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INVITED REVIEW

γ-Glutamylamines and neurodegenerative diseases

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Abstract Transglutaminases catalyze the formation of γ-glutamylamines utilizing glutamyl residues and amine-bearing compounds such as lysyl residues and polyamines. These γ-glutamylamines can be released from proteins by proteases in an intact form. The free γ-glutamylamines can be catabolized to 5-oxo-L-proline and the free amine by γ-glutamylamine cyclotransferase. Free γ-glutamylamines, however, accumulate in the CSF and affected areas of Huntington Disease brain. This observation suggests transglutaminase-derived γ-glutamylamines may play a more significant role in neurodegeneration than previously thought. The following monograph reviews the metabolism of γ-glutamylamines and examines the possibility that these species contribute to neurodegeneration.

Keywords γ-Glutamylamine · Transglutaminase · Neurodegeneration · γ-Glutamylamine cyclotransferase

This review is dedicated to the memory of the late Dr. Jack Folk.

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Introduction

γ-Glutamylamines are a series of compounds bearing an amide linkage between the γ carboxamide of a glutaminyl (Q) residue or γ-carboxylate of glutamate and an amine group (Fig. 1). An example of such a molecule is γ-glutamylcysteinylglycine or glutathione. This tripeptide is one of the most abundant molecules in the body and indicates that the formation of γ-glutamylamide bonds is a frequent biochemical event. The enzymes responsible for the formation of glutathione are glutamate cysteine ligase and glutathione synthetase. Free γ-glutamylamines can also arise through the combined actions of transglutaminases and proteases (Chung and Folk 1972; Abe et al. 1977; Folk and Finlayson 1977; Fink et al. 1980; Folk et al. 1980; Fink and Folk 1981, 1983; Folk 1983; Fésüs et al. 1985; Beninati et al. 1988a, b, Piacentini et al. 1988; Martinet et al. 1990). In contrast to glutathione, the biology of transglutaminase-derived γ-glutamylamines is poorly understood. There is a compelling case for considering a role for these γ-glutamylamines in neurodegeneration. Transglutaminases are thought to contribute to neurodegeneration primarily by cross-linking proteins known to be involved in these diseases (Zemaitaitis et al. 2000; Wilhelmus et al. 2008; Iismaa et al. 2009; Jeitner et al. 2009a, b; Caccamo et al. 2010; Hoffner et al. 2010; Ricotta et al. 2010; Wilhelmus et al. 2012). These proteins include amyloid-β (Aβ), tau, α-synuclein and huntingtin in the diseases described by Alzheimer (AD), Parkinson (PD) and Huntington (HD), respectively (Selkoe et al. 1982; Zhang et al. 1998; Kim et al. 1999; Singer et al. 2002; Junn et al. 2003; Nemes et al. 2004; Konno et al. 2005; Zainelli et al. 2005; Wilhelmus et al. 2009). Aggregates containing these proteins, however, are found at sites other than the affected regions in the brain and suggest that other transglutaminase

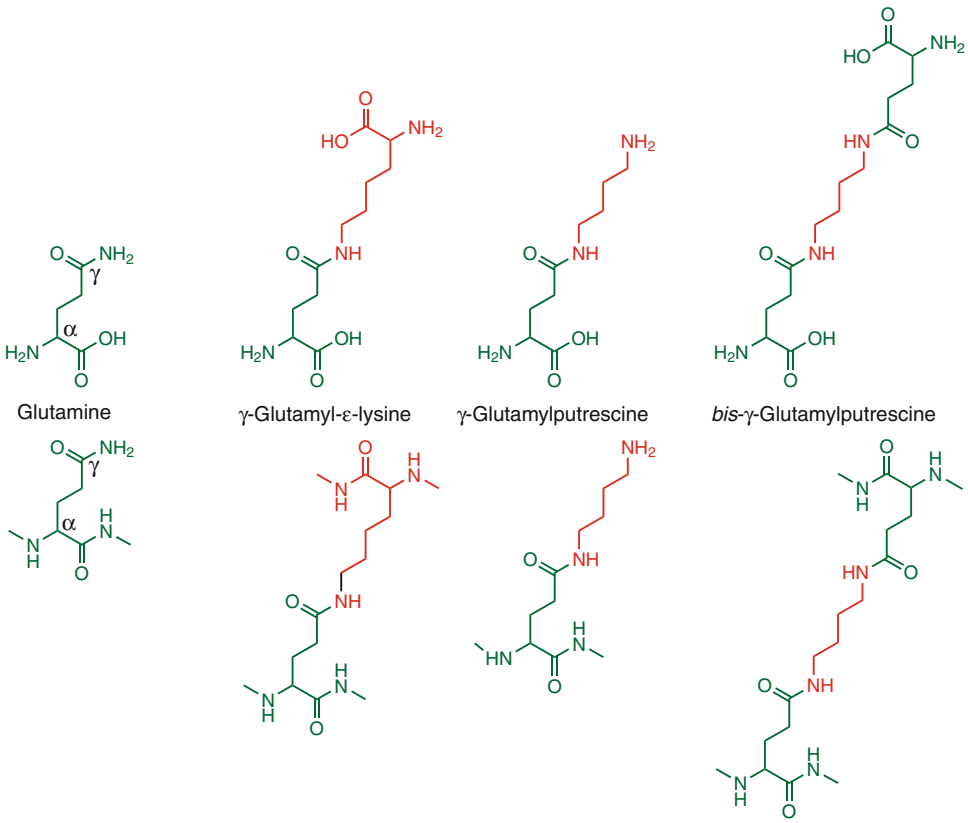

Fig. 1 γ-Glutamylamines. The structures for the free and protein-bound forms of glutamine and some γ-glutamylamines are shown in the upper and lower panels, respectively

products—namely the free γ-glutamylamines—may contribute to the toxic processes associated with these enzymes. The fact that free γ-glutamylamines accumulate in the diseased brain lends support to this notion (Jeitner et al. 2001; Dedeoglu et al. 2002; Jeitner et al. 2008). The following discussion briefly reviews the current knowledge concerning the metabolism of transglutaminase-derived γ-glutamylamines in the brain and the possible role of these molecules in neurodegenerative disorders.

Cerebral transglutaminases and formation of γ-glutamylamine bonds

Mammals produce eight active transglutaminases, of which transglutaminase 1–3 (Kim et al. 1999; Citron et al. 2001; Wilhelmus et al. 2009) and 6 (Grenard et al. 2001; Hadjivassiliou et al. 2008) are currently known to be expressed in the brain. Transglutaminases catalyze various modifications of the carboxamide moiety [–C(O)NH_2] of Q residues. These modifications include transamidation (Iismaa et al. 2003; Lorand and Graham 2003) (Fig. 2), deamidation (Molberg et al. 1998; van de Wal et al. 1998; Pinkas et al. 2007; Stamnaes et al. 2008) and esterification (Nemes et al. 1999), which convert the carboxamide group to [–C(O)NHR], [–CO_2^-] and [–C(O)OR] moieties, respectively. Transamidation is the only reaction attributed thus far to cerebral transglutaminases and results in the formation of γ-glutamylamine isopeptide bonds.

To date, all transglutaminase structures studied in detail share a common architecture of four ellipsoidal domains comprising an N-terminal β sandwich, a catalytic core and two C-terminal β barrels as shown for transglutaminase 2 in Fig. 3 (Pinkas et al. 2007). In the closed orientation, the C-terminal β barrels drape across the active site to prevent catalysis. The enzyme is subsequently activated by the binding of two calcium ions, which cause the hinge region between the catalytic core and β barrels to assume a helical conformation and extend the β barrels away from the catalytic site. This open conformation allows access to the catalytic quartet of Asp^{358}, His^{335}, Cys^{277} and Trp^{241} [residue designations as per human transglutaminase 2 (Pinkas et al. 2007)]. The conformational changes also juxtaposition Cys^{277} to Trp^{241} resulting in the formation of a thiolate–imidazolium ion pair oriented by Asp^{358} (Fig. 2). These changes allow a nucleophilic attack by the thiolate anion on the electron-deficient carbonyl of substrate γ-carboxamide group to generate an oxyanion intermediate (Iismaa et al. 2003). The charge on the oxyanion is stabilized by hydrogen bonding with the backbone nitrogen of Cys^{277} and the $N^{\varepsilon}1$ nitrogen of Trp^{241}. Subsequent acylation results in the release of NH_4^+ and the formation of an acyl-enzyme intermediate. This intermediate then undergoes a nucleophilic attack by an amine group and leads to the formation of a second oxyanion. Cys^{277} and Trp^{241} again stabilize the oxyanion through hydrogen bonding and deacylation

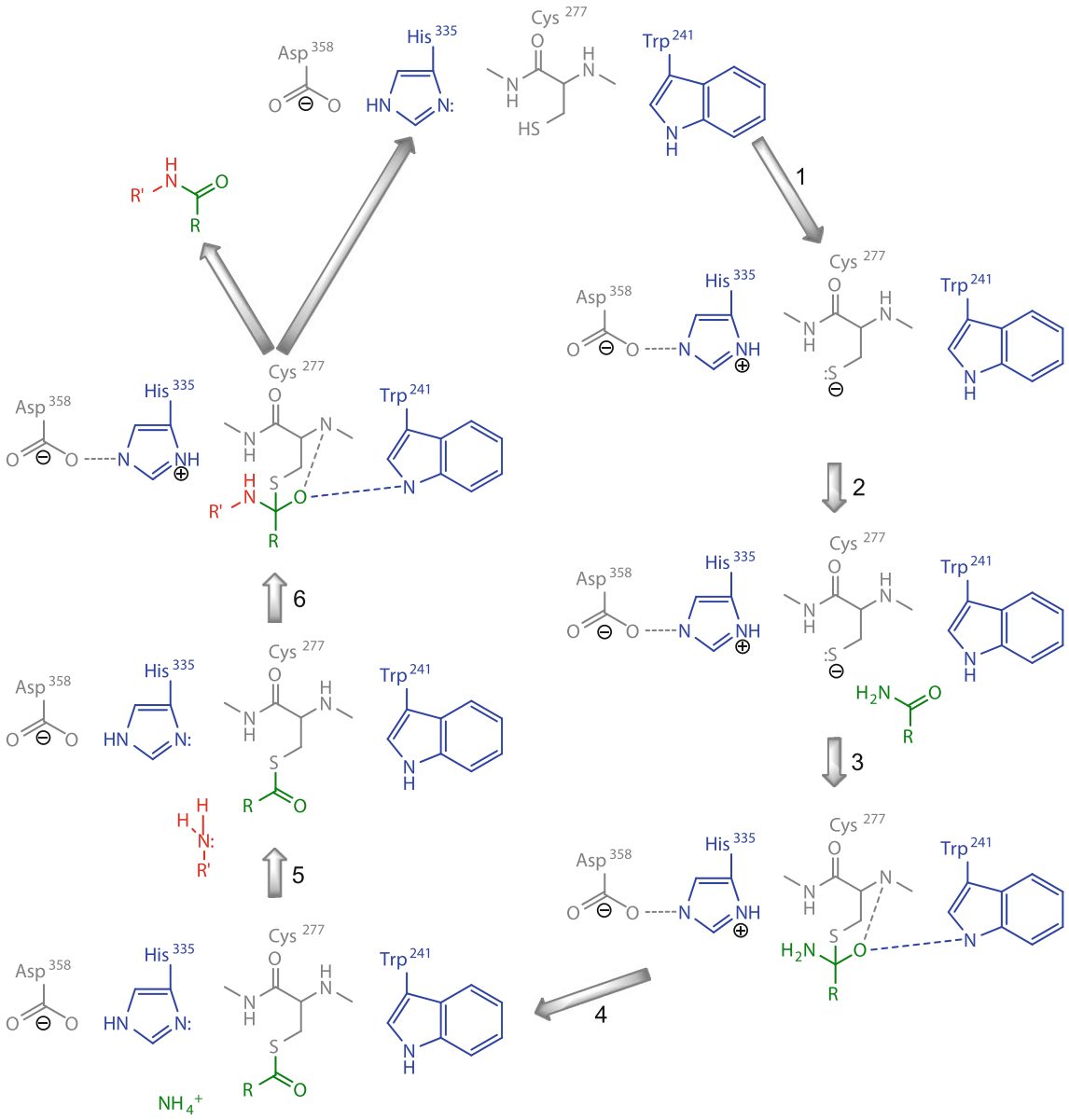

Fig. 2 Transamidation as catalyzed by transglutaminases. Adapted from IIsmaa et al. (2003). The *dashed lines* in this figure indicate a shared hydrogen atom

completes the reaction. The key residues and structural motifs described above are conserved among transglutaminases 1–3 and suggest that the reaction scheme given in Fig. 2 is shared by these enzymes.

γ-Glutamyl-ε-lysine cross-linking and neurodegenerative disorders

Monoamines, diamines, polyamines and the ε amino group of lysyl (K) residues all serve as amine-bearing substrates for the transamidation reaction (Lorand and Graham 2003). Of the possible reaction products, the bond formed between Q and K residues—the γ-glutamyl-ε-lysine or N^{ε}-(γ-L-glutamyl)-L-lysine isopeptide linkage—is the most commonly studied (Fig. 1). This bond can be formed within and between polypeptide chains. γ-Glutamyl-ε-lysine linkages between polypeptides can act to stabilize protein aggregates. In many cases, such as the creation of the extracellular matrix or the cornified envelope, formation of this linkage is a crucial event, and mutations that affect the activity of the relevant transglutaminases can result in life-threatening

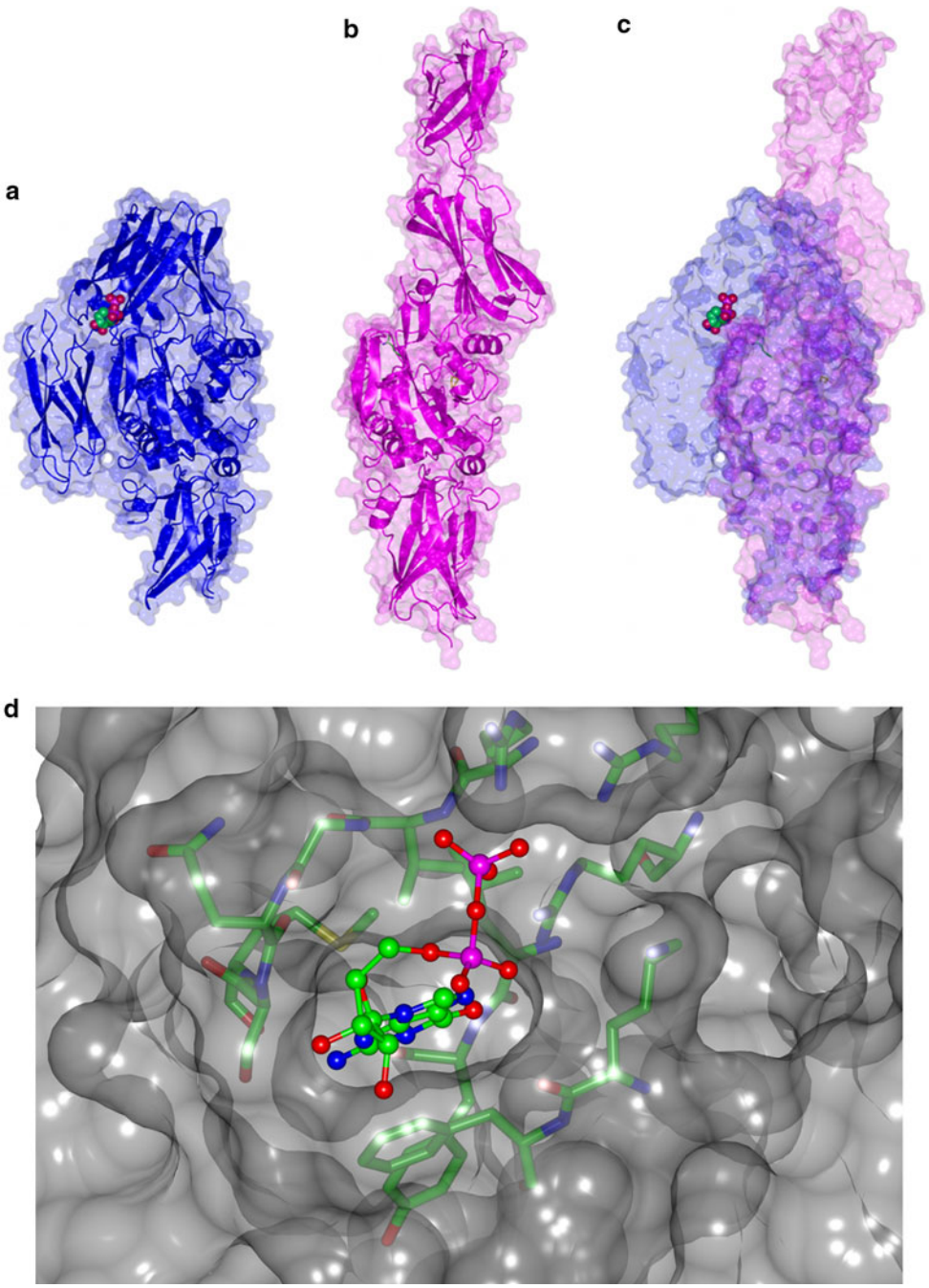

Fig. 3 Structure of transglutaminase 2. The *upper* illustrations represent the *closed* (**a**) and *open* (**b**) conformations of transglutaminase 2 as well as the merger of these conformations (**c**). The *lower diagram* shows a diagram of the active site with γ-glutamyl-ε-lysine in place (**d**). γ-Glutamyl-ε-lysine and some of the key active site residues are shown as stick and ball configurations, while the rest of the enzyme is depicted as ribbon diagram. These figures were generated using the program CCP4MG from the PDB coordinates IKV3 (*blue*) and ZQ3Z (*magenta*) (McNicholas et al. 2011)

disease (Cooper et al. 2002; Kim et al. 2002; Wilhelmus et al. 2008; Herman et al. 2009; Iismaa et al. 2009; Jeitner et al. 2009a, b; Caccamo et al. 2010; Hoffner et al. 2010; Ricotta et al. 2010). The aggregation of particular proteins in the brain, however, is thought to be a contributing factor in a number of important neurodegenerative disorders. Examples include Aβ in the amyloid plaques of AD (Zhang et al. 1998; Wilhelmus et al. 2009), α-synuclein in the Lewy bodies of PD (Junn et al. 2003; Nemes et al. 2004; Segers-Nolten et al. 2008; Nemes et al. 2009; Verhaar et al. 2011; Wilhelmus et al. 2011a, b), and huntingtin in the inclusion bodies of HD (Gentile et al. 1998; Kahlem et al. 1998; Cooper et al. 2002; Dedeoglu et al. 2002; Karpuj et al. 2002; Zainelli et al. 2003, 2005) [for a comprehensive list of these proteins see (Kim et al. 2002; Hoffner et al. 2010)].

It has been hypothesized that γ-glutamyl-ε-lysine cross-links help stabilize the aggregates found in neurodegenerative diseases. The following evidence supports this hypothesis: γ-glutamyl-ε-lysine cross-links colocalize with aggregates of aβ (Wilhelmus et al. 2009), hyperphosphorylated tau-containing paired helical filament (Zemaitaitis et al. 2000; Singer et al. 2002; Wilhelmus et al. 2009, 2012), α-synuclein (Junn et al. 2003) and huntingtin (Dedeoglu et al. 2002; Zainelli et al. 2003, 2005), as revealed by immunohistochemical staining of the affected areas of AD, PD, supranuclear palsy and HD, respectively. The presence of these cross-links in α-synuclein-containing oligomers isolated from the brains of AD and PD patients, and also from a detergent-insoluble fraction from the diseased regions in AD brains has been confirmed by other methods (Kim et al. 1999; Nemes et al. 2004, 2009). Moreover, the number of γ-glutamyl-ε-lysine cross-links present in these fractions is greater in the diseased brains (Table 1). For example, the detergent-insoluble fractions isolated from the cortex and cerebellum of AD patients contain between 30 and 50 γ-glutamyl-ε-lysine cross-links/10,000 residues as compared to 1 γ-glutamyl-ε-lysine cross-link/10,000 residues found in corresponding regions of age-matched controls (Kim et al. 1999). As revealed in Table 1, there is great disparity in the number of γ-glutamyl-ε-lysine cross-links reported to be associated with the detergent-insoluble proteins. Even so, the number of these cross-links is consistently higher in proteins extracted from individuals with neurodegenerative disorders and correlates with both the deposition of proteinaceous aggregates and the activation of transglutaminases in these diseases.

Bis γ-glutamylpolyamine residues in neurodegeneration

In addition to γ-glutamyl-ε-lysine cross-links, transglutaminases catalyze the formation of bis-γ-glutamylpolyamine bridges between polypeptide chains (Folk et al. 1980; Beninati et al. 1988a, b; Piacentini et al. 1988). These bridges are formed by two successive transamidations, the first of which involves the attack of a polyamine on a Q residue to generate a γ-glutamylpolyamine residue (e.g., γ-glutamylputrescine in Fig. 1). This moiety has a remaining free terminal amine which allows it to participate in a second transamidation, thereby producing a bis-γ-glutamylpolyamine cross-link (e.g., bis-γ-glutamylputrescine cross-link in Fig. 1). The role of bis-γ-glutamylamine cross-links in the stabilization of oligomerized proteins in plaques, tangles and inclusion bodies has not yet been investigated and may be important as a determinant of solubility of certain protein aggregates. Lai et al. (2004) and Konno et al. (2005) demonstrated that two proteins—thioredoxin bearing polyQ expansions and β-lactoglobulin—cross-linked with bis-γ-glutamylputrescine linkages formed insoluble aggregates, whereas those cross-linked with γ-glutamyl-ε-lysine bonds remained in solution. In particular, these researchers discovered that the formation of γ-glutamyl-ε-lysine cross-links by transglutaminases prevented the spontaneous generation of insoluble protein aggregates. The addition of polyamines in these experiments caused the proteins to precipitate implying that the formation of bis-γ-glutamylpolyamine or γ-glutamylpolyamine linkages favored the formation of insoluble aggregates. This idea has appeal because it suggests a reciprocal relationship between amounts of cerebral polyamines and insoluble aggregates as the brain ages. The amount of polyamines in the brain decreases with age, which may favor the formation of γ-glutamyl-ε-lysine linkages in certain proteins (Liu et al. 2008). The possibility that the ratio of bis-γ-glutamylputrescine to γ-glutamyl-ε-lysine linkages in aggregated protein determines the solubility of these aggregates is likely to be dependent on a number of factors including the size and composition of the

Table 1 Frequency of γ-glutamyl-ε-lysine cross-links in detergent-insoluble protein fractions of AD brains

Tissue	Group	γ-glutamyl-ε-lysine cper 10^6 residues	Fold change	References
Cerebellum/cortex	Control	0.10×10^3		(Kim et al. 1999)
	AD	$3–5 \times 10^3$	30–50	
Occipital lobe	Control	~2.00		(Nemes et al. 2004)
	AD	~2.00	1.00	
Frontal cortex	Control	~5.83		
	AD	~21.7	~3.71	
Hippocampus	Control	~8.33		
	AD	~31.7	~3.80	
Frontal cortex	Control	~0.11		(Nemes et al. 2009)
	AD	~0.58	~5.14	
Hippocampus	Control	~0.11		
	AD	~0.42	~3.71	

aggregates. For example, the low-n oligomers—dimers, trimers and tetramers of Aβ and α-synuclein cross-linked by γ-glutamyl-ε-lysine linkages—form large insoluble structures that are toxic to cells (Schmid et al. 2009, 2011). In the case of Aβ, these larger, insoluble polymers are known as protofibrils and amyloid fibrils. Although the toxicity of the low-n oligomers is well established (Ono and Yamada 2011), the mechanisms by which these structures kill cells has not been elucidated and may include the release of free γ-glutamyl-ε-lysine. It should be noted, however, that the toxicity of the soluble Aβ and α-synuclein oligomers—the dimers, trimers and tetramers—has not been investigated. The formation of these oligomers is not obligatory for the creation of a proportion of the large insoluble Aβ aggregates (Necula et al. 2007). Nonetheless, it is reasonable to assume that Aβ dimers, trimers and tetramers formed as precursors for the assembly of the larger aggregates, as described by Schmid and colleagues (2011), would be proteolyzed and thereby generate the free γ-glutamyl-ε-lysine dipeptide. These observations suggest that transglutaminase catalyze the formation of soluble oligomers stabilized by γ-glutamyl-ε-lysine linkages. The same proteins can be polymerized into insoluble assemblages cross-linked by bis-γ-glutamylpolyamine bonds or larger precipitates held together by a combination of hydrophobic interactions and γ-glutamyl-ε-lysine bonds.

Interestingly, the idea that transglutaminase-catalyzed modifications other than γ-glutamyl-ε-lysine bond formation affect the solubility of proteins has also been examined by Schmid et al. (2011) who demonstrated that the deamidation of Aβ by transglutaminase 2 promotes the solubility of this protein. As discussed below, solubility may be a crucial determinant of the toxicity of protein aggregates and highlights the importance of future studies on the contribution of bis-γ-glutamylpolyamine and γ-glutamyl-ε-lysine bridges to the solubility of proteinaceous aggregates.

Toxicity of plaques, tangles and inclusion bodies

The plaques, tangles, Lewy bodies and other inclusions observed in neurodegenerative disorders have long been assumed to be toxic to neurons by many researchers. This assumption led to the hypothesis that the processes creating these amalgamations—such as the activation of transglutaminases—are similarly detrimental to neurons. Green (1993) hypothesized that this might particularly be the case in CAG-trinucleotide expansion diseases because novel transglutaminase substrates—specifically stretches of polyQ residues—are produced in this group of neurodegenerative disorders. The CAG nucleotide encodes glutamine and expansion of this sequence in exons results in the insertion of polyQ residues into the expressed protein (Gusella et al. 1993; Rubinsztein et al. 1993). HD is an example of a CAG-expansion disease. This disease is caused by CAG-trinucleotide expansions in the *huntingtin* gene, which encodes huntingtin bearing varying lengths of polyQ (Hoogeveen et al. 1993). The number of CAG repeats, after a certain threshold, is inversely related to the age of onset and the severity of disease (Rubinsztein et al. 1993). Interestingly, the ability of polyQ residues to act as transglutaminase substrates increases as a function of polyQ length (Cooper et al. 1997a, b, Gentile et al. 1998; Kahlem et al. 1998; Cooper et al. 2002; Ruoppolo et al. 2003; Lai et al. 2004). Thus, the transglutaminase substrate activity of polyQ residues parallels the ability of these pathologically elongated sequences to cause HD, and strengthens the argument that transglutaminases play a role in CAG-trinucleotide expansion diseases. The assumption that insoluble aggregates cause the death of cells, however, is not universally accepted. For example, the distribution of inclusion bodies in the striata of HD patients does not coincide with that of the dead and dying neurons (Kuemmerle et al. 1999; Arrasate et al. 2004). Indeed, aggregates of mutant huntingtin are deposited throughout the body of the HD patients to no apparent ill effect in the early stages of the disease (Choi et al. 2000).[1] Similar observations have been made concerning the insoluble deposits in AD and PD, prompting the hypothesis that these structures are inert and act as a form of storage for cellular debris (Saudou et al. 1998, Arrasate et al. 2004). The possibility also exists that some of these structures may even play a hitherto undiscovered physiological function. For example, aggregates containing tau, parkin and α-synuclein are present in the laminated bodies or corpora amylacea found throughout the central nervous system of the elderly (Wilhelmus et al. 2011a, b). Corpora amylacea are thought to sequester debris accrued during aging. The aggregates found in these bodies colocalize with transglutaminase 1 and γ-glutamyl-ε-lysine, and indicate a role for this enzyme in the normal aging process (Wilhelmus et al. 2011a, b).

Insoluble aggregates can play other beneficial roles in the brain. An example of such an insoluble aggregate in the brain is melanin. This aggregate is formed from the oxidation products of dopamine (Bisaglia et al. 2007; Pham et al. 2009; Bisaglia et al. 2010). Melanic polymers occupy approximately 47% of the cytoplasmic volume of the neurons in the substantia nigra and are responsible for the characteristic black or *nigra* color of this tissue (Fedorow et al. 2006). Substantia nigra neurons do not express ferritin, and melanin serves instead to chelate iron and other adventitious metals (Zecca et al. 1996, 2008). The

[1] This is probably not the case for sporadic inclusion myositis. The increase in transglutaminase activity observed in this disease is accompanied by a remarkable accumulation of protein cross-linked with γ-glutamyl-ε-lysine linkages, in the periphery, which is thought to contribute significantly to the myositis (Choi et al. 2000).

deposition of melanin outside of these cells, however, is thought to contribute to the pathology of PD (Gibb 1992; Good et al. 1992; Jellinger et al. 1992; Kastner et al. 1992; Lopiano et al. 2000; Zecca et al. 2002; Faucheux et al. 2003; Fasano et al. 2006; Zecca et al. 2008). Other biologically useful precipitates include the extracellular matrix, fibrin clots and the cornified envelope, all of which are stabilized, in part, by γ-glutamyl-ε-lysine and bis-γ-glutamylpolyamine cross-links (Lorand and Graham 2003; Iismaa et al. 2009). Interestingly, in the one tissue where the frequency of these bonds has been compared, namely skin, the number of bis-γ-glutamylpolyamine exceeds that of the γ-glutamyl-ε-lysine bridges (Beninati et al. 1988a, b; Piacentini et al. 1988; Martinet et al. 1990). This observation supports the hypothesis that some of the insoluble aggregates bearing bis-γ-glutamylpolyamine, rather than γ-glutamyl-ε-lysine bridges, are not toxic.

On the other hand, studies performed with cell and animal models suggest that expanded polyQ-containing huntingtin aggregates may indeed be toxic under certain circumstances (Hoffner et al. 2010). This toxicity may occur when the precipitates sequester crucial proteins (Cooper et al. 1997a, b; Ruoppolo et al. 2003) or are sufficiently large enough to physically disrupt the cytoskeleton or organelles (Hoffner et al. 2010). The formation of such precipitates does not necessarily require the activation of transglutaminase (Hoffner et al. 2010). These precipitates are likely to have been formed at least in part by a mechanism involving non-covalent polar zippers (Perutz 1995; Hoffner et al. 2010). Many of the studies demonstrating a relationship between aggregate size and toxicity have relied on the explicit or implicit assumption that aggregation of the huntingtin or polyQ domains is by polar zipper formation rather than the actions of transglutaminases. Transglutaminase activity can prevent the generation of polar zippers (Lai et al. 2004; Konno et al. 2005), and polyQ and huntingtin-containing polyQ residues act as in vitro and in vivo transglutaminase substrates. As noted above, the propensity of these sequences to act as transglutaminase substrates also increases with the length of polyQ residues (Cooper et al. 1997a, b; Gentile et al. 1998; Kahlem et al. 1998; Cooper et al. 2002; Ruoppolo et al. 2003; Lai et al. 2004). Thus, it is possible that transglutaminases are activated in HD to prevent the formation of aggregates stabilized by polar zippers. This idea is consistent with the observed increase in transglutaminase activity in the diseased tissues of HD, particularly during the early stages of this disease (Lesort et al. 1999), the observation that the number of aggregates increases in mice that express mutant huntingtin, but not transglutaminase 2 (Mastroberardino et al. 2002), and the fact that the number of γ-glutamyl-ε-lysine isopeptides per 10^6 residues thought to be associated with protein aggregates is low (between 0.1 and 100 per 10^6 residues: Table 1). It may be that in HD, transglutaminases act to cross-link mutant huntingtin with γ-glutamyl-ε-lysine linkages to produce soluble and, presumably, toxic aggregates. If this is the case, then the number of γ-glutamyl-ε-lysine cross-links would be greater in the soluble rather than the insoluble form of huntingtin in HD brain. A comparable argument could be made for the cross-linking of α-synuclein in PD and Aβ in AD, given that some of the early oligomers—the dimers, trimers and tetramers—are soluble (Schmidt et al. 2004; Necula et al. 2007; Schmid et al. 2011). Investigating this possibility is a worthwhile endeavor, but first requires the development and testing of methods for measurement of γ-glutamylamines that do not produce the intra-laboratory variation illustrated in Table 1. One possibility is the LC–EC method we developed and validated with LC–MS (Jeitner et al. 2001; Jeitner et al. 2008), but as yet it has not been tested in other laboratories.

As noted above, total transglutaminase activity is increased in HD. The relevant measurements were of the ex vivo incorporation of polyamines into a Q-bearing transglutaminase substrate rather than an in vivo or in situ assessment of this activity (Lesort et al. 1999; Karpuj et al. 2002). The reported increases in total transglutaminase activity, however, were matched by increases in the amount of transglutaminase 2, as well as the amounts of bound and free γ-glutamylamines in these tissues (Lesort et al. 1999; Dedeoglu et al. 2002; Karpuj et al. 2002; Zainelli et al. 2005) (Table 1). Knocking out transglutaminase 2 in an animal model of HD prolonged the life of these animals. Nonetheless, the animals still die of a neurodegenerative disorder involving the continued deposition of huntingtin aggregates, stabilized in part by polar zippers. The question that arises from these considerations is whether the aggregates cross-linked with bis-γ-glutamylpolyamines are toxic as compared to those stabilized by polar zippers. Answering this question would aid in elucidating the role of transglutaminases in CAG-expansion disease, and also of the significance of bis-γ-glutamylpolyamine and γ-glutamyl-ε-lysine cross-links in protein aggregation. The implication from these considerations is that the disease process depends to some extent on either relative availability of polyamines or the affinity of the mutant substrates relative to polyamines for the transglutaminases. In this context, it is interesting to note that the amount of polyamines decreases in the aging brain (Liu et al. 2008) and the number of γ-glutamyl-ε-lysine cross-links increases (Nemes et al. 2004).

Free γ-glutamylamines and γ-glutamylamine cyclotransferase

γ-Glutamylamines are released intact from proteins during proteolysis, because proteases do not hydrolyze isopeptide

bonds formed between the γ carboxamide group of a Q residue and an amine. Free γ-glutamylamines can, however, be metabolized to 5-oxo-L-proline and an alkylamine by γ-glutamylamine cyclotransferase (Fig. 4) (Fink et al. 1980; Fink and Folk 1981, 1983; Chen 2008; Oakley et al. 2010). The crystal and solution structures of this enzyme were recently solved and, in combination with site-directed mutagenesis studies, revealed the critical residues required for catalysis by γ-glutamylamine cyclotransferase (Oakley et al. 2010; Serrano et al. 2010). These studies indicate that

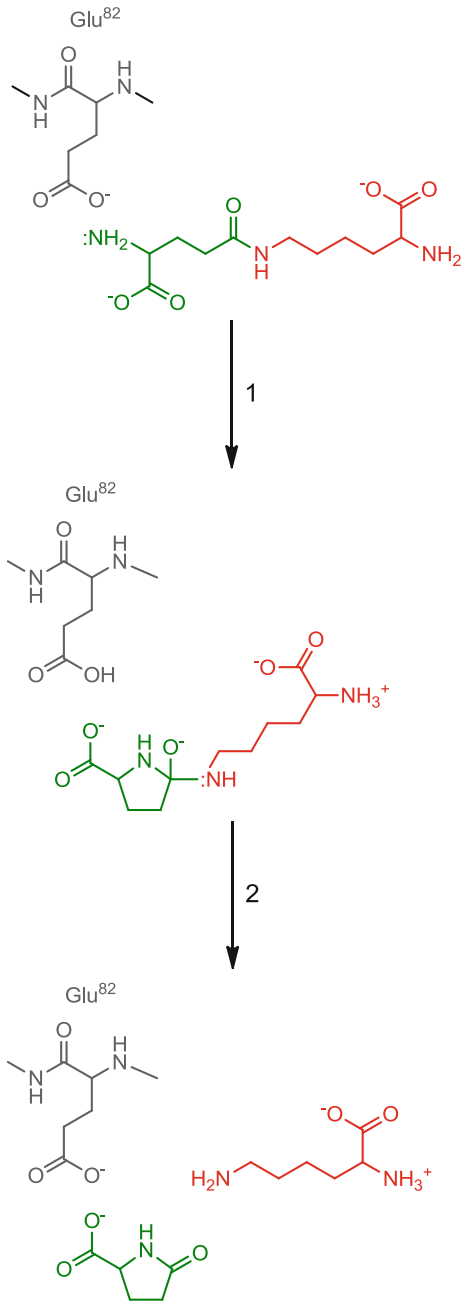

Fig. 4 Catalytic mechanism of γ-glutamylamine cyclotransferase. Adapted from Oakley et al. (2010)

γ-glutamylamine cyclotransferase possesses a five-strand β-barrel decorated with helices and connecting loops (Fig. 5). The barrel formed by this structure appears to be characteristic of cyclotransferases in general and features two β strands—β2a and β3b—that cross over each other (Oakley et al. 2010). A cavity is formed at the bottom of the barrel between the β1 and β5 strands that serves to juxtaposition substrates to the catalytic Glu^{82} residue. The cavity-forming residues are Tyr^{7}, Gly^{8}, Thr^{9}, Leu^{10}, Glu^{82}, Tyr^{88} and Tyr^{119}. Access to this cavity is restricted to substrates with extended aliphatic amines bound to a γ-glutamyl group, consistent with the known substrate preferences of this enzyme (Fink et al. 1980; Fink and Folk 1981, 1983). Substrates enter the cavity and are positioned by hydrogen bonding between the oxygen atoms of the glutamyl portion of the substrate and the amine moieties of Tyr^{7}, Gly^{8}, Thr^{9} and Leu^{10}. The formation of these bonds is accompanied by intra-strand movements that orient the α-amino group of the substrate γ-glutamyl moiety to the carboxyl group of Glu^{82} (Oakley et al. 2010). These movements culminate in the abstraction of a proton from the α-amino group by the carboxylate group of Glu^{82}. This proton abstraction accentuates the nucleophilicity of the amine and facilitates its attack on the side chain amide carbon atom to generate an oxyanion intermediate. This tetrahedral intermediate subsequently undergoes an elimination reaction and collapses to form 5-oxo-L-proline and the free amine. During this process, the proton initially abstracted from the amine of the substrate is donated to the amine of the amino portion of the substrate product (in the case of γ-glutamyl-ε-lysine, this would be the ε-linked amine of the lysine product) to complete the catalytic cycle.

It is interesting to compare γ-glutamylamine cyclotransferase to three other enzymes that can cleave or modify a γ-glutamyl peptide bond: γ-glutamylcyclotransferase, γ-glutamyltranspeptidase and transglutaminases. Although γ-glutamylamine cyclotransferase and γ-glutamylcyclotransferase catalyze very similar reactions, there is very little, if any, overlap in specificity of these enzymes. γ-Glutamylcyclotransferase, with rare exceptions, cleaves the isopeptide bond connecting the γ-glutamyl group of glutamate to the α-amino group of an α-amino acid (Meister 1985), whereas, γ-glutamylamine cyclotransferase cleaves the isopeptide bond connecting the γ-glutamyl group of glutamate to an amine that is not bound at the α-carbon of an amino acid (Fink et al. 1980; Danson et al. 2002). Oakley et al. (2010) proposed that this discrimination is due to the phenylalanine at position 81 in γ-glutamylamine cyclotransferase, which projects into the barrel made by the cyclotransferase fold and restricts the access of compounds other than extended aliphatic amines to the active site. This group of investigators further demonstrated that the residues

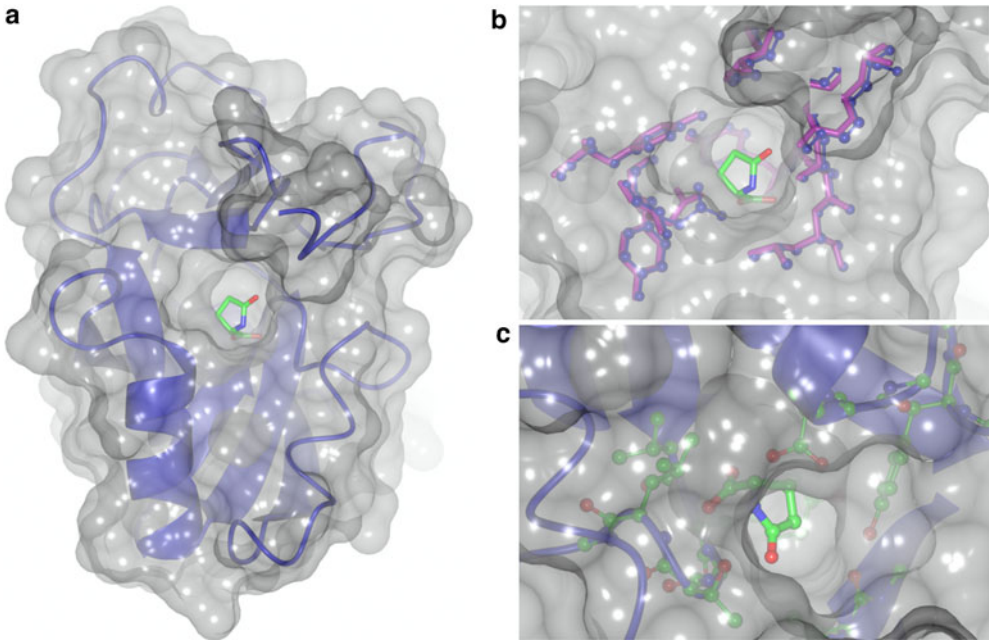

Fig. 5 Structural features of γ-glutamylamine cyclotransferase. The enzyme complexed with 5-oxo-L-proline is shown in **a**. The relative position of the active site residues in the absence (*magenta*) and presence (*blue*) is represented in **b**. **c** The details in **b** oriented at 180° degrees around the *x* axis and at higher magnification. These figures were generated using the program CCP4MG from the PDB coordinates 3JUB (*magenta*) and 3JUC (*blue*) (McNicholas et al. 2011)

responsible for substrate binding in these enzymes differed significantly, including those amino acids that bound the glutamyl moiety (Oakley et al. 2010). γ-Glutamyltranspeptidase catalyzes transformations of the γ-glutamyl bond in glutathione via nucleophilic attack of the amine group of an amino acid substrate, glutathione itself, or water, to generate a γ-glutamyl amino acid *plus* Cys-Gly in the first case, γ-glutamyl glutathione *plus* Cys-Gly in the second case and, finally, glutamate *plus* Cys-Gly (Tate and Meister 1985). In contrast to γ-glutamylamine cyclotransferase and γ-glutamylcyclotransferase where the nucleophilic attack is intramolecular, the nucleophilic attack catalyzed by γ-glutamyltranspeptidase is intermolecular. As noted earlier, transglutaminases can catalyze amide hydrolysis of Q residues of polypeptide substrates. This reaction contributes to the generation of the autoimmune forms of gluten in celiac disease (Molberg et al. 1998; van de Wal et al. 1998; Kim et al. 2002; Pinkas et al. 2007; Stamnaes et al. 2008). Transglutaminases can also catalyze the breakage of the γ-glutamyl bond of γ-glutamyl amines in the presence of high concentrations of amines such as ammonia (Folk 1969; Parameswaran et al. 1997). This observation suggests that the amidation reaction shown in Fig. 2 may be reversible at high ammonia levels. Elevated concentrations of ammonia are toxic to most cells in the brain, especially neurons. For this reason, the levels of ammonia are tightly regulated in the body and particularly so in the brain (Cooper and Plum 1987). The low concentration of ammonia—normally less than 0.2 mM in most tissues—ensures that the amidations catalyzed by transglutaminase are essentially irreversible events. It is also possible that free γ-glutamylamines can be enzymatically hydrolyzed by transglutaminases. Studies with transglutaminase 2 demonstrated that this enzyme has a minimum size requirement for binding of a Q-containing substrate. For example, glutamine is not a substrate and the K_m for the model transglutaminase 2 substrate carbobenzoxy γ-glutamyl glycine is very high (Folk and Cole 1966). Even so, it is possible that free γ-glutamylamines, at sufficiently high concentration, may participate in transglutaminase-catalyzed reactions in which the γ-glutamyl bond is hydrolyzed.

γ-Glutamylamine cyclotransferase is expressed throughout the body with the highest levels of activity occurring in the kidneys (Fink et al. 1980). Brain tissue contains 12% of the activity present in kidney on a per wet weight basis (Fink et al. 1980). The relatively low specific activity of cerebral γ-glutamylamine cyclotransferase results in an accumulation of γ-glutamylamines in the brain and cerebrospinal fluid (CSF) (Nakajima et al. 1976; Jeitner et al. 2001; Dedeoglu et al. 2002; Liu 2002; Nemes et al. 2004; Hoffner et al. 2008; Jeitner et al. 2008; Hoffner et al. 2009). Moreover, the amounts of these γ-glutamylamines correlate with the increases in transglutaminase activity noted with various neurological disorders (Jeitner et al. 2001; Dedeoglu et al. 2002; Nemes et al. 2004; Jeitner et al. 2008). The greatest increase in γ-glutamylamine levels in a neurodegenerative

disease measured thus far was for γ-glutamyl-ε-lysine in the caudate nucleus of HD patients, which increased from ~90 to ~760 pmol/mg protein as compared to the healthy individuals (Dedeoglu et al. 2002). This increase is thought to reflect the formation and proteolysis of soluble aggregates containing γ-glutamyl-ε-lysine bonds. Proteases are not able to release γ-glutamyl-ε-lysine from insoluble aggregates (Hartley et al. 2008). It has been argued that the catabolism of soluble cross-linked polypeptides may, after time, fatally congest the proteolytic machinery (Cooper et al. 2002; Nemes et al. 2004). Another possibility is that the aforementioned concentrations of γ-glutamyl-ε-lysine are toxic to neurons. This possibility is supported by studies demonstrating the toxicity of γ-glutamyl-ε-lysine to retinoic acid-differentiated SH SY5Y cells (Jeitner, unpublished observations).

The concentration of γ-glutamyl-ε-lysine or other γ-glutamylamines in peripheral tissues is not known and excludes the possibility of discussing the selective death of neurons in neurodegenerative diseases as a function of γ-glutamylamine content. Some inferences, however, can be made based on our observations of the concentration of γ-glutamylamine in body fluids. CSF contains γ-glutamyl-ε-lysine, γ-glutamylspermidine, γ-glutamylputrescine and bis-γ-glutamylputrescine, whereas blood contains γ-glutamyl-ε-lysine and γ-glutamylspermidine but no detectable γ-glutamylputrescine or bis-γ-glutamylputrescine (Jeitner et al. 2008). This disparity in composition suggests that γ-glutamylamines leave the brain primarily as constituents of the CSF. The lack of γ-glutamylputrescine and bis-γ-glutamylputrescine in blood is not due to a lack of synthesis as these compounds are made in skin (Folk and Finlayson 1977; Hennings et al. 1981; Beninati et al. 1988a, b; Piacentini et al. 1988; Martinet et al. 1990). It also unlikely that γ-glutamylputrescine and bis-γ-glutamylputrescine are preferentially cleared from the circulation by renal γ-glutamylamine cyclotransferase activity, since this enzyme hydrolyzes γ-glutamylputrescine, γ-glutamyl-ε-lysine or γ-glutamylspermidine at approximately the same rate (~30 μmol/h/mg) (Fink et al. 1980). γ-Glutamyl-ε-lysine, γ-glutamylspermidine, γ-glutamylputrescine and bis-γ-glutamylputrescine are present in urine (Jeitner et al. 2008) which points to the possibility of specific transporters for the renal clearance of these compounds. These observations suggest the existence of either multiple transporters to transfer γ-glutamylamines to the urine or a single transporter with varying selectivity for the γ-glutamylamines. The identity of these transporters or those regulating the export of γ-glutamylamines from cells is unknown.

CSF and blood contain ~160 and ~860 nM amounts of γ-glutamyl-ε-lysine, respectively (Jeitner et al. 2008). The amount of γ-glutamyl-ε-lysine in blood significantly exceeds that of CSF and indicates that this isodipeptide is actively made by tissues in the periphery and transported to blood for eventual clearance by the kidneys. The existence of γ-glutamylamine cyclotransferase and of clearance mechanisms suggest that the intracellular accumulation of γ-glutamylamines is regulated to a certain threshold concentration above which cellular metabolism may be compromised. This suggestion is supported by the observation that free γ-glutamyl-ε-lysine compromises the viability of differentiated SH SY5Y cells (Jeitner, unpublished observations). As in the case of γ-glutamyl-ε-lysine, the concentration of γ-glutamylspermidine in the blood significantly exceeds that in CSF. The concentration of γ-glutamylspermidine in blood is 41 μM, but only 0.7 μM in CSF (Jeitner et al. 2008). This difference coupled with the dissimilarity in γ-glutamylamine composition in blood and CSF (the preponderance of γ-glutamyl-ε-lysine and γ-glutamylspermidine versus γ-glutamylspermidine, γ-glutamylputrescine and bis-γ-glutamylputrescine, respectively) argues for a unique metabolism of these compounds in the brain. The difference is unlikely to be due to the activities of γ-glutamylamine cyclotransferase or transglutaminases in the brain as compared to other organs. For example, the specific activity of γ-glutamylamine cyclotransferase in brain is 12% that of the kidneys. This value is on par with the specific activities found in liver (11%), intestine (13%), and thyroid (15%) and adrenal gland (11%) (Fink et al. 1980). The lower amounts of γ-glutamyl-ε-lysine (160 vs. 860 nM) and γ-glutamylspermidine (0.7 vs. 41 μM) in CSF relative to blood suggests that these species are rapidly cleared from the brain and CSF. These lower values of γ-glutamylamines in brain also hint that neurons therein may be especially sensitive to excessive concentrations of these molecules.

Conclusions

The observation that γ-glutamylamines are differentially distributed among the various body fluids and tissues suggests that levels of these compounds are regulated in the body. This regulation implies the existence of transport mechanisms for the cellular elimination of γ-glutamylamines. γ-Glutamylamine cyclotransferase is likely to contribute to the maintenance of γ-glutamylamines within certain limits. Since 5-oxo-L-proline is produced as a result of this activity, it is also possible that γ-glutamylamine cyclotransferase acts primarily to provide this important metabolite. Our ability to distinguish between these possibilities is hampered by the current paucity of knowledge concerning γ-glutamylamine cyclotransferase and the metabolism of γ-glutamylamines in general. These considerations suggest that the levels of γ-glutamylamines may indicate more about the process of neurodegeneration than

the activation of transglutaminases per se. One important possibility is that the ratio of γ-glutamyl-ε-lysine to γ-glutamylpolyamines may be an important index of the solubility and toxicity of proteins cross-linked by transglutaminases in neurodegenerative disorders. Another possibility is that free γ-glutamyl-ε-lysine may be especially toxic to neurons. Given this and the other possibilities discussed in this review, studies of the metabolism and toxicity of γ-glutamylamines promise to yield important insights into neurodegenerative processes.

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Conflict of interest The authors declare that they have no conflict of interest.

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