Published in final edited form as:

J Neurochem. 2008 July ; 106(1): 37–44. doi:10.1111/j.1471-4159.2008.05350.x.

Increased Levels of γ -glutamylamines in Huntington disease CSF

Thomas M. Jeitner^{*}, Wayne R. Matson[†], John E. Folk[‡], John P. Blass^{§,¶,**}, and Arthur J. L. Cooper^{§,¶,††}

^{*}Red Anvil, LLC, Milwaukee, Wisconsin, USA

[†]Department of Systems Biochemistry, Bedford Veterans Administration Medical Center, Bedford, Massachusetts, USA

[‡]Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, NIH, Bethesda, Maryland, USA

[§]Burke Medical Research Institute, White Plains, New York, USA

[¶]Department of Neurology, Weill Medical College of Cornell University, New York, New York, USA

^{**}Department of Medicine, Weill Medical College of Cornell University, New York, New York, USA

^{††}Department of Biochemistry, Weill Medical College of Cornell University, New York, New York, USA

Abstract

Transglutaminases (TGases) catalyze several reactions with protein substrates, including formation of γ -glutamyl- ϵ -lysine cross-links and γ -glutamylpolyamine residues. The resulting γ -glutamylamines are excised intact during proteolysis. TGase activity is altered in several diseases, highlighting the importance of *in situ* enzymatic determinations. Previous work showed that TGase activity (as measured by an *in vitro* assay) and free γ -glutamyl- ϵ -lysine levels are elevated in Huntington disease (HD) and that γ -glutamyl- ϵ -lysine is increased in HD CSF. Although free γ -glutamyl- ϵ -lysine was used in these studies as an index of *in situ* TGase activity, γ -glutamylpolyamines may also be diagnostic. We have devised methods for the simultaneous determination of four γ -glutamylamines in CSF: γ -glutamyl- ϵ -lysine, γ -glutamylspermidine, γ -glutamylputrescine, and bis- γ -glutamylputrescine and showed that all are present in normal human CSF at concentrations of ~150, 670, 40, and 240 nM, respectively. The high γ -glutamylspermidine/ γ -glutamylputrescine and γ -glutamylspermidine/bis- γ -glutamylputrescine ratios presumably reflect in part the large spermidine to putrescine mole ratio in human brain. We also showed that all four γ -glutamylamines are elevated in HD CSF. Our findings support the hypotheses that (i) γ -glutamylpolyamines are reflective of TGase activity in human brain, (ii) polyamination is an important post-translational modification of brain proteins, and (iii) TGase-catalyzed modification of proteins is increased in HD brain.

Keywords

γ -glutamylputrescine; γ -glutamylspermidine; γ -glutamyl- ϵ -lysine; bis- γ -glutamylputrescine; CSF; Huntington disease

Transglutaminases (TGases) catalyze the formation of linkages between glutamyl and lysyl residues or between glutamyl residues and polyamines, generating γ -glutamyl- ϵ -lysine or γ -

glutamylpolyamine bonds, respectively. Mammals produce eight active TGases: factor XIIIa and TGases 1-7, of which at least three (TGases 1-3) are present in the human brain (Kim *et al.* 1999; Griffin *et al.* 2002; Lorand and Graham 2003). TGase activity is altered in a diverse group of diseases. One of the most profound of these diseases is lamellar ichthyosis (Kim *et al.* 2002), a life-threatening disorder of keratinization resulting from mutations that ablate TGase 1 activity. In contrast, TGase activity is increased in the affected brain regions of Alzheimer, Parkinson, and Huntington (HD) diseases (Karpuj *et al.* 1999; Kim *et al.* 1999; Lesort *et al.* 1999). This increase in activity is accompanied by greater amounts of TGase-derived products, such as proteins cross-linked by γ -glutamyl- ϵ -lysine linkages and free γ -glutamyl- ϵ -lysine (Kim *et al.* 1999; Dedeoglu *et al.* 2002; Zainelli *et al.* 2003; Nemes *et al.* 2004). One of the proteins cross-linked by γ -glutamyl- ϵ -lysine is mutated huntingtin (Zainelli *et al.* 2003), the product of the causative gene for HD, and aggregates that contain mutated huntingtin accumulate in affected striatum (Dedeoglu *et al.* 2002; and references therein). Strategies designed to lower TGase activity in murine models of HD or Parkinson disease are therapeutic, and suggest that TGases may play a significant role in the etiology of these diseases (Dedeoglu *et al.* 2002; Karpuj *et al.* 2002; Fox *et al.* 2004; Mastroberardino *et al.* 2002; Van Raamsdonk *et al.* 2005; Tremblay *et al.* 2006; Wang *et al.* 2005).

Many of the studies of TGase in disease have focused on γ -glutamyl- ϵ -lysine, presumably because methods are available to measure this compound. For example, antibodies that recognize γ -glutamyl- ϵ -lysine cross-links in histological sections are commercially available, as well as analytical techniques for the measurement of protein-associated and free γ -glutamyl- ϵ -lysine (Jeitner *et al.* 2001b; and references therein). As noted earlier, the γ -glutamyl- ϵ -lysine linkage is not the only bond formed by TGases (Griffin *et al.* 2002; Lorand and Graham 2003). These enzymes catalyze an acyl transfer by a ping-pong mechanism involving two successive nucleophilic substitutions. In the first substitution, a thiolate anion in the active site of TGase attacks the carboxamide moiety at the γ position of a glutamyl (Q) residue to form a thioester, with the concomitant release of ammonia. The thioester then undergoes a substitution during which a nucleophilic substrate attacks the thioester linkage to replace the sulfur and generate a covalent linkage between the glutamyl residue and the nucleophile. A variety of nucleophiles are capable of attacking the thioester in the TGase active site, accounting for the diversity of TGase products. These nucleophiles include H₂O, the hydroxyl moiety of a ceramide (in the case of TGase 1), as well as the amine moieties of polyamines and lysyl residues, generating a γ -glutamyl residue, a γ -glutamyl ceramide ester residue, γ -glutamylpolyamine residues, and γ -glutamyl- ϵ -lysine linkages, respectively (Folk and Finlayson 1977; Lorand *et al.* 1979; Folk *et al.* 1980; Molberg *et al.* 1998; Nemes *et al.* 1999). Both terminal amines on polyamines can act as TGase substrates. Thus, the attachment of a polyamine to a glutamyl residue via a γ -glutamylpolyamine linkage leaves the remaining amine free to be bound to a second glutamyl moiety, thereby generating a bis- γ -glutamylpolyamine bond.

The formation of γ -glutamylpolyamine and bis- γ -glutamylpolyamine residues deserves further study in the context of neurodegenerative diseases. Polyamines are excellent TGase *in vitro* substrates and are present in the brain in substantial concentrations. For example, it has been reported that the concentrations of putrescine, spermidine, and spermine in various regions of the human brain are 12-42, 219-780, and 75-200 nmol/g, respectively (Vivó *et al.* 2001). Moreover, the attachment of polyamines can profoundly alter protein function (Cordella-Miele *et al.* 1993; Poduslo and Curran 1996; Masuda *et al.* 2000). Finally, compounds that attenuate TGase activity are being developed as potential therapeutics for the treatment of neurodegenerative disorders. The efficacy of these compounds is often assessed in terms of their ability to blunt *in vitro* TGase-catalyzed γ -glutamylspermidine or γ -glutamylputrescine formation (see Jeitner *et al.* 2005; and references therein). An appropriate assessment of *in vivo* activity of such compounds would include a measurement of their effects on *in vivo* γ -

glutamylspermidine or γ -glutamylputrescine formation. Given these considerations, the aim of the present study was to develop a sensitive method for the detection of a selected group of γ -glutamylamines and to measure these in biological samples.

In this study, the γ -glutamylamines chosen as representatives were γ -glutamyl- ϵ -lysine, two γ -glutamylpolyamines (i.e. γ -glutamylputrescine and γ -glutamylspermidine), and one bis- γ -glutamylpolyamine (i.e. bis- γ -glutamylputrescine). Of the four γ -glutamylamines, only γ -glutamyl- ϵ -lysine is currently available commercially. A procedure for the synthesis of N^1, N^4 -bis(γ -glutamyl)putrescine in > 100 mg amounts has been published previously (Folk 1983), and this procedure was employed for the synthesis of the N^1, N^4 -bis(γ -glutamyl)putrescine used in this study. In the study of Folk (1983), the N^1 - and N^8 -positional isomers of γ -glutamylspermidine were synthesized by a combination of chemical and enzymatic steps. However, the yields were very small (< 1 mg in 0.5 mL) and the compounds thus obtained were suitable only as standards for HPLC identification. In the present work, we present a chemical method for the synthesis of N^1 - γ -glutamylspermidine in amounts (~1 g) suitable for biological studies. In addition, we provide a procedure for the chemical synthesis of γ -glutamylputrescine in amounts also suitable for biological studies. The procedure is based on a previously published method.

In the current work, we investigated the occurrence of γ -glutamylamines in plasma from normal volunteers, in CSF from patients with no known neurological symptoms and in CSF from HD patients.

Material and methods

CSF samples

The CSF samples for the control group were obtained from the Hospital for Special Surgery (New York, NY, USA) ($n = 19$; 8 males and 11 females). The age of sampled individuals ranged from 31 to 83 years and averaged 55.1 ± 3.2 years. At the time of sampling, the subjects in the control group were free of dementia. The HD CSF samples were obtained from SciCor (Princeton, NJ, USA) from patients, whose ages ranged from 25 to 72 years with an average age of 46.7 ± 2.4 . In accordance with the relevant privacy rules, the information pertaining to patient gender, duration of illness, or the length of huntingtin Q_n repeat was not supplied. All samples were stored at -80°C prior to analysis. Subject selection and experimental procedures were approved by the Institutional Review Boards of the Burke Medical Research Institute (protocol number BRL 278).

Plasma samples

Plasma samples were obtained from normal adult males and females 20–45 years of age, who had signed consent forms. Each plasma sample (0.2 mL) was deproteinized with 0.2 mL of methanol and stored at -80°C . The sample was centrifuged at 14 000 g for 10 min at 4°C . The supernatant was then re-centrifuged at 14 000 g (10 min at 4°C) and subsequently passed through a 0.45- μm filter (6000 g; 10 min at 4°C) and 0.25- μm filter (14 000 g; 10 min at 4°C). The treated plasma samples were analyzed for γ -glutamylpolyamines as described below.

Synthesis of γ -glutamylpolyamines

N^1 -(γ -L-Glutamyl)spermidine.3HCl was prepared from α -*tert*-butyl *tert*-butyloxycarbonyl- γ -(N^4, N^8 -di *tert*-butyloxycarbonylspermidine)-L-glutamate (**I**). Compound **I** was prepared as follows: Ethyl chloroformate (0.3 mL, 3.13 mmol) was added dropwise with stirring to a cooled solution (0°C) of α -*tert*-butyl *tert*-butyloxycarbonyl-L-glutamate (0.949 g, 3.13 mmol) and *tri-n*-butylamine (0.75 mL, 3.13 mmol) in a mixture of 3 mL each of dimethylformamide and tetrahydrofuran. After 30 min at 0°C , a solution of N^4, N^8 -di *tert*-butyloxycarbonylspermidine

(Goodnow *et al.* 1990) (1.08 g, 3.13 mmol) in the same mixture, was added. Stirring was continued for 30 min at 0°C followed by overnight stirring at 25°C. The solvents were removed under high vacuum, and the residue was dissolved in ethyl acetate and washed consecutively with cold 5% (w/v) citric acid, cold water, saturated sodium bicarbonate, and brine. After drying over magnesium sulfate, the solvent was removed under vacuum and the product was purified by flash chromatography (ethyl acetate/hexanes, 2/1) to yield 1.27 g (64%) of a glass after drying under high vacuum. **¹H NMR.** (CDCl₃) δ 1.40-1.54 (m, 36H), 1.57-1.65 (m, 6H), 1.92 (NH, 1H), 2.09-2.19 (m, 2H), 2.24-2.30 (m, 2H), 3.19 (t, 4H), 3.20-3.30 (m, 4H), 4.11-4.16 (m, 1H), 4.56 (NH, 1H), 6.90 (NH, 1H). **MS.** (E1) 630 (M).

Elemental analysis. *Calculated* for C₃₁H₅₈N₄O₉: C 59.02; H 9.27; N 8.88%; *Found*: C 59.16; H 9.27; N 8.66%.

*N*¹-(γ-L-Glutamyl)spermidine.3HCl (**II**) was prepared from **I** as follows. Protective groups were removed from **I** (0.347 g, 0.55 mmol) by stirring for 4 h under nitrogen at 25°C with a mixture of trifluoroacetic acid (2.2 mL, 28.6 mmol) and methylene chloride (4.4 mL) containing triethylsilane (0.88 mL, 5.5 mmol) as a carbocation scavenger (Mehta *et al.* 1992). After removal of volatiles under vacuum the product was purified by flash chromatography (methylene chloride/methanol/concentrated ammonium hydroxide; 2/2/1). The oil obtained upon evaporation of solvents was treated with ethanol and this was evaporated to remove the last traces of ammonia. After dissolution in water and adjustment of the pH to 3.0 with dilute HCl, the solution was lyophilized to afford 0.154 g (80%) of a somewhat hygroscopic solid (**II**). **¹H NMR.** (D₂O) δ 1.75-1.77 (m, 4H), 1.85-1.95 (m, 2H), 2.10-2.17 (m, 2H), 2.40-2.46 (m, 2H), 3.04-3.09 (m, 6H), 3.29 (t, 2H), 3.79 (t, 1H). **MS.** (High resolution FAB⁺, t-glyc) *Calculated* for MH⁺ 275.2083; *Found* 275.2080.

Elemental analysis. *Calculated* for C₁₂H₂₆N₄O₃.3HCl.H₂O.0.5 ethanol: C 36.76; H 8.07; N 13.19%; *Found*: C 36.37; H 8.08; N 13.50%.

*N*¹-(γ-L-Glutamyl)putrescine.HCl (**III**) was prepared from α-*tert*-butyl *tert*-butyloxycarbonyl-γ-(*N*⁴-*tert*-butyloxycarbonyl-1,4-diaminobutane)-L-glutamate using a procedure modified from that of Nakajima *et al.* (1976). α-*tert*-butyl *tert*-butyloxycarbonyl-γ-(*N*⁴-*tert*-butyloxycarbonyl-1,4-diaminobutane)-L-glutamate (**IV**) was prepared using the procedure described for **I** except that *N*⁷-*tert*-butyloxycarbonyl-1,4-diaminobutane (Krapcho and Kuell 1990) was employed as the amine in place of the spermidine derivative. The product was crystallized under pentane following flash chromatography and was obtained in 55% yield after recrystallization from ethyl acetate-pentane; melting point 88°C. Protective groups were removed from **IV** in a manner similar to that used to deprotect **I**. Following purification by flash chromatography (conditions used for **II**), the oil obtained was dissolved in ethanol and the pH adjusted to 5-6 with HCl in ethanol. Crystallization from water-ethanol afforded the compound **III** in 50% yield as the monohydrochloride. Elemental analysis was in accord with the empirical formula of *N*¹-(γ-L-glutamyl)putrescine.HCl.

*N*¹,*N*⁴-bis(γ-L-Glutamyl)putrescine was synthesized as described by Folk (1983).

HPLC-EC method for the detection of γ-glutamylamines

γ-Glutamylamines were analyzed as their isoindole derivatives, after separation by reversed phase HPLC, and identified by electrochemical detection (EC). The procedure is a modification of that used previously for the analysis of γ-glutamyl-ε-lysine in human CSF (Jeitner *et al.* 2001b). Two volumes of the CSF or plasma were mixed with one volume of 0.1 M sodium tetraborate buffer (pH 9.3) and centrifuged through a 10 000 M_r cut-off filter at 12 000 g for 15 min at 20°C. The derivatization reagent was prepared daily by diluting a *o*-phthaldialdehyde (OPA) stock solution composed of 27 mg of OPA, 1 mL of 100% methanol, 30 μL of b-

mercaptoethanol, and 9 mL of 0.1 M sodium tetraborate, with 0.1 M sodium tetraborate at a ratio of 1 : 3. Derivatization was initiated by mixing 50 μ L of the derivatization reagent with 50 μ L of filtered CSF sample for 4 min at 20°C, after which 50 μ L of the derivatized material was injected onto a C18 column (Xterra MS C18 5 mm, 4.6 $\times$ 250 mm; Waters, Milford, MA, USA). The derivatized γ -glutamylpolyamines were eluted using a linear gradient of 0-25% (v/v) acetonitrile in 20% (v/v) methanol and 50 mM sodium phosphate buffer (pH 6.6), developed over 21 min, and followed by a further 55-min elution with 25% (v/v) acetonitrile, 20% (v/v) methanol, and 50 mM sodium phosphate buffer, pH 6.6. All elutions were performed at a rate of 1 mL/min. The compounds were detected using a four-channel (260, 320, 760, and 800 mV) coulometric array system (CoulArray, Model 5600; ESA, Chelmsford, MA, USA) with the column and detectors maintained at 36 °C. The concentrations of γ -glutamylamines in the CSF samples were determined by comparison of peak heights against authentic standards carried through the analysis procedure. CSF samples were analyzed twice and each of the replicates was analyzed in random order to ensure the independence of the measures.

Statistical analysis

All data are reported as mean $\pm$ SEM (n) for $n \geq 3$ or mean alone for $n = 2$. In the case of the latter, the replicates did not vary by more than 5% of the mean. The statistical difference between various groups was assessed with the t -test. A p value of 0.05 was considered significant.

Results

Assay development

Our earlier method for the measurement of γ -glutamyl- ϵ -lysine in CSF (Jeitner *et al.* 2001b) was used to develop a method for the simultaneous detection of γ -glutamyl- ϵ -lysine, γ -glutamylspermidine, γ -glutamylputrescine, and bis- γ -glutamylputrescine. This method was modified as described in the Materials and methods section to improve the resolution among the peaks under consideration. Using these conditions, the recovery of γ -glutamyl- ϵ -lysine added at 91, 182, or 363 nM to pooled plasma was 103 ± 1.61 ($n = 3$), 108 ± 0.44 ($n = 4$), and $104 \pm 0.79\%$ ($n = 3$), respectively. Similarly, 99% ($n = 2$) of γ -glutamyl- ϵ -lysine added to pooled CSF was recovered using these conditions. As an additional test of the validity of the assay conditions, the recovery of 182 nM γ -glutamyl- ϵ -lysine added to pooled CSF chromatographed with gradients developed over 21, 22, and 23 min was measured. In each case, the peak assigned to γ -glutamyl- ϵ -lysine in the pooled CSF or CSF 'spiked' with 182 nM γ -glutamyl- ϵ -lysine eluted in the same position as reagent γ -glutamyl- ϵ -lysine, namely 23.9, 24.3, and 24.9 min for the 21, 22, and 23 min gradients, respectively. Moreover, the recovery of the 182 nM γ -glutamyl- ϵ -lysine 'spike' in these experiments averaged between 99% and 102% ($n = 2$).

The above assay of γ -glutamyl- ϵ -lysine relies on the derivatization of the amine moieties with OPA and is therefore applicable for the measurement of other amine-bearing species, such as γ -glutamylspermidine, γ -glutamylputrescine, and bis- γ -glutamylputrescine. OPA has an oxidizable group and its derivatives are therefore amenable to EC. This form of detection is a particularly useful tool for the simultaneous measurement of γ -glutamylpolyamines as the detector response is both sensitive and linear over the nano- to millimolar range of concentrations. For example, the detector response to γ -glutamylspermidine is linear between 4 nmol and 4.2 μ mol in the 50- μ L injectate (Fig. 1). In other experiments, the detector response was shown to be linear for γ -glutamylputrescine and bis- γ -glutamylputrescine between 1 pmol and 2 nmol in the 50- μ L injectate (not shown). The recovery of 651 nM γ -glutamylspermidine from pooled CSF was 99.4 and 95.9% ($n = 2$) using 22 and 23 min gradients, respectively. In plasma, the average recovery of 2.61 and 5.21 μ M γ -glutamylspermidine was 95.5% and 91.9% ($n = 2$), respectively. γ -Glutamylputrescine and bis- γ -glutamylputrescine were not detectable in

plasma (see below) by the described method and analysis of these compounds was restricted to CSF. The recovery of 287 nM bis- γ -glutamylputrescine from pooled CSF was 103% using either 22 or 23 min gradients. γ -Glutamylputrescine eluted late in the chromatography at 45 min and was present at low amounts in CSF.

The lowest concentrations used in the construction of standard curves, intra-run variance, and inter-run variance are shown in Table 1.

γ -Glutamylamines in human plasma

γ -Glutamyl- ϵ -lysine and γ -glutamylspermidine were detected in human plasma (Table 2). Neither γ -glutamylputrescine nor bis- γ -glutamylputrescine were detectable.

γ -Glutamylamines in human Huntington disease CSF

Of the γ -glutamylamines studied, γ -glutamylspermidine was the most abundant in CSF, followed by bis- γ -glutamylputrescine, γ -glutamyl- ϵ -lysine and then γ -glutamylputrescine (Fig. 2, Table 3). γ -Glutamylspermidine exhibited a significant change because of HD: an increase that was ~60% above the control γ -glutamylspermidine and comparable to fold increases of 1.4, 1.4, and 1.7 observed for bis- γ -glutamylputrescine, γ -glutamyl- ϵ -lysine, and γ -glutamylputrescine, respectively, in HD CSF. These increases were statistically significant ($p \leq 0.005$, Table 3). There were also significant differences among the γ -glutamylamines. bis- γ -glutamylputrescine was present in CSF at greater concentrations than γ -glutamyl- ϵ -lysine (control + HD bis- γ -glutamylputrescine vs. control + HD γ -glutamyl- ϵ -lysine; $p \leq 0.05$; control bis- γ -glutamylputrescine vs. control γ -glutamyl- ϵ -lysine and HD bis- γ -glutamylputrescine vs. HD γ -glutamyl- ϵ -lysine; $p \leq 0.005$). The concentration of bis- γ -glutamylputrescine also greatly exceeded the amount of γ -glutamylputrescine in CSF (control + HD bis- γ -glutamylputrescine vs. control + HD γ -glutamylputrescine; control bis- γ -glutamylputrescine vs. control γ -glutamylputrescine and HD bis- γ -glutamylputrescine vs. HD γ -glutamylputrescine; $p \leq 0.001$). Finally, the CSF content of γ -glutamylspermidine was significantly different from the other γ -glutamylamines measured ($p \leq 0.001$ for all comparisons).

Discussion

The above studies describe a method for the simultaneous measurement of γ -glutamyl- ϵ -lysine, γ -glutamylspermidine, γ -glutamylputrescine, and bis- γ -glutamylputrescine in biological fluids by HPLC-EC. This method was used to determine the concentrations of the aforementioned γ -glutamylamines in control and HD CSF and demonstrated that the CSF of HD patients contained significantly more γ -glutamyl- ϵ -lysine, γ -glutamylspermidine, γ -glutamylputrescine, and bis- γ -glutamylputrescine than did the CSF of individuals free of neurological disease. It was also shown that of the γ -glutamylamines measured, γ -glutamylspermidine was present in the highest concentration in human CSF. Correct assignment of HPLC peaks as OPA derivatives of γ -glutamyl compounds was assessed by three criteria. When CSF samples were spiked with authentic standards and the eluting gradients were varied, recoveries of standard plus endogenous γ -glutamyl compound were quantitative. This finding minimizes the possibility of additional amine-containing compounds eluting with the γ -glutamyl compounds under investigation. Moreover, for HPLC-EC analysis, an analyte has two characteristic signatures, namely retention time (R_f) and relative peak heights in each of the different channels. In the present work, we used a four-channel CoulArray system (Materials and methods). The relative heights in the four channels of peaks in the CSF samples assigned to the four γ -glutamyl compounds on the basis of their respective R_f -values were the same as those noted for authentic standards.

Although these studies focused on the analysis of CSF, the method described herein was also able to detect γ -glutamylpolyamines in the plasma, semen, and urine. Interestingly, the γ -glutamylamines were differentially distributed in these fluids. γ -Glutamyl- ϵ -lysine, γ -glutamylspermidine, γ -glutamylputrescine, and bis- γ -glutamylputrescine were present in CSF, whereas only γ -glutamyl- ϵ -lysine and γ -glutamylputrescine were detected in semen (data not shown). Similarly, γ -glutamyl- ϵ -lysine and γ -glutamylspermidine were identified in plasma but not γ -glutamylputrescine or bis- γ -glutamylputrescine (Table 2). Others have previously detected γ -glutamyl- ϵ -lysine in human plasma at concentrations of ~ 1.9 – 3.6 μM (Harsfalvi *et al.* 1992). Our values of ~ 0.9 μM (Table 2) are close to those reported by Harsfalvi *et al.* (1992). The lower mean value obtained in our samples may reflect either a difference in the collection of the plasma or the higher qualitative assurance and resolution afforded by compound-specific signatures obtained with the EC sensors used in this study. All of the measured γ -glutamylpolyamines were detected in urine, which suggests that γ -glutamylputrescine and bis- γ -glutamylputrescine are cleared from blood very effectively. The concentration of urinary γ -glutamylamines was far in excess of the amounts of γ -glutamyl- ϵ -lysine, γ -glutamylspermidine, γ -glutamylputrescine, and bis- γ -glutamylputrescine found in the other bodily fluids and indicates both a high rate of production and clearance. The relevance of this production awaits an understanding of the biological role of γ -glutamylamine formation.

The role of γ -glutamyl- ϵ -lysine linkage, however, has been extensively studied, although significant questions still remain concerning the overall metabolism of the free isodi-peptide in physiology and disease (Jeitner *et al.* 2001b; Cooper *et al.* 2002; Dedeoglu *et al.* 2002; Griffin *et al.* 2002; Kim *et al.* 2002; Lorand and Graham 2003).

γ -Glutamyl- ϵ -lysine isopeptide bonds serve to cross-link proteins. bis- γ -Glutamylpolyamine linkages also cross-link polypeptide chains, and based on the concentrations of CSF bis- γ -glutamylputrescine and γ -glutamylspermidine, bis- γ -polyamine linkages may be the more common cross-links formed by TGases. Significantly more bis- γ -glutamylputrescine was found in CSF than γ -glutamyl- ϵ -lysine and this may reflect the greater abundance of putrescine in the brain relative to the substrate lysyl residues available in the brain. As noted in the introduction, the human brain contains considerable amounts of putrescine, spermidine, and spermine (≥ 12 μM) (Vivó *et al.* 2001), while the concentration of lysyl groups acting as TGase substrates is likely to be in the micromolar range or less. It is not possible at present to directly compare the TGase substrate behavior of polyamines with lysyl residues because the appropriate kinetic information has not been reported. The formation of protein-bound γ -glutamylputrescine precedes the creation of the bis- γ -glutamylputrescine linkage. CSF contains significantly more bis- γ -glutamylputrescine than γ -glutamylputrescine and heuristically supports the hypothesis that the attachment of a polyamine at one amine to a glutaminy residue may increase the ability of the remaining amine to serve as a TGase substrate for the generation of a product in which glutaminy residues are attached at both ends of a γ -glutamylpolyamine bridge (Jeitner *et al.* 2001a). If this hypothesis is correct then the most frequent TGase-derived cross-link may be bis- γ -glutamylspermidine as CSF contains high nanomolar to low micromolar concentrations of γ -glutamylspermidine (Table 1, Fig. 1).

The extent to which the concentrations of free γ -glutamylamines reflect *in situ* bond formation is at present, difficult to ascertain. Free γ -glutamylamine concentrations reflect in part the rates of TGase transamidating and γ -glutamylamine cyclotransferase activities, proteolysis, and tissue clearance. In addition, there is some evidence that various TGases can catalyze the breakage of an isopeptide linkage *within* a polypeptide substrate (Parameswaran *et al.* 1997). However, to the best of our knowledge, TGases have not been shown to hydrolyze free γ -glutamylamines. At present, TGases are the only mammalian enzymes known to catalyze the generation of γ -glutamylamine residues. Nevertheless, it is interesting to note that the concentration of spermidine in human brain is considerably greater than that of putrescine

(Vivó *et al.* 2001), a finding that correlates with the greater concentration of γ -glutamylspermidine compared with γ -glutamylputrescine and bis- γ -glutamylputrescine in human CSF (Table 1). Although glutamine synthetase catalyzes a reaction similar to that of TGases, attempts to demonstrate γ -glutamylpolyamine formation using glutamine synthetase, polyamine, and glutamate have been unsuccessful (Jeitner unpublished observation and O. W. Griffith personal communication). Amide linkages at the γ position are not subject to hydrolysis by proteolytic enzymes, and consequently γ -glutamyl- ϵ -lysine, γ -glutamylpolyamines, or bis- γ -glutamylpolyamines are excised intact during proteolysis. γ -Glutamyl- ϵ -lysine and γ -glutamylpolyamines are substrates of γ -glutamylamine cyclotransferase, but the activity of this enzyme is limited in the brain (Fink *et al.* 1980; Fink and Folk 1981; Danson *et al.* 2002). Thus, CSF γ -glutamylamine concentrations may reflect *in vivo* TGase activity. This conclusion is consistent with the increases in cerebral TGase activities, and protein bound and free γ -glutamyl- ϵ -lysine reported for HD and Alzheimer disease (Karpuj *et al.* 1999; Kim *et al.* 1999; Lesort *et al.* 1999; Jeitner *et al.* 2001b; Dedeoglu *et al.* 2002). Nevertheless, tracer studies to evaluate the steady state concentrations of polypeptide-associated and free γ -glutamylamine pools are required to properly assess the relationship of these metabolites to the processes regulating their synthesis and elimination.

The concentration of γ -glutamyl- ϵ -lysine was previously reported to be significantly elevated in CSF in Alzheimer disease (Nemes *et al.* 2001; Sárvári *et al.* 2002) and in CSF in HD (Jeitner *et al.* 2001b). CSF γ -glutamyl- ϵ -lysine is also significantly elevated in vascular dementia, dementia with Lewy bodies, dementia with Parkinson disease, advanced Parkinson disease without dementia, and frontotemporal dementia/Pick's disease (Sárvári *et al.* 2002). It appears that increased cerebral TGase activity is a common phenomenon in a variety of neurodegenerative diseases and not just in HD.

One of the more interesting observations of this study is the approximate micromolar concentration of γ -glutamylspermidine in CSF (Table 1). The role of γ -glutamyl- ϵ -lysine formation in physiology and disease has been reviewed extensively (e.g. Cooper *et al.* 2002; Kim *et al.* 2002; Martin *et al.* 2006; Muma 2007; Ruan and Johnson 2007). In contrast, little is known about the consequences of γ -glutamylpolyamine bond formation. Two important functions have been described thus far. The first of these is a posttranslational modification of proteins to alter function, that is, as a signaling mechanism. Cordella-Miele *et al.* (1993) demonstrated the TGase-dependent binding of polyamines to phospholipase A2, markedly increased the activity of this enzyme. The second function may be to alter protein transport. Poduslo and Curran (1996) reported that proteins bearing γ -glutamylpolyamine bonds had a far greater ability to cross endothelial barriers. Thus, the attachment of polyamines to proteins via γ -glutamylamine bonds may have profound effects on the function and distribution of these proteins. Elucidating the physiological protein substrates for γ -glutamylamine formation, and the consequences of this bond formation, promises new insights into the role of TGase in physiology and disease.

Acknowledgements

The above studies were funded by National Institute of Health grant PO1 AG14930.

Abbreviations used

EC, electrochemical detection; HD, Huntington disease; OPA, *o*-phthalaldehyde; TGase, transglutaminase.

References

- Cooper AJL, Jeitner TM, Blass JP. The role of transglutaminases in neurodegenerative diseases: overview. *Neurochem. Int* 2002;40:1–5. [PubMed: 11738467]
- Cordella-Miele E, Miele L, Beninati S, Mukherjee AB. Transglutaminase-catalyzed incorporation of polyamines into phospholipase A2. *J. Biochem* 1993;113:164–173. [PubMed: 8096844]
- Danson JW, Trawick ML, Cooper AJL. Spectrophotometric assays for L-lysine α -oxidase and γ -glutamylamine cyclotransferase. *Anal. Biochem* 2002;303:120–130. [PubMed: 11950211]
- Dedeoglu A, Kubilus JK, Jeitner TM, et al. Therapeutic effects of cystamine in a murine model of Huntington's disease. *J. Neurosci* 2002;22:8942–8950. [PubMed: 12388601]
- Fink ML, Folk JE. γ -Glutamylamine cyclotransferase. An enzyme involved in the catabolism of ϵ -(γ -glutamyl)lysine and other γ -glutamylamines. *Mol. Cell. Biochem* 1981;38:59–67. [PubMed: 6117008]
- Fink ML, Chung SI, Folk JE. γ -Glutamylamine cyclotransferase: specificity toward ϵ -(L- γ -glutamyl)-L-lysine and related compounds. *Proc. Natl Acad. Sci. USA* 1980;77:4564–4568. [PubMed: 6107907]
- Folk JE. Synthesis of N^1 -(γ -glutamyl)spermidine, N^8 -(γ -glutamyl) spermidine, N^1,N^8 -bis(γ -glutamyl) spermidine, N^1 -(γ -glutamyl)spermidine, N^1 -(γ -glutamyl)spermine, N^1,N^{12} -bis(γ -glutamyl)spermine, and N^1,N^4 -bis(γ -glutamyl)putrescine. *Methods Enzymol* 1983;94:451–457.
- Folk JE, Finlayson JS. The ϵ -(γ -glutamyl)lysine crosslink and the catalytic role of transglutaminases. *Adv. Protein Chem* 1977;31:1–133. [PubMed: 73346]
- Folk JE, Park MH, Chung SI, Schrode J, Lester EP, Cooper HL. Polyamines as physiological substrates for transglutaminases. *J. Biol. Chem* 1980;255:3695–3700. [PubMed: 6102569]
- Fox JH, Barber DS, Singh B, et al. Cystamine increases L-cysteine levels in Huntington's disease transgenic mouse brain and in a PC12 model of polyglutamine aggregation. *J. Neurochem* 2004;91:413–422. [PubMed: 15447674]
- Goodnow R Jr, Konno K, Niwa M, Kallimopoulos T, Bukownik R, Lenares D, Nakanishi K. Synthesis of glutamate receptor antagonist philanthotoxin-433 (PhTx-433) and its analogs. *Tetrahedron* 1990;46:3267–3289.
- Griffin M, Casadio R, Bergamini CM. Transglutaminases: nature's biological glues. *Biochem. J* 2002;368:377–396. [PubMed: 12366374]
- Harsfalvi J, Tarcsa E, Udvardy M, Zajka G, Szarvas T, Fésüs L. Presence and possible origin of ϵ -(γ -glutamyl)lysine isodipeptide in human plasma. *Thromb. Hemost* 1992;67:60–62.
- Jeitner TM, Fuchsbauer HL, Blass JP, Cooper AJL. A sensitive fluorometric assay for tissue transglutaminase. *Anal. Biochem* 2001a;292:198–206. [PubMed: 11355851]
- Jeitner TM, Bogdanov MB, Matson WR, et al. $N^{\epsilon\epsilon}$ -(γ -L-glutamyl)-L-lysine (GGEL) is increased in cerebrospinal fluid of patients with Huntington's disease. *J. Neurochem* 2001b;79:1109–1112. [PubMed: 11739625]
- Jeitner TM, Delikatny EJ, Ahlqvist J, Capper HR, Cooper AJL. Mechanism for the inhibition of transglutaminase 2 by cystamine. *Biochem. Pharmacol* 2005;69:961–970. [PubMed: 15748707]
- Karpuj MV, Garren H, Slunt H, Price DL, Gusella J, Becher MW, Steinman L. Transglutaminase aggregates huntingtin into nonamyloidogenic polymers, and its enzymatic activity increases in Huntington's disease brain nuclei. *Proc. Natl Acad. Sci. USA* 1999;96:7388–7393. [PubMed: 10377424]
- Karpuj MV, Becher MW, Springer JE, Chabas D, Youssef S, Pedotti R, Mitchell D, Steinman L. Prolonged survival and decreased abnormal movements in transgenic model of Huntington disease, with administration of the transglutaminase inhibitor cystamine. *Nat. Med* 2002;8:143–149. [PubMed: 11821898]
- Kim SY, Grant P, Lee JH, Pant HC, Steinert PM. Differential expression of multiple transglutaminases in human brain. Increased expression and cross-linking by transglutaminases 1 and 2 in Alzheimer's disease. *J. Biol. Chem* 1999;274:30715–30721. [PubMed: 10521460]
- Kim SY, Jeitner TM, Steinert PM. Transglutaminases in disease. *Neurochem. Int* 2002;40:85–103. [PubMed: 11738475]
- Krapcho AP, Kuell CS. Mono-protected diamines. *N-tert*-Butyloxycarbonyl- α,ω -alkanediamines from α,ω -alkanediamines. *Synthetic Commun* 1990;20:2559–2564.

- Lesort M, Chun W, Johnson GVW, Ferrante RJ. Tissue transglutaminase is increased in Huntington's disease brain. *J. Neurochem* 1999;73:2018–2027. [PubMed: 10537061]
- Lorand L, Graham RM. Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nat. Rev. Mol. Cell Biol* 2003;4:140–156. [PubMed: 12563291]
- Lorand L, Parameswaran KN, Stenberg P, Tong YS, Velasco PT, Jonsson NA, Mikiver L, Moses P. Specificity of guinea pig liver transglutaminase for amine substrates. *Biochemistry* 1979;18:1756–1765. [PubMed: 35225]
- Martin NA, Romito G, Pepe I, De Vivo G, Merola MR, Limatola A, Gentile V. Transglutaminase-catalyzed reactions responsible for the pathogenesis of celiac disease: from basic biochemistry to clinic. *Curr. Med. Chem* 2006;13:1895–1902. [PubMed: 16842200]
- Mastroberardino PG, Iannicola C, Nardacci R, et al. 'Tissue' transglutaminase ablation reduces neuronal cell death and prolongs survival in a mouse model of Huntington's disease. *Cell Death Differ* 2002;9:873–880. [PubMed: 12181738]
- Masuda M, Betancourt L, Matsuzawa T, Kashimoto T, Takao T, Shimonishi Y, Horiguchi Y. Activation of Rho through a cross-link with polyamines catalyzed by *Bordetella* dermonecrotizing toxin. *EMBO J* 2000;19:521–530. [PubMed: 10675321]
- Mehta A, Jaouhari R, Benson TJ, Douglas KT. Improved efficiency and selectivity in peptide synthesis: use of a triethylsilane as a carbocation scavenger in deprotection of t-butyl esters and t-butoxycarbonyl-protected sites. *Tetrahedron Lett* 1992;33:5441–5444.
- Molberg O, McAdam SN, Korner R, et al. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat. Med* 1998;4:713–717. [PubMed: 9623982]
- Muma NA. Transglutaminase is linked to neurodegenerative diseases. *J. Neuropathol. Expt. Med* 2007;66:258–263.
- Nakajima T, Kakimoto Y, Tsuji M, Konoshishi H. Occurrence and formation of γ -glutamylputrescine in mammalian brain. *J. Neurochem* 1976;26:115–118. [PubMed: 1255160]
- Nemes Z, Marekov LN, Fésüs L, Steinert PM. A novel function for transglutaminase 1: attachment of long-chain ω -hydroxyceramides to involucrin by ester bond formation. *Proc. Natl Acad. Sci. USA* 1999;96:8402–8407. [PubMed: 10411887]
- Nemes Z, Fésüs L, Egerházi A, Keszthelyi A, Degrell IM. N^ε(γ -glutamyl)lysine in cerebrospinal fluid marks Alzheimer type and vascular dementia. *Neurobiol. Aging* 2001;22:403–406. [PubMed: 11378245]
- Nemes Z, Devreese B, Steinert PM, van Beeumen J, Fésüs L. Cross linking of ubiquitin, HSP27, parkin, and α -synuclein by γ -glutamyl- ϵ -lysine bonds in Alzheimer's neurofibrillary tangles. *FASEB J* 2004;18:1135–1137. [PubMed: 15132984]
- Parameswaran KN, Cheng X-F, Chen EC, Velasco PT, Wilson JH, Lorand L. Hydrolysis of γ : ϵ isopeptides by cytosolic transglutaminases and by coagulation factor XIIIa. *J. Biol. Chem* 1997;272:10311–10317. [PubMed: 9092583]
- Poduslo JF, Curran GL. Polyamine modification increases the permeability of proteins at the blood-nerve and blood-brain barriers. *J. Neurochem* 1996;66:1599–1609. [PubMed: 8627316]
- Ruan Q, Johnson GVW. Transglutaminases in neurodegenerative diseases. *Front. Biosci* 2007;12:891–904. [PubMed: 17127346]
- Sárvári M, Fésüs L, Nemes Z. Transglutaminase-mediated crosslinking of neural proteins in Alzheimer's disease and other primary dementias. *Drug Develop. Res* 2002;56:458–472.
- Tremblay ME, Saint-Pierre M, Bourhis E, Lévesque D, Rouillard C, Cicchetti F. Neuroprotective effects of cystamine in aged parkinsonian mice. *Neurobiol. Aging* 2006;27:862–870. [PubMed: 15913845]
- Van Raamsdonk JM, Pearson J, Bailey CDC, Rogers DA, Johnson GVW, Hayden MR, Leavitt BR. Cystamine treatment is neuroprotective in the YAC128 mouse model of Huntington disease. *J. Neurochem* 2005;95:210–220. [PubMed: 16181425]
- Vivó M, de Vera N, Cortés R, Mengod G, Camón L, Martínez E. Polyamines in the basal ganglia of human brain. Influence of aging and degenerative movement disorders. *Neurosci. Lett* 2001;304:107–111. [PubMed: 11335066]
- Wang X, Sarkar A, Cicchetti F, Yu M, Zhu A, Jokivarsi K, Saint-Pierre M, Brownell AL. Cerebral PET imaging and histological evidence of transglutaminase inhibitor cystamine induced neuroprotection

in transgenic R6/2 mouse model of Huntington's disease. *J. Neurol. Sci* 2005;231:57–66. [PubMed: 15792822]

Zainelli GM, Ross CA, Troncoso JC, Muma NA. Transglutaminase cross-links in intranuclear inclusions in Huntington disease. *J. Neuropathol. Expt. Neurol* 2003;62:14–24.

Fig. 1. CoulArray detector response to the indicated amounts of γ -glutamylspermidine injected onto the HPLC column.

Fig. 2.

Individual values for control and HD CSF γ -glutamylpolyamines. Note that the right-hand y-axis relates to the γ -glutamylspermidine data and has a different scale (μM) to that of the left-hand y-axis (nM). The left-hand axis pertains to values of CSF bis- γ -glutamylputrescine, γ -glutamyl- ϵ -lysine, and γ -glutamylputrescine. The bars indicate the mean values for each of the groups. The mean, median, SEM, and n values are given in Table 1, as are significance values.

Table 1

Intra-run variance, inter-run variance, and minimum sensitivity for detection of various polyamines by the OPA-derivatization - HPLC CoulArray procedure

Compound	Intra-run variance (%)	Inter-run variance (%)	Minimum sensitivity (pmol) ^a
bis- γ -Glutamylputrescine	3.0	3.1	0.70
γ -Glutamyl- ϵ -lysine	3.7	4.1	3.6
γ -Glutamylspermidine	2.8	4.5	5.1
γ -Glutamylputrescine	8.2	Not done	0.96

^aThe values represent the lowest amount analyzed per 50- μ L injectate in each standard curve. Thus, the true limit of detection is lower than the values shown in this column. OPA, *o*-phthaldialdehyde.

Table 2 γ -Glutamylamines in human plasma^a

Analyte	<i>n</i>	Concentration (μ M)	Range	SEM
γ -Glutamyl- ϵ -lysine	17	0.86	0.61-1.17	0.17
γ -Glutamylspermidine	17	40.8	21.1-66.5	12.1

^a Plasma samples were from normal individuals. No difference was noted between the values obtained for plasma from males versus females. Therefore, the values were combined. Neither γ -glutamylputrescine nor bis- γ -glutamylputrescine was detected ($< 0.1 \mu$ M).

Table 3

γ -Glutamylamine levels in HD and control CSF

Analyte	Group	Concentration	Range	Median	Mean	SEM	n	p value
γ -Glutamylspermidine ^a	Control	μ M	0.33-1.02	0.69	0.67	0.05	19	0.001
	HD	μ M	0.69-1.69	1.10	1.09	0.05	26	
Bis- γ -glutamylputrescine	Control	nM	85-524	215	242	25	19	0.003
	HD	nM	138-606	311	339	24	26	
γ -Glutamyl- ϵ -lysine	Control	nM	74-372	146	153	14	19	0.003
	HD	nM	133-383	196	212	12	26	
γ -Glutamylputrescine	Control	nM	9-101	38	39	5	19	0.003
	HD	nM	33-173	56	71	8	26	

^aThe designation γ -glutamylspermidine can represent two positional isomers in which the γ -glutamyl moiety is attached at either the N^1 or N^8 positions. We synthesized the N^1 positional isomer. The N^1 and N^8 isomers elute very closely on ion exchange chromatography (Folk 1983). Thus, it is possible that the peak in the CSF sample eluting in the HPLC with the same retention time as authentic N^1 -(γ -L-glutamyl)spermidine contains a mixture of both the N^1 and N^8 positional isomers. HD, Huntington disease.