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Cross-linking of ubiquitin, HSP27, parkin and α-synuclein by γ-glutamyl-ε-lysine bonds in Alzheimer's neurofibrillary tangles

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ABSTRACT

The accumulation of misfolded proteins in intracellular inclusions is a generic feature of neurodegenerative disorders. Although heavily ubiquitylated, the aggregated proteins are not degraded by the proteasomes. A possible reason for this phenomenon may be a modification of deposited proteins by transglutaminases forming γ-glutamyl-ε-lysine (GGEL) cross-links between distinct proteins. Here, we show that the frequency of GGEL cross-links is an order of magnitude higher in Alzheimer's brain cortex than in age-matched or younger controls. This difference is due to the accumulation of GGEL cross-links in ubiquitin-immunopositive protein particles present in both Alzheimer's brains and those from aged individuals. The highly cross-linked protein aggregates show immunoreactivity to antibodies against tau and neurofilament proteins, and partially also to α-synuclein, indicating that these structures are inherent in Alzheimer's neurofibrillary tangles and Lewy bodies. Using mass sequence analysis, we identified the same six pairs of peptide sequences cross-linked in both senile and Alzheimer's specimens: Gln^{31} and Gln^{190} of HSP27 protein are cross-linked with Lys^{29} and Lys^{48} of ubiquitin and HSP27 therefore may cross-link two (poly)ubiquitin chains. One lysine residue of parkin and one of α-synuclein were also found to be cross-linked. The data suggest that cross-linking of (poly)ubiquitin moieties via HSP27 may have a role in the stabilization of the intraneuronal protein aggregates by interference with the proteasomal elimination of unfolded proteins.

Key words: transglutaminase • neurodegeneration • proteasome • neurofibrillary tangles

Alzheimer's disease (AD) is the most common neurodegenerative dementia causing significant health care and social burden in aging societies. In spite of extensive genetic research, familial inheritance has only been demonstrated in less than one-tenth of cases

(1). The prevalence of AD is strongly correlated with aging. By extrapolation of community surveys, Americans older than 95 have a greater chance of suffering from AD than to be spared from this condition (2). Essential neuropathological features of AD involve senile plaque deposition, formation of neurofibrillary tangles and neuron loss, neither of which is unique to this type of dementia, but represents an exaggerated form of aging associated gray matter alterations (3). Indeed, AD is often regarded as a form of accelerated brain aging, where neurons cannot function and survive as long as other vital organs of the body (4).

Intraneuronal protein aggregates abundantly appear in AD brains as silver-stainable fibrillary structures, the neurofibrillary tangles (NFT), but other type of inclusions, like Hirano's bodies and granulovacuoles, are also well known (5).

The formation of NFT is modeled to start by the assembly of paired helical filaments from tau protein. The assembly of paired helical filaments could be demonstrated in vitro from an unphosphorylated tau isoform of bacterial expression (6); however, the stability of such in vitro aggregates is not particularly high (7). Moreover, tau from neurofibrillary tangles is heterogeneous in terms of splice isoform (8), sequence truncation (9) and phosphorylation state (10), and this heterogeneity is unlikely to strengthen tau cohesion per se. In contrast, ex vivo NFT are partially insoluble in detergents and other nonproteolytic lysis buffers (11), a fact which has hitherto hampered the biochemical characterization of insoluble NFT ingredients and favored scientific preoccupation with the extractable tau fractions (12).

Previous data indicate a possible role for transglutaminase-mediated cross-linking of neurofilaments (13, 14) and tau (13–15) in the formation of insoluble neurofibrillary lesions. Transglutaminases (TGases, E.C. 2.3.3.13.) are enzymes catalyzing the exchange of the γ-amido group from protein-bound glutamines to primary amines. If the glutamyl acceptor is an ε-amino group of a protein-bound lysyl moiety, the product of the reaction is a γ-glutamyl-ε-lysine (GGEL) isopeptide bond between two protein sequences, colloquially named a cross-link. Nine TGase genes are known in humans (16), some of which are expressed in splice variants (15). TGases are ubiquitous in tissues and the expression of different isoforms is overlapping (17). Five different TGase isoenzymes are expressed in human brain (TGase 1, 2, 3 (18), 6 and 7 (19)), and some brain regions express mRNA for several TGases simultaneously (18). Recombinant tau was shown to be a substrate for TGase2 and in vitro reactive residues within its repeat domains were identified (20). Using a monoclonal antibody against GGEL, cross-links were demonstrated in neurofibrillary tangles (21) and Huntington inclusions (22). The concentration of free GGEL isodipeptide, a marker of cross-linked protein turnover, was shown to be elevated in the cerebrospinal fluid of patients with AD (23) Huntington's chorea (22, 24) and other neurodegenerative dementias (25).

To characterize protein transglutamination in Alzheimer's diseased brains and in controls, we analyzed the frequency and location of GGEL cross-links in selected brain regions.

MATERIALS AND METHODS

Determination of the relative abundance of GGEL cross-links in brain proteins

Left hippocampus, orbitofrontal, and occipital cortex from patients with histologically verified Alzheimer's dementia were obtained from West Los Angeles VA Medical Center Neurological Research Specimen Bank (*n*=5, mean age 73.8±6.3 years). Age-matched (*n*=6, mean age 75.2±5.7 years) and younger adult (*n*=6, mean age 34.2±7.4 years) nondemented, brain specimens from similar anatomical locations were collected from autopsy cases of patients without any known history of neurological disease from the Department of Pathology, University of Debrecen, and stored at –80°C as ~0.5 g blocks. The postmortem intervals did not exceed 20 h. Cortical tissue blocks were homogenized and delipidized by a Teflon-head homogenizer in 5 ml TriPure (Roche). Insoluble material was always pelleted at 30,000 g and the TriPure (phenolic guanidine HSCN) solubilized brain proteins were recovered by 2-propanol precipitation of the aqueous phase according to the manufacturer's instructions. TriPure insoluble and soluble fractions were subjected to limited acid hydrolysis (0.5 N trifluoroacetic acid (TFA) at 95°C for 16 h), dried and consecutively digested with proteinase K, and the GGEL content was determined exactly as published (26). Briefly, the digests were competed for binding to a monoclonal antibody (81D4, Covalab, Lyon, France) to GGEL isodipeptide with a biotinylated, and GGEL cross-linked peptide of known concentration and the decrease of competitor binding was measured by a chromogenic reaction through streptavidine-coupled alkaline phosphatase in a plated enzyme-linked immunosorbent assay (ELISA) format. Amino acid content was determined by acid hydrolysis and analysis on a Beckman 6300 amino acid analyzer. When measuring GGEL from trypsin- or V8 proteinase solubilized proteins, acidic and proteinase K cleavage were omitted and PBS-redissolved peptide fractions were directly applied into the ELISA wells.

Isolation of ubiquitin-immunopositive, insoluble protein particles (UPP) from brain cortex

The TriPure-insoluble material from brain homogenates was re-extracted by suspension through pellet, incubation, and centrifugation of the material with the following sequence of solvents. TriPure was added at ambient temperature again, then Laemmli buffer (2% sodium dodecylsulfate, 100 mM DTT in Tris-Cl buffer, (27)) 20 min at 121°C, followed by 5M guanidine-HSCN in water at 121°C for 20 min, and Schweizer's reagent (30% tetraminoCu$_{[II]}$(OH)$_2$ in H_2O) overnight at room temperature, and 20 min at 121°C. The latter three treatments were repeated once. The remaining particulate material was washed with PBS containing 5 mM EDTA-Na and resuspended in 2 ml PBS with 1% BSA and was shaken overnight with 1:50 diluted rabbit anti-ubiquitin antiserum (Sigma Chemical Company, St. Louis, MO) at 4°C. Particles were cleared from unbound antiserum by washing with PBS-BSA buffer and then reacted with iron-microbead coupled goat anti-rabbit immunoglobulins (Miltenyi Biotech, Bergisch Gladbach, Germany). Immunoreactive antibodies were captured, washed and eluted on a Macs LS column (Miltenyi) according to the manufacturer's protocol. Eluted particles were stripped of bound antibodies by two extractions with hot Laemmli buffer and washed with water.

Immunofluorescent analysis of UPP

UPP were suspended in PBS + 1% BSA, spotted onto silanized glass slides and post-fixed with 1% paraformaldehyde in PBS for 30 min, and washed with PBS. Slides were blocked with 10% normal swine serum in PBS for 30 min, then double-stained with two antibodies: one was always monoclonal anti-ubiquitin (NCL-1, Novocastra, 1:200), the other was either rabbit anti-tau (1:200, Sigma), anti neurofilament 200 (1:100, Sigma), anti-α-synuclein (1:200, Sigma) or anti-apoE (1:100, Calbiochem Novabiochem Corp., San Diego, CA). Primary antibodies were detected with fluorescein-conjugated anti-mouse and rhodamine-coupled anti-rabbit immunoglobulin antibodies from swine (DAKO A/S. Glostrup, Denmark). Cross-reactivity was checked by controls, where the anti-ubiquitin antibody was omitted, or nonimmune rabbit antiserum was used.

Slides were mounted with antifade and observed at 1600× magnification under a Zeiss Axiovert microscope equipped with a dual (fluorescein+rhodamin) epi-illumination filter. Images were recorded by a CCD camera and stored digitized in RGB mode.

Cleavage of UPP and enrichment of GGEL cross-linked peptides

UPP from identical brain regions were pooled within the AD and AMND donor groups. 20–70 μg of UPP were shaken overnight in 70% formic acid containing 0.1% CNBr, dried in vacuum and digested with 50U/μg bacterial alkaline phosphatase (Sigma type III) in 0.1 M ammonium-bicarbonate buffer (ABB) at 37°C for 60 min, prior to the addition of 4 μg trypsin (Roche) or 5 μg *S. aureus* V8 protease (Roche). Proteolysis was carried out overnight at 37°C, the protease was then inactivated by boiling and the enzymatic digestions were repeated. Tryptic and V8 peptides were dried, dissolved in 0.1% TFA (buffer A) containing 5% MeCN and fractionated on a 250 × 4.6 mm Beckman Ultrasphere C_{18} column at a flow rate of 0.22 ml/min using a continuous gradient from 5 to 50% buffer B (0.1% TFA in MeCN). Column eluate was collected in 30 s fractions. Part (10 μl) of the fractions was used for amino acid analysis, another part for GGEL cross-link assay. The fractions harboring GGEL cross-links were neutralized with an equal volume of ABB containing 0.5% octyl glucoside and were immunocaptured with the same antibody (1:50 final dilution, 60 min at 37°C) used before for the detection of GGEL. The samples were then transferred into a 0.5 ml PBHK spin-concentrator (Millipore, Billerica, MA, USA) and the solvent was removed. The residual fluid was diluted with ABB and washed again. The same protease used for the generation of the peptides was added to the samples (2 μg each in 100 μl ABB), they were incubated for 2 h at 37°C and spun through the membrane again. Samples were dried, oxidized with 3% performic acid in 70% formic acid at –20°C for 30 min, and dried again.

Sequence identification of GGEL cross-linked peptides

The peptides were concentrated by ZipTip C18 (Millipore) and further analyzed by mass spectrometry using a Voyager2 MALDI-TOF apparatus (Applied Biosystems, Foster City, CA) and sinapinic acid matrix. A fraction of the sample was spotted on Platinum foil and applied to the sample compartment of a Porton 3000 gas-phase microsequencer (Beckman Coulter, Fullerton, CA), where it was dried and flushed with chloroacetylchloride and trimethylamine vapors as published by Vath and Biemann (28) and then analyzed by MALDI-TOF as before.

This chemical derivatization was used for the screening of peptides with two N-terminal amino-reactive groups. Peptides showing abundant double-trimethylaminoacetyl modification were sequenced by Q-TOF fitted with Z-spray (Micromass, Whytenshawe, United Kingdom) using nanoelectrospray capillaries (Protana, Odense, Denmark). Read sequences were searched against Swiss-Prot, TrEMBL, TrEMBL New, PDB, and translated EST databases. Nonhuman and hypothetical proteins were ignored.

Relative frequencies of GGEL cross-linked sequence pairs

After clarifying the tryptic and V8 sequences found in the HPLC fractions, coeluting cross-linked tryptic and V8 peptides showed to be different. GGEL cross-link content was quantified from these fractions as mentioned above. Relative abundance ratios between the peptide pairs were calculated from the GGEL contents of the time fractions in which the concerned (1, 2, or 3, Table 1) sequence pairs were eluted.

Statistical analysis

Means represent the average of 5 independent determinations, unless otherwise indicated. Statistical comparison of data sets was done by 2-way ANOVA (ANOVA) using SPSS 11.5. $P<0.05$ was considered significant, unless otherwise stated.

RESULTS

Frequency of GGEL cross-links in AD cortex is higher than in controls

The proteins extracted from brain cortex were cleaved to soluble oligopeptides and amino acids by protease, and concentrations of GGEL were assayed using an ELISA method with a detection threshold of 1 GGEL in 10^{13} amino acids (26). The relative abundance of GGEL bonds in brain proteins was measurably high in all samples. Middle-aged control specimens contained 11–29 GGEL cross-links/10^9 amino acids, and differences between hippocampus, frontal and orbital cortex were not significant ($P>0.05$). In the age-matched (to the AD group) nondemented (AMND) control group, GGEL density of either brain region was significantly ($P<0.01$) higher in tissue proteins than in either sample of the younger control group. The frequency of GGEL bonds was highest in the hippocampus and showed statistically significant ($P<0.01$) differences in this order: 1) hippocampus 2) frontal cortex 3) occipital cortex (Fig. 1). AD specimen showed the same ranking, and the mean abundance of GGEL cross-links was more than threefold higher in the hippocampus and frontal cortex as compared with AMND specimens ($P<0.001$); however, the occipital tissue showed only modest elevation of cross-link content ($P=0.03$).

Our method of delipidization and chaotropic lysis of brain proteins by phenolic guanidine thiocyanate was originally developed for extraction of RNA from tissue homogenates (29). This method yields two protein fractions when working with tissues: one that is soluble and one that is not. This prompted us to determine the relative abundance of GGEL cross-links separately for these two fractions.

Cultured cells can be completely solubilized in chaotropic salts and the Laemmli lysis buffer routinely used for denaturing polyacrylamide electrophoresis; however, tissues containing ubiquitous basement membranes, collagen or elastin fibers are not. The resistance is conferred by

covalent cross-linking of proteins, like that of connective tissue fibers, which is accomplished overwhelmingly by covalent bonds different from GGEL cross-links (30), although transglutamination of extracellular matrix components may be dramatically increased in special pathological conditions (30). The guanidine salt-insoluble fraction accounted for 1.3–2.2% of total tissue proteins in either AD or AMND specimen. A significant (P<0.01) increase of insoluble proteins was noted between AD and younger control groups in both hippocampus and the neocortical samples. The AMND control group was intermediate between AD and younger control samples, but differences between the latter groups did not reach statistical significance (P>0.05, Fig. 2). The relative abundance of GGEL in the chaotrope-soluble proteins varied between 3–14 /10^9 without showing statistically significant differences between donor groups or brain regions. The guanidine–thiocyanate-insoluble proteins, however, showed the same statistically significant differences among the three sample groups as noted for the total (soluble+insoluble) tissue proteins (Fig. 2). The highest relative abundance of GGEL within the insoluble fraction reached 57/10^6 amino acids in the hippocampus of an AD patient (Fig. 2*B*), indicating that every 9000th amino acid is involved in GGEL cross-link formation in this fraction. Given that the average protein consists of 100–1000 amino acids, this might mean that up to 1–10% of the chaotrope-insoluble fraction might be GGEL-cross-linked.

Neurodegeneration-associated cross-links copurify with ubiquitin immunopositive protein aggregates

To extract putative, and noncovalently bonded ingredients from the guanidine–thiocyanate-insoluble brain proteins, the latter were extensively washed with detergents, thiols, and chaotropic salts in hot water. Even if these harsh treatments can effectively disrupt highly insoluble and resistant material held together by hydrogen, ionic or disulfide bonds (e.g., silk, cotton, or wool), this procedure did not void more protein (21–36%) from the samples than GGEL cross-links (24–39%) and thus did not result in a further enrichment of transglutaminated products (Fig. 3*A*, top stack). Further fractionation of this particulate material was accomplished by immunocapturing ubiquitylated particles with antiubiquitin antibodies and separation of the ubiquitin-immunoreactive material by a batch method routinely used for cell sorting (magnet-assisted cell sorting). By processing 10–25 mg of pooled insoluble brain protein, ubiquitylated particles (UPP) containing 0.03–26 × 10^{-8} M amino acid could be recovered after removal of the bound antibodies. From the younger control brains, we could not purify sufficient quantities of UPP to subject them to further biochemical or immunological analysis, either because such particles were not extant in these specimens or because the UPP were too small to be recoverable by the centrifugation steps. This is in agreement with the observation that ubiquitylated inclusions in young adult brain are uncommon (31). The frequency of GGEL cross-links was assayed from the particulate fraction before affinity-separation and from the flow-through and immunocaptured particles after stripping off the primary and secondary antibodies (Fig. 3). The relative abundance of GGEL cross-links in the flow-through was 174–313 /10^9 and showed no significant differences (P<0.05) between samples of either patient group or anatomical region (Fig. 3). The UPP from AD brains contained 1527–1955 (mean 1793±176 s.d., n=4)/10^6, and those from AMND controls 1969–2614 (mean 2311±285 s.d., n=6)/10^6 GGEL cross-links. No statistically significant differences were seen between UPP from hippocampi and (pooled) neocortex. These data show that the formation of highly cross-linked UPP is more extensive in AD, but the intensity of their cross-linking is lower (P<0.01).

Antigenicity of UPP is similar to neurofibrillary tangles

We explored the immunophenotype of UPP using polyclonal antibodies to common neurofibrillary antigens. The bias resulting from disregarding the unstained particles and digital image capturing was circumvented by dual fluorescence recording using one standard immunolabeling in order to compare relative staining intensities between AD and AMND groups. In this setting, all UPP show green, unless they are stained with the other (red) label, which is recorded as yellow. A remarkable observation is that UPP from AD brains showed elongated, angular structures, reaching up to 2–3 μm size and having sharp margins, which showed strong positivity with anti-tau and anti-neurofilament antibodies, but were only weakly stained with anti-α-synuclein antiserum. UPP from AMND controls, on the contrary, were typically roundish particles of 0.5–1 μm diameter revealing strong immunopositivity to tau, neurofilament, and α-synuclein antibodies. Such particles were also present in AD specimens. Neurofibrillary tangles are known to be immunoreactive to antibodies against tau protein, ubiquitin, and neurofilament chains (32). Some reports indicate that ApoE (33) is also present in neurofibrillary structures, but we did not detect apoE immunoreactivity on UPP from either group. These findings indicate that Alzheimer's neurofibrillary tangles and UPP harbor common epitopes and are likely to be related structures; however, association of α-synuclein and tau positivity is more typical for Lewy bodies (34).

Isolation, sequencing, and identification of GGEL-cross-linked peptide sequences from UPP

UPP from hippocampus and cortex were fragmented into soluble peptide fragments by CNBr cleavage, dephosphorylation, and protease digestion. The resulting mixture of straight and cross-linked peptides was fractioned by HPLC, and time fractions that showed no measurable GGEL content were discarded. The cross-linked peptides from UPP from either AD or AMND control groups eluted in the same time fractions (Fig. 5, Table 1). Both of these fractions contained hundreds of overlapping mass peaks as evidenced by mass analysis (not shown). To reduce the number of interfering peptides, cross-linked ones were immunocaptured with the same monoclonal antibody that was used for their GGEL content determination. After repeated digestion with the same proteinase applied for the generation of peptides, the cross-linked peptides were recovered being contaminated with fragments from a monoclonal antibody, BSA, and the proteinase, thus giving a well-reproducible background of peptides for further mass analysis. GGEL-cross-linked peptides were identified by virtue of their two amino termini using trimethylaminoacetyl modification of unprotonated amino groups. This (partial) modification is known to label peptide amino termini without significant modification of lysine ε-amino groups (28). Cross-linked peptides were identified by the appearance of a novel, doubly charged ion peak at M+202/2 (mass/charge). Six tryptic and six V8 peptide masses were identified by this method as being cross-linked. These were sequenced using electrospray ionization MS/MS. Remarkably, identical sequences were found in cross-linked peptides of both AD and AMND control specimens. Sequencing of GGEL-cross-linked tryptic peptide pairs revealed two concurrent legible sequences in all cases (Fig. 6.). Moreover, the GGEL isopeptide bond did not break using acceleration voltages resulting in the selective fragmentation at the peptide bonds.

Fragmentation of the GGEL bond could only be achieved by applying anode voltages that caused sidechain fragmentations as well. Such fragmentation profiles were used to locate the Glu and

Lys residues to each sequence and to pair the two amino- and carboxy terminal sequences of the peptide pairs, respectively (data not shown). Thus, apart from Ile/Leu ambiguities, cross-linked tryptic peptides were equivocally sequencable. V8 peptides revealed only partial carboxy and amino-terminal ("y" and "b") ion series, but the created fragment ion masses were unambiguously attributable to only one possible pair of GGEL-cross-linked sequence after exploring the tryptic sequence pairs and assuming that V8 peptide pairs contain fragments from the same protein sequences as the tryptic ones.

A search of the found sequences against human protein and EST databases yielded only one full match for each of the twelve GGEL cross-linked sequences, if hypothetical proteins and entries containing fragments with high homology to fully sequenced proteins were ignored.

Our data show that two lysines of the ubiquitin (Ub) protein, Ub-Lys^{29} and Ub-Lys^{48}, which are also used for coupling ubiquitins to each other, are used for transglutaminase-mediated GGEL cross-linking, whereas Ub-Lys^{11} and Ub-Lys^{63}, which also form inter-ubiquitin bonds (35), were not cross-linked. Lys^{48} (P-Lys^{48}) is a structural position-equivalent of Ub-Lys^{48} in the ubiquitin-homologous domain of parkin proteins. Glutamine-donor sequences for the cross-linking are provided by two sites of small heat shock protein HSP27 (HSP27-Gln^{31} and HSP27-Gln^{190}) and one of α-synuclein (S-Gln^{99}). Again, S-Gln^{99} is located in a sequence environment strongly similar to that of HSP27-Gln^{190}.

Relative abundance of transglutaminated residues differ in AD and senile brains

Some cross-linked peptides from both tryptic and V8 digested UPP coeluted from the HPLC column (Table 1). As the association of coeluting, cross-linked tryptic and V8 peptide sequences was different, we could calculate the relative abundance of each transglutaminated peptide pair by comparing the cross-link content in the HPLC fractions of tryptic and V8 digests.

HSP27-Gln^{31}+Ub-Lys^{29} bonds were most frequent in all of the four sample groups and accounted for more cross-links than all other types combined (Fig. 7). The second most frequent cross-link was HSP27-Gln^{190}+Ub-Lys^{48} in AD and controls, accounting for 26–38% of the cross-links. Cross-links involving S-Gln^{99} and P-Lys^{48} were 7–12% and 5–8%, of AMND controls, respectively, but made up only 2–5% and 1–3% of GGEL cross-links in AD, respectively.

DISCUSSION

Neurodegenerative diseases are manifold, but all share the common feature of forming intraneuronal (or intraglial) protein inclusions generated from the deposition of misfolded, self-aggregating protein waste. Most globular proteins tend to self-aggregate and precipitate from solution when unfolded. Therefore, eukaryotic cells maintain intricate ways of chaperoning, quality control and waste disposal to manage such events. It is being recognized that the impairment of clearance mechanisms is more likely to account for aggregate deposition and cellular pathology than the existence of unusually strong cohesion between specific misfolded proteins (36). Application of proteasome inhibitors prompted inclusion formation in animal brain (37) and cell culture (38). These inclusions were shown to incorporate proteasomes and their anchoring cytoskeletal elements. Inhibition of the ubiquitin–proteasome system (UPS) appears downstream of ubiquitin-attachment mechanisms (the "quality control") as both pathological and

experimental inclusions carry massive ubiquitin labeling (39). The enzymes of the UPS and the chaperone proteins are present in the neurodegenerative lesions, and, along with other random proteins, they get clogged into these piles of protein waste (40). The overload of, block of access to, or inhibition of, proteasomes may be key features for the formation of ubiquitylated neuronal inclusions encountered in normal senescence as well as in neurodegenerative diseases (41). It is heavily disputed whether misfolding-induced aggregation alone might be sufficient to block proteasomal breakdown of the affected proteins (42). In vitro aggregates from tau (7) and α-synuclein (43) are not unusually stable and Huntington inclusions formed by overexpression of pathological proteins are resorbable by neurons (44).

Here, we show that GGEL cross-linking of brain proteins occurs in the AD cortex by an order of magnitude more than what occurs in the age-matched and younger controls. Our results systematically corroborate previous reports (14, 15, 18). Kim et al. (18) reported a greatly increased level of cross-links in insoluble proteins of Alzheimer's disease cortex and cerebellum of unspecified location, relative to normal brain cortex. Their report indicates that 5–10% of brain proteins in AD might be insoluble in Laemmli buffer and that the frequency of cross-links within this fraction might reach up to 5–10% of protein-bound lysines. In our samples the bulk of insoluble material was extracellular matrix-derived, and its amount did not show order-of-magnitude differences between AD and control specimens. The number of cross-links reported by Kim et al. (18) in insoluble fractions of both normal and AD specimen was much greater than that found by us.

Our data also indicate that transglutamination of proteins is not specific to AD, as it is also apparent in senile neuronal degeneration, and shows only quantitative differences with the latter.

Cross-linking is present in young adult brains that are apparently unaffected by any neurological disease, and the frequency of GGEL in chaotrope-soluble proteins did not differ in AD vs. controls. This fact argues for a physiological role of GGEL cross-link formation in the nervous system.

We found that the increased cross-link formation in brains affected by either aging or AD is confined to organelle-sized, ubiquitylated protein inclusions bearing remarkable similar antigenicity to neurofibrillary tangles. Though these particles seemed bigger and more elongated in AD than in aged controls, and α-synuclein immunopositivity was markedly weaker in the elongated particles as compared with the smaller (AMND-like) ones, we did not recognize any qualitative difference between these structures. Senile UPP might therefore mature into AD-like UPP with time. However, it is also possible that small particles with strong α-synuclein immunopositivity represent a different pool of UPP which might develop into bigger, Lewy body-like structures by further accumulation of α-synuclein protein, as α-synuclein is abundantly present in Lewy bodies (45). We allocated six GGEL cross-links of UPP to four proteins; ubiquitin, HSP27, α-synuclein, and parkin. Unlike ubiquitin, α-synuclein and HSP27 (46–48), parkin has not yet been shown to participate in the formation of neurofibrillary tangles. Parkin is an ubiquitylation-regulating protein (49), which contains a ubiquitin-homologous N-terminal domain. Mutant (dysfunctional?) parkin is known to cause Parkinson's disease and Lewy body pathology (50) and interfere with the proteolytic processing of tau (51).

Our most striking observation was that putative core proteins of neurofibrillary deposits, like tau or neurofilament components, were not found to be directly cross-linked, though a direct cross-linking of tau was proposed by previous studies based on indirect evidence and impressive immunocytochemical data (14, 21, 52, 53). However, our data indicate that tau protein, which is heavily ubiquitylated in neurofibrillary lesions (46, 54, 55), might be cross-linked to itself, or other misfolded proteins via its ubiquitin decoration.

Whereas the tertiary structure of ubiquitin is known (56), the crystal structure of the other three cross-linked proteins has not yet been published. Given the obvious sequence homology, the N-terminal domain of parkin is predicted to adopt an ubiquitin-like conformation with an exposed P-Lys^{48}. Because of sequence homology to crystallins, the small heat shock protein HSP27 is predicted to have a structured amino-terminal and a randomly coiled carboxy-terminal part, the latter involving HSP27-Gln^{190} (57). By virtue of this randomly coiled tail, HSP27 protein, being cross-linked at Gln^{31} and Gln^{190}, may be capable of bridging through a wide range of spatial distances. The α-synuclein sequence harboring S-Gln^{99} is also predicted to have a wholly random coil structure (58), and it is surrounded by amino acids similar to HSP27-Gln^{190}; therefore, it may react with the same partners as the other residue.

Our data reveal that not only may the aggregated proteins branch by N$^{\varepsilon}$(α-glycyl)lysine isopeptide bonds, but also by ubiquitin itself by forming N$^{\varepsilon}$(α-glycyl^{76})lysine$^{11/29/48/63}$ isopeptide cross-links leading to polyubiquitin chains (35), as well as γ-glutamyl-ε-lysine$^{29/48}$ isopeptide cross-links with HSP27 or α-synuclein.

The cross-linkage of P-Lys^{48} and of S-Gln^{99} may indicate a role for parkin and α-synuclein as chain terminators, that is, an endpoint in growth of cross-linked aggregates, as these proteins revealed only one potential cross-linkable residue. Parkin colocalizes with α-synuclein (59), although the biological roles for these two proteins have not yet been joined. The common feature of chain termination may explain how parkin might protect neurons from the toxicity of mutant α-synuclein (60).

Although three reactive glutaminyl and lysyl residues allow nine possible pairings, neither HSP27-Gln^{190}+Ub-Lys^{29}, nor HSP27-Gln^{31}+P-Lys^{48}, nor S-Gln^{99}+P-Lys^{48} combinations were detected, even after targeted searching for the predicted masses. As HSP27-Gln^{31} was not seen to react with any other acceptor but Ub-Lys^{29}, it is possible that the regions around these residues are initially positioned close to one another by a noncovalent type of interaction between Ubiquitin and HSP27 and become transglutamylated afterwards. It is predictable that eventual cross-linkage between parkin and α-synuclein would not form a larger structure than the size of average proteins, and therefore must have been lost during the isolation of UPP.

The abundance of parkin and α-synuclein cross-links was markedly less in AD as compared with elderly controls, but the relative frequency of Ub+HSP27 cross-links was similar for AD and senile UPP. This may be an argument to postulate qualitative differences between AD and senile neuropathology. However, senile UPP may also be the predecessors of AD-like UPP, as GGEL cross-links involving parkin and α-synuclein are only relatively less abundant and not quantitative, considering the total pool of cross-linkages (Fig. 3 and 7).

The aperture of the multiproteolytic 20S proteasome subunit is ~13Å wide and the penetration of the substrate must be preceded by the removal of the polyubiquitin branches (61). Proteasomes require the prior ATP-dependent winding of the substrate proteins into the cavity of the 20S proteasome subunit (62); therefore, covalently branched meshworks of proteins may be too large or tangled to enter the proteolytic cavity.

Microscopically apparent intraneuronal inclusions hallmark the biochemical process of waste protein deposition, which at a younger age are successfully eliminated by the UPS. Our data suggest that GGEL cross-linking of the (poly)ubiquitin chains of waste proteins by transglutaminases are a decisive mechanism for the stabilization of these aggregates possibly rendering them resistant to proteasomal degradation and causing neuronal pathology.

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Table 1

GGEL cross-linked peptide pairs of ubiquitylated brain aggregates

Peptide (mass)	HPLC Fraction (retention time)	Ms/Ms Sequence	Matching Swiss-Prot Sequence (from human proteins)	Transglutaminated Residues
Da	Min		***Tryptic peptides***	
T1 1244.3	13.5–14	A**K**LQDK D**E**LGK	Ubiquitin 26–33: (K)AKIQDK α-synuclein 96–102: (K)DQLGK	**Lys^{29}** **Gln^{190}**
T2 1625.9	15.5–16	A**K**LQDK A**E**LGGPEAAK	Ubiquitin 26–33: (K)AKIQDK HSP27 187–198: (R)AQLGGPEAAK	**Lys^{29}** **Gln^{99}**
T3 1955.1	21.5–22	VLFAG**K**QLEDGR A**E**LGGPEAAK	Parkin 44–54: (R)VIFAGKQLEDGR HSP27 187–198: (R)AQLGGPEAAK	**Lys^{29}** **Gln^{190}**
T4a 1888.2	23–24.5	LLFAG**K**QLEDGR D**E**LGK	Ubiquitin 41–54: (R)LIFAGKQLEDGR α-synuclein 96–102: (K)DQLGK	**Lys^{48}** **Gln^{99}**
T4b 1847.6	23–24.5	A**K**LQDK LFD**E**AFGLPR	Ubiquitin 26–33: (K)AKIQDK HSP27 26–37: (R)LFDQAFGLPR	**Lys^{29}** **Gln^{31}**
T4c 2270.9	23–24.5	LLFAG**K**QLEDGR A**E**LGGPEAAK	Ubiquitin 41–54: (R)LIFAGKQLEDGR HSP27 187–198: (R)AQLGGPEAAK	**Lys^{48}** **Gln^{190}**
			V8 peptides	
V1 1714.8	12.5–13	NVKA**K**LQD **E**LGKNEE	Ubiquitin 25–32: (E)NVKAKIQD α-synuclein 98-105: (D)QLGKNEE	**Lys^{29}** **Gln^{99}**
V2 1812.4	15.5–16	NVKA**K**LQD SRA**E**LGGPE	Ubiquitin 25-32: (E)NVKAKIQD HSP27 186-195: (E)SRAQLGGPE	**Lys^{29}** **Gln^{190}**
V3 2430.9	21–21.5	NVKA**K**LQD **E**AFGLPRLPEE	Ubiquitin 25-32: (E)NVKAKIQD HSP27 30-41: (D)QAFGLPRLPEE	**Lys^{29}** **Gln^{31}**
V4a 2056.3	22.5–23	QLRVLFAG**K**E SRA**E**LGGPE	Parkin 40-50: (D)QLRVIFAGKE HSP27 186-195: (E)SRAQLGGPE	**Lys^{48}** **Gln^{190}**
V4b 2230.0	22.5–23	QQRLLFAG**K**QLE **E**LGKNEE	Ubiqitin 39-51: (D)QQRLIFAGKQLE α-synuclein 90–105: (D)QLGKNEE	**Lys^{48}** **Gln^{99}**
V5 2328.4	24.5–25	QQRLLFAG**K**QLE SRA**E**LGGPE	Ubiqitin 39-51: (D)QQRLIFAGKQLE HSP27 186-195: (E)SRAQLGGPE	**Lys^{48}** **Gln^{190}**

Cross-linked peptides recovered from HPLC fractions of trypsin ($T_{1–4}$) and V8 proteinase ($V_{1–5}$) fragments of ubiquitylated brain protein particles were obtained as described under Experimental Procedures, identified by mass sequencing, and aligned to known protein sequences by database searching. Nonhuman and hypothetical protein sequences were ignored. The sequence pairs obtained from AD and age-matched nondemented control specimen were identical. The amino acids in brackets precede the cleavage site of the protease used and are shown to demonstrate the specificity of the protease. L in sequenced peptide pairs denotes either leucine or the isobaric isoleucine.

Fig. 1

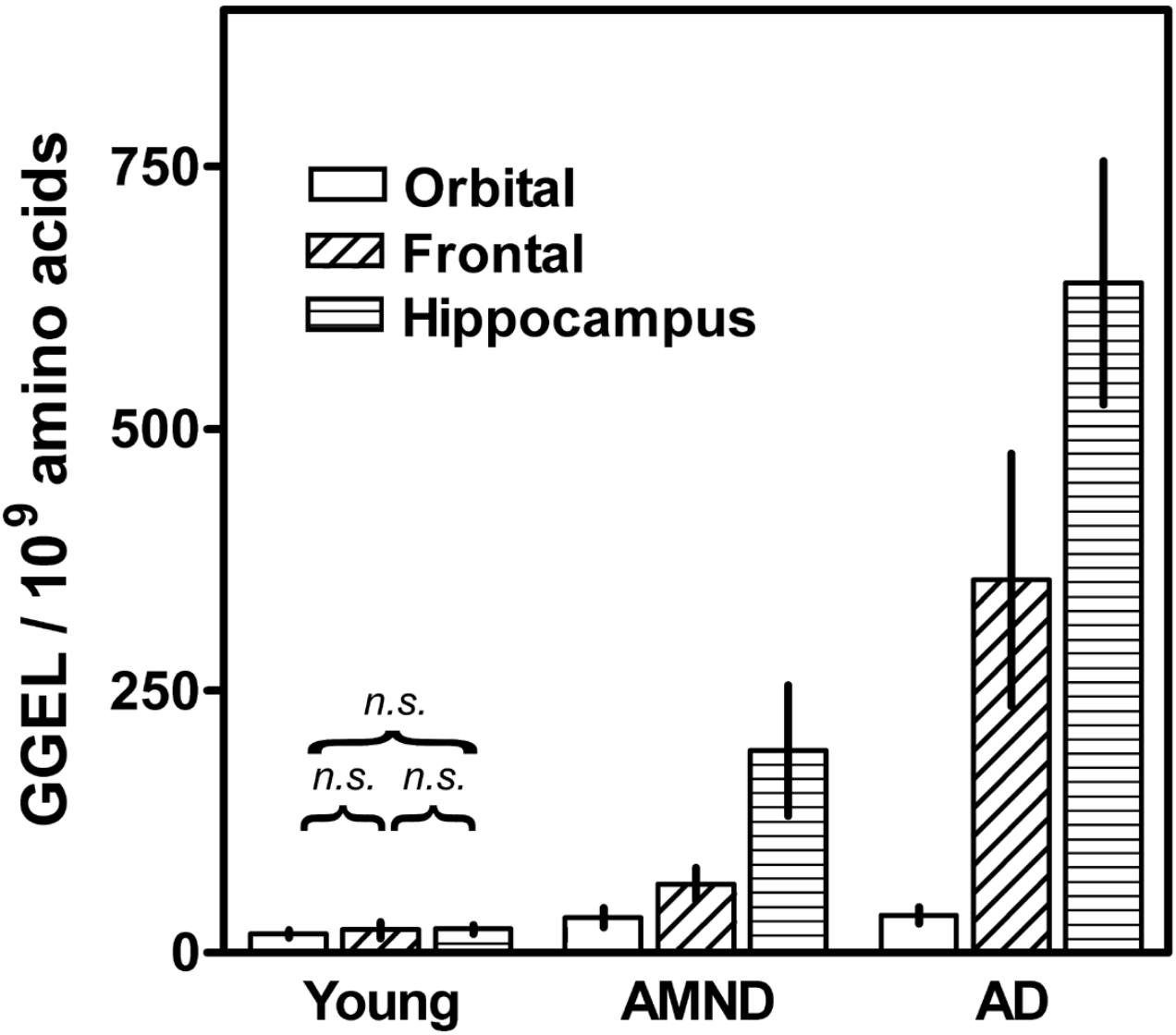

Figure 1. Accumulation of cross-links in brains from Alzheimer's diseased and aging persons. The frequency of transglutaminase-mediated γ-glutamyl-ε-lysine (GGEL) cross-linking was measured in the hippocampus, frontal and occipital lobe cortex of Alzheimer's diseased (AD) patients, age-matched nondemented (AMND) controls (average age 75.2 years) and middle-aged nondemented (young) controls (average age 34.2 years) from guanidine thiocyanate solubilized and residual brain cortex proteins digested by proteinase K and pooled. GGEL content was determined by a plated ELISA method using a monoclonal antibody to GGEL. The content of GGEL is related to the protein mass as determined by amino acid analysis. The relative abundance of GGEL cross-links is higher in AMND than in young controls, but lower than in AD samples of the same anatomical regions. The differences between frequencies of GGEL cross-links reached statistical significance ($P < 0.05$) either among brain regions or among the young, AMND, and AD brains unless indicated with *n.s.* (not significant). Higher values denote brain regions that are typically affected by AD type neuronal degeneration. Error bars represent the mean ± S. D. of five independent measurements.

Fig. 2

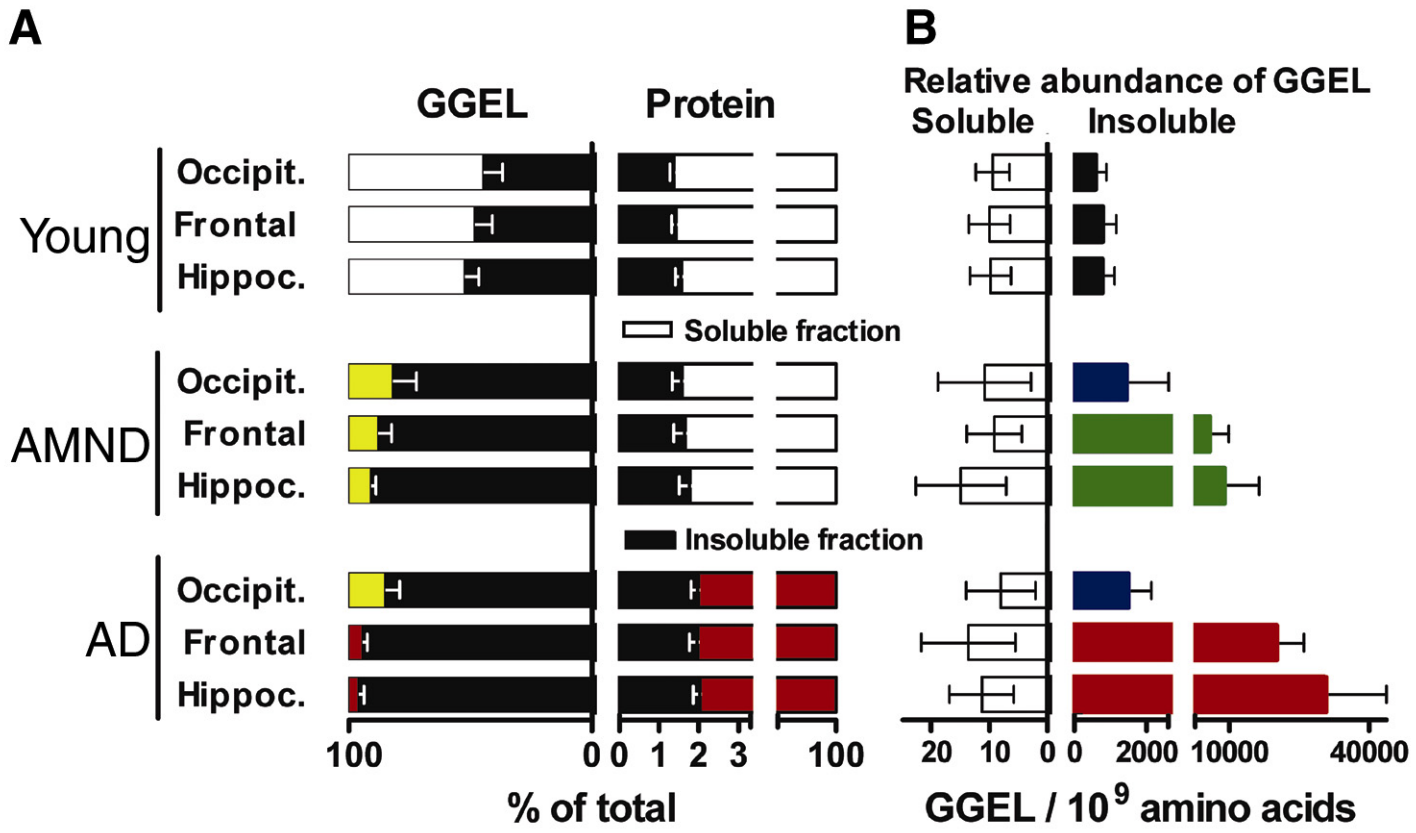

Figure 2. Higher frequency of cortical GGEL cross-links in AD and old-age subjects confined to the guanidine thiocyanate-insoluble protein fractions. GGEL and amino acid content was determined separately from the guanidine thiocyanate-soluble and insoluble proteins, as in **Fig. 1**. ***A*)** Amino acid mass and GGEL cross-links in the insoluble (resistant to phenolic guanidine HCSN lysis), and soluble fractions of proteins from the various brain cortex regions indicated. ***B*)** Calculated frequency of GGEL cross-links in the insoluble and soluble fractions of brain cortex proteins. The frequency of GGEL cross-links in the various soluble proteins is not statistically different either between brain regions or between the young, AMND and AD brains. Abbreviations as per **Fig. 1**. Error bars represent the mean ± S.D. of 5 independent measurements. Identical bar colors indicate that the differences between the data are not statistically significant ($P>0.05$).

Fig. 3

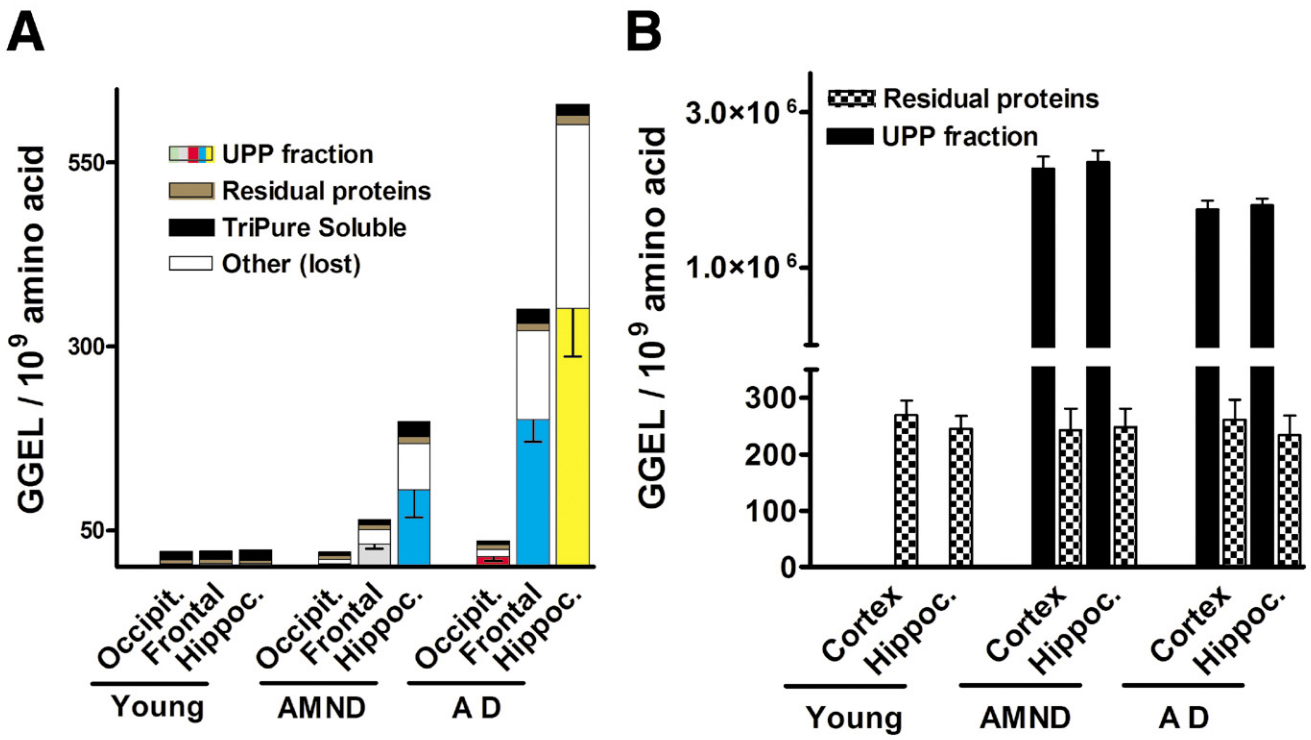

Figure 3. GGEL cross-links in AD and old-age subjects coimmunoseparated with ubiquitylated protein particles (UPP) form insoluble brain proteins. The insoluble particulate fraction remaining after exhaustive chaotropic extraction of brain proteins was immunoseparated by ubiquitin antiserum to ubiquitylated and residual fractions, as described under Experimental Procedures. ***A*)** Distribution of the total tissue GGEL content between solubilizable, UPP and other fractions. No detectable UPP fraction was obtained from insoluble proteins of middle-aged (young) cortex. Identical colors of the bars indicate that the differences between the data are not statistically significant ($P>0.05$). ***B*)** Relative abundance of GGEL cross-links in the UPP and residual particulate fractions. Abbreviations as per **Fig. 1**. Stacked bars represent the means of 3 determinations. Error bars represent the mean ± S.D. of 3 independent measurements.

Fig. 4

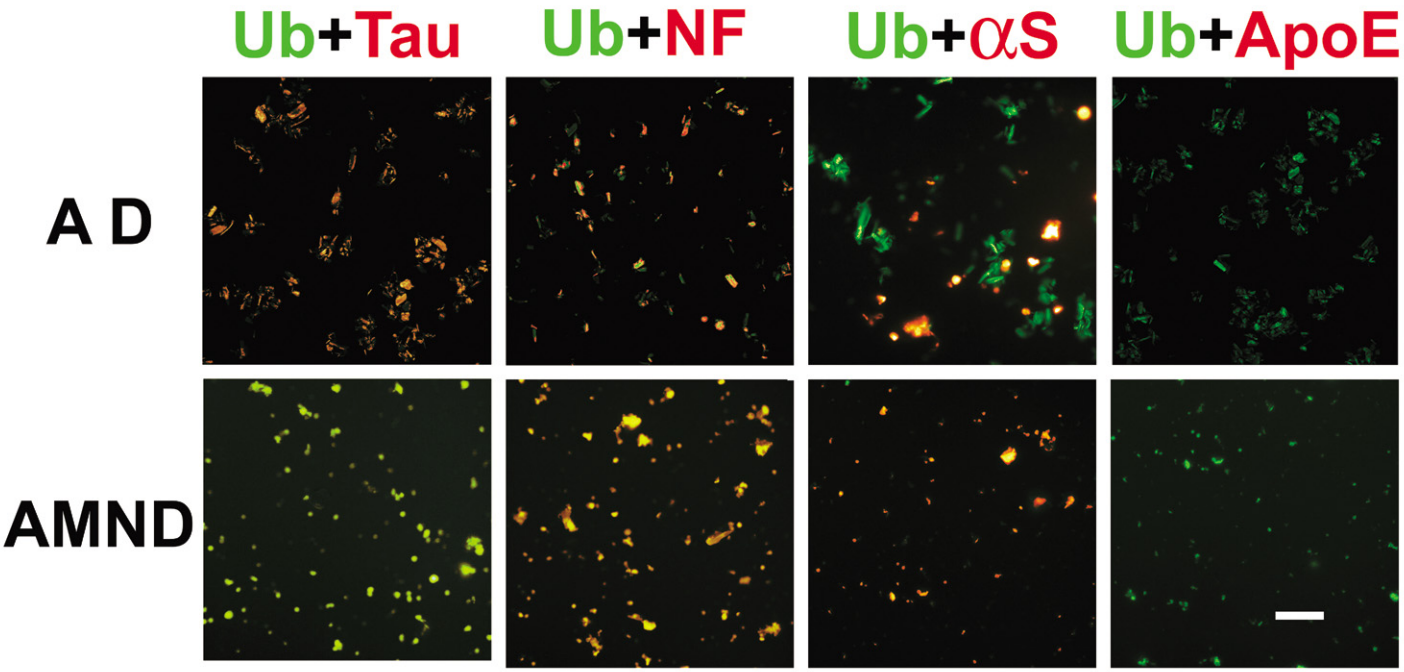

Figure 4. Immunostaining of ubiquitin-immunocaptured insoluble protein particles (UPP) from AD and senile hippocampi. UPPs were coimmunostained with antiubiquitin and tau, neurofilament, α-synuclein, and ApoE antiserum, respectively. Absence of immunopositivity is indicated by the green color (ubiquitin only), whereas positive staining is indicated by yellow (green+red) color on merged images. UPP from AD brain contain bigger, elongated, angular structures; those from elderly controls are smaller and rounded off. UPP from both sample groups reveal tau, neurofilament, but not apoE antigens. Immunostaining of bigger and elongated particles is markedly weaker for α-synuclein. Similar results were observed with UPP from neocortical specimen. Bar: 4 μm.

Fig. 5

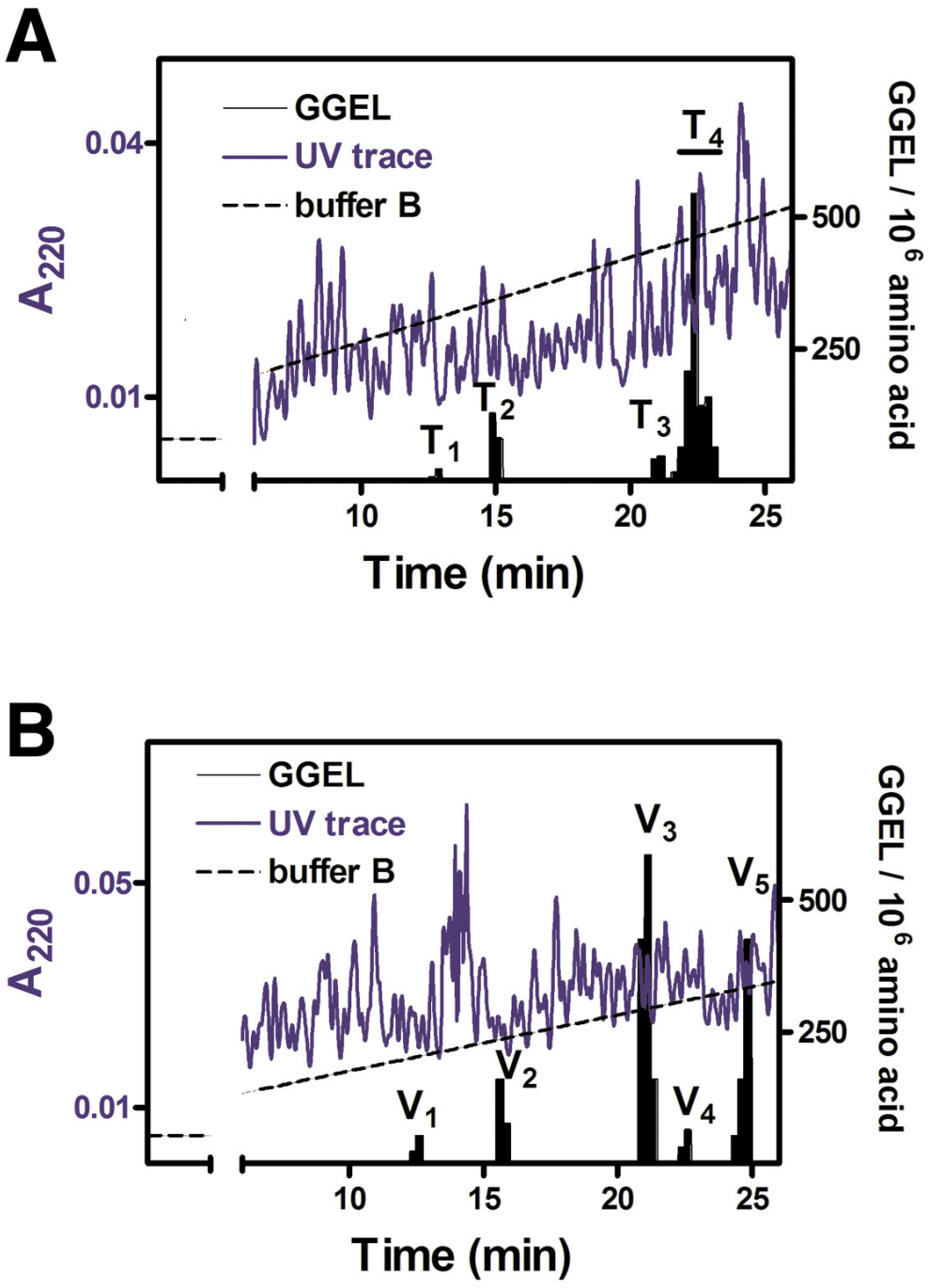

Figure 5. Cross-linked tryptic and V8 peptides from UPP eluted by reverse-phase HPLC in distinct time fractions. UPP (10 μg) from AD brains were cleaved into soluble fragments by trypsin (***A***) and V8 proteinase (***B***), and fractionated by C_{18} reverse-phase adsorption chromatography. Elution of GGEL-containing peptides was assessed from 30 s eluate fractions. GGEL cross-linked peptides eluted from the column in four distinct fractions for the tryptic peptides and in five fractions for the V8 peptides. The relative abundance of different cross-linked sequences was calculated by the distribution of total GGEL between the fractions.

Fig. 6

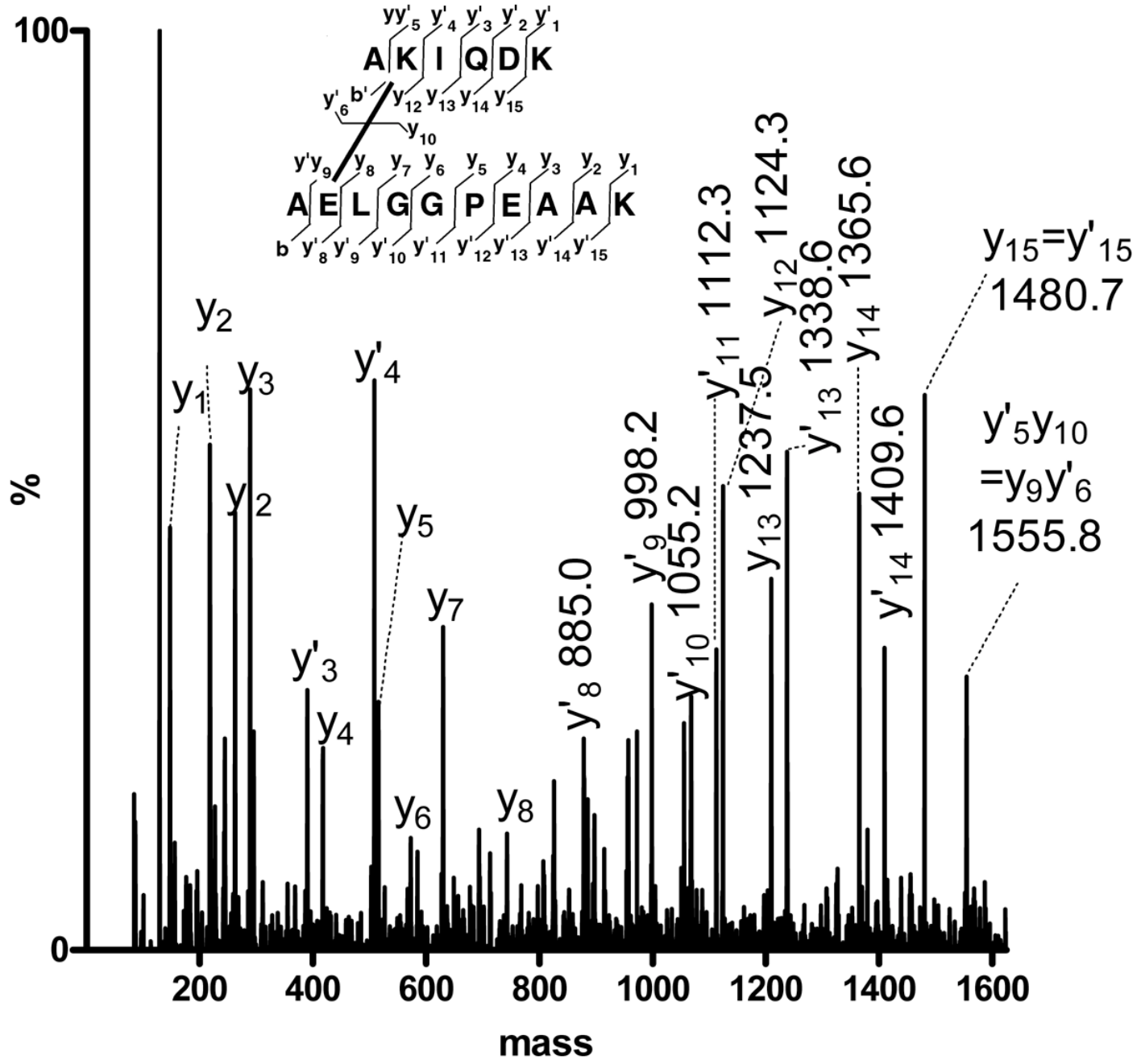

Figure 6. Tandem mass spectrum of a GGEL cross-linked tryptic peptide pair. The peptide bonds fragment into two charged C-terminal ions (y and y′) yielding sequence information preceding and following the GGEL cross-link (y_{10} and y'_6 ions are not present). The sequence concerned is mentioned on top, giving the expected fragmentation pattern according to the nomenclature of Roepstorff (63). Deconvoluted masses are shown.

Fig. 7

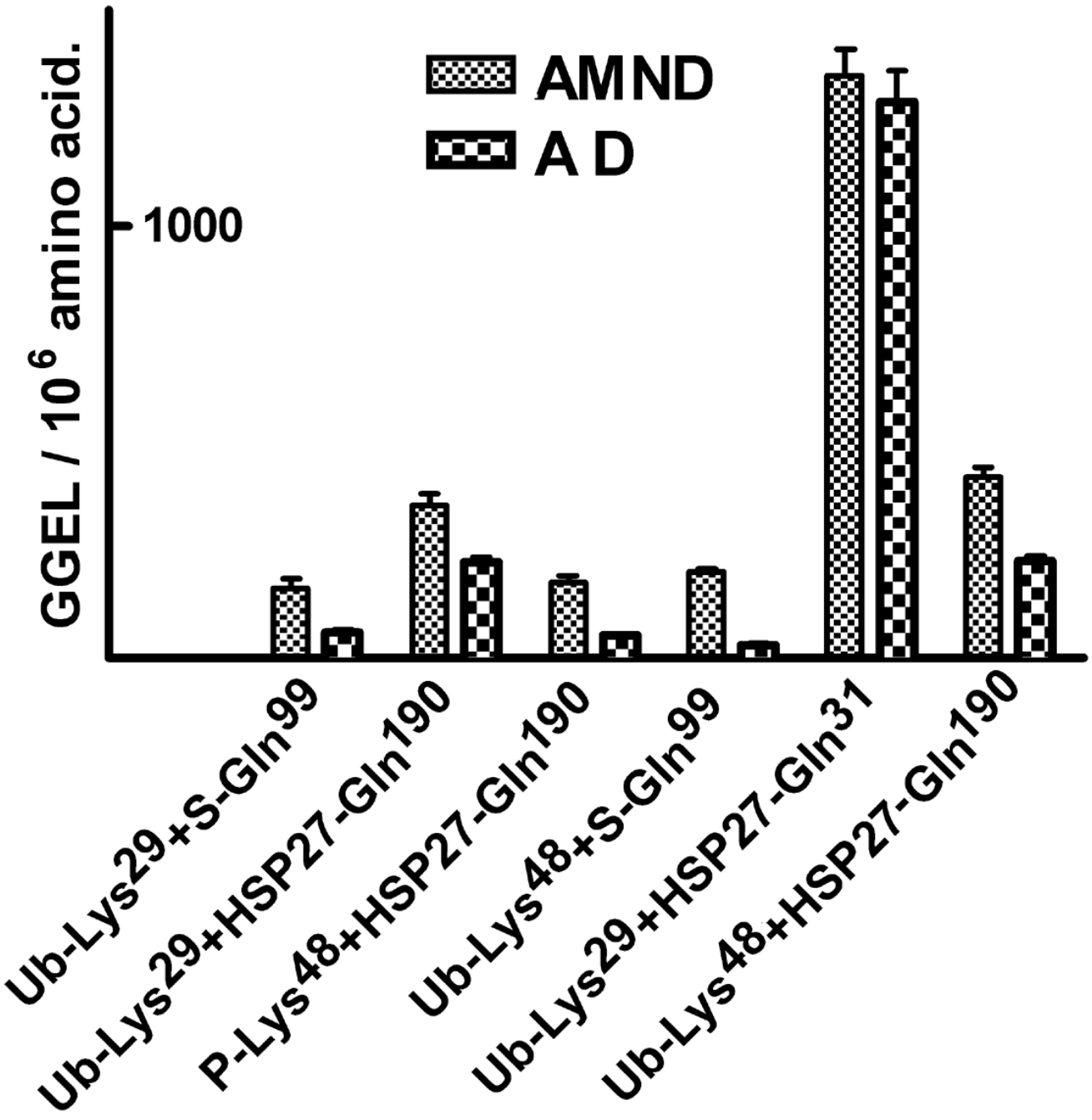

Figure 7. The relative abundance of GGEL cross-linked substrates is different in UPP from AD and senile brains. Relative ratios of transglutaminated residue-pairs was calculated from the cross-link content of fractions separated by HPLC (of **Fig. 5**) after identification of the coeluting sequence pairs by mass sequence analysis. Ubiquitin (Ub)+HSP27 cross-links are more frequent in AD, whereas UPP from AMND controls harbor more cross-linked parkin (P) and α-synuclein (S) ($P<0.05$). Other abbreviations as per **Fig. 1**. Stacked bars represent the means from 3 independent determinations.

Fig. 8

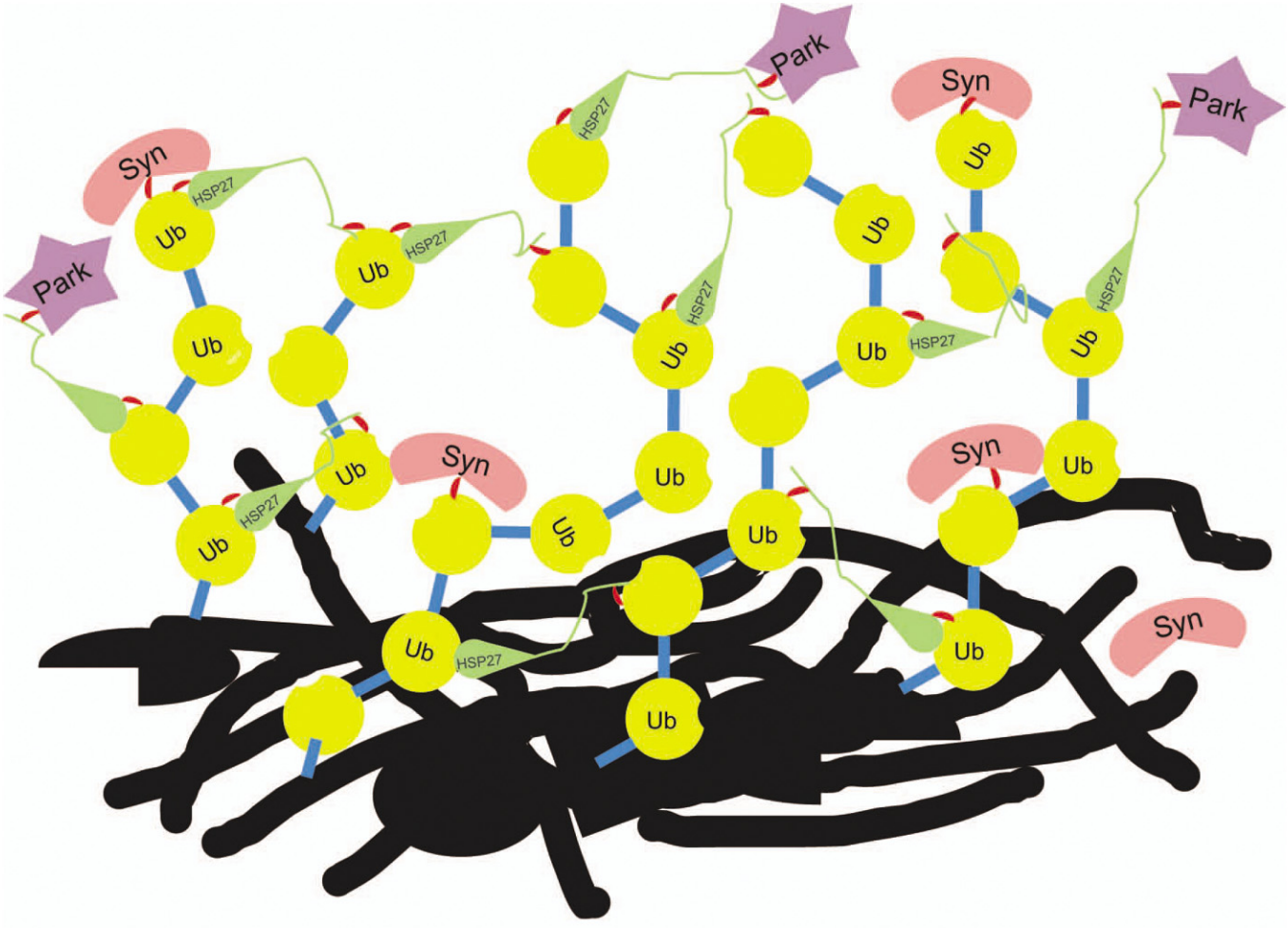

Figure 8. Model for the assembly of intraneuronal protein aggregates. Unfolded and aggregated waste proteins get ubiquitylated by N^{ε}(α-glycyl)lysine isopeptide bonds (blue), and ubiquitin (yellow) is assembled into polyubiquitin chains by similar bonds. Transglutamination forms GGEL isopeptide bonds (red) between two polyubiquitin chains through means of HSP27 protein (green). The monovalent cross-linking partners parkin (lilac) and α-synuclein (pink) act as chain terminators and limit the size of the isopeptide-bonded meshworks. Lower abundance of chain terminator proteins may result in the assembly of larger aggregates in AD.