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Biochemistry

Protease nexin-1, an antithrombin with neurite outgrowth activity, is reduced in Alzheimer disease

(glial-derived nexin/protease inhibitor/protease nexin-1–protease complexes)

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ABSTRACT **Protease nexin-1 (PN-1) is a cell-secreted protein that inhibits certain proteases, particularly thrombin, by forming SDS-stable complexes with the catalytic site serine of the protease. PN-1 was recently shown to be identical to a glial-derived neurite-promoting factor/glial-derived nexin present in rat brain. Its neurite outgrowth activity depends on inhibition of thrombin, presumably because thrombin brings about neurite retraction. Here we show that human brain contains PN-1 and that PN-1 activity in brains of individuals with Alzheimer disease (AD) was only 14% of control values (total of 14 AD patients and 7 control individuals). PN-1 activity in the hippocampus, a region with marked neuropathology in AD, was 15% of control values (10 AD patients and 4 control individuals). Western blot analysis indicated a large decrease in free PN-1 protein and an increase in PN-1-containing complexes that comigrated with PN-1–thrombin complexes. Northern blot analysis indicated that PN-1 mRNA levels were about equal in brains from AD patients and control individuals. Thus these results suggest that the decreases in PN-1 activity and free PN-1 protein are due to formation of PN-1–protease complexes.**

Alzheimer disease (AD) is associated with profound memory loss due to progressive and irreversible brain pathology. Its primary target is individuals over the age of 60 and the incidence increases with age, reaching up to 37% of the population over the age of 80 (1). The AD brain undergoes massive and selective neuronal loss and an accumulation of neuritic plaques and neurofibrillary tangles. Plaques consist of dystrophic neurites, amyloid fibrils, and astrocytic processes (2). Recent findings indicate that proteases and protease inhibitors play important complementary roles in the nervous system (3, 4) and that imbalances in proteolysis might be involved in the etiology of AD (5–9). For example, one form of the β-amyloid precursor protein contains a sequence homologous to the Kunitz family of serine protease inhibitors (5–7). A second serine protease inhibitor, α_1-antichymotrypsin, is associated with plaque amyloid deposits in AD (8).

Protease nexin-1 (PN-1) was shown to be identical to a glial-derived nexin (10, 11), which stimulates neurite outgrowth in neuroblastoma cells (12, 13) and primary sympathetic neurons (14) and is present in rat brain (15). PN-1 is a 44-kDa protease inhibitor that is synthesized and secreted by a variety of cultured cells including astrocytes (16–18). It rapidly inhibits thrombin, urokinase, and plasmin by forming SDS-stable complexes with the catalytic site serine of the protease (16, 19). The PN-1–protease complexes bind to the cells, through the PN-1 moiety of the complex, and are rapidly internalized and degraded (20). PN-1 binds to the extracellular matrix (21), which accelerates its inhibition of thrombin (22, 23) and blocks its inhibition of urokinase and plasmin (24). This suggests that PN-1 is primarily a thrombin inhibitor in interstitial fluids where much of it would be bound to extracellular matrices. Its neurite outgrowth activity is dependent on inhibition of thrombin since (*i*) hirudin, a potent thrombin inhibitor from leeches, stimulates neurite outgrowth from neuroblastoma cells to the same extent and with the same kinetics as PN-1 (12, 13), and (*ii*) thrombin blocks (12) and reverses (13) the neurite outgrowth activity of PN-1 and hirudin. The thrombin-mediated retraction of neurites is not a general proteolytic effect since neurite retraction does not occur with much higher concentrations of other target proteases of PN-1 (13).

These considerations prompted us to evaluate the possibility that alterations involving PN-1 might be observed in AD. We analyzed autopsy brain samples for PN-1 activity, free PN-1, PN-1–thrombin complexes, and PN-1 mRNA and found that PN-1 activity and free PN-1 protein were markedly reduced and that PN-1 containing complexes were increased in AD. In contrast, PN-1 mRNA levels were about equal in AD and control brains. These results indicate that the decrease in PN-1 is possibly due to the formation of PN-1–protease complexes.

MATERIALS AND METHODS

Materials. Monoclonal antibodies (mAbs) to PN-1 were prepared as described (25). PN-1 was purified by immunoaffinity chromatography from serum-free culture medium from human foreskin fibroblasts (26). Antithrombin III (ATIII) and heparin cofactor II (HCII) were provided by D. H. Farrell (University of Washington). Human α-thrombin was provided by J. W. Fenton, II (New York State Department of Health). Thrombin was labeled with $Na^{125}I$ using the chloroglycouril method described in ref. 16 to a specific activity of 25,500 cpm/ng. Human brains of control individuals and AD patients were removed at autopsy and bisected midsagitally. One hemisphere was fixed in 10% (vol/vol) buffered formalin; the AD diagnosis was confirmed using the autopsy criteria of Khachaturian (27). The control individuals were age-matched to the AD individuals and showed no evidence of neurological disease. The other hemisphere was dissected into appropriate regions, frozen immediately in powdered dry ice, and stored at −70°C.

PN-1 Activity Measurements. Dissected areas of control and AD brain samples were homogenized at 4°C in 10 vol of 0.32 M sucrose/10 mM Hepes, pH 7.5; the homogenates subsequently were centrifuged for 20 min at 2000 × *g*. All

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Abbreviations: PN-1, protease nexin-1; AD, Alzheimer disease; mAb, monoclonal antibody; ATIII, antithrombin III; HCII, heparin cofactor II.

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detectable PN-1 activity resided in the supernatant. Protein concentration was measured according to Lowry *et al.* (28). Aliquots of brain supernatants (90 μl) containing protein concentrations of ≈5 mg/ml were incubated with or without anti-PN-1 mAb (25) (220 μg/ml) for 60 min at 37°C; then, ^{125}I-labeled thrombin (25,500 cpm/ng) was added at a final concentration of 5 μg/ml and the incubation was continued for 30 min. The reactions were quenched using an equal volume of Laemmli sample dilution buffer (29) and the mixtures were analyzed by SDS/gel electrophoresis using 7.5% polyacrylamide gels. Protein concentrations in supernatants of AD and control homogenates did not vary by more than 10%. ^{125}I-labeled proteins were visualized by autoradiography.

Immunoblot Analysis. Human brain supernatants, 290 μg of protein per lane, were electrophoresed on 7.5% polyacrylamide gels. The proteins were then transferred to nitrocellulose and probed with anti-PN-1 polyclonal serum at a dilution of 1:200. Biotinylated donkey anti-rabbit antibody and streptavidin–biotinylated horseradish peroxidase preformed complexes (Amersham) were used at 1:200 and 1:400 dilutions, respectively.

Northern Blot Analysis. Poly(A)$^+$ RNA was isolated from AD and control brain tissue and cultured human glioblastoma cells (U138-MG, American Type Culture Collection) using the guanidinium thiocyanate procedure (30) followed by oligo(dT)-cellulose chromatography. Poly(A)$^+$ RNA was electrophoresed in 1.4% agarose gels containing 2.2 M formaldehyde, 20 mM Hepes (pH 7.6), and 1 mM EDTA and transferred to a nitrocellulose membrane overnight. Hybridizations were carried out in 50% (vol/vol) formamide, 5× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0)/5% (wt/vol) dextran sulfate/5× Denhardt's solution (31)/0.1% SDS/50 mM sodium phosphate, pH 6/polyadenylic acid (100 μg/ml). PN-1 and β-actin mRNA were analyzed using ^{32}P-labeled complementary RNA probes. For the PN-1 probe two complementary 30-base oligonucleotides were synthesized corresponding to amino acids 360–369 of human PN-1 (11). The oligonucleotides were annealed and inserted into the *Hin*cII site of pBS (Stratagene). β-actin cDNA was provided by P. Ponte (32). A 1100-base-pair *Bam*HI–*Sal* I fragment was subcloned into pBS (Stratagene). ^{32}P-labeled complementary RNA probes to both PN-1 and β-actin were synthesized using T7 RNA polymerase (Stratagene).

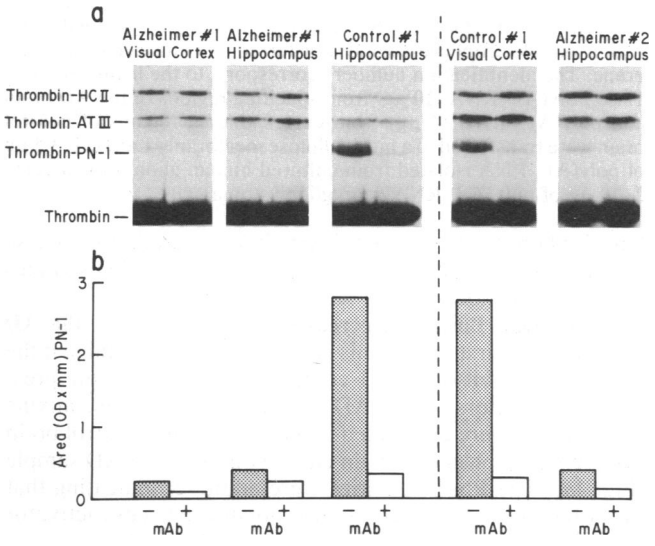

FIG. 1. (*a*) Autoradiograms of electrophoresed ^{125}I-labeled thrombin–protease inhibitor complexes resulting from incubation of various human brain extracts with ^{125}I-labeled thrombin. (*b*) Results of densitometric scans of ^{125}I-labeled thrombin–PN-1 complexes on the autoradiograms. The autoradiograms were scanned and integrated with an LKB Ultrascan laser densitometer. The differential value obtained in the presence (+) and the absence (−) of the mAb was normalized to a positive control containing known amounts of PN-1. The ages of the patients were as follows. AD 1, 81 years; AD 2, 89 years; and control 1, 70 years. The intervals between death and freezing of tissue were as follows. AD 1, 4 hr; AD 2, 3 hr; and control 1, 20 hr.

RESULTS

Measurements of PN-1 Activity in AD and Control Brain Extracts. PN-1 activity was quantitated in human brain tissue based on its ability to form SDS-resistant complexes with ^{125}I-labeled thrombin (16, 25). The homogenized tissue also contained two plasma thrombin inhibitors, ATIII and HCII, released from blood vessels upon homogenization. It, therefore, was necessary to distinguish complexes formed between ^{125}I-labeled thrombin and each of the three inhibitors. This was accomplished by SDS/polyacrylamide gel electrophoresis using an anti-PN-1 mAb that blocks the inhibition of target proteases by PN-1 (25).

PN-1 activity was markedly reduced in brains from 14 AD patients compared to 7 control individuals (see identification numbers in Fig. 1 and Table 1). Two autoradiograms of ^{125}I-labeled thrombin–protease inhibitor complexes (separated by a dotted line in Fig. 1*a*) revealed that the activity of PN-1, but not plasma ATIII or HCII, was much lower in three regions of AD brain compared to control brain. To measure the relative amounts of ^{125}I-labeled thrombin–PN-1 complexes formed in each of the samples, the autoradiograms were scanned and integrated using a laser densitometer (Fig. 1*b*). This confirmed the much reduced PN-1 activity in the AD brain samples.

Table 1. Protease nexin-1 activity in various anatomical regions of AD and control brains

Patient	Tissue	PN-1, pmol/mg
Control 2	Hippocampus	78.8
Control 3	Hippocampus	71.9
Control 4	Hippocampus	43.1
Control 7	Hippocampus	85.4
Control 2	Visual cortex	110
Control 3	Visual cortex	62.6
Control 4	Visual cortex	33.3
Control 5	Cingulate cortex	40.7
Control 6	Cingulate cortex	84.2
Mean ± SEM		67.8 ± 25.1
AD 3	Hippocampus	1.51
AD 4	Hippocampus	4.86
AD 6	Hippocampus	14.5
AD 7	Hippocampus	13.3
AD 9	Hippocampus	17.0
AD 10	Hippocampus	0.00
AD 11	Hippocampus	9.89
AD 12	Hippocampus	24.6
AD 13	Hippocampus	14.2
AD 14	Hippocampus	5.98
AD 3	Visual cortex	3.48
AD 10	Visual cortex	3.66
AD 5	Cingulate cortex	10.3
AD 7	Cingulate cortex	15.0
AD 8	Cingulate cortex	6.80
Mean ± SEM		9.67 ± 6.77

PN-1 activity was measured as described in Fig. 1 in brain tissues obtained from control individuals, aged 39–88 ($\bar{X}$ ± SEM = 67.7 ± 6.5 years), and AD patients, aged 59–89 (75.7 ± 3.2 years). The interval between death and freezing of the tissue was 4–20 hr (9.7 ± 2.3 hr) for the control group and 3–21 hr (7.9 ± 1.8 hr) for the AD patients.

The remaining AD and control brain samples were analyzed using this assay. In addition, a standard curve was constructed employing known quantities of purified PN-1, so that the concentration of active PN-1 in the brain samples could be determined. Analysis focused on the hippocampus since neuropathology in this structure shows a high correlation with clinical diagnosis of AD (33). PN-1 activity was lower in all AD brain samples compared to controls (Table 1). There was no significant correlation between the much reduced PN-1 levels in the AD samples and age, sex, or postmortem delay. Preliminary analyses were also carried out on select samples from other brain areas. Reduced PN-1 activity was not restricted to the hippocampus but was also observed in other cortical regions examined. The decreased activity was specific to PN-1, however. There was no significant variation in the activity of ATIII or HCII between the AD and control brain samples (data not presented). Moreover, the activity of protease nexin-2 (34), which is also synthesized by glial cells, did not vary significantly among these samples (W. E. Van Nostrand and D.D.C., data not presented).

The immunoblots in Fig. 2 show that there was a large decrease in the amount of free PN-1 protein in two of the AD samples; in fact, every AD sample tested showed a significant decrease in free PN-1 protein (see also Fig. 5). The amounts of free PN-1 protein estimated in the control samples by the immunoblots of Fig. 2 approximated the amounts indicated by the above activity measurements. Thus, most of the PN-1 in the control samples was active.

Measurements of PN-1 mRNA in the AD and Control Brain Samples. The basis of decreased PN-1 levels in the AD samples was explored by measuring levels of PN-1 mRNA. These measurements (Fig. 3) were conducted on poly(A)$^+$ RNA prepared from the hippocampus, parietal cortex, or cingulate cortex of four control individuals (control, 2, 3, 4, and 5 of Table 1) and five AD patients (AD 3, 4, 5, 9, and 14 of Table 1). Levels of β-actin mRNA were also measured to provide a marker for general mRNA degradation. Analysis of the hippocampus samples (Fig. 3*a*, lanes 4–7) showed that the PN-1 mRNA level of AD 4 (lane 6) was about the same as the control cases. PN-1 mRNA was barely detectable in AD 3 (lane 7); this was undoubtedly due to RNA degradation since this same sample did not contain detectable levels of β-actin mRNA. Cingulate cortex mRNA samples from two control individuals (control 4 and 5) and two AD patients (AD 5 and 9) also showed no significant difference in the amount of PN-1 mRNA between control and AD samples (Fig. 3*a*, lanes 10–13). Analysis of superior parietal cortex samples (Fig. 3*a*, lanes 8 and 9) showed that control 4 and AD 14 contained about the same levels of PN-1 mRNA. Additional brain regions of these two individuals were analyzed in a separate experiment (Fig. 3*b*). As shown, the levels of PN-1 mRNA in the brain sections examined were about equal in the control and AD samples, with the exception of the control sample in lane 5 where there was significant RNA degradation. These results indicate that the decreased PN-1 in the AD samples analyzed was not due to a lack of PN-1 mRNA.

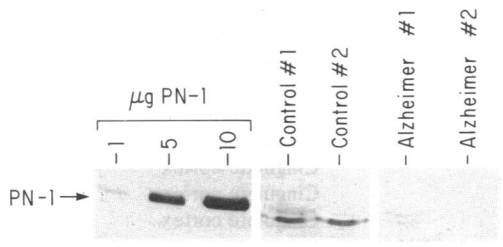

FIG. 2. Immunoblots of supernatants from AD or control hippocampus probed with polyclonal anti-PN-1 sera (1:200 dilution). The indicated amounts of immunopurified PN-1 were blotted and probed with the anti-PN-1 serum. Each lane contained 290 μg of brain supernatant protein.

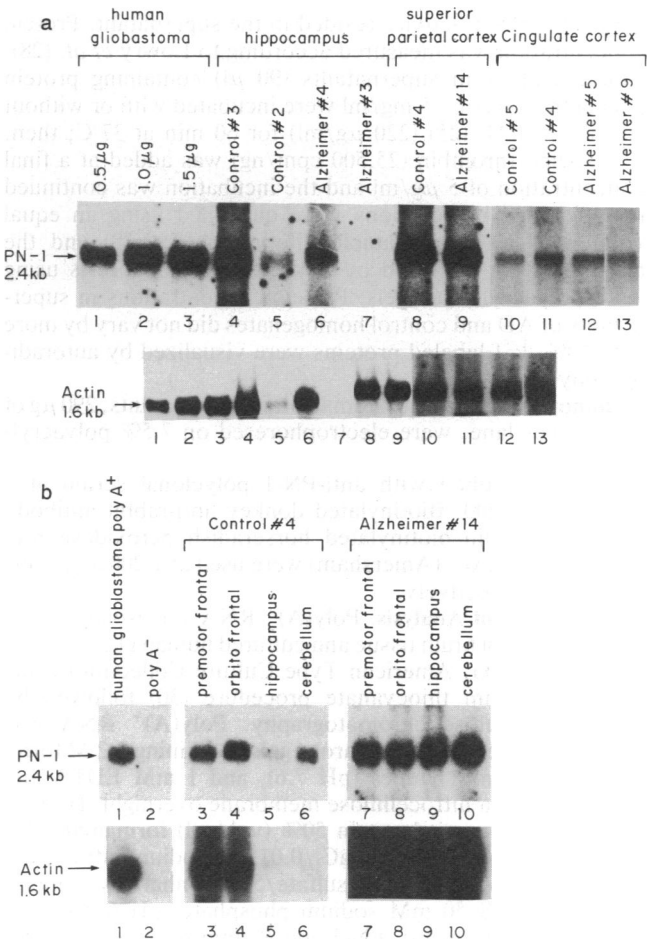

FIG. 3. Northern blot analysis of PN-1 and β-actin mRNA in poly(A)$^+$ RNA of AD or control brain. The PN-1 and β-actin riboprobes were hybridized to poly(A)$^+$ RNA at 60°C and 65°C, respectively. (*a*) Poly(A)$^+$ RNA (8 μg) from each specified brain region was electrophoresed and transferred to a nitrocellulose membrane. The identification numbers correspond to the listing in Table 1. (*b*) Poly(A)$^+$ RNA (10 μg) from specified regions of the AD brain and poly(A)$^+$ RNA (4 μg) from corresponding regions of control brain were transferred to a nitrocellulose membrane. Lanes: 1, 3.5 μg of poly(A)$^-$ RNA isolated from cultured human glioblastoma cells; 2, 10 μg of poly(A)$^-$ RNA from glioblastoma cells.

Biochemical Basis of Decreased PN-1 Activity in the AD Samples. The first step in this analysis was to determine if the decreased activity of PN-1 could partly be due to the presence of inhibitors in the AD extracts. To test this, mixing experiments were conducted in which 50% of a control brain sample was replaced with an equal volume of an AD sample (Fig. 4*a*). The PN-1 activities were additive, indicating that the difference in PN-1 activity was not due to excess activator [e.g., heparan sulfate (23)] in the control sample or excess inhibitor of PN-1 (e.g., thrombin) in the AD sample. This was confirmed by showing that added PN-1 was similarly recovered from both AD and control samples (Fig. 4*b*).

It should be emphasized that the above mixing experiments would not detect a PN-1 inhibitor that was present at a substoichiometric level and that bound irreversibly to PN-1. Target proteases of PN-1 would represent such an inhibitor. One possible source of these proteases could be from blood that was released upon homogenization of the brain tissue. To test the possibility that the AD samples contained more blood than the control samples, hemoglobin levels were

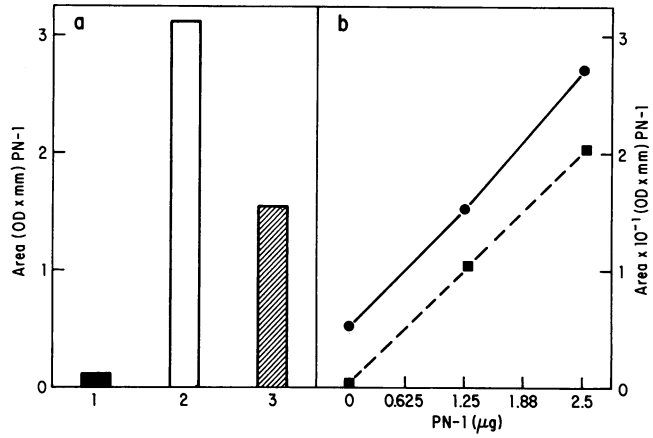

FIG. 4. Analysis of AD and control brain supernatants for inhibitors of PN-1 activity. (*a*) Additivity of PN-1 activity. Bars: 1, PN-1 AD 4 hippocampus (90 μl); 2, control 2 hippocampus (90 μl); 3, AD 4 hippocampus (45 μl) plus control 2 hippocampus (45 μl). (*b*) Recovery of added PN-1 activity from hippocampus samples of AD (■) or control (●) brain. ^{125}I-labeled thrombin–PN-1 complexes were measured by densitometric analysis (see Fig. 1). Note difference in scale between the two panels.

measured by CO difference spectroscopy. These levels did not vary significantly (control, 1.5 ± 0.06 μM; AD, 1.6 ± 0.07 μM), indicating that the AD samples did not contain more blood. Another possible source of increased proteases in the AD samples could be proteases that were present in the brain tissue before homogenization. Such proteases could possibly be released from dying neurons. They could also result from breakdown of the blood–brain barrier that appears to occur in some individuals with AD (35, 36). This might not be detected by the above hemoglobin assay since extravasation of plasma proteins could occur without extravasation of erythrocytes. Although direct assays for these proteases were not feasible after homogenization, we checked for them indirectly by immunoblot analysis of a series of samples that revealed not only free PN-1 but also PN-1-containing complexes that comigrated with PN-1–thrombin complexes (Fig. 5). In view of the presumed importance of hippocampus lesions in AD (33), these studies were conducted on hippocampus samples from seven AD and three control samples. The immunoblots of Fig. 5 show that there was a consistent decrease in free PN-1 protein in the AD samples. Moreover, PN-1-containing complexes that comigrated with PN-1–thrombin complexes were present in most of the AD samples. PN-1-containing complexes of smaller size and unknown identity were observed in one of the control samples (lane 1) and one of the AD samples (lane 7). These results suggested that the decrease in free PN-1 in the AD samples might partly be due to the presence of thrombin or a thrombin-like protease present in the samples before homogenization.

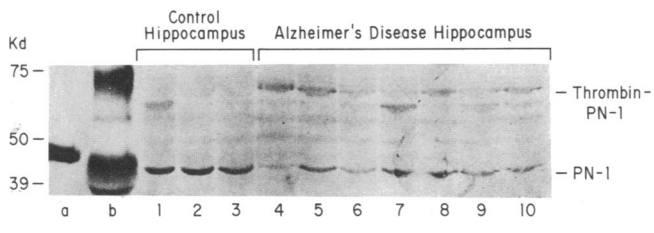

FIG. 5. Immunoblots of supernatants from AD (lanes 4–10) or control (lanes 1–3) hippocampus. Each lane contained 290 μg of brain supernatant protein. Lanes: a, PN-1; b, thrombin–PN-1 complexes; 1–3, control 2, 3, and 4, respectively; 4–10, AD 3, 4, 6, 7, 9, 11, and 12, respectively.

DISCUSSION

The present studies demonstrated the presence of PN-1 in human brain and revealed large decreases in PN-1 activity and free PN-1 protein in brain tissue from individuals who died of AD. Although conducted with autopsy tissue, the measurements of PN-1 activity and PN-1 protein indicated that most of the PN-1 in the control samples was active. Moreover, there was no significant correlation between levels of PN-1 activity and age, sex, or postmortem delay in either the AD or control groups in any of the brain regions examined. Large decreases were observed in the hippocampus and two cortical regions, all of which exhibit differing degrees of pathology in AD (33). The invariant decline in the hippocampus is important because neuropathology in this structure shows an 88% correlation with clinical diagnosis of AD (33). It will be important in future studies to conduct detailed regional analyses correlated with clinical and neuropathological severity.

Previous studies showed that brain amyloid deposits of AD contain α_1-antichymotrypsin (8) and that the amyloid precursor protein contains a trypsin inhibitor sequence (5–7). These interesting results indicate that an imbalance of proteases and protease inhibitors may be involved in the etiology of the disease (5–9). Several recent studies suggest the potential importance of thrombin or a thrombin-like protease. (*i*) The neurite outgrowth activity of PN-1 depends on thrombin inhibition (12, 13); also, PN-1 appears to be primarily a thrombin inhibitor (24). The latter conclusion comes from studies that have shown that PN-1 binds to the extracellular matrix (21) and that this interaction regulates its activity and target protease specificity. It accelerates the PN-1 inactivation of thrombin (22, 23) and blocks its inactivation of urokinase or plasmin (24). (*ii*) Thrombin blocks neurite outgrowth from cultured neuroblastoma cells and sensory ganglia (12–14) and retracts neurites in neuroblastoma cells (13). (*iii*) Specific high-affinity binding sites have been detected on neuroblastoma cells (37); binding of thrombin has also been detected in primary brain cultures (38) and in homogenates of brain and spinal cord (39). These results suggest a potential importance for interaction of thrombin or a thrombin-like protease with specific brain components.

An important goal of the present studies was to examine the molecular basis of the PN-1 decrease in AD. This may involve glial cells since they secrete PN-1 in culture (18). The alteration(s) appears to be posttranscriptional, since PN-1 mRNA levels were about equal in the AD and control samples examined. The immunoblots in Figs. 2 and 5 revealed decreased free PN-1 protein in all of the AD samples examined, consistent with altered mRNA translation, altered PN-1 secretion, or with posttranslational modification of PN-1. The latter possibility was supported by the finding in Fig. 5 of increased PN-1-containing complexes in AD samples that comigrated with PN-1–thrombin complexes. The most likely explanation for this finding is increased thrombin, although the present data do not exclude another protease of similar size. We considered possible sources of increased thrombin that could lead to increased PN-1–thrombin complexes in the AD compared to control hippocampus samples. One was increased blood released from vessels upon homogenization of the tissue. This seems unlikely because hemoglobin levels were about equal in the AD and control homogenates. Another possibility is based on previous findings that the blood–brain barrier is compromised in some individuals with AD (35, 36). This could lead to release of prothrombin from capillaries followed by conversion to thrombin. A final possibility is that thrombin or a thrombin-like protease could be produced locally in the brain.

These results suggest a model to explain some of the symptoms and lesions of AD based on the ability of thrombin

to bring about neurite retraction in neuroblastoma cells (13), on findings that the neurite-outgrowth activity of PN-1 and the identical glial-derived nexin depends on their ability to inhibit thrombin or a thrombin-like protease (12, 13), and on the fact that thrombin also inactivates a neurotrophic factor, acidic fibroblast growth factor (40). The model is that reduced levels of PN-1 and increased levels of thrombin could bring about alterations in neurons, disrupt interactions among neurites, and alter neurite morphologies. It is noteworthy that degenerating neurites have been observed around the periphery of angiopathic blood vessels in AD (41). This is consistent with the above model and suggestions that breakdown of the blood–brain barrier might play some role in the pathology of AD (35, 36).

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