those with dendrite length at least twice the diameter of the cell body.

Detection of apoptosis in the cell cultures. Twenty-four hours after the treatment, cells were fixed with 4% paraformaldehyde and labeled with Hoechst 33258 (Sigma). The apoptosis was analyzed using an Olympus microscope and documented with an Olympus DP70 camera. Apoptotic nuclei were counted in comparison with total nuclei (Hoechst stained). At least 300 nuclei in random, nonoverlapping fields per condition were counted in every experiment.

Densitometry

The density of the immunoreactive bands was determined by densitometry analysis using a GS-800 Calibrated Densitometer (Bio-Rad). Pixel values for problem samples were compared with control values in at least four separate Western blots.

Pro-NGF in Vivo Administration

Male laboratory mice were anesthetized with isoflurane for 1 to 2 minutes and then injected with 2% Rompun (Bayer) and 10% ketamine (WDT) in 0.9% NaCl (10 μ l/g). Two μ g (in a maximum volume of 4 μ l) of GO-pro-NGF or GO-modified-bovine serum albumin, as a control solution were injected in the right ventricle (A 0.0 mm; L +0.8 mm; V +2.2 mm) 1 μ l/min speed. The needle was held in place for 1 minute before retraction and retracted slowly with 1 mm/min speed. The incisions were sutured and the mice left for 1 week to recover.

Behavioral Tests

All of the behavioral procedures were conducted at the same time of day in an isolated room every day for 5 days. The mice were trained to find a 50 cm² platform hidden under the water surface in a water tank of 150 cm diameter, with four different geometrical forms attached to the four sides of the water tank wall. Four trials per day with start positions close to the four geometrical signs were performed, and latency in reaching the platform was recorded. Cut-off time to find the platform was 90 seconds, and mice failing to find the platform were placed on it and left there for 15 seconds. Each trial for a single animal was 30 minutes apart from the previous.^{29,30}

Statistical Analysis

Statistical significance between groups was calculated using Student's *t*-test.

Results

Pro-NGF Nonenzymatic Modifications Are Increased in AD in a Stage-Dependent Manner

As we previously reported, pro-NGF isolated from AD-affected brains (ADhbi-proNGF) differs functionally from pro-NGF isolated from control brains (Chbi-proNGF) at comparable ages. Chbi-proNGF was susceptible to be processed to mature NGF when it was added to cell cultures.¹⁴ We also reported that AGE/ALEs are common nonenzymatic modifications of proteins in AD,³ but the question whether the differences in post-translational modifications between control and AD-affected brain pro-NGF may account for the differences in resistance to protease degradation remains open.

To test whether pro-NGF is modified *in vivo* in human brain, we immunoprecipitated this protein from hippocampal and entorhinal cortex lysates obtained from AD-affected and age-matched control human brain samples. Using Western blot we analyzed the immunoprecipitates with five different antibodies that recognize different AGE/ALEs (Figure 1 and 2). The analysis realized by a specific antibody raised against CEL, which is known to be generated from modification of lysine by methylglyoxal, revealed an increase in the amount of modified pro-NGF in hippocampus that corresponded to the progression of the disease. Using the same antibody in entorhinal cortex we observed an increase in the levels of modification in AD samples that was maintained in relatively similar levels during the progression of the disease. The pattern of CEL modifications was identical for both 35 and 50 kDa pro-NGF bands (Figure 1A). CML can be a product of both lipoxidation and glycoxidation modifications of the proteins. With anti-CML antibody we observed a strong increase in the levels of the modification of pro-NGF, but only in advanced stages of the disease—III/IV and V. Similarly to CEL, the pattern of modifications of CML was the same as well for 35 and 50 kDa bands as for both anatomical areas of the hippocampus and entorhinal cortex (Figure 1B). Anti-AGE is an antibody against advanced glycation end products and with this antibody we observed an increase in the levels of AGE in the advanced stages of the disease that was more evident for 35 kDa band (Figure 1C). Western blots of immunoprecipitates using antibodies against lipoxidation markers such as MDAL (Figure 2A) and NKTls (Figure 2B) also display an augmentation in the nonenzymatic modifications of the 35 kDa band of pro-NGF from AD-affected patients when compared with controls, and again the pattern of increase of the levels of modifications that corresponds with the stages of the disease was prominent. Fifty kDa pro-NGF band in Figure 2, A and B, is masked by the immunoglobulin signal detected by the secondary antibody (anti-goat IgG-horseradish peroxidase). Figure 1D shows a Western blot of the same amount of the immunoprecipitates as in the Figures 1, A–C, and 2, A and B, blotted with anti-mature NGF anti-

body (H20) that was used as a loading control. Figure 2C shows a Western blot of the immunoprecipitates using an antibody raised against the pro-domain of pro-NGF, indicating that the 35- and 50-kDa bands of Figures 1 and 2 correspond to pro-NGF and not to mature NGF aggregates. These data suggest a general increase in glycooxidation and lipoxidation of pro-NGF in AD-affected human brains during the progression of the disease.

Rh-Pro-NGF Modified by GO, MGO, and OML Increases Its Resistance to Degradation and Processing

Since we have seen that pro-NGF from AD-affected human brain was more modified by nonenzymatic post-translational modifications than the pro-NGF from control brains, we wanted to assess whether these modifications could be effective in blocking its processing by convertases. Furin processing accounts for the most of the intracellular physiological maturation of pro-NGF, giving rise to the mature NGF form (13.5 kDa).³¹ At the extracellular level, plasmin and matrix metalloproteinase-7 are able to process pro-NGF, yielding 13.5 kDa NGF and fragments from 18 to 30 kDa.¹⁵

We used recombinant human pro-NGF (rh-pro-NGF) and modified it *in vitro* with GO, MGO, and OML, as described in the *Materials and Methods*. We performed the Western blots with anti-CEL antibodies that recognize the modifications by methylglyoxal, anti-CML that recognizes the modifications by glyoxal, and anti-MDAL that recognizes the modifications by MDA, to assess the obtained levels of modifications (Figure 3A). We incubated the modified and the non-modified rh-pro-NGF in the presence of PC12 cells for 24 hours, then we concentrated the media, and, to evaluate the levels of pro-NGF degradation, we performed another Western blot with anti-mature NGF antibody (Figure 3, A and B). We could not detect any mature NGF in MGO-rh-pro-NGF and only a very small amount in GO- and OML-rh-pro-NGF. Only faint bands of molecular weights from 17 to 30 kDa were seen with anti-mature NGF antibody in modified proteins, compared with the more evident corresponding bands in non-modified rh-pro-NGF lines. These data suggest that the modifications by MGO, and to lesser extent GO, may protect the rh-pro-NGF from processing.

The lack of signal given with anti-CEL, anti-CML, and anti-MDAL antibodies at 14 kDa in lanes labels as MGO, GO, and MDA, may indicate that only the non-modified proportion of pro-NGF is susceptible to being processed

Figure 1. Glycooxidative modifications of pro-NGF from hippocampus and entorhinal cortex in AD patients. Samples of hippocampus and entorhinal cortex were pooled and immunoprecipitated with anti-mature NGF antibody, followed by immunostaining with markers of nonenzymatic glycooxidation: anti-carboxyethyl-lysine (CEL), anti-carboxymethyl-lysine (CML), and anti-advanced glycation end products (AGE). Blots shown are representative of three repetitions. **A:** Western blot analyses demonstrated gradually increased glycooxidation of pro-NGF, with the progression of the disease, in the pool from hippocampus compared with the control pool. Differences are observed from stages I/II in entorhinal cortex but not in hippocampus. The increase in hippocampus is more evident from stages III, IV both in 35- and 50-kDa molecular weight bands. **Arrows** indicate apparent molecular weight. **B:** Western blot analyses of pro-NGF from AD samples show a progressive increase in the concentrations of Nε-(carboxymethyl)-lysine, a mixed marker of glycooxidative and lipoxidative modifications, in immunoprecipitates from hippocampus and entorhinal cortex. **Arrows** and numbers indicate apparent molecular weight. **C:** Pro-NGF from entorhinal cortex compared with the control group in both modified forms of the protein, depicting gradual augmentation in the amounts of AGE modification. The immunoprecipitates from hippocampus show an increase in the AGE modifications only in the 35-kDa form with the progression of the disease, whereas there is no evident change compared with the control pool for the 50-kDa form of pro-NGF. **Arrows** and numbers indicate apparent molecular weight. **D:** Immunostaining with anti-mature NGF antibody of the same immunoprecipitates used in Figures 1 and 2 was used as immunoprecipitation loading control.

to mNGF (Figure 4E). Using the same protocol of incubation of pro-NGF in the presence of PC12 cells, we used pro-NGF isolated from control human brains (Chbi-pro-NGF) and from human brains affected by advanced stages of AD (ADhbi-pro-NGF). As described in a previous work,¹⁴ ADhbi-pro-NGF, but not Chbi-pro-NGF, is able to induce apoptosis in cell cultures. In Figure 3D, there can be observed a high degree of degradation in Chbi pro-NGF and not in ADhbi-pro-NGF, when the protein is incubated with PC12 cells for 24 hours.

We verified whether reactive carbonyl compounds were able to block specific processing of pro-NGF by

Furin (which gives fragments of 14, 18, and 30 kDa) *in vitro*. The band corresponding to NGF was visible only in non-modified rh-pro-NGF (Figure 3C), which suggests that GO modification is able to completely block the processing of pro-NGF by this enzyme *in vitro*.

GO and MGO Modified rh-Pro-NGF Are Physiologically Active in Inducing Apoptosis through p75NTR and Do Not Differentiate PC12 Cells

An indicator of pro-NGF processing by convertases is the ability of their product mature NGF to differentiate PC12 cells in culture for 48 hours.^{13,14} A 100 ng/ml solution of hr-pro-NGF is able to differentiate PC12 cells to a similar extent as an equivalent molarity of NGF during 48 hours treatment. This differentiation is due to the processing of pro-NGF to mature NGF caused by the action of the convertases present in culture. By contrast, neither glycooxidated nor lipoxidated hr-pro-NGF is able to differentiate PC12 cells (Figure 4A). These results indicate that the processing of modified pro-NGF is blocked, and as a consequence mature NGF is not formed during the 48-hour treatment.

In the same experiment, we examined whether GO-, MGO-, or OML- modified hr-pro-NGF is functional and can induce apoptosis through its interaction with p75NTR. We counted the number of PC12 cells with pyknotic nuclei 48 hours after the treatment. GO- and MGO-modified hr-pro-NGF was seen to be significantly more active in inducing apoptosis compared with non-modified hr-pro-NGF and control (Figure 4B). To verify whether the apoptotic effect of GO- and MGO-hr-pro-NGF was due to its interaction with p75NTR, we blocked the receptor with anti-p75NTR (antibody raised against extra cellular domain of p75NTR). The apoptosis induced by GO- and MGO-hr-pro-NGF was reduced to the basal apoptosis caused by serum deprivation (Figure 5, A and B). We also performed pre-incubation with antibody raised against the pro-part of pro-NGF molecule that blocks pro-NGF activity. The results were very similar to those obtained with anti-p75NTR antibody pre-incubation (Figure 5). This demonstrates that GO- and MGO-hr-pro-NGF induce apoptosis by interacting specifically with the p75NTR.

Figure 2. Lipoxidation of pro-NGF from hippocampus and entorhinal cortex of patients with AD. The immunoprecipitates with anti-NGF antibody anti-mature NGF from pool samples of different stages of AD and control pool were immunoblotted with two marker of lipoxidation: Anti-malondialdehyde-lysine (MDAL) and NKTLs. Immunostaining with anti-mature NGF antibody used as loading control is shown in Figure 1D. Blots shown are representative of three experiments. **A:** Western blot for lipoxidation of pro-NGF from AD samples shows increases in lipoxidation-derived damage, by measurement of MDAL in the 35-kDa form. Malondialdehyde is the main oxidative breakdown product of polyunsaturated fatty acids that are abundant in the brain tissue. **Arrows** and numbers indicate apparent molecular weight. **B:** Western blot analyses with anti-NKTLs demonstrate gradually increased lipoxidation of pro-NGF, which is correlative with the progression of the disease, in both pools from hippocampus and entorhinal cortex, as compared with control pools. **Arrows** and numbers indicate apparent molecular weight. **C:** Western blot with anti-pro-NGF antibody was used as immunoprecipitation specificity control. **Arrows** and numbers indicate apparent molecular weight.

Figure 3. Degradation resistance of GO, MGO, and OML modified rh-pro-NGF. **A:** Western blots of rh-pro-NGF modified by glyoxal (GO), methylglyoxal (MGO), and OML solution (OML), as well as the controls of non-modified rh-pro-NGF revealed with anti-CEL, anti-CML, and anti-MDAL, incubated in the presence of PC12 cells for 24 hours. The same membranes were reblotted with anti-mature NGF (anti-H₂O). **B:** Densitometry of the 14 kDa band from the membranes revealed with anti-CEL, anti-CML, and anti-MDAL. Density of 14-kDa band of modified rh-pro-NGFs (gray bars) compared with non-modified rh-pro-NGF (black bars). Values represent the mean $\pm$ SEM of three independent experiments.

Figure 4. Apoptosis induction by physiologically active GO and MGO modified rh-pro-NGF through p75NTR in PC12 cells. PC12 cells were serum deprived and treated for 48 hours with 100 ng/ml of rh-pro-NGF, rh-pro-NGF modified by glyoxal (GO), methylglyoxal (MGO), and OML solution (OML). The same volume of the corresponding modification reaction buffers were used as controls. After 48 hours the cells were fixed and stained with Hoechst. Differentiation (**A**) and apoptosis (**B**) were quantified and expressed as percentage of the total number of cells. Values represent the mean $\pm$ SEM of three independent experiments. * P < 0.05; ** P < 0.01; Student's *t*-test.

In Vivo Administration of Modified rh-Pro-NGF and of ADhbi-Pro-NGF Induces Learning Impairment in Mice

It was described that intraventricular administration of A β peptide in rodents produces memory and cognitive alterations and that these animals can be used as an AD-model. These alterations can be evaluated by measuring learning capacity in spatial navigation tests (latency in finding a hidden platform in the water maze during training).³⁰ To examine whether either hr-pro-NGF or GO-hr-pro-NGF could be active in inducing similar behavioral alterations we administered 2 μ g of the neurotrophin in a

Figure 3 continued. * P < 0.05; ** P < 0.01; Student's *t*-test. **C:** GO-hr-pro-NGF and hr-pro-NGF control incubated for 30 minutes in the presence of recombinant Furin. Western blot of the incubation mixture, using anti-mature NGF antibody, shows that modified pro-NGF is not processed by the enzyme. **D:** Western blot analyses with anti-mature-NGF of 10 ng hr-pro-NGF (first lane) and 10 ng mature-NGF (second lane), revealed with anti-mature NGF. **E:** Western blots of Chbi-pro-NGF and ADhbi-pro-NGF either incubated in the presence of PC12 cells (+PC12) or with PBS for 24 hours, revealed with anti-mature NGF. Arrows and numbers indicate apparent molecular weight.

Figure 5. Blocking of GO- and MGO-modified rh-pro-NGF induced apoptosis by anti-p75NTR and anti-pro-NGF antibodies. PC12 cells were serum deprived and pretreated for 2 hours with 50 ng/ml anti-p75NTR or anti-pro-NGF antibodies, or left without blocking, and treated for 48 hours with 100 ng/ml of rh-pro-NGF modified by glyoxal (GO) (A) and methylglyoxal (MGO) (B). After 48 hours the cells were fixed and stained with Hoechst. Apoptosis was quantified and expressed as a percentage of total cells. Values represent the mean $\pm$ SEM of three independent experiments. * $P < 0.05$; ** $P < 0.01$; Student's *t*-test.

single injection as described in methods. Mice injected with hr-pro-NGF had significantly impaired learning in the first 3 days (Figure 6A), but afterward they presented learning and memory skills indistinguishable from control animals (injected with GO-modified bovine serum albumin, which was modified *in vitro* in parallel to hr-pro-NGF) that learned the spatial navigation tasks in about 2 days. Animals injected with GO-hr-pro-NGF behaved during the first 3 days like hr-pro-NGF injected mice, but in contrast to them they did not succeed in learning to find the hidden platform after 5 training days (Figure 6A). This impairment was maintained for at least the following 2 days (data not shown). To examine whether ADhbi-pro-NGF and Chbi-pro-NGF used by us in previous studies¹⁴ can produce similar behavioral alterations we injected two groups of four mice each with 2 μ g protein purified from AD-affected and control brains respectively. We observed significant differences in the learning capacities between the groups (Figure 6b). The animals injected with ADhbi-pro-NGF showed significant difficulties in finding the platform from day 3 to day 5 of the training.

Figure 6. Learning difficulties in mice injected intracerebroventricularly with modified rh-pro-NGF. Mice intracerebroventricularly injected with GO-rh-pro-NGF (filled circle), non-modified rh-pro-NGF (open triangle), and the same volume and amount of GO-modified-bovine serum albumin (open square) present significant differences in learning retardation, measured as seconds needed to find the submerged platform in the water maze test (A). Mice intracerebroventricularly injected with ADhbi-proNGF (filled circle) and Chbi-proNGF (open square) present significant differences in learning retardation, measured as seconds needed to find the submerged platform in the water maze test (B). Values represent the mean $\pm$ SD of five animals. Error bars represent SEM. * $P < 0.05$; ** $P < 0.01$ Student's *t*-test for GO-rh-pro-NGF. * $P < 0.05$; ** $P < 0.01$ Student's *t*-test for rh-proNGF. Values result from three independent experiments.

This effect was not attributable to changes in the locomotor activity of the animals; and as expected, intraventricular administration of pro-NGF did not induce adverse effects other than cognitive impairment.

Discussion

In recent decades, the information about oxidative stress changes in living cells has grown continually. Oxidative stress is a natural process that affects all aerobic living cells and can cause different modifications of macromolecules. As a consequence, loss and/or alteration of the normal functions of modified molecules can occur. Some of the oxidative changes in the proteins are due to non-enzymatic chemical modifications caused by low-molecular-weight reactive carbonyl compounds such as GO, MGO, and MDA. These compounds arising from dam-

aged carbohydrates or unsaturated fatty acids are able to modify primarily Lys, Arg, and Cys residues. The molecule of pro-NGF contains several Lys and Arg residues that can be modified by glycooxidation and lipoxidation reactions. Most importantly, the target sequences for Furin and other convertases contain Lys, Arg, and Cys residues, making them suitable sites for AGE/ALEs formation.²² The maturation of pro-NGF to 13.5 kDa mature NGF is obtained by Furin processing in arg-ser-lys-arg103/ser-ser-thr site.³¹ The nonenzymatic chemical modifications in these sequences could block the convertase cutting and make the pro-NGF molecule especially stable and resistant to degradation and maturation. In the present work, immunoprecipitates using anti-mNGF antibody, show 32- and 53-kDa bands of pro-NGF. In a previous work,¹³ we described these bands as the most abundant forms of pro-NGF in the human brain, their being significantly increased in AD. It has been described that the 53 kDa form is glycosylated,^{13,30} as it varies its apparent molecular weight when it is deglycosylated *in vitro*. So far, no other posttranslational enzymatic and/or nonenzymatic protein modifications for pro-NGF have been described *in vivo*. The low-molecular-weight, reactive carbonyl compounds, in addition to modification in Lys, Arg, and Cys residues, can produce inter- and intramolecular cross-link formation in the proteins. Since normally pro-NGF is secreted as a dimer, the cross linking between the two peptides in the dimer is a probable event, and it could stabilize the molecule and make it even more refractory to the access of convertases and degradation.

The increase in glycooxidation and lipoxidation has been described in a variety of neurodegenerative diseases and the extent of AGE/ALEs in a given protein is indicative for oxidative stress, as they are present in relevant proteins such as ATP synthase and glial fibrillary acidic protein in AD.³

Previous data have demonstrated that several growth factor receptors, such as epidermal growth factor receptor, platelet-derived growth factor receptor, and insulin receptor, are modified by MGO,^{32,33} but this is the first evidence of a nonenzymatic modification *in vivo* of a soluble signaling protein. In AD, with aging, and under conditions of oxidative stress, the levels of reactive carbonyl compounds continuously increase. Increased protein damage by glycooxidation and lipoxidation has been implicated in neuronal cell death leading to AD.³⁴ Accumulating carbonyl levels might be caused by an impaired enzymatic detoxification system. The major dicarbonyl detoxifying system is glyoxalase I, which removes methylglyoxal to minimize cellular impairment. This enzyme plays a critical role in the detoxification of dicarbonyl compounds and thereby reduces the formation of advanced glycation end products. In mice AD models, an increased expression in neurons of glyoxalase I has been described, and populations with glyoxalase I polymorphisms present a higher susceptibility to developing AD.³⁵ Taking all these data into account, it could be possible that MGO modifications in pro-NGF are more frequent in neuronal patterns of altered glyoxalase I.

It has been revealed that cross-linking modifications of tau protein are likely to contribute to the characteristic features of paired helical filaments, including their insolubility and resistance to proteolytic degradation in the context of AD.³⁶ MGO, GO, and MDA are some of the most reactive compounds in terms of formation of tau dimers and higher molecular-weight oligomers. These modifications have been suggested as accelerating tangle formation *in vivo*, and as a consequence interference with the formation or the reaction of these reactive carbonyl compounds could be a promising way of inhibiting tangle formation and neuronal dysfunction in AD.³⁶

The entorhinal cortex is the earliest cortical region affected by AD (stages I/II), followed by the hippocampus (stages III/IV).²⁰ In our study we observed a clear augmentation in the levels of nonenzymatic oxidative modifications in pro-NGF molecules in hippocampus and entorhinal cortex samples from AD-affected brains. This increase is present for both lipoxidation and glycooxidation. The increase in the pro-NGF levels in the human brains affected by the AD cortex described in our previous work,¹³ together with the increase in the oxidative modifications in the molecule and as a consequence increased stability of pro-NGF, can account for the neuronal loss observed in AD. More importantly, the increase in the levels of pro-NGF modifications corresponds to the progression of the disease for the majority of oxidative modifications examined by us.

We used different *in vitro* approaches to further investigate the possible pathological relevance of the oxidatively modified pro-NGF. First, we modified recombinant human pro-NGF *in vitro* with GO, MGO, and OML, and our study demonstrated that this nonenzymatically modified pro-NGF was more resistant to convertase processing. This was demonstrated with Western blot, and confirmed with the response of PC12 cell culture to treatment with modified and non-modified pro-NGF. As suggested by our results, only the non-modified proportion of pro-NGF is susceptible to being processed to mNGF. In fact, the proportion of pro-NGF that could escape the modification is susceptible to being processed by cellular proteases raising mNGF and other low-molecular-weight fragments and it would not give a positive signal with anti CML, CEL, or MDAL, as can be observed in our results. This also reinforces the idea that mNGF raised from samples subjected to modification is non-modified and as a consequence modifications in mNGF cannot be responsible for the *in vitro* or *in vivo* effects of modified pro-NGF described here.

We observed that PC12 treatment with GO- and MGO-modified pro-NGF was unable to induce differentiation of the cells and more importantly we observed an increase in the percentage of pyknotic nuclei 48 hours after the treatment, as compared with the non-modified pro-NGF. The differences in the response of PC12 cells to the treatment with modified and non-modified pro-NGF can be explained with the rapid degradation of the hr-pro-NGF to mNGF, due to the action of the plasmin and matrix metalloproteinases from PC12 cells. Thus, the produced mNGF interacts with trkA and induces the differentiation and sustains the survival of PC12 cells. In contrast, GO-

and MGO-modified rh-pro-NGF are more resistant to this processing and are able to induce apoptosis through their interaction with p75NTR. Moreover, we demonstrated that Chbi-pro-NGF and not ADhbi-pro-NGF is susceptible to being processed by convertases. These data reinforce the idea that AGE/ALEs modifications of pro-NGF in AD affected human brain could be the responsible for stabilizing the molecule and protect it from processing.

More importantly, we showed that rh-pro-NGF delays the learning of a spatial navigation task when injected *in vivo* in mice and that this cognitive impairment is exacerbated when the protein is GO-modified. The fact that the animals injected with rh-pro-NGF or with Chbi-pro-NGF demonstrate significant cognitive impairment only in the first 3 days of the training could be associated with the higher susceptibility of non-modified rh-pro-NGF to degradation, when compared with GO-modified rh-pro-NGF or ADhbi-pro-NGF modified, as shown in the present work.

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