

Up-regulation of tissue-type transglutaminase after traumatic brain injury

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Abstract

Tissue-type transglutaminase (tTG, EC 2.3.2.13) has been implicated in various disease paradigms including neurodegenerative disease. In these studies, tTG induction after traumatic brain injury was studied using a rat cortical impact model. Using western blots, two forms of tTG protein expression were identified – a ~79-kDa primary form (tTG-L) and a less abundant ~70-kDa form (tTG-S). Both forms of tTG protein were elevated after injury. In ipsilateral cortex, peak induction of tTG-L protein [561% ± 80% of control ($n = 5$)] was observed five days after injury, with expression remaining elevated after two weeks. Peak induction of tTG-S protein [302% ± 81% of control ($n = 5$)] was observed three days after injury. Lesser tTG protein induction was observed in

hippocampus. Northern blot analysis demonstrated two tTG transcripts in the ipsilateral cortex with peak induction of tTG-L mRNA three days after injury. However, tTG-S mRNA was not identified in control samples and only faintly detected in injured tissue. To facilitate analysis of low abundance transcripts in smaller tissue samples, a semiquantitative real-time PCR strategy was used. Semi-quantitative PCR analysis of tTG-L mRNA induction in ipsilateral cortex (peak after three days; 414% ± 21% of control, $n = 3$) confirmed tTG-L mRNA induction determined by northern blot (410% of control).

Keywords: quantitative PCR, transglutaminase, traumatic brain injury.

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Tissue-type transglutaminase (type II transglutaminase, transglutaminase C, tTG) has been implicated and may play a role in apoptotic cell death, neurodegenerative disease and in protein aggregation associated with neurodegenerative disease. Although apoptosis is a prominent feature of cell death following TBI and TBI, it is a risk factor for a number of neurodegenerative diseases, such as Alzheimer's disease, no studies have examined the alteration in tTG following CNS injury (CNS).

tTG is a Ca^{2+} dependent enzyme and is a member of a family of transglutaminases (TG) that catalyzes the transamidation of glutamine residues. tTG can catalyze the incorporation of a polyamine into a polypeptide-bound glutamine leading to the formation of a (γ -glutamyl) polyamine bond. Alternatively, when this reaction occurs between the γ -carboxamide group of a peptide-bound glutamine residue and the ϵ -amino group of a peptide-bound lysine residue, ϵ -(γ -glutamyl) lysine isopeptide bond is formed (Greenberg *et al.* 1991). This isopeptide bond is resistant to proteolytic cleavage, and may serve not only to

stabilize proteins against degradation, but also to alter their function. There are numerous substrates for tTG, including structural proteins, enzymes, and elements of signal transduction pathways (Chen and Mehta 1999).

Though there are currently seven known TGs, only TG 1 (106 kDa), tTG (80 kDa) and TG 3 (77 kDa) have been identified within the brain (Selkoe *et al.* 1982). Importantly, Kim *et al.* (1999) revealed that only TG 1 and tTG were expressed within the cerebral cortex, with tTG most abundantly expressed.

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Abbreviations used: APP, amyloid precursor protein; ECL, enhanced chemiluminescence; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBI, traumatic brain injury; tTG, tissue-type transglutaminase.

Additionally, tTG functions as a G-protein, designated $G\alpha_h$, that interacts with phospholipase C- $\delta 1$ (Nakaoka *et al.* 1994; Feng *et al.* 1996; Park *et al.* 1998). $G\alpha_h$ was originally identified as the signal transducer between epinephrine binding to α_1 -adrenoreceptor and phospholipase C (PLC) activation (Im *et al.* 1990; Im and Graham 1990; Das *et al.* 1993), specifically PLC- $\delta 1$ (Feng *et al.* 1996). The identity of $G\alpha_h$ as tTG was clarified by peptide foot-print analysis between tTG and $G\alpha_h$ and cross-reactivity between antibodies for rat liver $G\alpha_h$ and antibodies for guinea pig liver tTG (Nakaoka *et al.* 1994). The C-terminal region of tTG is important for its ability to interact with PLC (Hwang *et al.* 1995; Feng *et al.* 1996) and with receptor molecules (Feng *et al.* 1999).

Previous reports have described changes in overall TG activity during the development of mouse brain (Maccioni and Seeds 1986) and rat brain (Gilad and Varon 1985; Perry and Haynes 1993). Other proposed functions include involvement in cell-matrix interactions, wound healing, regeneration and neurodegenerative disease (for a review see Lesort *et al.* 2000). Among its many possible functions, tTG has been implicated as a mediator of apoptosis. In the neuroblastoma cell line SK-N-BE(2), tTG overexpression stimulated apoptosis while inhibition of tTG expression with an antisense construct decreased both spontaneous and retinoic acid-induced apoptosis (Melino *et al.* 1994). tTG-mediated apoptosis may involve multiple target substrates. During apoptosis tTG modifies several proteins including histone protein (Ballestar *et al.* 1996), actin (Nemes *et al.* 1997), troponin (Gorza *et al.* 1997), and retinoblastoma protein (Oliverio *et al.* 1997). Given the complexity with which tTG interacts with numerous proteins, the role of tTG in apoptosis is not yet understood (for a review see Melino and Piacentini 1998).

tTG mRNA and protein expression have been demonstrated in cultured astrocytes from two-day postnatal rats (Monsonogo *et al.* 1997). In astrocyte cultures treated with IL-1 β , two transcripts encoding tTG were identified – a full-length long form (encoding a ~77-kDa protein), and a less abundant short form (encoding a ~73-kDa protein) that lacked a portion of the C-terminus coding region and had a truncated 3' untranslated region. Interestingly, the enzymatic activity of the tTG short form lacked the GTP-dependence observed in the tTG long form (Monsonogo *et al.* 1997). The functional significance of the two types of tTG expressed in cultured astrocytes is not yet clear.

Induction of tTG expression by IL-1 β and TNF- α *in vitro* may prove relevant to the *in vivo* response to traumatic injury. Traumatic brain injury (TBI) induces the expression of both IL-1 β and TNF- α (Woodrooffe *et al.* 1991; Taupin *et al.* 1993; Rostworowski *et al.* 1997; Herx *et al.* 2000). Using a microdialysis probe to induce mechanical trauma as well as measure cytokine levels, Woodrooffe *et al.* (1991) showed a 15-fold induction of IL-1 within a two-day period.

Using a fluid percussion model of traumatic brain injury, Taupin *et al.* (1993) demonstrated IL-1 induction in cortex and hippocampus up to 18 h after injury and TNF- α induction 3–8 h after injury. After stab injury, IL-1 β mRNA is induced 3–12 h after injury, while TNF- α mRNA is induced from 3 to 96 h after injury (Rostworowski *et al.* 1997). After corticectomy, both IL-1 β and TNF- α transcripts were increased three hours after injury (Herx *et al.* 2000). If injury-induced IL-1 β and TNF- α elevate tTG expression *in vivo* as has been shown for *in vitro* astrocytes, then tTG may play a role in the subsequent CNS response to trauma. In particular, the pro-apoptotic effects of tTG expression may contribute to the post-traumatic apoptotic phenotype seen in CNS injury. The apoptotic components of cell death after experimental TBI have been examined in models of fluid compression (Rink *et al.* 1995; Yakovlev *et al.* 1997; Conti *et al.* 1998) and cortical impact (Colicos and Dash 1996; Newcomb *et al.* 1999).

The aim of this study was to determine whether tTG is up-regulated as a result of TBI, and if so, to determine the time course of induction. Using western blot analyses, we found that both forms of tTG [tTG-L (~79 kDa) and tTG-S (~70 kDa)] were strongly induced in ipsilateral cortex after traumatic injury with predominant expression of tTG-L. These two forms are similar to the long and short forms seen in cytokine-induced astrocytes (Monsonogo *et al.* 1997). To further analyze this phenomenon, induction of specific tTG transcripts was verified by northern blot and semiquantitative PCR analysis.

Materials and methods

Surgical preparation and controlled cortical impact traumatic brain injury

A previously described cortical impact injury device was used to produce TBI in adult rats (Dixon *et al.* 1991; Pike *et al.* 1998). Cortical impact TBI results in cortical deformation within the vicinity of the impactor tip associated with contusion, and neuronal and axonal damage that is constrained in the hemisphere ipsilateral to the site of injury (Gennarelli 1994; Meaney *et al.* 1994). Adult male (280–300 g) Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were anesthetized with 4% isoflurane in a carrier gas of 1 : 1 O₂/N₂O (4 min) followed by maintenance anesthesia of 2.5% isoflurane in the same carrier gas. Core body temperature was monitored continuously by a rectal thermistor probe and maintained at 37° ± 1°C by placing an adjustable temperature controlled heating pad beneath the rats. Animals were mounted in a stereotaxic frame in a prone position and secured by ear and incisor bars. A midline cranial incision was made, the soft tissues were reflected, and a unilateral (ipsilateral to site of impact) craniotomy (7 mm diameter) was performed adjacent to the central suture, midway between bregma and lambda. The dura mater was kept intact over the cortex. Brain trauma was produced by impacting the right cortex (ipsilateral cortex) with a 5-mm diameter aluminum impactor tip (housed in a pneumatic cylinder) at a velocity of 3.5 m/s with a

1.6-mm compression and 150 ms dwell time (compression duration). Velocity was controlled by adjusting the pressure (compressed N2) supplied to the pneumatic cylinder. Velocity and dwell time were measured by a linear velocity displacement transducer (Lucas Shaevitz™ model 500 HR, Detroit, MI, USA) that produces an analogue signal that was recorded by a storage-trace oscilloscope (BK Precision, model 2522B, Placentia, CA, USA). Sham-injured animals underwent identical surgical procedures but did not receive an impact injury. Appropriate pre- and post-injury management was maintained and these measures complied with all guidelines set forth by the University of Florida Institutional Animal Care and Use Committee and the National Institutes of Health guidelines detailed in the *Guide for the Care and Use of Laboratory Animals*.

Tissue lysis and protein purification

Cortical and hippocampal tissues were collected from animals at 1–14 days after sham-injury or TBI. At the appropriate time-points, TBI or sham-injured animals were killed with CO₂ and subsequent decapitation. Ipsilateral and contralateral (to the impact site) cortices and hippocampi were rapidly dissected and snap-frozen in liquid nitrogen. Tissue samples were stored at –80°C. Cortices were homogenized in a glass tube with a Teflon dounce pestle in 15 volumes of ice-cold triple detergent lysis buffer (20 mM HEPES, 1 mM EDTA, 2 mM EGTA, 150 mM NaCl, 0.1% SDS, 1.0% IGEPAL 40, 0.5% deoxycholic acid, pH 7.5) containing a broad range protease inhibitor cocktail (cat. no. 1-836-14; Roche Molecular Biochemicals, Indianapolis, IN, USA). Samples were sonicated and centrifuged at 800 *g* for 5 min at 4°C. The supernatant was then collected for western blot analysis.

Western blot analysis

Protein concentrations of tissue homogenates were determined by bicinchoninic acid microprotein assays (Pierce Inc., Rockford, IL, USA) with albumin standards. 20 µg aliquots of each sample were prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) by addition of 2× loading buffer (1× loading buffer contains 125 mM Tris-HCl (pH 6.8), 100 mM DTT, 4% SDS, 0.01% bromophenol blue, and 10% glycerol). Samples with loading buffer were heated for 10 min at 100°C, centrifuged for 1 minute at 10 000 *g*, and were resolved by SDS–PAGE on 7.5% Tris/glycine gels at 200 V for 1 h at 4°C. Following electrophoresis, fractionated proteins were transferred to Immobilon-*p* polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) in a transfer buffer containing 192 mM glycine, 25 mM Tris-HCl (pH 8.3), and 10% methanol at 100 V for 1 h at 4°C. Ponceau Red (Sigma, St Louis, MO, USA) was used to stain membranes to confirm successful transfer of protein and to insure that an equal amount of protein was loaded in each lane. Blots were blocked for 1 h at room temperature in 5% non-fat milk in TBST (20 mM Tris-HCl, 150 mM NaCl, and 0.005% Tween-20, pH 7.5).

Immunoblots were probed with one of two monoclonal anti-tTG antibodies, designated CUB 7402 or TG100 (LabVision, Fremont, CA, USA). Following overnight incubation at 4°C with the primary antibody (1 : 3000), blots were incubated for 1 h at room temperature (22°C) in 3% non-fat milk/TBST containing a horse-radish peroxidase-conjugated goat anti-mouse IgG (1 : 3000; Bio-Rad, Hercules, CA, USA). Bound antibodies were visualized with

enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ, USA) on Kodak Biomax ML chemiluminescent film.

RNA purification

Total RNA was isolated from control and injured samples of cortical or hippocampal tissue using TRIzol reagent (Gibco BRL, Rockville, MD, USA), isopropanol precipitation, and ethanol washes according to the manufacturer's instructions. Samples were resuspended in 50–100 µL DEPC-treated water.

Reverse transcription

Total RNA (3 µg) was incubated with 1 µL oligo(dT) (0.5 mg/mL, Gibco BRL) at 70°C for 10 min, then at 4°C for five minutes. A reverse transcription reaction mixture was then added to the RNA-oligo(dT) sample for a final volume of 20 µL, containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM MgCl₂, 500 µM dNTP's, 10 mM DTT, 50 units SuperScript II reverse transcriptase (Gibco BRL) and 40 units RNaseOUT recombinant ribonuclease inhibitor (Gibco BRL). The sample was incubated at 42°C for 55 min, 70°C for 15 min for enzyme denaturation, and then transferred to 4°C. Each sample was diluted to a final volume of 100 µL with DEPC-treated water.

Primer selection

TTG-specific primers

All base pair designations refer to GeneBank locus AF106325, rat tissue-type transglutaminase (TGaseII). *5'tTG* (accttgacgtgtttgccac; bp 1470–1489) recognizes an upstream homologous sequence in tTG-L and tTG-S transcripts. *3'tTG-L* (caatatcagtcgggaacaggtc; bp 1961–82) recognizes a downstream tTG-L mRNA-specific sequence. *3'tTG-S* (gctgagctctgggtgaagacacag; bp 1861–1872 and 2083–93) recognizes a downstream tTG-S mRNA-specific sequence. *3'tTG-S* primer bridges the junction created absence of bp 1873–2082 (a sequence present exclusively in tTG-L). The underlined 3' half of the 3'tTG-S sequence will hybridize to both tTG-L- and tTG-S-specific sequence, while the full length primer will hybridize only to tTG-S mRNA sequence.

GAPDH-specific primers

All base pair designations for GAPDH-specific primers refer to GeneBank locus AF106860. The upstream primer is designated *5'GPD* (ggctgcctctctgtgac; bp 903–921) and the downstream primer is designated *3'GPD* (ggccgcctgcttcaccac; bp 1624–1641).

Standard PCR

A PCR reaction buffer was added to 2 µL of reverse transcription product for a final volume of 25 µL containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 200 µM dNTP's, 0.5 µM dNTP's, 6% DMSO, 1.25 units Taq DNA polymerase (Gibco BRL). The mixture was then transferred to a PCR apparatus for amplification: each denaturation, annealing, and extension step was held for 30 s (two cycles of 95°, 65°C, and 72°C; then two cycles of 95°C, 62.5°C, and 72°C; then 32 cycles of 95°C, 60°C, and 72°C). Aliquots of PCR products were loaded onto 1.5% agarose gels and separated by electrophoresis in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.5) containing 5 µg/mL ethidium bromide. To assay for genomic DNA contamination, RNA samples

underwent PCR amplification without prior reverse transcription. Any samples showing genomic contamination underwent repurification and repeat assay for genomic contamination prior to PCR analysis for transcript expression.

Northern blot analysis

Total RNA (10 µg) was size-fractionated by electrophoresis in 1.5% agarose gels containing 6% formaldehyde, then transferred onto Hybond N + nylon membrane (Amersham), and fixed by baking at 80°C for 1 h under vacuum. Membranes were hybridized in a solution containing 5× SSPE (1× SSPE contains 180 mM NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.7), 5× Denhardt's solution (1× Denhardt's solution contains 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone), 0.5% SDS, 20 µg/mL sheared denatured salmon sperm DNA, and random primer-generated probe at 65°C overnight. DNA probes were radiolabeled to a specific activity of 1×10^9 dpm/µg by random-primed labeling (Gibco BRL) with [α -³²P]dCTP (3000 Ci/mmol; DuPont NEN, Boston, MA, USA). After hybridization, membranes were washed sequentially with 2× SSPE/0.1% SDS at room temperature, then 1× SSPE/0.1% SDS at 65°C, and then 0.1× SSPE/0.1% SDS at 65°C. Membranes were exposed to film with one intensifying screen at -80°C.

TTG and GAPDH cDNA

For northern blot analysis of tTG expression, a 414-bp tTG-cDNA was generated via PCR. This fragment corresponds to 403 bp of tTG-L coding sequence and to 414 bp of tTG-S coding sequence. To monitor GAPDH expression, a 1.2-kb EcoRI fragment from HHCMC32 (American Type Culture Collection, Manassas, VA, USA) was used.

Semi-quantitative/light cycler PCR

Real time PCR was performed using a Light Cycler rapid thermal cycler system (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. Reactions were performed in a 10 µL volume with 0.5 µM primers, 2.5 mM MgCl₂. Other reagents including nucleotides, FastStart Taq DNA polymerase, and buffer were used as provided in the LightCycler-FastStart DNA Master SYBR Green I reaction mix (Roche Diagnostics). Amplification protocol included a 5 minute 95°C denaturation; one cycle with 95°C denaturation for 5 s, 65°C annealing for 10 s, and 72°C extension for 35 s; one cycle with 95°C denaturation for 5 s, 62.5°C annealing for 10 s, and 72°C extension for 35 s; then 30–40 cycles of 95°C denaturation for 5 s, 60°C annealing for 10 s, and 72°C extension for 35 s. Detection of the fluorescent product occurred at the end of the 72°C extension periods. Specificity of the amplification product from each primer pair was confirmed by melting curve analysis of the PCR product and subsequent gel electrophoresis.

Quantification was performed by online monitoring for identification of the exact time point at which the logarithmic linear phase could be distinguished from the background (crossing point). The crossing point is expressed as a cycle number.

Standard curve preparation and semiquantitative PCR analysis

From the northern blot analysis, maximal tTG-L and tTG-S mRNA expression was identified three days after injury. The reverse

transcription product from ipsilateral cortical RNA collected three days after injury underwent serial dilution, creating a standard curve of 100%, 33%, 11% and 3.7% of original RT product. Each dilution from the standard curve was analyzed with the LightCycler PCR using primer sets for tTG-L, tTG-S, or GAPDH mRNA. For each primer set, a crossing point cycle number was determined for each dilution of the standard curve. Linear regression analysis of the logarithm of the dilution factor vs. the crossing point cycle number generated a standard curve for each transcript-specific primer set. From each primer set's standard curve, a crossing point cycle number could be converted to a relative amount of RNA.

For individual samples, the crossing point cycle number was identified with the LightCycler PCR. Then, using the standard curve for each primer set, the amount of tTG-L, tTG-S, or GAPDH mRNA was determined. The amount of each transcript in sham animals was set at 100%, and the level of expression in an experimental sample was calculated as a percentage of sham expression.

Statistical analyses

For the western and northern blots, semiquantitative analysis was performed by computer-assisted densitometric scanning (AlphaImager 2000 Digital Imaging System, San Leandro, CA, USA). Data were acquired as integrated densitometric values and transformed to percentages of the densitometric levels obtained from sham-injured animals visualized on the same blot. PCR data was evaluated by least squares linear regression followed by ANOVA and Dunnett's multiple comparison test. All values are given as mean ± SEM. Differences were considered significant if $p < 0.05$.

Results

Western blot analysis of tTG expression in cortex after traumatic injury

Total protein was prepared from rat ipsilateral cortex after cortical impact injury. Expression of tTG was examined using two monoclonal anti-tTG antibodies, TG-100 and CUB-7402. Figure 1(a) shows western blot analyses for tTG expression in rat ipsilateral cortex one to 14 days after injury. Using the TG-100 antibody, two distinct tTG protein bands were identified: a ~79-kDa band corresponding to the tTG long form (tTG-L) and a ~70-kDa band that may represent the tTG short form (tTG-S). The presence of tTG-S was not detected with the CUB-7402 antibody; however, with prolonged film exposure of the TG-100 blots, tTG-S was more clearly identified (Fig. 1b).

Western blot analyses of ipsilateral cortex samples using TG-100 were quantified by densitometric analysis (Fig. 1c). Levels of tTG-L and tTG-S were expressed as percentage of sham levels. Sham-operated animals showed no change in tTG-L or tTG-S expression one day and five days after craniotomy (Fig. 1a). tTG-L induction was observed 1–14 days after cortical injury. tTG-L expression peaked at $561\% \pm 16\%$ ($n = 5$) five days after injury, with statistically significant elevated expression at 3–14 days. tTG-S induc-

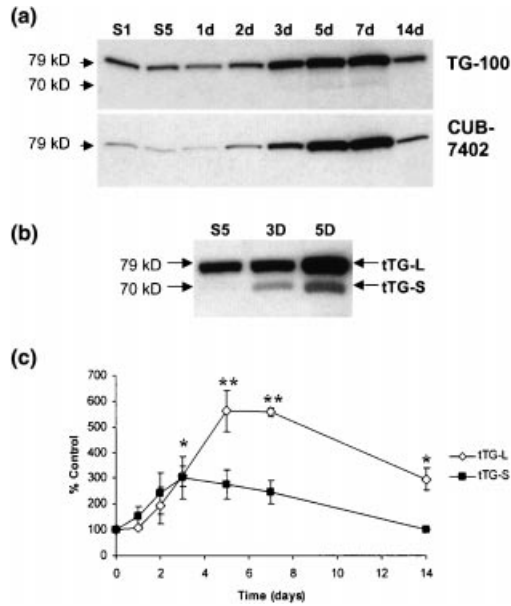


Fig. 1 Western blot analysis for tTG expression in ipsilateral cortical protein samples using the anti-TG-II antibodies TG-100 and CUB-7402. (a) Samples were collected from sham-operated animals one day and five days after surgery (S1, S5) as well as from injured animals from one day to 14 days after injury (1d–14d). (b) Longer exposure of the western blot using TG-100 antibody suggests co-expression of tTG-L (~79 kDa, full length tTG) and tTG-S (~70 kDa, a short form of tTG). (c) Quantitative analysis: western blot analyses using TG-100 antibody were quantified by densitometry, and the level of tTG (tTG-L and tTG-S) expression in the ipsilateral cortex of injured animals was calculated as a percentage of sham (control) tTG expression. For each condition, $n = 5$ animals. One-way ANOVA was performed, followed by Dunnett's multiple comparison test to evaluate statistical significance. Comparing tTG-L expression from injured animals to control/sham animals, increased levels of expression were statistically significant at three, five, seven, and 14 days ($*p < 0.05$, $**p < 0.01$). Comparing tTG-S expression from injured animals to control/sham animals, increased levels of expression were not statistically significant.

tion was observed 1–7 days after cortical injury, with peak expression ($302\% \pm 81\%$, $n = 5$) at three days. However, levels of tTG-S induction did not reach statistical significance (Fig. 1c). No changes in tTG-L or tTG-S expression were observed in the contralateral cortex (not shown).

Western blot analysis of tTG expression in hippocampus after traumatic injury

Total protein was prepared from rat ipsilateral hippocampus after cortical impact injury. Expression of tTG was examined using TG-100 and CUB-7402 antibodies. Figure 2(a) shows western blot analyses for tTG expression in rat ipsilateral hippocampus one to 14 days after injury. Compared to ipsilateral cortex, lower levels of tTG induction were identified in the hippocampus, and tTG-S was not detected with either antibody. Quantitative densitometric analysis of

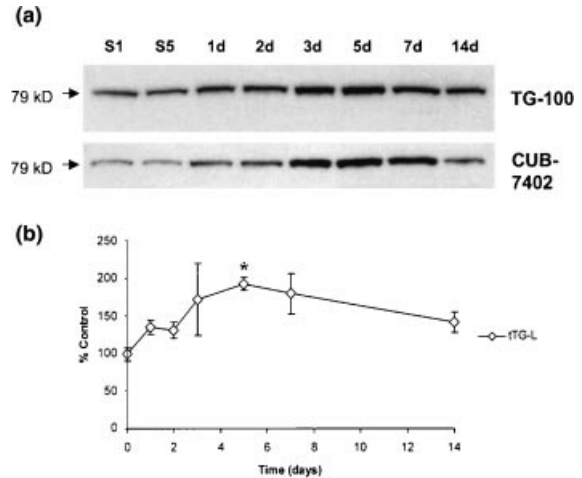


Fig. 2 Western blot analysis for tTG expression in ipsilateral hippocampal protein samples using the anti-tTG antibodies TG-100 and CUB-7402. Samples were collected from sham-operated animals one day and five days after surgery (S1, S5) as well as from injured animals one day to 14 days after injury (1d–14d). tTG-S, the shorter form of tTG (70 kDa), was not identified in these samples even at longer exposures. (b) Quantitative analysis: western blot analyses using TG-100 were quantified by densitometry, and the level of tTG expression in the ipsilateral hippocampus of injured animals was calculated as a percentage of sham (control) tTG expression. Only tTG-L was quantified, as tTG-S expression was not identified in every sample. For each condition, $n = 5$ animals. One-way ANOVA was performed, followed by Dunnett's multiple comparison test to evaluate statistical significance. Comparing tTG-L expression from injured animals to control/sham animals, increased levels of expression were statistically significant at five days ($*p < 0.05$).

tTG-L expression revealed peak levels ($194\% \pm 9\%$, $n = 5$) five days after injury. Increased tTG-L expression was observed 1–14 days after injury, but statistically significant increases were only detected five days after injury (Fig. 2b).

tTG transcript PCR analysis

Given the previous report of two types of tTG expressed in rat brain astrocytes (Monsonogo *et al.* 1997), and the current findings suggesting long (tTG-L) and short (tTG-S) forms of tTG in ipsilateral rat cortex after injury, the molecular analysis of tTG transcripts was performed. Figure 3(a) provides a schematic diagram of the two tTG transcripts previously described (Monsonogo *et al.* 1997). The tTG-L transcript contains an additional 209 bp sequence (bp 1873–2082) absent in tTG-S mRNA. To investigate which tTG transcripts were expressed after injury, total RNA was purified from rat ipsilateral cortex five days after injury. The sample was then reverse transcribed and underwent standard PCR to detect tTG-L, tTG-S and GAPDH transcripts. Figure 3(b) shows the results of the PCR analysis, indicating the presence of both tTG-L and tTG-S mRNA in rat ipsilateral cortex five days after injury. The identity of the

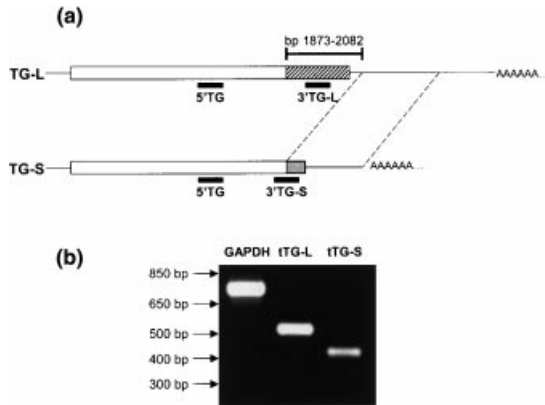


Fig. 3 (a) Schematic diagram of tTG transcripts. tTG-L mRNA contains an additional sequence (bp 1873–2082) not present in tTG-S mRNA. This sequence encodes additional coding region and 3' untranslated region. PCR primer sites are designated: 5'TG (bp 1470–1489) recognizes homologous sequence in tTG-L and tTG-S transcripts. 3'TG-L (bp 1961–82) recognizes tTG-L mRNA-specific sequence. 3'TG-S recognizes the junctional sequence created by the absence of the tTG-L-specific sequence (bp 1861–1872 and 2083–93). All base pair designations refer to GeneBank locus AF106325, rat tissue-type transglutaminase (TGaseII). (b) PCR analysis of ipsilateral cortex RNA. GAPDH, 739 bp PCR product generated with rat GAPDH-specific primers. (5'GAPDH primer includes bp 903–921; 3'GAPDH primer includes bp 1624–1641, as designated in GeneBank locus AF106860). tTG-L, 513 bp PCR product generated with tTG-L-specific primers. tTG-S, 414 bp PCR product generated with tTG-S-specific primers.

PCR products were verified by restriction endonuclease analysis (not shown).

Northern blot analysis of ipsilateral cortex RNA postinjury

Given the observation of both tTG-L and tTG-S transcripts in ipsilateral cortex after injury by PCR, northern blot analysis was performed to assess the temporal profile of tTG induction (Fig. 4a). Under sham conditions tTG-L mRNA was barely detectable. tTG-L mRNA expression was elevated 1–5 days after injury, with maximal tTG-L transcript expression (410% of sham expression) observed three days after injury. The northern blot analysis of tTG-L expression in ipsilateral cortex samples was quantified by densitometry and total RNA loading was corrected using GAPDH expression (Fig. 4b). Although maximal tTG-S transcript expression was also observed three days after injury, the degree of induction cannot be calculated, as tTG-S mRNA was undetectable under sham conditions.

Northern blot analysis was able to demonstrate the temporal profile of tTG-L transcript induction in response to cortical injury. However, clear identification and analysis of tTG-S transcript was not possible due to background signal on the northern blots, as well as the low levels of

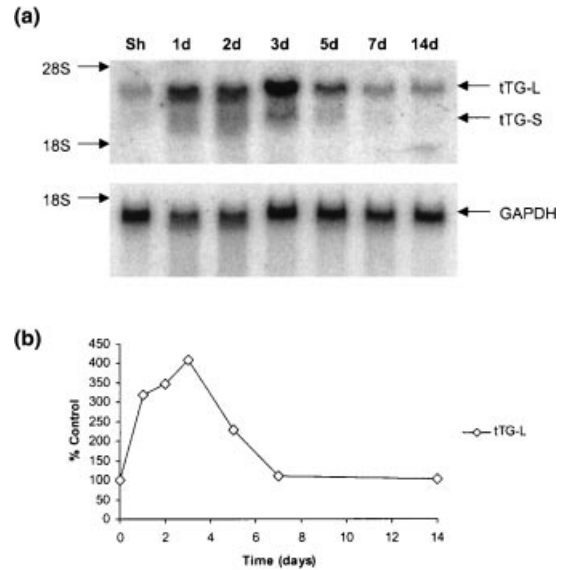


Fig. 4 Northern blot analysis of ipsilateral cortex RNA after injury. (a) Above: northern blot using tTG-S PCR product as template for random hexamer-generated probe. Positions of tTG-L mRNA (3.7 kb) and tTG-S mRNA (2.4 kb) are noted. Below: northern blot using GAPDH cDNA as template for random hexamer-generated probe. Position of GAPDH (2 kb) mRNA is noted. (b) Northern blot analysis for tTG-L mRNA expression (\diamond) were quantified by densitometry and corrected for loading by the level of GAPDH expression. tTG-L mRNA expression is represented as a percentage of sham expression. For each condition, tissue from three replicate animals was combined prior to northern analysis.

tTG-S transcript expression. To address these limitations, a semiquantitative PCR strategy was developed to independently measure tTG-L and tTG-S transcript levels. This approach has two major advantages: (1) PCR amplification allows for detection of much lower levels of transcript expression and (2) a PCR-based approach decreases the amount of total RNA required for analysis, thereby facilitating analysis of smaller tissue samples and obviating the need to pool RNA samples prior to analysis.

Standard curve generation for semiquantitative PCR using serially diluted cDNA

As the highest level of tTG-L and tTG-S transcript expression was observed in ipsilateral cortex three days after injury, ipsilateral cortex total RNA was collected three days after injury, reverse transcribed, and serially diluted to generate a standard curve of relative amounts of RNA. These samples underwent analysis using the LightCycler PCR with primer sets for tTG-L, tTG-S, or GAPDH mRNA. For each dilution sample (100%, 33.3%, 11.1% and 3.7%), the PCR analysis yielded a crossing point cycle number for each primer pair (tTG-L, tTG-S or GAPDH-specific). Figure 5 shows the linear regression analysis of each primer set's crossing point cycle number vs. the logarithm of the dilution factor. For

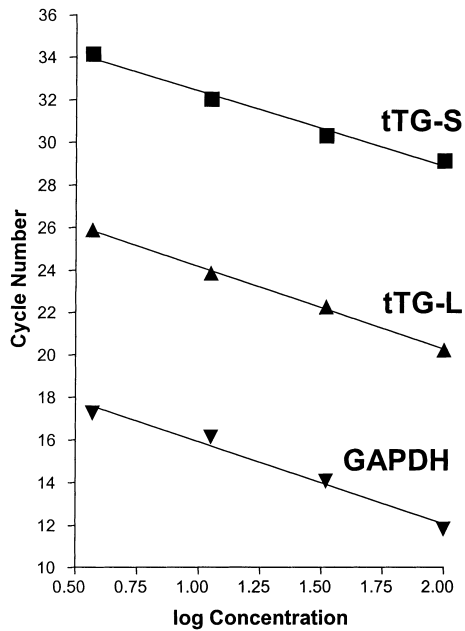


Fig. 5 Standard curve generation for tTG-L-, tTG-S-, and GAPDH-specific quantitative PCR. Total RNA was extracted from rat ipsilateral cortical tissue three days after injury. RNA samples were reverse transcribed, and then the cDNA's were serially diluted. Aliquots of the cDNA dilution curve underwent real-time PCR using primer pairs for tTG-L mRNA, tTG-S mRNA, or GAPDH mRNA. For each dilution and each primer set, the cycle number at which the PCR amplification entered the log-linear region was identified (crossing point cycle number). Standard curves were generated by plotting the log concentration of total RNA versus the crossing point cycle number, and a linear regression analysis was performed (r^2 ranged from 0.980 to 0.998). For quantitation, individual sham and experimental RNA samples underwent real time PCR, generating a crossing point cycle number for each primer set. Using the standard curves, the cycle number was converted to an amount of RNA. These amounts were then expressed as percentage of control/sham RNA.

each primer set, the range of crossing point cycle numbers required to cover the serially diluted standard curve varied: 11–18 cycles for GAPDH, 20–26 cycles for tTG-L, 29–35 cycles for tTG-S. These differences reflect primarily the abundance of the transcripts. GAPDH mRNA is the most abundant transcript and requires the fewest cycles, whereas tTG-S mRNA is the least abundant transcript and requires the most cycles. Furthermore, each primer set amplified its target transcript with different efficiencies (GAPDH 79%, tTG-L 80%, and tTG-S 90%).

Semi-quantitative PCR analysis of experimental samples

Experimental RNA samples were analyzed with the Light-Cycler PCR system, generating crossing point cycle numbers for tTG-L-, tTG-S- and GAPDH-specific primers. Using the standard curves, the crossing point cycle numbers were

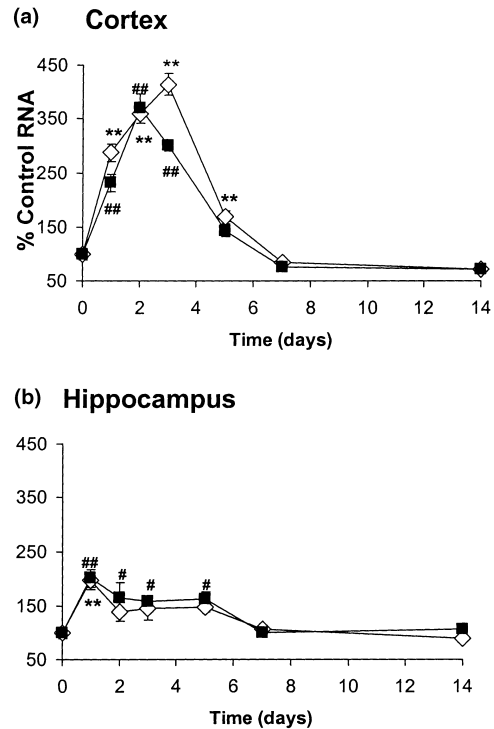


Fig. 6 Semi-quantitative PCR analysis of tTG-L and tTG-S mRNA expression in cortex (a) and hippocampus (b). tTG-L (\diamond) and tTG-S (\blacksquare) mRNA levels are expressed as a percentage of control/sham animal expression. One-way anova was performed, followed by Dunnett's multiple comparison test, to evaluate statistical significance. Comparing tTG-L mRNA expression from injured animals to control/sham animals. * $p < 0.05$; ** $p < 0.01$. Comparing tTG-S mRNA expression from injured animals to control/sham animals. # $p < 0.05$; ## $p < 0.01$.

converted to relative amounts of RNA. These relative amounts were then expressed as percentage of control/sham levels. Figure 6 shows the time course of tTG-L and tTG-S RNA expression in ipsilateral cortex and hippocampus after cortical injury. In cortex, maximal tTG-L mRNA expression was observed ($414\% \pm 21\%$ ($n = 3$) of control) three days after injury, while maximal tTG-S mRNA expression was observed [$369\% \pm 28\%$ ($n = 3$) of control] two days after injury. In hippocampus, maximal tTG-L and tTG-S mRNA levels were observed one day after injury, $196\% \pm 14\%$ ($n = 3$) of control, and $201\% \pm 16\%$ ($n = 3$) of control, respectively.

Linear regression analysis of tTG-L mRNA quantification by PCR versus northern blot

To verify that semi-quantitative PCR methods would yield results similar to northern blot analysis, a linear regression analysis was performed to compare tTG-L mRNA expression determined by PCR and northern blot (Fig. 7). Results from northern blot and PCR analyses fit a linear correlation with a slope = 1.01, $r^2 = 0.95$. This comparison is unavailable for

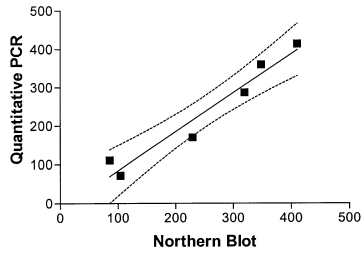


Fig. 7 Linear regression analysis of tTG-L mRNA expression in ipsilateral cortex, comparing quantification by PCR and by northern blot analysis. The linear relation with 95% confidence interval lines are shown. Regression analysis revealed a slope of 1.01 and $r^2 = 0.95$.

tTG-S mRNA since the transcript was not clearly quantified on the northern blot.

Discussion

This study describes the increased expression of tTG in cortex and hippocampus after traumatic brain injury. Two forms of tTG were expressed, tTG-L and tTG-S, that differ in their C-terminal regions. Western blot analysis with two separate antibodies for tTG identified tTG-L as the predominant form. In cortex, tTG-L protein expression peaked five days after injury and remained elevated at least two weeks after injury. tTG-L protein expression in the hippocampus also peaked five days after injury, but the degree of induction was more modest. tTG-L protein induction was supported by northern blot and semiquantitative PCR transcript analysis. The relatively delayed response of tTG to TBI suggests that it is not a major contributor to the more acute pathophysiological events such as those mediated by activation of calcium-dependent proteases. The contribution of these more delayed changes in tTG to more prolonged mechanisms of TBI recovery remained to be examined.

tTG-S expression was more difficult to analyze, since tTG-S protein was identified with only one of the two anti-tTG antibodies and tTG-S protein was less abundant than tTG-L protein. Although northern blot analysis detected tTG-S transcript three days after injury, tTG-S mRNA was undetectable in sham animals and at later time points after injury. Semi-quantitative PCR presents the most compelling evidence that tTG-S mRNA induction occurs. These studies provide the first evidence of tTG induction in response to traumatic CNS injury.

In a spinal cord ischemia model, over all TG activity underwent a transient increase that declined to control levels after one week (Fujita *et al.* 1995). In the superior cervical ganglion, TG activity was increased within one hour after axotomy and returned to baseline after 24 h (Gilad *et al.* 1985; Ando *et al.* 1993). TG activity also increased in the vagus nerve after crush injury (Tetzlaff *et al.* 1988). These previous studies measured over all TG activity and did not

measure expression of tTG protein or transcript. While the current studies did not address tTG activity, TBI induction of tTG activity may have been further potentiated by the increased intracellular calcium flux consistent with traumatic injury (Hubschmann and Nathanson 1985; Katayama *et al.* 1995), as tTG is a Ca^{++} dependent enzyme.

The role of tTG induction in the neural response to injury is not known; however, based on other experimental injury models, both damaging and protective functions can be proposed. For example, application of TG to injured optic nerve promoted axonal regeneration and recovery of visual evoked responses (Eitan *et al.* 1994). It was proposed that the supportive effects of TG treatment were due to stabilization of matrix proteins or protection of growing axons from oligodendrocyte-mediated growth inhibition. It had been previously shown that TG dimerizes interleukin-2 and that IL-2 dimers were cytotoxic to cultured oligodendrocytes (Eitan and Schwartz 1993).

Future studies of TBI-induced tTG expression will need to examine the cell type localization of tTG induction as it relates to the postinjury time course. This question is best answered by immunohistochemical analysis or *in situ* hybridization experiments. Also, the pathophysiology of tTG induction bears further examination. tTG may not only be important for subsequent cell death after traumatic injury, but also for compensatory mechanisms of other brain systems as they respond to the injury.

TG expression has been implicated in the cellular pathogenesis of Alzheimer's disease (for a review see Lesort *et al.* 2000). Increased over all TG activity and tTG-specific immunoreactivity have been observed in prefrontal areas of Alzheimer's disease brains compared to controls, with no differences observed in cerebellar expression (Johnson *et al.* 1997). Tau, the microtubule-associated-protein present in neurofibrillary tangles of Alzheimer's disease, is readily cross-linked by tTG (Dudek and Johnson 1993; Miller and Johnson 1995; Appelt *et al.* 1996; Appelt and Balin 1997; Murthy *et al.* 1998). TG also cross-links amyloid α -protein *in vitro*, producing amyloid protein oligomers similar to those seen in the neuritic plaques of Alzheimer's disease (Ikura *et al.* 1993; Dudek and Johnson 1994; Ho *et al.* 1994). tTG-specific immunoreactivity has been observed in neuritic plaques and amyloid cores from Alzheimer's disease brain (Zhang *et al.* 1998).

If TG activity is pathophysiologically relevant to the development of Alzheimer's disease, then TG induction after traumatic brain injury may contribute to the epidemiological observation that a subset of individuals who experience a traumatic brain injury are at a greater risk for developing Alzheimer's disease. Individuals with the apolipoprotein- $\alpha 4$ allele are at higher risk for developing Alzheimer's disease than other genotypes (Saunders *et al.* 1993). When the risks of Alzheimer's disease were examined in an elderly population, it was found that the apolipoprotein- $\alpha 4$ allele

conferred a two-fold increase in risk while the apolipoprotein- $\alpha 4$ allele combined with a history of traumatic head injury was associated with a 10-fold increase in risk (Mayeux *et al.* 1995). Subsequent studies have shown that the frequency of apolipoprotein- $\alpha 4$ expression is higher in individuals with amyloid α -protein deposition after head injury than in head-injured individuals without amyloid α -protein deposition (Nicoll *et al.* 1995). Although the pathophysiological mechanism linking head trauma and Alzheimer's disease is not known, it has been shown that traumatic head injury increased levels of amyloid precursor protein (APP) and this correlated with the presence of activated microglia expressing IL-1 (Griffin *et al.* 1994; Griffin *et al.* 1994; McKenzie *et al.* 1994; McKenzie *et al.* 1994). The induction of both APP and tTG by traumatic brain injury provides a potential mechanism by which head injury may contribute to Alzheimer's pathology. This hypothesis awaits further *in vivo* and *in vitro* experiments.

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