Transglutaminase potentiates ligand-dependent proteasome dysfunction induced by polyglutamine-expanded androgen receptor

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Expansion of the CAG trinucleotide repeat encoding glutamine in the androgen receptor gene leads to spinobulbar muscular atrophy (SBMA), a neurodegenerative disorder in a family of polyglutamine diseases with enigmatic pathogenic mechanisms. One established property of glutamine residues is their ability to act as an amine accepter in a transglutaminase-catalyzed reaction, resulting in a proteolytically resistant glutamyl-lysine cross-link. To examine underlying disease mechanisms we investigated the relationship between polyglutamine-expanded androgen receptor and transglutaminase. We found androgen receptor N-terminal fragments are a substrate for transglutaminase. Western blots of the proteins following incubation with transglutaminase show that several different epitopes of the AR appear to be lost. We propose that this is due to the transglutaminase cross-linking of the AR, which interferes with antibody recognition. Furthermore, HEK GFP-1 cells expressing polyglutamine-expanded androgen receptor and transglutaminase exhibit ligand-dependent proteasome dysfunction; this effect was not observed in the presence of cystamine, a transglutaminase inhibitor. In addition, transglutaminase-mediated isopeptide bonds were detected in brains of SBMA transgenic mice, but not in controls, suggesting involvement of transglutaminase-catalyzed reactions in polyglutamine disease pathogenesis. Our hypothesis is that cross-linked AR cannot be degraded by the proteasome and obstructs the proteasome pore, preventing normal function. Because of the central role the ubiquitin–proteasome degradation system plays in fundamental cellular processes, any alteration in its function could cause cell death, ultimately contributing to SBMA pathogenesis.

INTRODUCTION

Spinobulbar muscular atrophy (SBMA) is one of a family of at least eight neurodegenerative diseases caused by an expanded polyglutamine (polyGln) tract including Huntington’s disease (HD), spinocerebellar ataxias (SCA) 1–3, 6 and 7 and dentatorubralpallidolusian atrophy (DRPLA) (1). Although different proteins harbor the polyGln tract in each disease, a common pathology is observed, albeit in different neuronal populations. In SBMA, caused by a CAG repeat expansion in exon one of the androgen receptor (AR) gene (2), motor neurons in the anterior horn of the spinal cord and in motor nuclei in the brainstem are selectively affected, resulting in progressive muscle weakness and atrophy (3,4).

At the cellular level, the majority of polyGln diseases are characterized by the formation of ubiquitin-containing, but proteolytically resistant, protein aggregates. Several hypotheses regarding polyGln disease pathogenesis revolve around the formation of these aggregates, although the mechanism of their formation remains elusive (reviewed in 5). It has been postulated that polyGln stretches act as polar zippers, aggregating through hydrogen bonding, or that the aggregates...
are a result of endogenous nucleation similar to prion disease. Proteins with expanded glutamine tracts have also been shown to be substrates for transglutaminase activity.

Transglutaminases (TG) are calcium-dependent enzymes that catalyze an acyl transfer reaction between the $\gamma$-carboxamide group of a peptide-bound glutamine residue and the $\varepsilon$-amino group of a lysine in another protein (6). This transfer leads to the formation of isopeptide bonds that cross-link proteins into polymers resistant to chemical and enzymatic degradation (7). TGs, including tissue transglutaminase (tTG), are expressed in the mammalian nervous system, and in the human brain tTG is localized primarily within neurons (8). Although tTG is primarily an extracellular and cytosolic protein, a small percentage of cellular tTG is situated in the nucleus, and found to be activated in situ (9).

It has been hypothesized that polyGln-containing aggregates resist degradation, preventing ubiquitin recycling and disrupting proteasome function (10,11). More specifically, intracellular protein aggregates have recently been shown to inhibit the ubiquitin-proteasome system (UPS) (12). Taken together, these results suggest that impairment of the UPS contributes to polyGln disease pathogenesis. We propose that the UPS targets TG cross-linked AR for destruction, but since the proteasome is unable to cleave the TG isopeptide bond, the AR effectively inhibits proteasome activity. The proteasome pore is 13 Å in diameter (13), thus the proteasome must unfold proteins before they can be degraded. However, we hypothesize that cross-linked proteins pose a serious challenge since the proteasome would be unable to unfold them, preventing their degradation and inhibiting the proteasome from recognizing and processing other targets. Proteasomes thus engaged would be unavailable for participation in normal degradation processes, disrupting regular cellular metabolism and allowing the formation of intracellular aggregates.

Here we report that the AR is a substrate for TG in vitro and can be induced to form both inter- and intramolecular bonds. Transfection experiments showed that both TG and polyGln-expanded AR are required to induce ligand-dependent proteasomal dysfunction, and aggregate formation and proteasome dysfunction can occur independently of each other. This phenotype can be rescued by culturing the cells in the presence of cystamine, a potent TG inhibitor. Furthermore, we found SBMA model mice have elevated levels of TG-isopeptide bonds in the neurons of the anterior horn, the area affected in SBMA, compared to normal mice, suggesting that TG activity contributes to the SBMA disease pathogenesis in vivo.

RESULTS

AR is a transglutaminase substrate in vitro

To determine whether AR N-terminal fragments could be cross-linked by TG in vitro we used purified thrombin-cleaved glutathione-S-transferase (GST)-AR fusion proteins containing amino acids 11–341, with tracts of 0 Gln (Q), 20Q or 50Q. All fragments were found to be substrates of TG, regardless of the glutamine tract length (Fig. 1A). It should be noted that, although the 0Q fragments have no glutamines corresponding...
AR forms both inter- and intramolecular cross-links

To determine whether TG catalyzes both inter- and intramolecular AR bond formation we varied the reaction conditions. Intramolecular cross-linking should be favored by diluting the reaction. Thrombin-cleaved AR N-terminal fragments of differing glutamine tract lengths were incubated with TG in a larger reaction volume than previously. Along with a high molecular weight smear visible only in western blots (results not shown), bands migrating lower than the parental bands were observed on SDS-PAGE gels (Fig. 1B). We hypothesized these bands represented different intramolecularly cross-linked AR species, with altered conformations and therefore abnormal migration patterns. Western blotting (using an N-terminal antibody) and mass spectrometric analyses of tryptic digests of these lower bands (results not shown) confirmed their identity as AR. Reactions performed in the presence of protease inhibitors verified that the lower band is not the result of proteolytic cleavage. The absence of any intermediate intramolecular product formation in dilute experiments combining 0Q and 50Q fragments further supports the hypothesis that intramolecular cross-linking is occurring.

To assess the differences between the behavior of the normal and expanded forms of the AR N-terminus, time trials were performed. Reactions stopped after 30 min clearly demonstrate the formation of intramolecularly cross-linked species for the 0Q and 20Q fragments, but no lower molecular weight band was observed for the polyGln-expanded AR (Fig. 1C). However, the absence of the band corresponding to the parental AR50Q fragment in lane 7 indicates that a reaction has definitely occurred. We speculate that the lack of intramolecularly cross-linked product may correspond to an increase in intermolecularly-linked products, since the AR50Q fragment may aggregate on its own in vitro, making our attempts to promote intramolecular cross-links by diluting the reaction futile. Aggregation, probably due to hydrogen bonding between the polyGln tracts, would alter the effective concentration of the protein substrates and promote intermolecular cross-links. This hypothesis correlates with our results when inducing intramolecular bond formation, because less AR50Q intramolecular-linked product was always observed (Fig. 1B).

Identification of reactive residues in the AR

Flanking amino acids affect the ability of TG to cross-link lysine residues. In general, a glycine or an aspartic acid before the amine donor lysine has the strongest adverse effects on substrate reactivity, while valine, arginine and phenylalanine have an enhancing effect (14). Following sequence analysis, the AR N-terminal was determined to have one extremely likely TG reactive lysine residue (at position 311), and several lesser ones. To determine whether or not this lysine was used in TG cross-linking we performed western blots of normal and expanded GST-AR fusion proteins after incubation with TG. If the lysine residue at position 311 in the AR 441 epitope was modified by isopeptide bond formation it might be recognizable by specific antibodies. Using an antibody that recognizes AR amino acids 299–315, we found TG cross-linking of the AR did indeed mask the AR441 antibody recognition site within the protein (Fig. 2C) when compared with the proteins detected in Figure 2A. Linkage of the glutamines in the polyGln tract also masked the 1C2 epitope (expanded polyGln tract) in the normal, but not in the expanded, AR (Fig. 2B). Even if cross-links are formed within the expanded polyGln tract it appears that enough unlinked glutamines remain to be recognized by the 1C2 antibody (15). It is important to bear in mind that within cells, the AR could be cross-linked to many other proteins other than itself, especially given the large number of proteins known to interact with the AR in vivo (16).

AR and TG co-transfected cells exhibit ligand-dependent proteasomal dysfunction

To examine the effects of TG cross-linking of the AR on proteasome function, we used HEK GFP-u-1 kidney cells, which stably express an unstable green fluorescent protein (GFP)12 that accumulates if proteasomes are dysfunctional. Normally, the cells exhibited a low level of green fluorescence (Fig. 3A). However, when cultured in the presence of 10 μM MG132 (a proteasome inhibitor) the cells were bright green due to a build-up of GFP12 (Fig. 3B).

To determine whether TG alone could cause proteasomal dysfunction, cells were transiently transfected with human tTG.
and incubated in the presence of mibolerone, a synthetic non-
metabolizable androgen. Twenty-four hours post-transfection,
fluorescent microscopy showed no change in GFP\(\textsuperscript{u}\) levels
(Fig. 3C). Similarly, transient transfections with blue fluorescent
protein (BFP)-tagged human full length AR\(\textsuperscript{20Q}\), in the presence
of ligand, had no effect on proteasome activity (D, E), nor did
cotransfections of BFP-AR\(\textsuperscript{20Q}\) and TG (F, G). Cells transfected with BFP-AR\(\textsuperscript{50Q}\) in the presence of ligand showed GFP\(\textsuperscript{50}\) accumulation, demonstrating
proteasome malfunction (H, I). Greater GFP\(\textsuperscript{50}\) accumulation is seen in ligand-treated cells co-transfected with BFP-AR\(\textsuperscript{50Q}\) and TG (J, K), indicating that
the combination of polyGln-expanded AR and TG alters proteasome function. When identical transfection experiments were carried out in the absence of
ligand, no GFP\(\textsuperscript{50}\) accumulation occurred when cells were transfected with BFP-AR\(\textsuperscript{50Q}\) (L, M) or co-transfected with TG (N, O). Note: aggregates were seen
in some cells without proteasome malfunction (D, F), and GFP\(\textsuperscript{50}\) accumulation can be seen in some cells without aggregates, indicating that TG-mediated pro-
teasome malfunction and aggregate formation can occur independently.

Figure 3. HEK GFP\(\textsuperscript{u}-1\) cells transfected with BFP-tagged polyGln-expanded full length AR and TG exhibit ligand-dependent proteasomal dysfunction.
Mock-transfected cells (A), as well as those transfected with TG alone (C), express a low level of green fluorescence when compared with those treated with
proteasome inhibitor MG132 (B). Transient transfections in the presence of ligand with BFP-AR\(\textsuperscript{20Q}\) had no effect on proteasome activity (D, E), nor did
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in ligand-treated cells co-transfected with BFP-AR50Q and TG (Fig. 3I and K), indicating that polyGln-expanded AR and TG together alter proteasome function. Endogenous TG in the HEK GFPu-1 cell line may be responsible for GFPu accumulation seen in cells transfected solely with BFP-AR50Q. Importantly, an increase in GFPu was visible in many cells where no aggregates were present, indicating that proteasomal dysfunction and aggregate formation are independent processes.

Strikingly, when identical transfection experiments were carried out in the absence of ligand, no GFPu accumulation occurred when cells were transfected with BFP-AR50Q (Fig. 3L and M) or co-transfected with BFP-AR50Q and TG (Fig. 3N and O).

TG inhibition prevents proteasome dysfunction

Transfection experiments identical to those described above were carried out in the presence of 100 μM cystamine, a transglutaminase inhibitor, to definitively correlate TG activity with proteasomal dysfunction. Mock transfected cells, and those treated with MG132, appeared pale and bright green, respectively (Fig. 4A and B), demonstrating that cystamine itself does not alter proteasome function. In the presence of cystamine, cells transfected with TG alone showed no GFPu build-up, nor did those transfected with BFP-AR20Q or co-transfected with BFP-AR20Q and TG. We found that cells transfected with BFP-AR50Q or co-transfected with BFP-A50Q and TG and incubated with cystamine (Fig. 4C-F), had no increase in GFPu (compared with the GFPu build-up visible in Fig. 3I and K). These results indicate that TG, either endogenous or exogenous, is required for the proteasomal dysfunction seen in our transfection experiments. The presence of aggregates in some cystamine-treated cells indicates aggregate formation may occur independently of TG cross-linking.

TG cross-linking of AR does not affect 20S proteasome catalytic activity

To quantitate the degree to which the combination of polyGln-expanded AR and TG inhibited the proteasomes we utilized a fluorogenic proteasome substrate which specifically measures activity of the 20S catalytic core of the proteasome (17). HEK GFPu-1 cells were mock transfected, treated with MG132 or transfected with TG, green fluorescent protein (GFP)-AR20Q or GFP-AR50Q, or co-transfected with GFP-AR20Q/50Q and TG. GFP-AR fusion proteins were used because BFP excites and emits at the same wavelengths as the fluorogenic substrate. Twenty-four hours post-transfection (in the presence of ligand), cells were counted, lysed and incubated with the fluorogenic substrate. Proteasome activity levels were measured after 30 min. The activity level of mock-transfected cells was set as 100% and, surprisingly, 20S catalytic activity was not substantially decreased by the transfection of GFP-AR50Q or co-transfection of GFP-AR50Q and TG in repeat experiments (results not shown).

Brain tissue from mice transgenic for SBMA shows TG isopeptide bond formation

To determine if TG cross-linking is associated with the SBMA disease phenotype in vivo, transgenic mice carrying a full-length AR with a repeat length of 112 glutamines were analyzed for the presence of Nε-(γ-glutamyl) isopeptide bonds. Immunohistochemical staining clearly demonstrated the presence of isopeptide bonds in the neurons of the anterior horn in the transgenic mice when compared with the wild-type controls (Fig. 5). Furthermore, the isopeptide bond staining appears to co-localize with cytoplasmic inclusions, although it is unknown at this point whether the AR is present and cross-linked to itself or to other proteins in the aggregates. Preliminary immunohistochemical analysis of brain tissue from presymptomatic transgenic mice (aged 2 months) demonstrates the presence of TG cross-links in the brain before aggregate formation is visible.

DISCUSSION

A link between TG and pathogenesis has been established for several polyGln-expanded diseases. Huntingtin fragments containing expanded polyGln tracts have been found to be substrates for TG (18), and TG enzyme activity levels are elevated in HD brain nuclei (19,20). TG γ-glutamyl lysine
bonds have been documented previously to co-localize with huntingtin in nuclear inclusions in immunohistochemical studies of HD brain, suggesting that TG plays a role in the formation of such inclusions in HD (21). Experiments with COS-7 cells transfected with polyGln fusion proteins and TG demonstrated that aggregate formation was largely dependent on the length of the glutamine tract, and treatment with the TG inhibitor cystamine reduced the percentage of all aggregates and decreased cell death (22,23). More recently, cystamine-treated HD transgenic mice were found to have an extended life span and a reduction in tremor and abnormal movements (24). Furthermore, when R6/1 (transgenic for HD) mice were crossed with tTG knock-out mice there was a reduction in neuronal cell death and a significant improvement in both motor performance and survival (25). Intriguingly, in neither of these model systems was a reduction in the number or frequency of aggregates found, suggesting that TG cross-linking of polyGln proteins is not required for aggregate formation.

On the basis of these and other results, we undertook experiments to determine whether the AR is a substrate for TG and, if so, how TG modification of polyGln-expanded AR might contribute to the pathogenesis of SBMA. In keeping with other studies on polyGln-expanded proteins, we found the AR N-terminal is indeed a substrate for TG in vitro. Cross-linking of the AR fragment occurs both inter- and intramolecularly, and differences in the cross-linking pattern exist between the normal and polyGln-expanded AR, providing clues as to how TG-modified AR could contribute to the disease phenotype. Our results with the 1C2 (polyGln) antibody support the recent model of polyGln as a linear lattice, in which the number of binding epitopes increases with length (15), since the polyGln-expanded proteins were still recognized by the antibody after cross-linking, indicating the presence of at least one functional epitope.

Previous results demonstrating that protein aggregates inhibit proteasome function (12), and that expanded AR is proteolytically resistant (26), prompted us to examine the effect of TG-mediated cross-linking of AR on proteasome activity. Our transfection studies clearly demonstrate that co-expression of TG and AR50Q disrupts normal proteasome function in a ligand-dependent fashion. This disruption in cellular metabolism due to UPS dysfunction could be an important primary event in the pathogenesis of expanded-polyGln diseases.

Cystamine, a potent TG inhibitor and inactivator, has recently been found to alleviate symptoms in several polyGln disease models (22,24), but has not been studied in relation to SBMA. The observation that the presence of cystamine prevented proteasomal dysfunction in cells transfected with the polyGln-expanded AR led us to hypothesize that TG activity is required for altering UPS function. Alternatively, it has been suggested that cystamine prevents polyGln pathogenesis not by inhibiting TG, but by increasing transcription of genes such as Hsp 40 that aid in protein degradation (24).

The most striking aspect of our transfection studies is that the proteasome malfunction we documented is ligand-dependent. Our findings are in accord with recent results which found polyGln-expanded AR aggregation in cells transfected with the polyGln-expanded AR led us to hypothesize that TG activity is required for altering UPS function. Alternatively, it has been suggested that cystamine prevents polyGln pathogenesis not by inhibiting TG, but by increasing transcription of genes such as Hsp 40 that aid in protein degradation (24).

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ligand may expose additional or more favorable sites for TG cross-linking. In agreement with recent results demonstrating polyGln peptides are only toxic to cells when they contain a nuclear localization signal (31), proteasome dysfunction may result not from a ligand-induced conformational change, but from the ligand-induced nuclear translocation of the AR. Possibly, AR may have to be translocated to the nucleus before it can be cross-linked by TG and induce proteasome dysfunction. Mutant huntingtin only induces apoptosis when the protein is localized to the nucleus (32), and it has been suggested that the intricate structure of the nucleus might concentrate mutant proteins in subdomains that foster aggregation (33). This idea supports our in vitro studies, which demonstrate the importance of effective concentration in the formation of intermolecular TG cross-links.

Our use of a fluorogenic proteasome substrate, which directly measures the 20S proteasome catalytic activity (17), indicates that cross-linked AR does not inhibit 20S proteasome activity directly, but likely interferes with proteasome function in some other way, such as substrate recognition. Recent results suggest that the proteasome can digest proteins containing up to three cross-links, depending on the flanking sequence (34). When a protease-resistant domain (such as a disulfide bond) stops the sequential degradation of a protein, the next cleavage site must be within a specified number of amino acids (~90) for the protein to be degraded (34). In pathogenic length polyGln proteins the next cleavage site beyond the polyGln tract may be too far for the proteasome to continue processing the protein. Situations where more than one polypeptide chain may pass through the proteasome degradation channel are well documented (35), thus, it is likely that we continue to see 20S proteasome cleavage activity because the fluorogenic substrate used in our assay is extremely small (five residues). Since 20S proteolytic degradation of small peptides is unaltered, it is conceivable that the overall proteasome dysfunction seen in our transfection experiments results from inhibition of a different aspect of proteasome activity. We propose that the undegradable, bulky polyGln-expanded protein blocks the proteasome pore and probably prevents the 19S regulatory subunits of the proteasome from recognizing additional substrates. This hypothesis is supported by the fact that polyGln-expanded AR has been found to associate with the 19S proteasome subunits (36,37).

Proteasome inhibition has been shown to activate apoptosis (38), and apoptosis can induce TG activity (39), thus, in cells undergoing apoptosis, TG concentration may be increased, resulting in cross-links. Alternatively, endogenous TG activity (either nuclear or cytoplasmic) could cross-link the polyGln-expanded AR, causing UPS malfunction when the proteasome attempts to degrade the cross-linked protein. This proteasome dysfunction could induce apoptosis and activate more TG, perpetuating the cycle and possibly contributing to the neuronal loss seen in SBMA.

The presence of N°(γ-glutamyl) isopeptide bonds in brains of SBMA transgenic mice suggests that TG cross-linking could be an important pathogenic mechanism in vivo. Our preliminary immunohistochemical studies in presymptomatic mice suggest that TG cross-linking occurs early in pathogenesis, although further studies are required to determine whether this is a primary disease process or a secondary outcome.

Importantly, tTG undergoes alternative processing (a phenomenon common to genes expressed in the CNS) which results in a short isoform associated with more active cross-linking activity (40). This isoform requires less Ca²⁺ for activation and has been found to be associated with increased neuronal loss in affected areas in the brain. The release of Ca²⁺, associated with mitochondrial dysfunction seen early in HD pathogenesis (41), could cause increased TG cross-linking activity, which could result in proteasomal dysfunction. There are further links between mitochondrial dysfunction and increased tTG activity, as depleting ATP and GTP levels and increases in oxidative stress conditions can increase in situ TG cross-linking activity (42,43). In addition, mitochondrial dysfunction also leads to free radical release, which can be a contributing factor to increases in TG levels (43).

It has been reported that a polyGln tract on its own can be degraded by the proteasome (44), indicating that some protein modification must occur to prevent the proteasome from degrading polyGln-containing proteins. Our results allow us to propose the following model (Fig. 6) for TG cross-linking of the AR as the protein modification that prevents normal degradation. Upon ligand binding, the AR becomes a target for inter- or intramolecular TG cross-linking. When the proteasome attempts to degrade the aberrantly cross-linked protein, the 19S proteasome cap structure is prevented from recognizing other degradation substrates. Unable to unfold and degrade the polyGln-containing protein because the next cleavage site is too far from the catalytic core, but also unable to dissociate from it, the proteasomes would be unable to keep up regular proteolytic turnover in the cell. Proteasomes occupied with cross-linked expanded-polyGln AR would have decreased ability to degrade other key proteins such as p53 (45) and allow misfolded proteins to aggregate into the characteristic inclusions seen in expanded-polyGln diseases.

We have found that AR is a substrate for TG and clearly correlate TG-mediated cross-linking activity with proteasome dysfunction. Our results add to the collection of expanded polyGln disease proteins known to undergo TG-catalyzed reactions, indicating TG potentiated proteasome dysfunction could be a unifying pathogenic mechanism of the polyGln diseases.

**MATERIALS AND METHODS**

**TG cross-linking of the AR**

Androgen receptor N-terminal fragments were purified from E. coli using a GST fusion protein expression system (46). To remove the GST tag, proteins were cleaved with thrombin (Sigma) using standard manufacturer’s protocols for 2 h at 4°C. The TG cross-linking reaction buffer contained 10 mM HEPES, 0.2 mM EDTA, and 0.25 mM CaCl₂. To promote intramolecular bond formation the reaction volume was doubled. Three milliliters of purified guinea pig liver tissue transglutaminase (Sigma) were used in 25 μl reactions containing 10 μg of AR protein, and incubated at 37°C for 1 h. The reaction products were subjected to electrophoresis on 8% SDS–PAGE gels and visualized by Coomassie blue staining.
The human embryonic kidney cell line HEK GFP u-1, kindly provided by Dr Ron Kopito, was cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were plated in 24-well plates with coverslips, transiently transfected the following day with SuperFect transfection reagent (Qiagen) at a ratio of 1μg DNA:3μl Supercfect per well and analyzed after 24 h (for ligand-dependent studies, transfection media contained 3 nM mibolerone). Mock transfections contained pcDNA3 vector DNA. Human TG and BFP-AR plasmids have been previously described (47,48) and were transfected at a ratio of 0.1 μg pSG5-tTG to 1 μg pBFP-AR. For treatment with proteasome inhibitor, cells were incubated with 20 μM MG132 (Sigma) for 4 h prior to fixation. Certain cells were cultured and transfected in media containing 100 μM cystamine (Sigma). A 48 h rinsing in PBS and fixation in 4% paraformaldehyde, fluorescence microscopy was performed using a Zeiss-Axioskop fluorescent microscope and Eclipse software.

Proteasome activity assay

Cells were plated on six-well plates and the following day were transiently transfected with SuperFect (Qiagen). Prior to analysis, treatment with MG132 and cystamine was performed as described above. After 48 h, cells were resuspended in 50 mM Tris (pH 7.4) and 0.5 mM EDTA, counted and lysed by centrifugation through a QiaShredder (Qiagen), followed by one cycle of freeze-thaw lysis. The proteasome substrate Suc-Leu-Leu-Val-Tyr-AMC (Bostem Biochemistry, Inc., Cambridge, MA, USA) was added to a final concentration of 50 μM and the cell lysate were incubated for 30 min at 37°C. Reactions were stopped by placing cell lysates on ice and fluorescence was read immediately in a fluorometer (excitation at 380 nm, emission at 440 nm).

Immunohistochemistry

Brain tissue from transgenic mice, kindly provided by Dr Diane Merry, was prepared as follows: 10% buffered formalin, 2 h; 70% ethanol, 1 h; 95% ethanol, 2 × 1 h; 100% ethanol, 2 × 1 h; xylene, 2 × 1 h; paraffin, 3 × 1 h at 60°C and embedded in Tissue Prep 2 (Fisher). Primary antibody incubation with mouse monoclonal anti-Nterminal glutamyl) lysine antibody (Novus Biologicals, Inc., Littleton, CO, USA) was carried out at 4°C overnight at a dilution of 1:50. Secondary rabbit anti-mouse IgM antibody (Sigma) was diluted 1:200 and incubated for 1 h at room temperature. Staining was visualized with the ABC Vectastain kit (Vector Laboratories, Burlingame, CA, USA).

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