In order to understand the mechanism for insoluble neurotoxic protein polymerization in Alzheimer's disease (AD) brain neurons, we examined protein and gene expression for transglutaminase (TGase 2; tissue transglutaminase (tTG)) in hippocampus and isocortex. We found co-localization of tTG protein and activity with tau-positive neurofibrillary tangles, whereas mRNA and sequence analysis indicated an absolute increase in tTG synthesized. Although apoptosis in AD hippocampus is now an established mode of neuronal cell death, no definite underlying mechanism(s) is known. Since TGase-mediated protein aggregation is implicated in polyglutamine ((CAG)_n) mediated protein aggregation is implicated in polyglutamine ((CAG)_n) expansion) disorder apoptosis, and in neuronal tau aggregation in Alzheimer's disease.

Intron-exon swapping of transglutaminase mRNA and neuronal tau aggregation in Alzheimer's disease

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In order to understand the mechanism for insoluble neurotoxic protein polymerization in Alzheimer's disease (AD) brain neurons, we examined protein and gene expression for transglutaminase (TGase 2; tissue transglutaminase (tTG)) in hippocampus and isocortex. We found co-localization of tTG protein and activity with tau-positive neurofibrillary tangles, whereas mRNA and sequence analysis indicated an absolute increase in tTG synthesized. Although apoptosis in AD hippocampus is now an established mode of neuronal cell death, no definite underlying mechanism(s) is known. Since TGase-mediated protein aggregation is implicated in polyglutamine ((CAG)_n) expansion) disorder apoptosis, and expanded Q non-repeats are excellent TGase substrates, a role for TGase in AD is possible. However, despite such suggestions almost 20 years ago, the molecular mechanism remained elusive. We now present one possible molecular mechanism for tTG-mediated, neurotoxic protein polymerization leading to neuronal apoptosis in AD that involves not its substrates (like Q non-repeats) but rather the unique presence of alternative transcripts of tTG mRNA. In addition to a full-length (L) isoform in aged non-demented brains, we found a short isoform (S) lacking a binding domain in all AD brains. Our current results identify intron-exon “switching” between L and S isoforms, implicating G-protein-coupled signaling pathways associated with tTG that may help to determine the dual roles of this enzyme in neuronal life and death processes.

Transglutaminases (TGases, EC 2.3.3.13) are a gene family of transamidinating enzymes that, under the influence of calcium, catalyze protein cross-linking through acyl transfer of specific glutamine residues to lysines. These enzymes are involved in a variety of key metabolic processes that range from blood coagulation to cell death. Expression for the most ubiquitous intracellular member, tissue TGase (tTG), is highly regulated. It is induced in cultured cells by various agents including cytokines, such as interleukin-6 (IL-6) (1, 2), cyclic AMP (3–5), activation of the transcription factor, NFκB (6–8), and DNA methylation (9). The most potent inducers of tTG gene expression are retinoids (10–12), which also promote apoptosis in various cells (13, 14), including neurons (15–17).

In this context, the history of tTG in Alzheimer's disease (AD) pathogenesis began almost 20 years ago with the report that brain tTG catalyzed cross-linking of neurofilament molecules (18). A decade later, Aβ peptide (19, 20) as well as the β-amyloid precursor protein (21) were shown to be cross-linked by tTG. Subsequently, tTG protein was demonstrated within amyloid plaques in AD brains (22). By using immunohistochemistry and an antibody to coagulation factor XIII, an extracellular TGase that cross-reacts with tTG, co-localization with paired helical filaments, the major components of neurofibrillary tangles (NFTs) in AD neurons were reported further suggesting a role for tTG in AD pathogenesis (23). Consistent with this notion, the phosphorylated microtubule protein, tau, a molecular component of NFTs and paired helical filaments, associates with tTG to form insoluble filaments (24). In recent studies, tTG activity was found to be increased in the most severely affected brain region in AD, the hippocampus (25).

In the last few years, tTG was shown to be a bifunctional enzyme, a G-protein possessing GTPase activity (26, 27) capable of binding GTP (and ATP) (28–30), in addition to its cross-linking activity. This also suggests dual roles in programmed cell death as well as death. GTP binds to the COOH-terminal of the full-length protein (27–32), which is designated Gαt/TG. An important discovery was the appearance of an alternatively spliced short (S) form lacking the GTP-binding site when rat brain astrocytes in culture were treated with the cytokines interleukin 1β (IL-1β) or tumor necrosis factor-α. With translation of this S form, cross-linking activity would no longer be negatively regulated by GTP (33). This loss-of-function might impact on AD and other neurodegenerative diseases, where apoptosis is prominent (34–37). This finding led us to consider first whether alternatively spliced variants were produced in response to injury. We have found evidence of such alternative transcripts in rat spinal cord within the first 8 h after controlled contusion injury (2). We reasoned that if injury could rapidly induce S isoforms that are more associated with apoptotic cell death, a search for

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∥∥∥ The abbreviations used are: TGase, transglutaminase; tTG, tissue transglutaminase; NFT, neurofibrillary tangle; IL, interleukin; AD, Alzheimer's disease; NFTs, neurofibrillary tangles; DAB, diaminobenzidine; FITC, fluorescein isothiocyanate; qRT-PCR, quantitative reverse transcription coupled to polymerase chain reaction; HEL, human erythrocytes; RT-PCR, reverse transcriptase-polymerase chain reaction.

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similar splice variants in neurodegenerative diseases might be revealing. Therefore, we examined cortical and hippocampal regions of AD patients and non-demented matched controls for tTG, isodipeptide bonds, TGGase enzymatic activity, and gene expression for the full-length tTG gene (TGM2) in parallel with tau in NFTs. Finally, using specific oligonucleotide primers, we determined which mRNA transcripts were produced in AD, and we compared these with non-demented, aged brains. Our results confirm several recent reports that TGase enzymatic activity is increased in hippocampal regions of neuropathologically confirmed AD brains (25, 39). We further found that increased activity correlates with increased expression of TGM2 in these same brain regions. Finally, we report the novel finding in human tissue that alternative mRNA processing for TGM2 occurs in AD. This alternative splicing is accompanied by protein precipitation in the form of insoluble inclusions, in situ isodipeptide bond formation, and increased TGase activity levels.3

**EXPERIMENTAL PROCEDURES**

**Materials—**Brain samples were obtained from the Alzheimer’s Disease Center Neuropsicmen Brain Bank at the University of Kansas Medical Center. All AD brains came from patients with the clinical, ante-mortem diagnosis of AD. The 9 AD brains used in these studies ranged in age at death from 69 to 93 years, except for one 51-year-old patient with both trisomy 21 and AD. Numerous neuritic plaques and NFTs were identified in modified Bielschowsky-stained sections of cortex as well as hippocampus. The neuritic plaques were in sufficient quantities for the diagnosis of AD using Consortium to Establish a Registry for Alzheimer’s Disease criteria (40, 41). Controls came from age-matched, non-demented individuals. All samples for biochemical studies had been rapidly frozen in liquid nitrogen within 8 h of death and preserved at −70 °C. Sections for immunohistochemistry were processed routinely with formalin fixation and paraffin embedding. Oligonucleotide primers were synthesized at the University of Kansas Medical Center Biotech support facility. Bicinchoninic acid kit was purchased from Pierce. Guinea pig liver tTG, N,N-dimethylcasein, and dianisobenzofuran (DAB) chromogen were bought from Sigma. [3H]Putrescine (7.77 TCi/mmol) was obtained from New England Nuclear. Trizol reagent was bought from Life Technologies, Inc. PCR mix was from PerkinElmer Life Sciences; reverse transcriptase, Superscript II, was from Life Technologies, Inc. Antibody to tTG was purchased from the Neomarkers Division of Lab Vision Corp. (Fremont, CA). Rabbit anti-human tau polyclonal antibody was from Dako (Carpenteria, CA) as was a labeled streptavidin-biotin kit used for visualization. Monoclonal antibodies recognizing the (g-glutamyl)lysine isodipeptide bonds produced by the action of TGase activity (42) was 811-MAG purchased from Covabla (Lyon, France).

**Analysis of AD Versus Control Brain tTG Immunocytochemistry—**Both frozen and fixed tissue reacted equally well with tTG and isodipeptide antibodies. The results and photomicrographs reported here are from formalin-fixed and paraffin-embedded tissue that were deparaffinized and microwaved in citrate buffer for antigen retrieval. For double labeling experiments, the sections were formalin-fixed and embedded in paraffin. The primary antibodies were applied sequentially; the first antibody was a rabbit anti-human tau, made against four repeated sequences involved in microtubule binding from the COOH-terminal of the human tau protein (43). The secondary antibody was a biotinylated anti-rabbit IgG, and this was followed by streptavidin conjugated to alkaline phosphatase and revealed by Fast Red as chromogen for visualization. The TGGase antibody was a mouse monoclonal antibody made against purified guinea pig liver TGase 2. A secondary biotinylated anti-mouse antibody was followed by streptavidin labeled with horseradish peroxidase. The DAB chromogen used for horseradish peroxidase visualization gave a brown precipitate.

Fluorescence was performed with 7-μm formalin-fixed, paraffin-embedded sections. Mouse monoclonal anti-tTG was used at 1:100 dilution and visualized with goat anti-mouse secondary antibody conjugated to CY3 (42, 44). Fluorescence was captured on a Nikon Eclipse TE-300 fluorescence microscope linked to a Bio-Rad MicroRadiance Plus confocal system utilizing HyQ filters for direct observation on the microscope and 543, 588, and 678 nm laser lines utilizing control software for CY3 and FITC, respectively. In some experiments, stained sections were also viewed on a Leitz Orthoplan microscope under epifluorescence using Ploem filters. Double-stained sections were photographed on a Nikon UFX automatic camera and digitized, and images were collected and analyzed on a Macintosh G3 computer using Adobe Photoshop 4.0.

**Brain tTG Assay—**Brain tissue samples weighing 35–55 mg were homogenized in 10 μl of 10 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose, 1 mM EDTA, 4 mM dithiothreitol, 125 mM potassium thiocyanate, 1% Lubrol-PX, with a Polytron (Brinkman, Westbury, NY) at 4 °C 3 times at 5-s bursts. Protein concentrations were determined with a micro-bicinchoninic acid assay (45). TGase activity was determined by a modified assay (46, 47) in which brain protein extracts (50-μl aliquots) were added to 0.7 μl of 50 mM Tris-HCl, pH 7.4, containing 1.3 mM CaCl2, 10 mM dithiothreitol, 5 mM MgCl2, N,N-dimethylcasein, 0.4 mM [3H]Putrescine and incubated for 150 min at 37 °C, after confirmation of linearity over the period. Guinea pig liver tTG was used for the standard curve. The incorporated putrescine was measured by precipitation with 20% trichloroacetic acid, resuspension in 10% TCA, and scintillation counting of triplicate samples in a Packard 2200CA Tri-Carb Liquid Scintillation Analyzer. Nonspecific reactions were included reactions without substrate, and blanks were without enzyme source.

**mRNA Analyses—**RNA was extracted (48) with denaturing phenol (Trizol reagent). Tissue was powdered under liquid N2 and homogenized on ice with a Polytron. Nucleoprotein complexes were dissociated at room temperature for 5 min and then 0.2% volume of chloroform was added, and the solution was first agitated for 15 s and then incubated at room temperature for 3 min. The aqueous phase, obtained after centrifugation at 15,000 × g for 15 min at 4 °C, was precipitated with 2-propanol, washed with 75% ethanol, and resuspended in H2O. The RNA concentration was determined by absorbance at 260 and 280 nm and electrophoresis in formaldehyde-agarose gels.

**RNA Extracts were reverse-transcribed to produce cDNA with the downstream primer (5'-TGGTAGATGAGGCCTGTGTTG). These were performed in 20-μl reactions with 1 μg of total brain RNA, 1 μM dNTPs, 1× PCR mix, 10 pmol of primer, and 200 units of reverse transcriptase (Superscript II). Primers were annealed at room temperature for 10 min followed by cDNA synthesis at 42 °C for 30 min, then extended at 95 °C for 1 min and then quick cooled. The DNase was amplified in PCR brought to 100 μl containing a total of 50 pmol of each of the upstream (5'-GCTGCTCCTGAGAGGTTG) and downstream primers and 200 μM dNTPs. The reactions were first denatured at 98 °C for 10 min, cooled to 90 °C for addition of 2.5 μl Taq polymerase (AmpliTaq, PerkinElmer Life Sciences or Biolase, Bioline), and followed by 30–55 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 45 s. After amplification, 10 μl were visualized on a 1.5% agarose gel resol fluorescently labeled and quenching dye-labeled internal oligonucleotides by the PCR amplification and a real time system (ABI Prism 7700, Applied Biosystems, Foster City, CA) used according to manufacturer’s recommendations.

**tTG mRNA Sequences—**Amplifications with primers spanning the entire coding region as well as primers directed toward the COOH-terminal region were employed to analyze for alternative tTG trans- scripts in AD versus non-demented control brain mRNA. The primers utilized in these experiments included the upstream primer (5'-GGAGCGATTATCCACAG) and the downstream primer (5'-GGGTTATAAAATGGAGGACG). The RT-PCR amplifications were performed as described above.

**Amplimer Sequencing—**To identify unambiguously the potential alternative tTG transcripts, we isolated RT-PCR products, removed primers with two ethanol precipitations, and sequenced these on an ABI fluorescent automated sequencer, Prism model 377. Nested primers were included for the sequencing reactions, or RT-PCR products were subcloned into pCRII utilizing the TOPO T/A cloning kit (Invitrogen, Valencia, CA) according to the manufacturer’s directions, and plasmids were purified with a spin column miniprep procedure (Qiagen, Carlsbad, CA). Data were examined with Applied Biosystems Editview soft-

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RESULTS

\textbf{tTG Immunolocalization in Control and AD Brain—} To establish the distribution of tTG in our AD patient samples (ranging in age from 65 to 81, \( n = 5 \)), brain sections were characterized using tTG, and tau immunohistochemistry sections examined included the hippocampus at the level of the lateral geniculate nucleus, cingulate gyrus, superior temporal lobe, mid-frontal gyrus, and striate cortex. All of the AD patients showed severe NFT formation in all sections examined except the occipital cortex consistent with Braak and Braak stage 5 out of 6 (52). Using tau immunohistochemistry, NFTs were not seen in any location in the control brains. Frequent neuritic plaques were also present in all sections of cortex examined, and these were in sufficient quantities to confirm the clinical impression of AD using Consortium to Establish a Registry for Alzheimer’s Disease criteria.

With tTG immunohistochemistry, staining of blood vessels was present in all brains; of interest, vascular staining was less prominent in AD brains. In stark contrast, neuronal staining was only seen in the AD brains. The most obvious tTG-positive staining was present in the hippocampus and adjacent regions (subiculum, transentorhinal, and entorhinal cortex). In addition, with tTG antibody many pyramidal neurons showed fine granular staining, whereas a minority showed additional weak diffuse staining. Isocortical neuronal tTG staining was also present in each AD brain examined, and these were in sufficient quantities to confirm the clinical impression of AD using Consortium to Establish a Registry for Alzheimer’s Disease criteria.

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Fig. 1 shows that hippocampal brain regions of AD patients contained significant numbers of diffuse and neuritic plaques, numerous intraneuronal NFTs (Fig. 1, C and D, small arrows), significant cell loss, and gliosis. Antibody to tTG, visualized by the brown DAB stain, was detected in both hippocampal regions (Fig. 1, C and D, arrowheads) as well as in the cortex (Fig. 1, A and B, arrowheads). Differences in tTG immunoreactivity between AD and control brains were detected, since both endothelial cells (Fig. 1A, linear small arrows) and neurons stained in AD, whereas staining was exclusively in endothelial cells in control brains (not shown).

\textbf{tTG Is Associated with Cross-links in Tau-Positive Brain Regions—} The next important question concerned whether tTG was functioning as a cross-linking agent at these brain locations. As evidence that the localized tTG in these AD neurons was active in situ to cross-link one or more neuronal proteins, novel immunohistochemical evidence of isodipeptide bond formation was detected in regions showing increased immunoreactive tTG (Fig. 2A). As found with tTG, in some neurons isodipeptide immunoreactivity was in the form of diffuse neuronal staining (Fig. 2A, arrowhead), but in other hippocampal neurons granulovacuolar material was stained (Fig. 2A, arrows). Significantly, in no non-demented brain could significant anti-isodipeptide bond staining be found (Fig. 2B).

As shown in the figure, double labeling enabled the determination that isodipeptide bonds were co-localized with intraneuronal inclusions (NFTs). To confirm this further, we examined fluorescently labeled samples with laser scanning confocal microscopy. Through fluorescent staining techniques, we were also able to confirm co-localization (Fig. 3) of isodipeptide bonds (Fig. 3D) with tTG antigen (Fig. 3C), and these occurred along with intraneuronal inclusions (NFTs, Fig. 3, A and B, arrows) in these tau-positive regions.

\textbf{Increased tTG Cross-linking Activity in AD Brains—} In support of these immunocytochemical results, we found that tTG cross-linking enzymatic activity was increased in hippocampal regions of AD patients (53, 54) (Fig. 4), confirming a previous report (25). Enzymatic activity was increased 1.5–8 fold in these AD brains compared with non-demented controls (Fig. 4), with a statistically significant p value (<0.04). Of interest, relative to unaffected AD brain regions, AD brain hippocampus and cortex had elevated tTG activity by a factor of 3.5 ± 1.2.

\textbf{tTG Gene Expression in AD Brains—} It still remained to be shown to what extent alterations in tTG expression would account for the elevated cross-linking activity. Activity measurements cannot distinguish among the genes now known to transcribe proteins with TGase activity (55). This is particu-
A man microscopy (Hoffman) microscopy (A and B), visible neurons containing NFTs (arrows) stained positively with both anti-tTG (visualized with FITC, C) and anti-isodipeptide (visualized with Texas Red, D)-conjugated secondary antibody under confocal microscopy.

**Fig. 3.** Anti-tTG and anti-isodipeptide bond immunostained AD tissue from the cortex area. By using modulation contrast (Hoffman) microscopy (A and B), visible neurons containing NFTs (arrows) stained positively with both anti-tTG (visualized with FITC, C) and anti-isodipeptide (visualized with Texas Red, D)-conjugated secondary antibody under confocal microscopy.

Newly relevant given the recent report of TGase 1 and 3, in addition to tTG, in human brain (39). Consequently, we employed specific primers to examine message corresponding to TGase C/TGase 2 (intracellular tTG), encoded by the gene TGM2 (56), and confirmed the amplified products by DNA sequence analysis. Brain cortical RNA extracts were examined by RT-PCR analysis (Fig. 5), and appropriately sized products were obtained corresponding to tTG. Although intended to detect both long (L) and short (S) isoforms of tTG (Fig. 7), with these human-specific primers, we analyzed the products derived from cortical samples of non-demented and AD brains. In this manner, we could only detect long form in non-demented brain RNA. In contrast, in AD brain samples we were able to detect both long (L) and short (S) isoforms of tTG (Fig. 7), with tTG-specific primers spanning the COOH-terminal region of human tTG. This intriguing result suggested that like traumatic central nervous system injury, neurodegeneration induced tTG alternative transcription.

**Fig. 4.** tTG activity measurements in human brain samples. AD and age-matched control brain samples (A–D) were obtained and assayed for tTG activity as described under “Experimental Procedures.”

Real Time Quantitative RT-PCR (qRT-PCR)—With the electrophoretic analyses of mRNA levels indicating significant increases in the AD brains, we then determined the quantitative changes in tTG with a precise system monitoring tTG sequence present at every cycle. With the ABI Taqman system, we used two human tTG RT-PCR primers with an internal human tTG primer conjugated to a fluorescent probe. Included in the reaction was a quenching molecule such that extension of template releasing it and resulting in a fluorescent signal proportional to the amount of template present at each cycle of amplification. In this manner, tTG mRNA levels were determined for RNA extracts from the frontal cortex for three age-matched controls and four AD brains. The relative tTG mRNA per total RNA present was normalized to the non-demented aged control samples giving them a value of 1.0 ± 0.16 (S.D.), and the tTG mRNA levels were elevated 4.1 ± 1.31-fold (p = 0.011, Student unpaired t test) in this specific assay (Fig. 6).

**Fig. 5.** RT-PCR analysis of tTG mRNA in human cortex. Total RNA, from normal and AD samples in Fig. 2, was reverse-transcribed and amplified by PCR to subplateau levels with tTG-specific primers and separated by agarose gel electrophoresis (B) as under “Experimental Procedures.” Ratios between the products obtained produced relative tTG mRNA levels summarized in A.

**DISCUSSION**

We have demonstrated that tTG protein and activity accumulate with tau-containing NFTs in affected regions in AD brain. The evidence indicates that tTG is up-regulated and...
subsequently participates in neuronal apoptosis in AD not through its action on substrates (like Qm repeats), but rather through the unique presence of alternative transcripts of tTG mRNA. Analysis of RNAs from affected AD tissues demonstrates that elevated levels, including intron/exon switching that produce a shortened S isoform of tTG, are commonly found. In contrast to AD brain, S isoform message for tTG is not found in aged, non-demented human brain to any extent. The lack of a GTP binding domain on the S isoform implicates it as a neuron-specific variant, as we demonstrated in earlier reports of the presence of isodipeptide bonds in AD brains (61). TGase enzymatic activity was reported elevated in AD brain (25). At what level transcriptional or posttranscriptional regulation of increased tTG activity occurred in AD pathogenesis was not addressed in those studies.

Previous studies have established that tTG is capable of cross-linking neuronal proteins such as NFs (18), Aβ peptide (19), β-amyloid precursor protein (21), the non-amyloid component, α-synuclein (58), and tau (24), all implicated in the pathogenesis of AD and other neurodegenerative diseases. Furthermore, using an antibody to factor XIII, an extracellular TGase that antigenically cross-reacts with TGase 2 (tTG), tTG was detected in hippocampal neurons in AD but not control brains (23), and TGase enzymatic activity was reported elevated in AD brain (25). At what level transcriptional or posttranscriptional regulation of increased tTG activity occurred in AD pathogenesis was not addressed in those studies.

Our novel results with isodipeptide bonds in the human brain build on earlier reports of the presence of isodipeptide bonds in situ in cultured neurons (59), as well as in mutant rodent central nervous system (60). Relevant also to our current AD results, a recent report showed that patients with progressive supranuclear palsy also express tau proteins in brain with significant tTG-catalyzed isodipeptide bonds (61). Furthermore, insoluble tau found in the AD brain also harbor these isodipeptide bonds, and human tau can be cross-linked by tTG in vitro (62, 63). The detection of these γ-glutamyl-e-lysine cross-links represents unambiguous evidence for the in situ activity of one or more TGases in the tissue where they are found (42, 64). Our present results also confirm that tTG-specific (using an anti-TGase 2 antibody, distinct from factor XIII) immunoreactivity is localized to neurons in hippocampus and cortex (23). We also corroborate that TGase cross-linking activity is increased, from 2- to 8-fold, in extracts of affected brain regions from AD patients but not in non-demented brains (Fig. 4). In this context, we found that tTG was one of the TGases increased since we found a semi-quantitative increase in specific TGase 2 (tTG) mRNA (Fig. 5) using specially designed primers. These results suggest that regulation of tTG in AD brain was pretranslational. Real-time qRT-PCR confirmed this result, as we identified a 4-fold increase (Fig. 6) in tTG copy number per μg of RNA.

Thus, TGase 2/tTG gene expression is clearly increased in brain regions affected by the brunt of the disease process in AD. In support of this finding, a recent report indicated differential expression of TGases 1–3, with increased expression for both TGase 1 and 2 (tTG) in AD brain (39).

What might be the basis for this up-regulation? Although experiments to address this in AD are in progress, it is known that retinoic acid up-regulates tTG, acting through one of several retinoic acid receptors and response elements (3, 11, 12, 65, 66). Other transcription factor recognition sites are present in the promoter regions such as AP-1 and SP-1 (67). In development, Tgm2 expression appears to be essential for “appropriate” apoptosis (68–72). Since apoptotic neuronal loss has been documented in AD and related disorders (34–37), “inappropriate” AD-associated apoptotic neuronal death may be due, at least in part, to up-regulation and increased activity of tTG (73). It might also relate to changes in the dual function of the tTG/Gαi2 subunit. In this regard, the “switch” from GTPase to unregulated cross-linking activity (29) may be critical in nervous system degeneration (33).

The dual enzymatic functions of tTG may be essential to its divergent action in cellular life as well as death (26, 28–30, 44, 74, 75). GTP-independent tTG may be more involved with apoptotic neuronal death as evidenced from studies of trophic deprivation in primary rat forebrain cells in culture (44). When ligand is provided, coupling of the αi-adrenergic receptor to phospholipase C 81 (29, 76, 77) via Gαi2 and down-regulated cell activity. In contrast, when trophic ligand is deprived, a loss of peripheral distribution of tTG protein occurs with an increase in isodipeptide bonds, the in situ product of the TGase reaction in the cytoplasm of cells undergoing apoptosis (44). Suggesting that deprivation coincided with the onset of GTP-independent tTG activity, photoaffinity labeling revealed reduced binding to Gαi2/tTG in deprived cultures, whereas formation of inositol triphosphate and mobilization of [Ca2+]i were reduced. Thus, loss of Gαi2/tTG signal transduction during a period when cell survival is reduced following withdrawal of αi-adrenergic agonist supports the hypothesis that Gαi2/tTG might represent a switch operating with either programmed cell life or death and that this might result from altered synthesis of mRNA transcripts. The switch to S isoform might reflect a reduction in regulation that takes place at the level of transcription.

Such evidence for alternative tTG transcripts as we present here has not previously been detected in human tissue. In fact, our report of S form in injured spinal cord is the first example in vivo. However, a TGase homologue was earlier described in HEL cells (79), and then a third was reported (57), which also was found to hydrolyze GTP. The latter finding suggests that this form is more homologous to L than to S isoforms; although when expressed in E. coli, this homologue had a Kₘ value several orders of magnitude less than the L form for binding GTP (26). More importantly, the human AD tTG short form we have identified results from alternative splicing at precisely

![Fig. 6. Quantitative RT-PCR for cortical tTG mRNA levels in AD brains.](http://www.jbc.org/figure/6/) We quantitatively determined the increase in tTG message seen in AD relative to aged-matched controls as described under “Experimental Procedures.”

![Fig. 7. Variant TG messages present in AD brains.](http://www.jbc.org/figure/7/) By using human-specific primers spanning the GTP binding region, in TGM2 and designed to identify L and S isoforms, we examined TG messages present in normal and AD-affected brain samples by RT-PCR (RT), as described under “Experimental Procedures.” RNAs from AD brains contained greater amounts of tTG S form relative to L form when compared with non-demented aged control (Normal) brains.
the same intron/exon boundaries first seen in these HEL cells (79). This alternative message results from a substitution of intron X for exon 11 (80), a differential processing analogous to the alternate splicing seen in cultured rat astrocytes treated with either IL-1β or tumor necrosis factor-α (33) and similarly results in the loss of a GTP-binding domain (81). Thus, increased GTP-independent tTG might be involved with concomitant neuronal death via an apoptotic pathway in AD brains as it is after catecholamine deprivation in primary rat forebrain cells in culture (44).

Strong evidence implicates intraneuronal protein aggregation in the pathogenesis of the CAG (polyglutamine tract) repeat diseases, which like AD also exhibit late onset neuronal death (82, 83). These CAG diseases all involve specific gene products exhibit long polyglutamine repeats that are excellent substrates for tTG cross-linking (83–86). In Huntington’s disease, for example, neuronal nuclear inclusions are the result of elevated tTG and tTG-catalyzed aggregations as evidenced by tTG cross-links (87). Protein aggregation in neurodegeneration has recently been extended to include AD and related disorders in which long polyglutamine repeats are not present and in which cross-linking may be due to some other feature of the protein, such as point mutation, oxidative injury, or mis-folding and altered chaperone function (88–91) and/or, as we suggest, alternative tTG RNA processing.

The definitive test for the role of Gα0/tTG in neurodegeneration will likely come from total or tissue-specific conditional mutant (knockout) studies in mice. Cloning of the mouse Gα0/ tTG gene (Tgm2), homologous to TGM2 in humans, and coding for the dual-function protein that binds and catalyzes trans-gamma-glutamylcyclization (TGase) reactions has been accomplished (92). Amidation of glutamine residues have been accomplished (92). The definitive test for the role of Gα0/tTG in neurodegeneration will likely come from total or tissue-specific conditional mutant (knockout) studies in mice. Cloning of the mouse Gα0/tTG gene (Tgm2), homologous to TGM2 in humans, and coding for the dual-function protein that binds and catalyzes trans-gamma-glutamylcyclization (TGase) reactions has been accomplished (92). Amidation of glutamine residues have been accomplished (92). However, publication of Gα0/tTG knockout mice has not yet appeared. On the other hand, mice null for the keratinocyte TGase (TGase 1), also recently found increased in AD brain (39), do not exhibit neurological abnormalities but do present severe epidermal deficiencies (93). In humans, celiac disease is a non-demented aged brain, is most dramatic in association with NFTs stained with tau in AD brains, and not at all in control brains. We have demonstrated for the first time that these tau-containing NFTs are the site of γ-glutamyl-ε-lysine cross-links in these same locations in brains from AD patients. This may be common to another rare neurodegenerative disorder, progressive supranuclear palsy, where a recent report shows these cross-links in NFT tau (61). A first clue to the marked increase in tTG activity is the unique identification in de-mented human brain of tTG messages with a swapped intron-exon. Since our sequence analysis confirms that the known inhibitory GTP binding domain in this protein is absent in this AD alternatively spliced form, this should serve as a potential molecular mechanism for production of neurotoxic aggregates and neuronal cell death characteristic of AD.

Conclusions—By analysis of protein immunoreactivity, enzymatic activity, and mRNA levels, we have shown that tTG is elevated in AD brains and co-localizes with tau in NFTs, prominent inclusions that are one of the hallmarks of neuronal degeneration. This elevation approaches 5-fold compared with non-demented aged brain, is most dramatic in association with NFTs stained with tau in AD brains, and not at all in control brains. We have demonstrated for the first time that these tau-containing NFTs are the site of γ-glutamyl-ε-lysine cross-links in these same locations in brains from AD patients. This may be common to another rare neurodegenerative disorder, progressive supranuclear palsy, where a recent report shows these cross-links in NFT tau (61). A first clue to the marked increase in tTG activity is the unique identification in de-mented human brain of tTG messages with a swapped intron-exon. Since our sequence analysis confirms that the known inhibitory GTP binding domain in this protein is absent in this AD alternatively spliced form, this should serve as a potential molecular mechanism for production of neurotoxic aggregates and neuronal cell death characteristic of AD.

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REFERENCES

![FIG. 8. Alternate splicing in AD brains.](image-url)