Transglutaminase Activity, Protein, and mRNA Expression Are Increased in Progressive Supranuclear Palsy

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Abstract. Transglutaminases catalyze the covalent cross-linking of substrate proteins to form insoluble protein complexes that are resistant to degradation. Our previous studies demonstrated that transglutaminase-induced cross-linking of tau proteins occurs in Alzheimer disease and progressive supranuclear palsy (PSP). The current study was designed to measure transglutaminase enzyme activity and the mRNA and protein levels of 3 transglutaminase isoforms that are expressed in human brain. Overall, transglutaminase activity was significantly increased in the globus pallidus (182% of control) and pons in PSP (171% of control) but not the occipital cortex (a region spared from pathology). Using a Spearman rank correlation test, we found that tissues with more transglutaminase-activity had more neurofibrillary tangles. Protein and mRNA levels of transglutaminase 1 were increased in globus pallidus of PSP as compared to controls. There were also significantly higher mRNA levels of the short form of transglutaminase 2 in globus pallidus of PSP (974% of control). Transglutaminase 1 mRNA and the long isoform of transglutaminase 2 mRNA (2212% of control) were significantly higher in PSP in the dentate of cerebellum. Together, these findings suggest that transglutaminase 1 and 2 enzymes may be involved in the formation and/or stabilization of neurofibrillary tangles in selectively vulnerable brain regions in PSP. These transglutaminases may be potential targets for therapeutic intervention.

Key Words: Cross-linking; Neurodegeneration; Progressive supranuclear palsy (PSP); Tauopathy; Transglutaminase.

INTRODUCTION

Progressive supranuclear palsy (PSP), also known as Steele-Richardson-Olszewski syndrome, is the second most common form of parkinsonian movement disorder after Parkinson disease (1). Clinically, PSP is associated with early postural instability and supranuclear vertical gaze palsy (2, 3). Neuropathologically, PSP is characterized by neuronal loss, gliosis, neuropil threads, tau immunoreactive astrocytes, and neurofibrillary tangle (NFT) formation (4–9). NFTs are composed of straight filaments or a mixture of straight filaments and paired helical filament-like structures containing tau protein (10). The mechanisms involved in the abnormal formation of tau filaments and NFTs are unclear. However, there is mounting evidence that transglutaminases are involved in the formation and/or stabilization of NFTs in tauopathies, including Alzheimer disease (AD) and progressive supranuclear palsy (PSP). Previous studies demonstrated that tau can be cross-linked by tissue transglutaminase (i.e. transglutaminase 2) in vitro (11–14). Appelt et al and Yamada et al demonstrated increased transglutaminase immunoreactivity and localization of transglutaminase with NFTs in AD (15, 16). Transglutaminase-induced cross-linking was observed in paired helical filament (PHF)-tau in AD and PSP (14, 17), suggesting that transglutaminase may be associated with the neurodegenerative processes characteristic of AD and PSP.

Transglutaminases are a large family of calcium-activated enzymes that catalyze the covalent cross-linking of peptide bound glutamine residues of proteins to the γ-amino group of lysine residues (18–23). Transglutaminases are responsible for a number of biological functions, including fibrin clot formation (24), ionophore-induced hardening of the erythrocyte membrane (25), and formation of cornified envelope of the epidermis and hair structure (26–29). In each of these examples, the transglutaminase-catalyzed cross-linking reaction results in the formation of protein polymers and renders these proteins insoluble and resistant to degradation (19).

Although transglutaminases share homologous sequences, they are derived from several distinct genes. There are at least 7 known transglutaminase isoforms (23), three of which are present in normal brain. The 7 known members of the transglutaminase family identified include transglutaminase 1 (also known as keratinocyte transglutaminase [TGK], 106 kDa, mostly membrane bound, widely expressed in the epithelia); transglutaminase 2 (cellular or tissue transglutaminase [TGC], 80 kDa, soluble/cytosolic, ubiquitously expressed); transglutaminase 3 (epidermal or hair follicle transglutaminase [TGE], 77 kDa, soluble, mostly in the epithelia); transglutaminase 4 (prostate transglutaminase, 80 kDa, soluble, mostly in the prostate); factor XIIIa (80 kDa, soluble, in the circulating blood (22, 30); band 4.2 (structural protein in...
some cells) (31–32); and transglutaminase X (expressed in the epithelia) (33). Recently, Aeschlimann et al suggested an additional form of transglutaminase in humans (transglutaminase Z) (34).

Transglutaminases have been found to be localized in a variety of tissues including the peripheral and central nervous system (35–39). Korner and colleagues previously reported transglutaminase activity in the peripheral nervous system in sympathetic postganglionic motor neurons (36). Increased transglutaminase activity was also found in sheath cells in the distal portion of peripheral nerves following nerve injury (40). In central nervous system, immunohistochemical studies by Thomazy and Fesus identified transglutaminase 2 in human brain (37). Immunohistochemical studies by Akiyama et al and Iwaki et al have shown factor XIIIa and transglutaminase 2 occur in human brain (41, 42). Also, Facchiano and his group reported presence of numerous bands in nervous tissue homogenates on the blots incubated with antitransglutaminase antibody (39); however, the types of transglutaminases present in those tissues have not yet been well described. More recently, Citron et al and Kim et al investigated the isoforms of transglutaminase enzyme expressed in human brain. They found that transglutaminase 2 enzyme was most abundant, but significant amounts of transglutaminase 1 and transglutaminase 3 were also present in the brain tissues. In addition, an alternatively spliced form of transglutaminase 2 mRNA was found in hippocampus and isocortex of AD cases, suggesting an additional subtype of the transglutaminase family (43). Previously, Monsonego et al (44) demonstrated the presence of 2 different types of transglutaminase 2 in rat brain astrocytes. The longer form had molecular mass of 77 kDa. The novel transglutaminase 2-like isoform encoded for an enzyme with molecular mass of 73 kDa. In this shorter isoform, the C-terminal region was replaced with a novel transglutaminase peptide sequence (44). Monsonego's results suggest that 2 forms of transglutaminase 2 resulted from differential splicing of the same gene.

To date, only 2 studies have examined specific isoforms of transglutaminases expressed in neurodegenerative disorders. First, Kim et al showed that the transglutaminase 1 and 2 are significantly up-regulated in AD in cerebellum and transglutaminase 2 was also increased in frontal cortex of AD cases (45). This region-selective activation of transglutaminases, in turn, might be due to differential availability of transglutaminase activators and substrates. Then, Citron et al found an alternatively spliced isoform of transglutaminase 2 in the cortex of AD patients (43). So far there have been no studies done to differentiate between the types of transglutaminases enzymes expressed in selectively vulnerable regions in PSP brain. Previously, we examined transglutaminase 2 protein levels in PSP and control cases and found no significant differences in the enzyme expression levels in globus pallidus and pons, selectively vulnerable brain regions of PSP, and controls (17).

The current study was designed to determine which transglutaminase isoforms, if any, are upregulated in selectively vulnerable regions in PSP in comparison to the normal controls. We examined several brain regions, including globus pallidus, pons, and the dentate of the cerebellum, which contain NFTs, and occipital cortex, a region spared from neurofibrillary pathology in PSP.

Since we are proposing that the transglutaminase-induced cross-linking of tau protein could lead to the development and progression of PSP, studies of transglutaminase isoform expression would be beneficial to a better understanding of the disease process. The appropriate transglutaminase isoform then may be a target for therapeutic intervention in diseases with neurofibrillary pathology such as PSP.

**MATERIALS AND METHODS**

**Human Tissue**

Fresh, frozen human brain tissue from brain regions with lesions, including globus pallidus, pons (tegmentum and basis pontis), dentate of the cerebellum, and a region spared from lesions, occipital cortex, was obtained postmortem from neurologically normal individuals (controls) and individuals with PSP. PSP cases were a generous gift from the Harvard University Brain Tissue Resource Center and control cases were obtained from the Loyola University Chicago Brain Bank. Tissue from the occipital cortex from PSP and control cases was obtained from Johns Hopkins University Brain Research Center. The diagnosis of PSP was made based on established clinical and neuropathological criteria at the time of autopsy (46). Globus pallidus, pons, cerebellum (dentate), and occipital cortex were examined to determine transglutaminase cross-linking activity and isoforms expressed in PSP and control cases. However, due to limitations in the availability of tissue, not all assays were performed for every brain region. All of the PSP cases are matched to normal controls with respect to the age and postmortem interval (PMI). The demographics of the PSP and matched control cases are as follows: the mean age of PSP individuals were 77.5 years for pons and 77 years for globus pallidus and dentate of cerebellum. The mean age of control cases was 88.8, 74.7, and 75.5 years for respective brain regions. The mean PMI of the PSP and control cases were 10.8 and 16 hours for globus pallidus, 15.2 and 16 hours for pons, and 13.6 and 16 hours for dentate of cerebellum, respectively. Tissues were stored at −80°C until use.

**Transglutaminase Activity**

Enzymatic activity of transglutaminase in pons and globus pallidus from PSP (n = 6 for pons and globus pallidus) and control cases (n = 5 and n = 4 for pons and globus pallidus, respectively) was determined using a modified version of methods previously published by Ando et al (47), Johnson et al (48), and others.
and Citron et al (43). We also measured transglutaminase activity in the occipital cortex from 8 PSP cases and 6 control cases. This method utilizes an exogenous protein acceptor (casein) and a radioisotopic polyamine donor (H-putrescine) to measure the amount of functional enzyme present (47). PSP and control brain tissue samples weighing 35 to 55 mg were homogenized in 2 ml of homogenization buffer (250 mM sucrose, 1 mM EDTA, 1 M NaCl, 50 mM Tris HCl, pH 7.4, 1% lubrol, protease inhibitors [10x cocktail, 1:1,000]) using a homogenizer. A particle-free supernatant was prepared from the homogenate by centrifugation at 17,500 g for 15 min at 4°C. Protein concentrations were determined with a Protein Assay Reagent, BCA (Pierce, Rockford, IL). Aliquots of tissue extracts containing 100 to 150 μg protein in 10 to 50 μl homogenization buffer were added to individual microfuge tubes containing 50 mM Tris-HCl, pH 7.4, 1.3 mM CaCl2, 10 mM dithiothreitol, 5.0 mg/ml N, N-dimethylcasein, 0.4 mM H-putrescine (in 0.7 ml assay reaction volume). Background values were obtained by incubation of the tissue supernatant in a buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM dithiothreitol, 5 mM EGTA, 0.4 mM H-putrescine with no CaCl2 or N, N-dimethylcasein. All measurements were done in triplicate. The samples were incubated at 37°C for 150 min. The reaction was terminated by the addition of 20% trichloroacetic acid (TCA) to precipitate the dimethylcasein. The tubes were centrifuged at 9,000 x g for 15 min, and the resulting pellet was washed twice with a rinsing buffer (50 mM Tris-HCl, pH 7.4, 1.3 CaCl2, 10 mM dithiothreitol, 0.4 mM putrescine, 20% TCA). The final pellet was dissolved in 1 ml of 2 N NaOH over night. Aliquots (250 μl) of each sample were removed and the amounts of incorporated tritiated putrescine were determined in triplicate samples by liquid scintillation counting. Transglutaminase activity was calculated after subtracting background (i.e. values obtained in the absence of CaCl2 and casein) and was expressed as pmol H-putrescine incorporated per mg protein.

NFT Counts

NFTs were counted in the globus pallidus (n = 6) and pons (both, tegmentum and basis pontis) (n = 6) from PSP cases on sections that were stained with Bielschowsky silver stain. NFTs were rated on a 3-point scale: 0 corresponded to no NFTs; + to scattered and very rare NFTs; ++ to many NFTs; and +++ to frequent NFTs.

Western Blot Quantitation

Western blot analysis was performed on homogenized tissue samples from globus pallidus, pons, and occipital cortex from PSP (n = 6, n = 5, and n = 7, respectively) and control cases (n = 6, n = 6, n = 7, respectively). All samples were separated on 10% SDS-PAGE (10 μg protein/lane) and electrophoretically transferred to nitrocellulose membranes. Immunoblotting was performed using antibodies directed against different isoforms of transglutaminase: TG-100 (anti- TG2, 1:1,000; Lab Vision, Fremont, CA), TG-1 (1:200), and TG-3 (1:200) (gifts from Dr. Kim at Weill Medical College of Cornell University Burke Medical Research Institute, White Plains, NY). Equivalent loading of protein in each well was verified by reprobing each blot for actin. Signal was detected with Chemiluminescent Immuno blot Western-Star Kit (TROPIX, Inc. Foster City, CA). The optical densities and areas occupied by the protein bands on films were obtained with Scion Image analysis program. Grey scale density readings were calibrated using a transmission step wedge standard fitted to the Rodbard equation. An integrated optical density (IOD) was calculated by summing the optical densities within the outlined area and subtracting the background optical density of an adjacent area of the film. Tissue homogenates from PSP and control samples from a single brain region were loaded on the same blot. The relative levels of transglutaminase proteins in PSP and controls from each blot were compared using a Student t test and GB-Stat software program (Dynamic Microsystems, Silver Spring, MD). These measurements were repeated in triplicate and the mean values were calculated for each brain region for each PSP and control case.

Tissue Preparation for RT-PCR

To prepare the total RNA samples for RT-PCR, PSP and control tissues were homogenized with a glass homogenizer with Tri reagent (1.0 ml for 50–100 mg of tissue) (Sigma, St. Louis, MO) then incubated at room temperature for 5 min. The tissue samples were then centrifuged at 12,000 rpm for 10 min at 4°C to pellet insolubles. We then added CHCl3 to the samples and shook the samples for 15 s. Samples were incubated at room temperature for 15 min and followed by centrifugation at 12,000 rpm for 15 min at 4°C. A second phenol:chloroform (pH 5.2) extraction was done to minimize DNA contamination of RNA samples. Next glycogen (20 μg) and isopropanol were added and samples were incubated at room temperature for 10 min followed by centrifugation at 12,000 rpm for 10 min at 4°C. The final RNA pellet was dissolved in diethylprocarbonate (DEPC)-treated water. Spectrophotometric methods were used to determine RNA concentration and to analyze DNA and protein contamination. RNA samples were stored at -80 until use.

Quantitative RT-PCR Analyses for Transglutaminase 1, 2, and 3

To perform quantitative PCR data we used a competitive PCR method that uses separate sets primers for an internal (mimic) standard and the target mRNA/cDNA (49, 50). This process involves co-amplification of the mimic standard and the target of interest and uses primers that yield products of different sizes to distinguish between the two. By varying the amount of the mimic standard, the ratio between the 2 products of the PCR reaction affords a measure of the initial amount of the target mRNA species. The mimic templates of human transglutaminases 1 and 2, and β-actin were constructed by PCR. The PCR primers for the targets were transglutaminase 1 sense strand (5'-ACC CTC ACC AAC GAT GTC TGC TTC C-3'); transglutaminase 1 antisense strand (5'-TTA GCA TCT GTT CCC CCA GTG C-3'); transglutaminase 1 sense strand (5'-CCT CGT GGA GCC AGT TAT CAA C-3'); and antisense transglutaminase 2 (5'-TAT GGT GGG TGG TCA ATG GC-3'); β-actin sense strand (5'-TTT GAG ACC TTC AAG ACC CCA-3'); and β-actin antisense (5'-TTG GTG AAT GCC ACA GGA CTC-3'). The products of RT-PCR give 455 base pairs (bp) for
target transglutaminase 1, and 355 bp for mimic transglutaminase 2; 698 bp for target transglutaminase 2, and 600 bp for mimic transglutaminase 2; 460 bp for target β-actin, and 308 bp for mimic β-actin. The quantitation of initial amount of target cDNAs for transglutaminases 1 and 2 were measured in total RNA samples from human tissue. PCR reactions were done with conditions of 1 cycle of 95°C (2 min); 25, 30, or 35 cycles of 95°C (30 s), 58°C (30 s); 72°C (30 s); and 1 cycle of 72°C (7 min) with a Perkin Elmer Biosystems (Foster City, CA) 9600 machine. All PCR reactions contained in 20 μl: 1.5 mM MgCl₂; 200 μM dATP, dGTP, dTTP; 100 μM dCTP; 10 μCi 32P-dCTP (3,000 Ci/mmol); 0.2 μM of each sense and antisense primers; 0.5 unit of Taq polymerase; templates from 0.05 μg total RNA; and different concentrations of mimic standard (1.0 pM–1 fM). The RT-PCR products were separated on 6% TBE gel (Novex, San Diego, CA) and scanned for quantitation by a phosphoimager (Molecular Dynamics, Sunnyvale, CA). We calculated the ratio of target amount per standard mimic amount. The values are means of 3 different amplifications, and were within <10% standard deviation. To confirm the cDNA sequences, the products of RT-PCR were excised from the gel, ligated into a T-vector (Novagen, Madison, WI), and sequenced. The sequences matched the human transglutaminase 1 and 2 cDNA sequences.

Quantitative RT-PCR Analysis of Short and Long Isoforms of Transglutaminase 2

To identify the alternatively spliced transglutaminase 2 isoforms in PSP, we designed primers to co-amplify the long and short isoform using the human transglutaminase 2 mRNA sequence (GenBank™ accession number NM 004613). The long isoform has been previously found to be present in both aged controls and AD cases; however, alternatively spliced short isoform of the transglutaminase 2 was shown to exist only in AD cases (43). The primers used in these experiments included an upstream primer for both short and long forms: 5'-GGG CGA CCT GAG CAC CAA-3', downstream primer for a long isoform of transglutaminase 2: 5'-AGC CCT TCA CAG CCT TCA-3', a downstream primer for a short isoform: 5'-GGC GGT CAA CAA ATG CTC-3'. Histone primers were included as an internal control (51). We performed a 2-step RT-PCR using a GeneAmp® Gold RNA PCR Reagent Kit (Perkin Elmer Biosystems). All RT reactions contained in 20 μl: 1X RT-PCR buffer, 6 mM MgCl₂, 1 mM dNTP (250 μM of each dNTP), 10 mM DTT, 1.25 μM random hexamer, 15 units of MultiScribe reverse transcriptase, 10 Units of RNase inhibitor, and 1 μg of total RNA. Cycling parameters for the RT step were hybridization for 10 min at 25°C and reverse transcription for 12 min at 42°C. All PCR reactions contained in 50 μl: 25 pmol of each primer, 6 mM MgCl₂, 0.8 mM dNTP (200 μM of each dNTP), 2.5 units of AmliTaq Gold DNA polymerase, 2 μCi 32P (10 μCi/μl), and 10 μl of cDNA. The PCR conditions were as follows: AmliTaq Gold activation for 10 min at 95°C; and 30 cycles of 1 min at 94°C (denaturation), 2 min at 68°C (annealing/extension) for PCR step, followed by 7 min final extension at 72°C. Cycle number and starting amount of cDNA used in PCR amplified both transglutaminases and histone cDNA within in a linear range. To analyze the RT-PCR products, we added 10 μl of the 6X loading buffer (0.25% Bromophenol blue, 0.25% Xylene Cyanol FF, 15% Ficoll [type 400, Pharmacia, Piscataway, NJ], water) to the final reaction mix and loaded 20 μl per lane onto a 6% nondenaturing polyacrylamide gel (29:1 acrylamide:bisacrylamide, Bio Rad, Hercules, CA). After the initial experiments, we found that we had to load 2 times more reaction product for the PSP cases than in the control lanes in order to quantify the reaction products for the globus pallidus. The RT-PCR amplification resulted in a long isoform product of 893 bp and a short isoform product of 510 bp.

RT-PCR Data Analysis

Gels were dried and the amount of radioactivity incorporated into each band was estimated after exposure to x-ray film. Scion Image analysis program was used for quantification of transglutaminase 2 cDNA bands. The measurements for the short and long transglutaminase isoform band were divided by the measurement for the histone band from the same line to normalize for starting amount of RNA and variability in loading of each lane of the gels. These measurements were repeated in triplicate and the mean percentage of control values was calculated for each case. These data were analyzed using a nonparametric Mann-Whitney U-test to determine whether there was a difference in mRNA levels encoding for 2 isoforms in PSP compared to control cases. We used Mann-Whitney U-test due to a high nonhomogeneity of variance.

RESULTS

Transglutaminase Activity and Correlation to NFTs

Since transglutaminase catalyzed cross-linking was previously found to be present in neurons with NFTs in PSP, we were interested in the transglutaminase enzyme itself. We first measured the activity of transglutaminase in 2 selectively vulnerable brain regions, the globus pallidus and pons, and in a region spared from neurofibrillary pathology, the occipital cortex. We determined transglutaminase activity by a modification of previously published methods that use radioactive putrescine (14C-putrescine) incorporation into an exogenous casein acceptor (43, 47). With this assay we were able to quantify the activity of transglutaminase isoforms present in the tissue samples (43, 47).

We found that transglutaminase activity was significantly increased in the globus pallidus and pons in PSP as compared to the controls (p < 0.05). The mean transglutaminase activity levels, expressed as percent of control values, were 182% for both globus pallidus and 171% for the pons (Fig. 1). There was no significant difference in the transglutaminase activity between PSP and controls in the occipital cortex, a brain region without neurofibrillary pathology in PSP (Fig. 1).

We examined NFT density in globus pallidus and pons regions of PSP cases in order to determine whether there is a relationship between the amount of transglutaminase enzyme activity and the amount of NFT pathology in PSP...
Expression of Transglutaminase 1 and 3 Protein Levels

To investigate which transglutaminase isoforms are predominantly expressed in the brain regions with extensive neurofibrillary pathology in PSP, we compared protein levels of transglutaminase 1 and 3 in the selected brain regions in PSP cases with the control cases. We performed Western blot analysis on the homogenates from the globus pallidus, pons, and occipital cortex from PSP and control brains. We found a significant increase in transglutaminase 1 expression in PSP in the globus pallidus (p < 0.05) from PSP cases as compared to the age-matched control cases (Fig. 2). Interestingly, transglutaminase 1 protein levels were not found to be increased in the pons of PSP cases as compared to the control cases. The mean transglutaminase 1 protein levels in PSP cases were 320% of control for globus pallidus and 90% of control for the pons. There was no difference in the expression of transglutaminase 1 in the occipital cortex in PSP cases as compared to the control cases (Fig. 2). Comparable comparisons for the transglutaminase 3 isoform were not possible because protein expression levels were too low to be detected and measured.

Expression of Transglutaminase 1, 2, and 3 mRNA Levels

RT-PCR was performed to measure the amount of mRNA encoding transglutaminase 1, 2, and 3, in normal as well as PSP brain. The products of RT-PCR give 455 bp for target transglutaminase 1, and 355 bp for mimic transglutaminase 1; 698 bp for target transglutaminase 2, and 600 bp for mimic transglutaminase 2; 460 bp for target β-actin, and 308 bp for mimic β-actin. The quantitation of initial amount of target cDNAs for transglutaminases 1 and 2 was measured in total RNA samples from human tissues. The means of 3 different experiments were within the <10% standard deviation. Transglutaminase 1 mRNA levels were increased in the globus pallidus and dentate of the cerebellum in PSP as compared to the control brain regions (Fig. 3A).

Transglutaminase 1 and 2 mRNA levels were not found to be increased in the pons in PSP cases despite an increase in transglutaminase activity and the presence of neurofibrillary pathology in this brain region (Fig. 3B). Transglutaminase 3 mRNA levels were too low to be detected by RT-PCR in the globus pallidus and pons brain regions of PSP and control cases although Kim et al (45) were previously able to detect transglutaminase 3 in cortex and cerebellum.

Long and Short Isoform of Transglutaminase 2 mRNA

We next asked the question whether the alternatively spliced variant of transglutaminase 2 was also present in our PSP samples but not in the controls as previously seen in AD (43) and in rat brain astrocytes (44). We performed RT-PCR using primers specifically designed to amplify the 3’ region of previously published sequence of the short form of transglutaminase 2 (43). We found that there were significantly higher levels (974% of control) of short form of transglutaminase 2 in the globus pallidus of PSP cases as compared to the controls (p = 0.0176) (Fig. 4C, D). We found both the long and short isoforms of transglutaminase 2 to be present in both aged controls and PSP globus pallidus (Fig. 4E); however, the short transglutaminase 2 isoform mRNA was expressed in much higher levels in PSP compared to controls (Fig. 4C).
Fig. 2. Comparison of transglutaminase 1 enzyme protein expression levels in the pons and globus pallidus from PSP and control cases. Transglutaminase 1 protein levels are significantly higher in globus pallidus from PSP as compared to the age-matched controls as determined by Western blot and Student t-test (p < 0.05). There was no difference in transglutaminase 1 protein levels in the pons and occipital cortex from PSP and control samples as determined using Student t-test. In the globus pallidus, the means ± SEM from 5 PSP cases and 6 age-matched control cases are shown. In the pons, the means ± SEM from 5 PSP cases and 6 age-matched control cases are shown. In the occipital cortex, the means ± SEM from 7 PSP cases and 7 age-matched control cases are shown.
Fig. 3. Expression of transglutaminase 1 in the globus pallidus (GP) and dentate (D) of the cerebellum (A) and transglutaminase 1 and 2 in the pons (B) in PSP cases as compared to the age-matched control cases. RT-PCR products were quantified by PhosphorImager analysis following [32P]dCTP labeling. A. Transglutaminase 1 mRNA levels were increased in the dentate and globus pallidus of PSP as compared to the control cases. These data are the average ± SD from 4 PSP cases and 4 age-matched control cases. B: There is no change in transglutaminase 1 and 2 mRNA levels in the pons in PSP as compared to the control cases as determined by RT-PCR. RT-PCR products were quantified by PhosphorImager analysis following [32P]dCTP labeling. These data are the average ± SD from 4 PSP cases and 4 age-matched control cases.

We also performed the same RT-PCR procedure using the dentate region of the cerebellum from PSP and control cases. In the dentate region of the cerebellum, we found significantly higher levels (p = 0.0176) of the long form of transglutaminase 2 in PSP cases (2212% of control) as compared to the control cases (Fig. 4A, B). We were unable to detect the presence of the short form of transglutaminase 2 in the dentate of the cerebellum (Fig. 4B).
DISCUSSION

The purpose of this study was to identify the isoform(s) of the transglutaminase enzyme that might be responsible for formation of abnormal tau protein inclusions in PSP. Previous work from our laboratory showed that transglutaminase-catalyzed cross-links are present in PHF-tau proteins in selectively vulnerable brain regions of PSP (17). Transglutaminase cross-linking causes the formation of stable polymers in other systems and tau polymers in vitro. Therefore, transglutaminase-catalyzed cross-linking of tau protein could lead to the development and progression of NFTs in PSP.

Transglutaminases are a family of calcium-activated enzymes. Ca$^{2+}$ acts as an intracellular regulator of many physiological processes. Ca$^{2+}$ can also play a role in a variety of pathological conditions. Loss of calcium homeostasis has been previously implicated in neurodegenerative disorders such as Alzheimer disease (52). In AD and PSP, increased calcium could lead to stimulation of kinases that phosphorylate tau. It is also reasonable to hypothesize that if there is an increase of intracellular calcium, it is likely that transglutaminase activity would be upregulated in susceptible brain regions under this condition. Indeed, we found that the activity of transglutaminases in 2 selectively vulnerable brain regions of PSP, globus pallidus and pons, was significantly increased in PSP as compared to the control samples. Also, we found a correlation between the enzyme activity and amount of NFTs in globus pallidus of individual PSP cases. Transglutaminase activity was not altered in PSP in the occipital cortex, a brain region spared of the NFT pathology in PSP, further suggesting a relationship between transglutaminase and NFT pathology.

We then asked the question whether this increase in activity could also result from an increase in the expression of transglutaminase enzymes in selectively vulnerable brain regions in PSP. We looked at 4 different regions: globus pallidus, pons, dentate of cerebellum, and occipital cortex. Globus pallidus, pons, and dentate regions contain NFT pathology in PSP; occipital cortex is spared from the pathology in PSP and therefore serves as a control region. To detect the amount of transglutaminase isoforms, PSP and control samples were analyzed using RT-PCR and Western blots with antibodies directed against specific transglutaminase isoforms. We found that there are 2 members of the transglutaminase family, transglutaminase 1 and transglutaminase 2, abundantly expressed in selectively vulnerable brain regions in PSP. In addition to our previous findings that transglutaminase 2 is expressed in numerous brain regions in PSP and controls (17), this study demonstrates presence of transglutaminase 1 in globus pallidus, dentate of the cerebellum, and pons of both PSP and control cases. Further, our data suggest that transglutaminase 3 is probably not involved in mechanisms leading to neuropathology since we were not able to detect transglutaminase 3 protein in the pons and globus pallidus or transglutaminase 3 mRNA in the pons using RT-PCR paradigms used to detect transglutaminase 3 mRNA in other brain regions.

Transglutaminase 1 mRNA and protein levels were significantly upregulated in globus pallidus and the cerebellum (dentate) in the PSP tissues as compared to the age-matched controls. There were no significant differences in the protein levels of the long isoform of transglutaminase 2 in globus pallidus, pons, and occipital cortex from PSP cases as reported in our previous studies (17). We also found no significant differences in the expression of mRNA encoding the long isoform of transglutaminase 2 in the globus pallidus and pons, but in the dentate of the cerebellum the mRNA levels were 2212% of control values.

Interestingly, when we examined the pons we found no change in transglutaminase 1 and 2 protein or mRNA levels between PSP and the age-matched controls. Pons, including the tegmentum and the basis pontis, have many structures that are not involved in the pathology, such as ascending and descending white matter tracks in addition to other nongray matter, which might dilute the signal in that region. Thus, the diffuse pathology and gross homogenization of the tissue in pons might not provide enough pathological sample to pick up potential transglutaminase changes. In contrast, globus pallidus and the dentate of cerebellum are anatomically well defined and condensed regions that have a high degree of neuropathology. Therefore, globus pallidus and the dentate of cerebellum are locations where one could expect easier detection of changes in transglutaminase isoform expression. Alternatively, other transglutaminase proteins in the

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Fig. 4. Expression of short and long isoforms of transglutaminase 2 in selectively vulnerable brain regions of PSP. A: There are significantly higher mRNA levels of the long form of transglutaminase 2 in the dentate region of the cerebellum of PSP as compared to the control cases as determined by RT-PCR and (B) a nonparametric Mann-Whitney U-test ($p = 0.0176$). C, D: Both short and long isoforms of transglutaminase 2 are present in globus pallidus in PSP and control cases. C: There are significantly higher mRNA levels of the short isoform of transglutaminase 2 in the globus pallidus in PSP as compared to the control cases as determined by RT-PCR. E: The long isoform of transglutaminase 2 is present in both aged controls and PSP globus pallidus. The RT-PCR amplification resulted in a long isoform product of 893 bp and a short isoform product of 510 bp. The means ± SEM from 5 controls and 6 PSP cases for dentate of the cerebellum and 6 controls and 6 PSP cases for globus pallidus are shown.
pons could underlie the increase in transglutaminase activity. A number of additional transglutaminase genes have been identified but their expression in the brain has not been examined (30, 33).

Next, we examined the alternative splicing of transglutaminase 2. Monsonego and colleagues (44) and Citron and his group (43) demonstrated the presence of an alternatively spliced short isoform of transglutaminase 2 in rat brain astrocytes and AD cortex, respectively. We show the presence of both long and short isoforms of transglutaminase 2 in globus pallidus from PSP and control cases. However, the mRNA levels of the short isoform of transglutaminase 2 were significantly greater in PSP cases (974% of control) compared to the control cases. The long isoform has been previously found to be present in both aged controls and AD cortex, although the short isoform of transglutaminase 2 was not detected in the cortex of the 2 control cases previously examined (43). We found that 2 of the 6 globus pallidus control cases expressed very low levels of the short isoform of transglutaminase 2. An increase in the short transglutaminase 2 isoform is important because in the short isoform of transglutaminase 2 the GTP-binding domain has been spliced out; thus there could be no negative regulation by GTP. Therefore, this short isoform could be constitutively active in the presence of calcium. These novel findings suggest an additional possible mechanism for the activation of transglutaminase not only in PSP but also other tauopathies. However, future studies are necessary to examine the function of the short isoform of transglutaminase 2 to confirm its potential pathogenic role in PSP and other neurodegenerative disorders.

The current findings support a hypothesis put forward by Kim et al that although there are multiple transglutaminase enzymes involved in numerous normal cellular processes, in disease states such as AD, the expression levels and cross-linking activity of specific transglutaminase isoforms are higher when compared to the normal controls (45). Our data also extend this hypothesis and suggest that transglutaminase isoforms are differentially and selectively altered in different brain regions in PSP. For example, the short isoform of transglutaminase 2 mRNA is over-expressed in the globus pallidus while mRNA encoding the long isoform is increased in the dentate of the cerebellum in PSP.

Selkoe and colleagues were the first to suggest that transglutaminase may be contributing to the development of pathology in AD by cross-linking neurofilament proteins, which at that time were thought to be a main component of NFTs in AD (53). Since then it has been demonstrated that hyperphosphorylated tau protein is a predominant component of NFT, and that tau is an excellent substrate for transglutaminase in vitro (12, 14). Next, numerous groups reported evidence suggesting that transglutaminase-catalyzed ε-(γ-glutamyl) lysine cross-linking of tau proteins may contribute to the formation of PHFs and NFTs in AD and PSP (14–17, 48, 54). In tauopathies, tau protein may be more available for cross-linking by transglutaminase. Hyperphosphorylation of tau that occurs in tauopathies inhibits binding of tau to microtubules. Expression of 4R tau is higher in PSP than control cases (55), suggesting that there may be an increase in the available pool of free tau that is not bound to microtubules. Therefore, tau phosphorylation and overexpression of 4R tau in PSP may make tau proteins available for transglutaminase-induced cross-linking into tau polymers. Transglutaminases are involved in apoptosis (30) and could also play a role in neuronal loss through apoptotic mechanisms in PSP.

In conclusion, our findings that transglutaminase activity is increased in the regions with pathology in PSP, in addition to the previous findings from our laboratories and others (12, 14–17, 48, 54) suggest that increased transglutaminase activity results in cross-linking of available tau proteins and contributes to the formation of stable NFTs. In this study, we demonstrated that both transglutaminase 1 and 2 are expressed in selectively vulnerable brain regions in PSP. Transglutaminase 1 is significantly upregulated in the globus pallidus and dentate of the cerebellum in PSP, a region rich in NFT pathology. In addition, we found increased expression of mRNA encoding both the long and short forms of transglutaminase 2 in selectively vulnerable brain regions in PSP. The short form of transglutaminase 2 is not inhibited by GTP and may therefore be more active and lead to the cross-linking of available tau protein. These studies further suggest that transglutaminase is altered in neurodegenerative diseases and may be involved in the formation of stable NFTs. Future studies with specific transglutaminase inhibitors are needed to confirm that transglutaminase isoforms 1 and 2 are important in the development and progression of PSP as well as other prevalent neurodegenerative disorders.

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