The effect of mutant ubiquitin on proteasome function in relation to neurodegenerative disease

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CHAPTER IV

Low levels of mutant ubiquitin are degraded by the proteasome in vivo

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Abstract

The ubiquitin-proteasome system fulfils a pivotal role in regulating intracellular protein turnover. Impairment of this system is implicated in the pathogenesis of neurodegenerative diseases characterized by ubiquitin-containing proteinaceous deposits. UBB^{+1}, a mutant ubiquitin, is one of the proteins accumulating in the neuropathological hallmarks of tauopathies, including Alzheimer's disease, and polyglutamine diseases. *In vitro*, UBB^{+1} properties shift from a proteasomal ubiquitin-fusion degradation substrate at low expression levels to a proteasome inhibitor at high expression levels. In this study we report on a novel transgenic mouse line expressing low levels of neuronal UBB^{+1}. In these mice, the UBB^{+1} protein is scarcely detectable in the neuronal cell population. Accumulation of UBB^{+1} only commences after intracranial infusion of the proteasome inhibitors lactacystin or MG262, showing that at these low expression levels the UBB^{+1} protein is a substrate for proteasomal degradation *in vivo*. In addition, accumulation of the protein serves as a reporter for proteasome inhibition. These findings strengthen our proposition that in healthy brain, UBB^{+1} is continuously degraded and disease-related UBB^{+1} accumulation serves as an endogenous marker for proteasomal dysfunction. This novel transgenic line can give more insight into the intrinsic properties of UBB^{+1} and its role in neurodegenerative disease.

Introduction

The ubiquitin-proteasome system (UPS) is the main intracellular pathway for regulated protein turnover and essential for maintaining cellular homeostasis (reviewed by (Glickman and Ciechanover, 2002)). Besides functioning as a protein quality control mechanism by degrading aberrant and misfolded proteins, ubiquitin (Ub) modification of proteins also regulates many other processes, including cell-cycle progression, endocytosis and intracellular signaling (reviewed by (Welchman *et al.*, 2005; Mukhopadhyay and Riezman, 2007)). Substrates are tagged for proteasomal degradation by covalent binding of the C-terminal glycine of a Ub moiety to an internal lysine residue in a substrate. Additional Ub moieties are successively attached to the substrate-bound Ub forming a poly-Ub chain (Pickart, 2004). Ubiquitinated substrates with a chain of four or more lysine-48 linked ubiquitins are selectively targeted for degradation by the 26S proteasome (Thrower *et al.*, 2000), a multi-subunit proteolytic complex composed of a barrel-shaped 20S core particle and two 19S regulatory complexes. The 19S particle, which forms a cap-like structure on the 20S core, is essential for substrate recognition, deubiquitination, unfolding and subsequent translocation into the 20S catalytic core. Three different proteolytic activities are present in the 20S particle; chymotrypsin-like, trypsin-like and peptidyl-glutamyl-peptide hydrolyzing (PGPH) activity residing in the β5, β2 and β1 subunits re-

spectively (reviewed by (Wolf and Hilt, 2004)).

Impairment of the UPS is implicated to play a role in the pathogenesis of a broad array of diseases, including neurodegeneration. This is exemplified by the presence of ubiquitin -positive pathology in many neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Ciechanover and Brundin, 2003; van Tijn *et al.*, 2008). Another indication of involvement of the UPS in neurodegeneration is the disease-specific accumulation of a mutant Ub (UBB^{+1}) in tauopathies, including Alzheimer's disease, and polyglutamine diseases such as Huntington's disease and spinocerebellar ataxia-3 (van Leeuwen *et al.*, 1998; Fischer *et al.*, 2003; De Pril *et al.*, 2004). UBB^{+1} is generated by a di-nucleotide deletion in the Ub mRNA which results in a mutant protein with a 19 amino acid C-terminal extension. The UBB^{+1} protein lacks the C-terminal glycine essential for substrate ubiquitination, however UBB^{+1} is a substrate for ubiquitination itself and targeted for degradation by the proteasome (Lam *et al.*, 2000). *In vitro,* degradation of UBB^{+1} occurs only at low expression levels; accumulation of UBB^{+1} commences after exceeding a threshold level, leading to dose-dependent inhibition of the UPS (van Tijn *et al.*, 2007). High expression levels of UBB^{+1} also result in cell cycle arrest, apoptotic-like cell death, expression of heat shock proteins (Hsp) and resistance to oxidative stress (De Vrij *et al.*, 2001; Lindsten *et al.*, 2002; Hope *et al.*, 2003). In human disease, accumulation of the UBB^{+1} protein in the disease-specific neuropathological hallmarks is proposed to be an endogenous reporter for UPS dysfunction (Fischer *et al.*, 2003; Hol *et al.*, 2005).

We previously reported on two UBB^{+1} transgenic mouse lines (lines 3413 and 8630) expressing high levels of neuronal UBB^{+1}, ranging from 50-67% of wildtype Ub mRNA levels (Chapter 3). In the 3413 transgenic mice, UBB^{+1} protein accumulation resulted in a significant decrease of chymotryptic proteasome activity ultimately leading to proteome changes and deficits in spatial reference memory (Chapter 3). As UBB^{+1} shifts from UPS substrate to inhibitor with increasing levels of expression *in vitro*, it is essential to know if these properties are conserved *in vivo* to extrapolate these findings to human disease. To this end, we investigated in the present study the effect of low-level UBB^{+1} expression *in vivo*. We generated a UBB^{+1} transgenic mouse line (line 6663) with neuronal UBB^{+1} expression at relatively low levels compared to the previously reported UBB^{+1} transgenic lines and studied the intrinsic properties of low-level UBB^{+1} expression *in vivo*.

Materials and Methods

Generation of transgenic mice

The murine Ca(2+)/Calmodulin-dependent Protein Kinase II alpha (CamKIIα) promoter (Mayford *et al.*, 1996) was used to drive UBB^{+1} expression. The *UBB^{+1}* cDNA, encoded by the first ubiquitin open reading frame and the C-terminus in the +1 reading frame (van

Leeuwen *et al.*, 1998) was cloned with a flanking 5' intron (Choi *et al.*, 1991) and 3' polyadenylation site (bovine growth hormone) in the CamKIIα cassette by NotI. Before injection, the insert was excised from the plasmid, purified from gel by electro-elution and ethanol precipitated. The construct was injected into fertilized oocytes of FVB/N mice. The line was maintained on its genetic background by breeding hemizygous mice to wild-type mice. From F2 onwards Mendelian ratios were observed in the offspring. Mice were kept in group housing on a 12/12 h light-dark cycle with food and water ad libitum in specific pathogen free conditions (Nicklas *et al.*, 2002). All mice were genotyped on DNA isolated from ear-snips using the QIAamp DNA mini kit (Qiagen), primers being GGTGAGTACTCCCTCTCAAAAGC (forward) and CTGCAGTTGGACCTGGGAGTGGA (reverse). The copy-number of the transgene (10 copies) was determined by Southern blotting and analysed on a Storm 860 phosphorimager (Molecular Dynamics). All animal experiments were performed conforming to national animal welfare law and under guidance of the animal welfare committee of the Royal Netherlands Academy of Arts and Sciences.

RNA isolation and qPCR

Mice (n=4 per group; 2 male, 2 female) were euthanized by carbon dioxide asphyxiation, the brain was immediately dissected and hemispheres were frozen in liquid nitrogen. RNA was isolated using Trizol (Invitrogen) and an Ultraturrax homogenizer and stored at -20° C. cDNA was synthesized from 2 µg of RNA using superscript II (Invitrogen). Real-time quantitative PCR was performed with SYBR-green mastermix (Applied Biosystems) on an ABI5700 (Applied Biosystems) as described previously (Hope *et al.*, 2003). The primer-set for the *ubiquitin-B* (*UBB*) target recognizes both the endogenous UBB mRNA and the transgene (forward: TACCGGCAAGACCATCACC, reverse: GGATGCCTTCTTTATCCTGGAT, efficiency 1.97), the bovine growth hormone polyA set recognizes the CamKIIα transgene (forward: GCCTTCTAGTTGCCAGCCAT, reverse: AGTGGGAGTGGCACCTTCC, efficiency 1.95), three house-keeping genes were used as normalizers (*EF1α, Ube2d2* and *rS27a* (Warrington *et al.*, 2000; Lee *et al.*, 2002)). Statistics were performed with Mann-Whitney U-test in SPSS 11 for Mac.

Radioimmunoassay

Mice were euthanized by carbon dioxide asphyxiation, the brain was immediately dissected and hemispheres were frozen in liquid nitrogen. A hemisphere was homogenized in suspension buffer: 100 mM NaCl, 50 mM Tris pH7.6, 1 mM EDTA pH8.0, 0.1% Triton-X-100, 10 mM DTT and protease inhibitors (Complete, Roche), samples were stored at -80°C. Total protein concentration was determined by means of a Bradford assay. UBB^{+1}

protein levels were measured in a radioimmunoassay (RIA) as described previously (Hol *et al.*, 2003) with Ub3 peptide (YADLREDPDRQ) and Ub3 antiserum (bleeding date 05/08/97, final dilution 1:24000, (Fischer *et al.*, 2003)). The final UBB^{+1} protein concentration in the transgenic mice was corrected for background levels in wild-type mice. For the 6663 transgenic mice; n=8 (5 males, 3 females) and for wildtype mice; n=6 (4 males, 2 females).

Implantation of osmotic pumps

Proteasome inhibitors lactacystin (BIOMOL International LP, UK) and MG262 ((Z-Leu-Leu-Leu-$B(OH)_2$) Boston Biochem Inc., Cambridge, MA) were dissolved in DMSO and diluted to their final concentration in Ringer's solution (Fresenius Kabi). 100 µl of dilution was applied to an osmotic minipump (Alzet 1003D, 3 day run-time, 1 ul/h, Alzet, CA). Osmotic minipumps were primed overnight at 37°C in 0.9% NaCl solution. Mice were anesthetized with 10 ml/kg FFM (0.0787 mg/ml fentanyl citrate, 2.5 mg/ml fluanisone, 0.625 mg/ml Midazolam in H_2O). The skull was exposed and stereotactic coordinates for infusion of proteasome inhibitors were read against bregma (-2.3 mm anterior-posterior; -1.5 mm lateral) (Paxinos and Franklin, 2001). A hole was drilled through the skull and the dura was punctured. A canula, 2.5 mm long with a sharp tip (Brain Infusion Kit II, Alzet) was implanted in the brain and glued to the skull with cyanoacrylate adhesive (Loctite 415). Polyvinylchloride tubing was used to connect the canula to the osmotic pump placed subcutaneously in a pocket under the skin in the flank of the mouse. The skin was sutured and mice were administered 0.05 mg/kg buprenorphine (Shering-Plough) intra-muscularly as an analgesic and 20 ml/kg 0.9% NaCl subcutaneously to prevent dehydration. Mice were kept at 37°C until they were recovered, and subsequently housed individually to prevent opening of the sutures. All experimental mice implanted with an osmotic pump were female, wild-type female littermates were used as controls.

Immunohistochemistry

Animals were given deep pentobarbital anaesthesia (intra-peritoneal) and were perfused intra-cardially with phosphate-buffered saline, pH7.4, followed by phosphate-buffered saline containing 4% paraformaldehyde. The brain was removed and cut on a vibratome (Leica VT1000S) in 50 µm coronal sections. Sections were immuno-histochemically stained overnight as described previously (Chapter 3) at 4°C using the peroxidase-anti-peroxidase method (Sternberger *et al.*, 1970) with rabbit polyclonal anti-UBB^{+1} antibody (Ub3; bleeding date 05/08/97, 1:1000 (Fischer *et al.*, 2003)), polyclonal anti-ubiquitin antibodies (#Z0458, DAKO, 1:1000 and #U5379, Sigma, 1:200) or a monoclonal anti-polyubiquitin conjugate antibody (LB112, 1:3, (Iwatsubo *et al.*, 1996)). Staining was visu-

alized with 3,3'-diaminobenzidine solution using nickel intensification. Images were made using a Zeiss Axioplan 2 or Wild Makroskop M420 imaging microscope and an Evolution MP digital camera (MediaCybernetics, Silver Spring, MD), analyzed with Image-Pro Plus software (version 5.1, MediaCybernetics).

Results

Generation of transgenic mice expressing low levels of UBB+1

We have generated a transgenic mouse line postnatally expressing low-levels of human UBB^{+1} in the brain under the neuronal CamKIIα promoter on a FVB/N background (line 6663). The relative expression levels of UBB^{+1} mRNA versus endogenous ubiquitin-B (UBB) mRNA were measured in adult mouse brain with real-time quantitative PCR using primers directed against UBB, recognizing both the endogenous UBB mRNA and the transgene, and a bovine growth hormone polyadenylation primer set recognizing the CamKIIα transgene. The expression level of the UBB^{+1} mRNA in the 6663 line amounted to

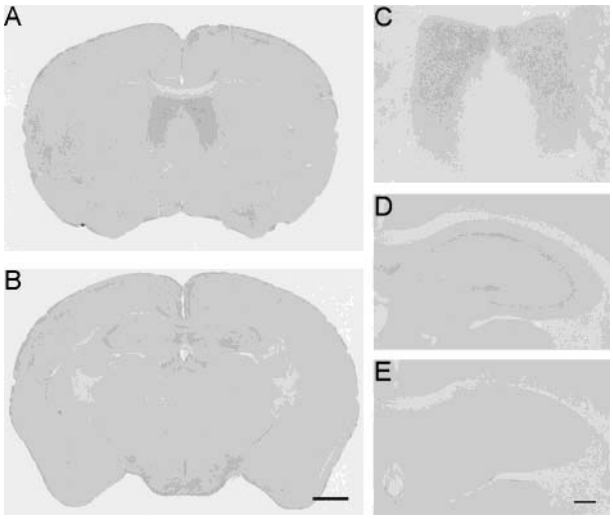

Figure 1 *Expression of UBB^{+1} in transgenic line 6663.* A-D. Expression pattern of the UBB^{+1} protein in the low-level expression transgenic line 6663. Coronal 50 µm vibratome sections of a 9-month-old 6663 transgenic male were stained with an anti-UBB^{+1} antibody. Minor UBB^{+1} reactivity is present in the brain mainly in neurons in the lateral septal area (A) and faintly in the pyramidal cell layer of the hippocampus (B). Magnifications of the lateral septal area and hippocampus are shown in (C) and (D). E. Wild-type littermate FVB/N control mice do not show any hippocampal UBB^{+1} reactivity when stained with anti-UBB^{+1}. Bar = 1 mm (A-B), bar = 250 µm (C-E).

17% of the endogenous UBB mRNA (n= 4 per group). Line 6663 UBB^{+1} heterozygous mice did not show any overt behavioral abnormalities and lifespan was not reduced compared to wild-type control mice. The gross morphology of the brain also did not differ between UBB^{+1} 6663 transgenic and FVB/N wild-type mice.

The expression pattern of the CamKIIα promoter is confined to neurons, located mainly in the hippocampus, neocortex, striatum and forebrain (Mayford *et al.*, 1996). The previously described UBB^{+1} transgenic line 3413 with high levels of UBB^{+1} expression from the same CamKIIα expression vector as line 6663 indeed showed neuronal UBB^{+1} protein expression in these brain areas (Chapter 3). Immunohistochemical analysis of the 6663 mice using an antibody directed against the C-terminal +1 extension of UBB^{+1} only showed very weak staining of UBB^{+1} positive neurons (fig. 1A-D), located mainly in the lateral septal area (fig. 1A, C) and also in the hippocampal pyramidal cell body layers (fig. 1B, D). Control mice did not show any UBB^{+1} reactivity (exemplified in fig. 1E). The UBB^{+1} protein could also be detected in brain homogenates of 6663 transgenic mice by radioimmunoassay (n=8 transgenic; n=6 wildtype). UBB^{+1} protein was present in a concentration of 116 ng/g total protein content in the 6663 transgenic mice, a concentration relatively low when compared to the levels of UBB^{+1} protein in the previously described UBB^{+1} high expression line 3413 (1080 ng/g total protein content, (Chapter 3)).

Low concentrations of lactacystin transiently inhibit the UPS and induce UBB^{+1} accumulation

In vitro, UBB^{+1} is ubiquitinated and degraded by the UPS at low expression levels (Lindsten *et al.*, 2002; van Tijn *et al.*, 2007). Therefore, we investigated whether the low UBB^{+1} protein expression levels in 6663 transgenic mice were attributable to proteasomal degradation of UBB^{+1} *in vivo*. We monitored UBB^{+1} protein levels in 6663 transgenic mice after exogenous inhibition of the UPS; an overview of the results is given in Table 1. Proteasome inhibition was achieved by intra-cranially administering UPS inhibitors using an Alzet osmotic mini-pump placed subcutaneously in the flank of the animal connected with an infusion probe placed unilaterally in the hippocampus.

We implanted the osmotic pump and infusion probe on day 1 and infused wild-type and 6663 transgenic mice with the irreversible proteasome inhibitor lactacystin (0.166 mM) for three consecutive days. Mice were sacrificed directly after the 3-day infusion of lactacystin (day 3) or seven days after the infusion had ended (day 10). Accumulation of UBB^{+1} was detected using an anti-UBB^{+1} antibody on 50 μm coronal vibratome sections. Lactacystin treatment resulted in moderate accumulation of UBB^{+1} in 6663 transgenic mice at day 3, located mainly in hippocampal neurons around the infusion site spreading slightly to the CA1 area of the contralateral hippocampus (fig. 2A), with a small area void of staining where the infusion needle was placed. The intensity of the UBB^{+1} staining var-

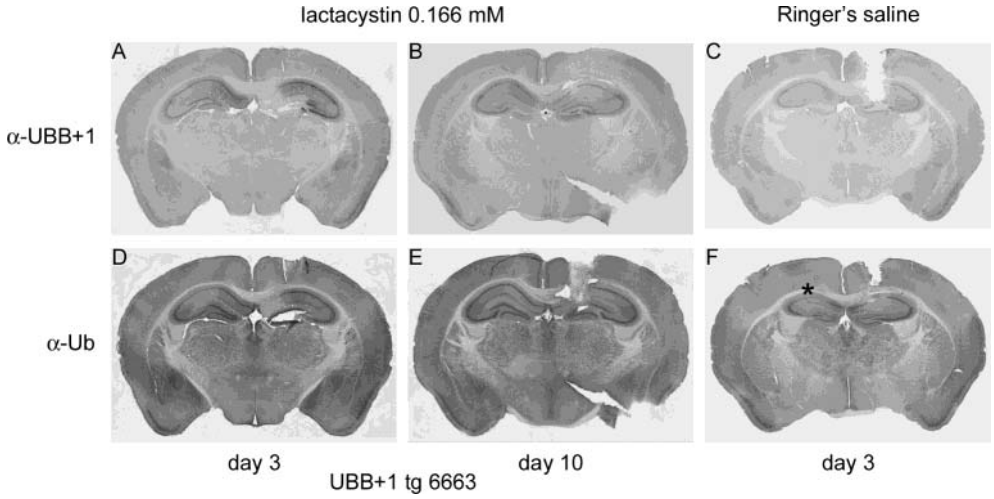

Figure 2 *Low concentrations of lactacystin induce reversible UBB^{+1} accumulation.* A, D. Hippocampal infusion of the irreversible inhibitor lactacystin (0.166 mM) for three days in 6663 transgenic mice gives rise to clear accumulation of the UBB^{+1} protein, mainly in the ipsilateral hippocampus (A). In the same animal, Ub positive cells are dispersed throughout the brain, only minor additional accumulation of Ub is present at the infusion site (D). The Ub reactivity is slightly decreased around the infusion site, marked by an asterisk. B, E. Mice were perfused one week after the 0.166 mM lactacystin infusion into the hippocampus ended (day 10). UBB^{+1} 6663 transgenic mice do not show accumulation of UBB^{+1} (B) or Ub (E) at this timepoint. C, F. Infusion of Ringer's saline in the hippocampus of 6663 UBB^{+1} transgenic mice for 3 days does not give rise to additional accumulation of UBB^{+1} (C) or of Ub (F). The Ub reactivity is slightly decreased in the contralateral CA2/3 area, marked by an asterisk. The infusion needle was placed in the right hippocampus.

ied slightly between mice (n=4), in one animal no increase in UBB^{+1} expression was seen. Accumulation of endogenous Ub was used as a general marker for UPS dysfunction. Ub-positive cells were present in a dispersed pattern throughout the brain. In the transgenic mice, only minor additional accumulation of Ub was present at day 3 (fig. 2D). However, these Ub levels increased to a much lesser extent compared to the UBB^{+1} levels; a decrease in expression was observed directly around the probe site (fig. 2D, decreased expression marked by an asterisk). In the wild-type control group, only two out of five mice showed a slightly higher hippocampal Ub expression after infusion of lactacystin (n=5, results not shown).

As a control for the proteasome inhibitor we unilaterally infused physiological saline (Ringer's solution) into the hippocampus. After infusion of Ringer's, no increase in UBB^{+1} reactivity could be detected in the 6663 transgenic mice (n=7, fig. 2C), showing that the accumulation of UBB^{+1} after infusion of lactacystin is specific for UPS inhibitor

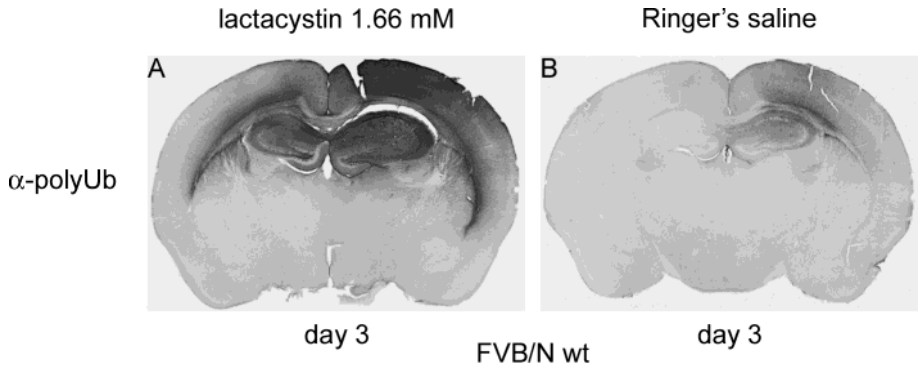

Figure 3 *Poly-ubiquitin chain accumulation after infusion of high concentrations of lactacystin.* A, B. Infusion of high concentrations of lactacystin (1.66 mM) in FVB/N wild-type mice leads to massive accumulation of poly-ubiquitinated proteins at day 3 in the ipsilateral hippocampus and cortex spreading to the contralateral side, shown by staining with an antibody directed against poly-ubiquitin chains (A). Infusion of Ringer's saline leads to only minor accumulation of poly-ubiquitinated proteins in wild-type mice at day 3 (B).

infusion. As wild-type mice do not endogenously express human UBB^{+1}, UBB^{+1} positive staining was completely absent in these mice in all experimental conditions (n=4, result not shown). Additional accumulation of Ub around the infusion site compared to the contralateral hippocampus was absent at day 3 in all wild-type (result not shown) and 6663 transgenic mice (fig. 2F). In some cases, Ub expression was even slightly decreased in the CA2/3 area of the contralateral hippocampus (fig. 2F, marked by an asterisk).

Considering the fact that lactacystin is an irreversible inhibitor of the UPS (Fenteany *et al.*, 1995), we expected that the UPS inhibition at day 3 would persist up to day 10. Contrary to our expectations, the additional accumulation of UBB^{+1} observed at day 3 in the 6663 transgenic mice had almost fully disappeared by day 10 (n=3, fig. 2B). Correspondingly, no clear accumulation of Ub was visible at day 10 in the 6663 transgenic mice (fig. 2E) or in the wild-type mice (n= 2, data not shown). The absence of both Ub and UBB^{+1} accumulation at day 10 suggests that in this setup UPS inhibition induced by 0.166 mM lactacystin was reversed from day 3 to day 10 and thus might not have been complete. Again, Ringer's saline infusion was used a control. Similar to the results obtained at day 3, infusion of Ringer's for 3 days did not induce accumulation of Ub or UBB^{+1} in a wild-type or a 6663 transgenic mouse at day 10 (results not shown).

Table 1 Results of UPS inhibitor infusion in wild-type and line 6663 transgenic mice (summary)

Condition			n	Ubiquitin staining	UBB^{+1} staining	Comments
Ringer's	day 3	wt	4	~	not present	
		tg	7	~	~	
	day 7/10	wt	5	~	not present	
		tg	3	~	~	
lactacystin 0.166mM	day 3	wt	5	↑ minor (in 2 out of 5 mice)	not present	
		tg	4	↑ minor (in 3 out of 4 mice)	↑ clear in ipsil. hippocampus (3 out of 4 mice) ↑ variable in contral. CA1/DG and cortex	different patterns UBB^{+1} and Ub
	day 10	wt	2	~ (only few darker cells)	not present	reversed inhibition of lacta.
		tg	3	~	~	
lactacystin 1.66mM	day 3	wt	1	↑ large directly at infusion site ↓ in a large ring around infusion site	not present	
		tg	4	↓ in a large ring around infusion site ↑ around ring (in 3 out of 4 mice) cells with abnormal morphology (clear in 1 mouse)	↑ very clear in contral. hippocampus and cortical/thalamic areas near probe (2 mice strong effect, 2 mice moderate effect)	tissue damage, different patterns UBB^{+1} and Ub
	day 7	wt	2	↓ in a large ring around infusion site ↑ around ring (clear in 1 out of 2 mice)	not present	large tissue damage,
		tg	3	↓ in a large ring around infusion site ↑ around ring	↑ variable from modest to strong (1 mouse strong effect, 2 mice moderate effect)	different patterns UBB^{+1} and Ub
MG262 1.66mM	day 3	wt	2	~ / ↑ minimal	not present	different patterns UBB^{+1} and Ub
		tg	3	↑ moderate in ipsil. DG and cortex	↑ in ipsil. hippocampus	
	day 7	wt	3	↑ in ipsil. to contral. hippocampus cells with abnormal morphology	not present	tissue damage, different patterns UBB^{+1} and Ub, sustained inhibition of MG262
		tg	2	↑ in ipsil. to contral. hippocampus cells with abnormal morphology	↑ in ipsil. to contral. hippocampus absent around infusion site	

~ unaltered; ↑ increased; ↓ decreased; Ub: ubiquitin; wt: wild-type; tg: transgenic; ipsil.: ipsilateral, contral.: contralateral, DG: dentate gyrus, lacta.: lactacystin

High concentrations of lactacystin irreversibly inhibit the UPS

The unexpected reversal of lactacystin-induced UPS inhibition over time might be attributed to the concentration of the inhibitor; hence, we also infused a ten-fold higher concentration of lactacystin (1.66 mM). Infusion of 1.66 mM lactacystin lead to widespread accumulation of poly-ubiquitinated proteins in a wild-type mice after three days of continuous infusion, visualized by increased reactivity for poly-ubiquitin chains (LB112, (Iwatsubo *et al.*, 1996)), indicating substantial UPS inhibition (fig. 3A). This large increase in the levels of poly-ubiquitinated proteins was not present after infusion of

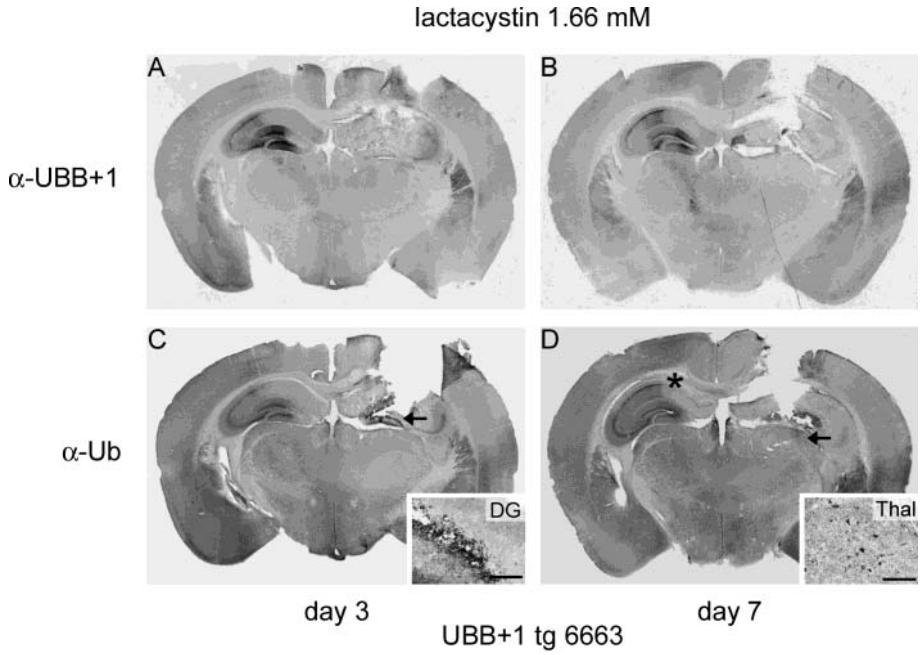

Figure 4 *High concentrations of lactacystin induce irreversible UBB^{+1} accumulation.* A, C. Treatment of 6663 transgenic mice with a high dose of lactacystin (1.66 mM) induces a clear accumulation of the UBB^{+1} protein at day 3, mainly in the contralateral hippocampus spreading to adjacent thalamic areas (A). A decrease in Ub expression was found in a large circular area around the infusion site, bordered by a rim of intensely stained cells (C). Magnification of area left of the arrow: some Ub positive cells with abnormal morphology are visible around the infusion site in the dentate gyrus (DG) in this animal (C, inset; bar = 100 μm). B, D. The UBB^{+1} accumulation present at day 3 persists up to day 7. The staining pattern of UBB^{+1}, mainly in the contralateral hippocampus, is similar to day 3 (B). At day 7, the staining pattern of Ub resembles day 3 as well, however no irregular shaped cells are present in the hippocampus. Decreased Ub staining in the contralateral dentate gyrus is marked by an asterisk (D). Magnification of area left of the arrow: a few cells with abnormal morphology can be seen in the thalamic area (Thal) (D, inset; bar = 100 μm).

Ringer's solution in comparable conditions (fig. 3B). Staining for Ub showed a somewhat different pattern; positive cells disappeared in a large ring around the infusion site bordered by darker stained Ub-positive cells in the transgenic mice (n=4, fig. 4C). In one animal, many Ub accumulating cells showing abnormal morphology appeared in the hippocampus, surrounding the infusion site (fig. 4C, inset). However, 1.66 mM lactacystin also caused extensive necrotic-like damage to the tissue of the ipsilateral hippocampus (fig. 4A, 4C). At day 3, considerable accumulation of UBB^{+1} was present in the 6663 transgenic mice, mainly in the contralateral hippocampus and also in several additional ispi- and contralateral brain regions at some distance from the infusion site, e.g. cerebral cortex and thalamic areas (fig. 4A). Here, UBB^{+1} accumulation was more prominent and widespread compared to the lower concentration of lactacystin, indicating a dose-dependent effect of lactacystin on the accumulation of UBB^{+1}. In contrast to the 0.166 mM lactacystin infusion, infusion of a ten-fold higher concentration of lactacystin (1.66 mM) induced UBB^{+1} accumulation which remained present for four days after the 3-day infusion of lactacystin ended (day 7). This response varied from modest (not shown) to substantial UBB^{+1} accumulation (n=3, fig. 4B). Also, the elevated Ub levels in these transgenic mice (fig. 4D) and in wild-type control mice (n=2, result not shown) persisted up to day 7 in ipsilateral areas spreading to the contralateral hemisphere. A few abnormally shaped cells were present in the ipsilateral thalamic area (fig. 4D, inset). The results obtained for UBB^{+1} and Ub at day 7 showed relatively high variability between animals. Unfortunately, tissue damage also increased over time, making it difficult to accurately compare protein expression patterns at day 3 and day 7. These results show that high concentrations of the proteasome inhibitor lactacystin result in irreversible UPS inhibition leading to UBB^{+1} accumulation which persists over time.

MG262 causes UBB^{+1} accumulation in vivo

In vitro, accumulation of UBB^{+1} induced by a reversible UPS inhibitor can be (partially) reversed after washout of the inhibitor (van Tijn *et al.*, 2007). To confirm these results *in vivo*, we infused the reversible inhibitor MG262 for three days (1.66 mM) and perfused the mice directly (day 3) or 4 days after ending administration (day 7), allowing reversal of MG262 induced UPS inhibition. Hippocampal administration of MG262 in 6663 transgenic mice caused only slight Ub accumulation day 3, resulting in darker cells in e.g. the cortex and dentate gyrus (n=3, fig. 5C). Only a minimal increase in Ub was present in the wild-type mice at day 3 (n=2, not shown). As MG262 is a reversible inhibitor, we expected that washout of MG262 would lead to a decrease in Ub levels. However, the Ub accumulation was increased in the wild-type (n=3, not shown) as well as the 6663 transgenic mice (n=2) at day 7 (fig. 5D). Ub wild-type accumulated in a circular border around a necrotic-like Ub negative area, spreading to contralateral CA1 and DG areas (fig. 5D).

Many dark cells with abnormal morphology were present directly around probe site, in between the damaged tissue (fig. 5D, inset). These results suggest sustained UPS inhibition over time even after washout of MG262. Furthermore, MG262 infusion in 6663 transgenic mice caused ipsilateral accumulation of UBB^{+1} at day 3 (n=3, fig. 5A) and this accumulation remained present up to four days after ending the MG262 administration (n=2, fig. 5B). We also observed that the UBB^{+1} accumulation spread to the contralateral CA1 area at day 7 (fig. 5B). The combined results for Ub and UBB^{+1}, as summarized in Table 1, suggest that using this setup UPS inhibition induced by MG262 was not only sustained after a period without continuous inhibitor treatment, but also was more widespread.

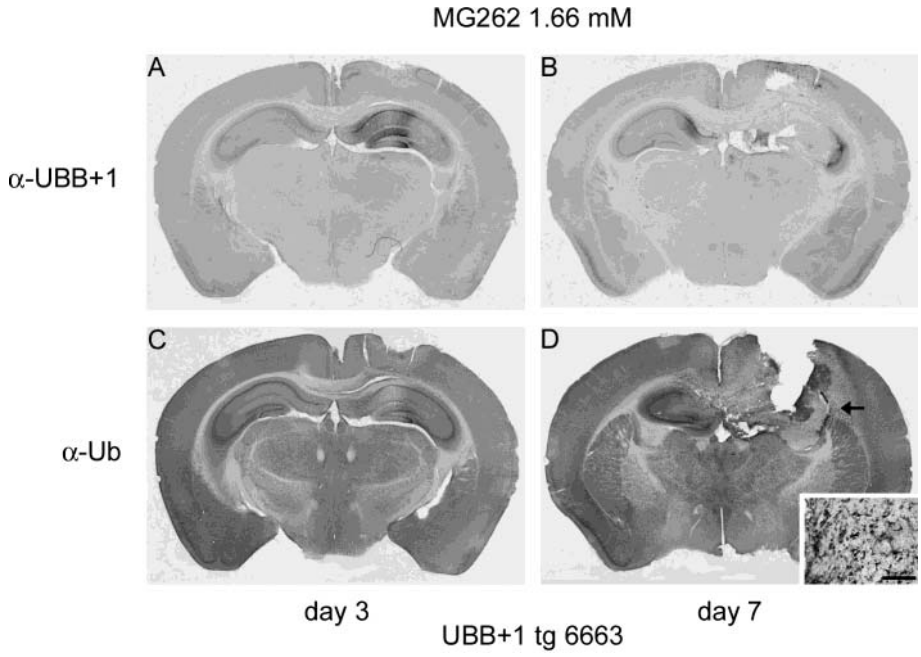

Figure 5 *UBB^{+1} accumulates irreversibly after UPS inhibition by MG262.* A, C. After treatment with 1.66 mM MG262, many UBB^{+1} positive cells are clearly visible in the ipsilateral hippocampus at day 3 (A). In the same 6663 transgenic animal, Ub accumulates slightly in the 6663 transgenic mice in cells mainly in the ipsilateral dentate gyrus. The area directly around the infusion needle is devoid of staining (C). B, D. After 4 days of MG262 washout (day 7), UBB^{+1} is absent in the area directly around the probe site, accompanied by substantial tissue loss. UBB^{+1} accumulates in a ring-like shape around the necrotic area dispersing to the outer parts of the ipsilateral hippocampus and into the contralateral CA1 and dentate gyrus areas. (B). A similar staining pattern could be observed for the Ub protein (D). Magnification of area left of the arrow: cells with abnormal morphology are present in the vicinity of the infusion site (D, inset; bar = 100 μm).

UBB+1 does not accumulate during aging

Proteasome function has been reported to decline during ageing in various tissue types, including in nervous tissue in rat (Keller *et al.*, 2000), and also in several brain regions in aged mice (Zeng *et al.*, 2005). We investigated if this reported decline in proteasome activity would result in an increase in UBB^{+1} protein levels in the brains of the 6663 transgenic mice, as we showed in this study that UBB^{+1} readily accumulates after exogenous proteasome inhibition (fig 2A, 4A and 5A). We examined all brain regions where UBB^{+1} protein expression could be expected due to the CamKIIα promoter expression pattern, including hippocampus, cortex, striatum and forebrain (Mayford *et al.*, 1996). However, we did not find an increase in the levels of neuronal UBB^{+1} in any brain region studied in 6663 transgenic mice up to 18 months of age (exemplified by the microphotographs of the hippocampus in fig. 6A-C).

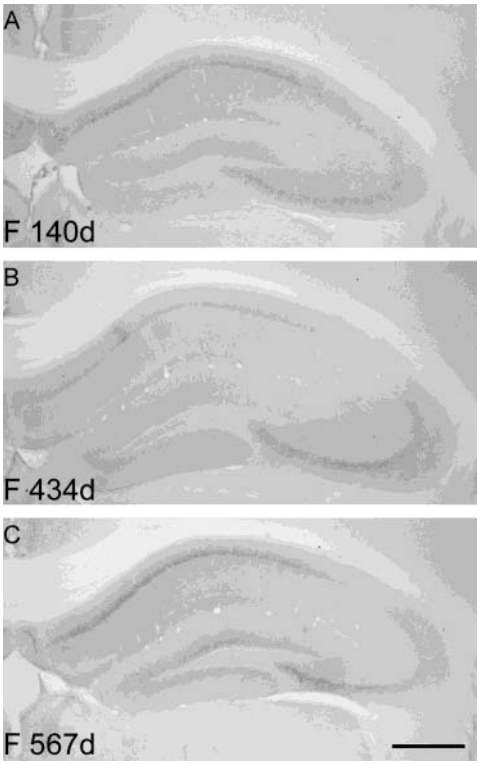

Figure 6 *UBB^{+1} does not accumulate during aging.* A-C. Expression pattern of hippocampal UBB^{+1} protein in 6663 transgenic mice during aging. Female 6663 transgenic mice of 140 days old (A), 434 days old (B) and 567 days old (C) were stained with anti-UBB^{+1} antibody. Scale bar for A-C = 500 μm.

Discussion

In this study, we generated and characterized a transgenic line with low-levels of neuronal UBB^{+1} expression (line 6663). In human, UBB^{+1} accumulates not only in the ubiquitin-positive neuropathological hallmarks of tauopathies (van Leeuwen *et al.*, 1998; Fischer *et al.*, 2003; van Leeuwen *et al.*, 2006), polyglutamine diseases (De Pril *et al.*, 2004) but also in non-neuronal diseases, including alcoholic liver disease (McPhaul *et al.*, 2002) and inclusion-body myositis (Fratta *et al.*, 2004). We show here that the UBB^{+1} protein is barely detectable in line 6663 UBB^{+1} transgenic mice. This suggests that the UBB^{+1} protein is efficiently degraded *in vivo*, similar to our earlier observations *in vitro* showing proteasomal degradation of UBB^{+1} at low expression levels (Fischer *et al.*, 2003; van Tijn *et al.*, 2007). Indeed, UBB^{+1} accumulated in the 6663 transgenic mice only after inhibition of the proteasome by intracranial infusion of the irreversible proteasome inhibitor lactacystin or the reversible inhibitor MG262 (results summarized in Table 1). These data confirm that at low-levels of expression, UBB^{+1} is normally degraded by the neuronal UPS *in vivo*. This strengthens our hypothesis that accumulation of UBB^{+1} in human neuropathology indicates a dysfunctional UPS (Fischer *et al.*, 2003; Hol *et al.*, 2005).

Strikingly, we found that 0.166 mM lactacystin-induced UPS inhibition had disappeared one week after the completion of the infusion, as shown by the absence of Ub and UBB^{+1} accumulation, even though lactacystin derived proteasome inhibition is considered to be irreversible (Fenteany *et al.*, 1995). Lactacystin is a potent naturally occurring proteasome inhibitor (reviewed by (Kisselev and Goldberg, 2001) of which the spontaneous derivative *clasto*-lactacystin β-lactone covalently binds to the active site threonine of the β5 subunit in the 20S core particle (Groll and Huber, 2004). The lactone has the highest affinity for the chymotryptic activity, although it also inhibits the trypsin-like (β2) and PGPH (β1) activities, the latter being a reversible process (Fenteany *et al.*, 1995). However, due to the relative minor contribution of the reduction of PGPH activity to lactacystin induced proteasome inhibition (Fenteany *et al.*, 1995), this reversibility is not likely to cause the loss of UPS inhibition in our setup. The reversed inhibition can possibly be attributed to restoration of proteasome activity over time via slow hydrolysis of the β-lactone-proteasome adduct in aqueous solutions by the formation of the inactive lactacystin analogue *clasto*-lactacystin dihydroxy acid (Dick *et al.*, 1996; Kisselev and Goldberg, 2001). Infusion of a higher concentration of lactacystin might overcome this latent reversibility of the inhibitor. Indeed, we show here that UPS inhibition induced by a 10-fold higher concentration of lactacystin (1.66 mM) was irreversible over time. Another possibility is that the natural turnover of 20S core subunits influences the effectiveness of the inhibitor. In chicken skeletal muscle it was shown that the 20S subunits are constitutively turned over, with ~55% of the β5 subunits being newly synthesized over 120 hours (Hayter *et al.*, 2005). It is thus conceivable that the lactone-modified β5 subunit in the

core proteasome is replaced over time via intrinsic turnover of β5 subunits followed by degradation of the lactacystin-modified β5 subunit. Newly incorporated β5 subunits will hold full proteolytic activity, and so reverse the lactacystin induced UPS inhibition.

In this study we also found sustained, and even increased, proteasome inhibition four days after MG262 infusion ended, although MG262 is considered to be a reversible inhibitor. MG262 belongs to the peptidyl boronic acid proteasome inhibitors (Adams *et al.*, 1998), a class of highly potent inhibitors. This class of inhibitors shows a slow dissociation rate of the boronate-proteasome adducts, leading to slow kinetics of inhibition reversal (reviewed in (Kisselev and Goldberg, 2001)). This slow on/off rate could very well explain the sustained UPS inhibition over time by MG262 we observed in our setup. Peptide boronates are highly specific towards inhibiting proteasome activity and are metabolically stable, making them excellent targets for drug development. The peptide boronate derivate PS-341, (Velcade, Millenium Pharmaceuticals), is indeed an FDA approved clinical drug to treat relapsed multiple myeloma, acting on e.g. cell cycle progression and tumor angiogenesis (Adams, 2004). We indeed also show in this study the long-lasting effects of MG262 treatment *in vivo*. The possibility that reversal of proteasome inhibition was unachievable in our setup using osmotic mini-pumps was negated, as proteasome inhibition by 0.166 mM lactacystin did reverse over time.

It was previously shown that infusion of saline into mice cerebral cortex resulted in necrosis of the cortical tissue surrounding the probe site (Jablonska *et al.*, 1993). We did not discern this in our study; infusion of Ringer's saline did not lead to tissue damage or substantial inhibition of the UPS, shown by the absence of UBB^{+1} or Ub accumulation. The minor accumulation of poly-ubiquitinated proteins found after Ringer's infusion (fig. 3B) could point to an activation of the intracellular stress response due to the probe implantation, leading to an upregulation of Hsp expression. Indeed, increased Hsp70 expression has previously been reported to occur after traumatic brain injury in rodents, e.g. (Brown *et al.*, 1989; Chen *et al.*, 1998) and human (Dutcher *et al.*, 1998; Seidberg *et al.*, 2003). Also in our experiments, upregulation of Hsp70 immunoreactivity was present around the infusion site (not shown). Induction of the heatshock response can be regulated via the UPS, with the ubiquitin-ligase enzyme CHIP mediating e.g. heatshock mediated substrate ubiquitination (Qian *et al.*, 2006). Implantation of the infusion probe could thus lead to a local stress response in the hippocampus, ultimately increasing the level of poly-ubiquitinated substrates.

In contrast to Ringer's infusion, high concentrations of lactacystin and prolonged exposure to MG262 did result in substantial tissue damage in the ipsilateral hippocampus. Lactacystin is known to exhibit dose-dependent toxic effects and ultimately induces apoptosis in several neuronal cell types, including rat cerebellar granule cells (Pasquini *et al.*, 2000), rat dopaminergic cells e.g. (Rideout *et al.*, 2001; McNaught *et al.*, 2002; Fornai *et al.*, 2003) and rat and mouse primary cortical neurons (Qiu *et al.*, 2000; Yew *et al.*, 2005).

These toxic properties possibly account for the increased tissue necrosis after infusion of high concentrations of lactacystin as opposed to low concentrations, as well as the increase in tissue damage after prolonged duration of the exposure. This substantial tissue damage likely abolishes UBB^{+1} accumulation in the ipsilateral hippocampus, explaining why the majority of UBB^{+1} accumulation resides in the contralateral hippocampus after inhibition with high concentrations of lactacystin. The observed UBB^{+1} accumulation does appear to be more widespread than the Ub accumulation following UPS inhibition, indicating that UBB^{+1} might be a more sensitive marker for UPS dysfunction than Ub in the 6663 transgenic mice.

Many studies report an age-dependent decline in UPS activity in various cell types including nervous tissue, accompanied by an increased amount of oxidized proteins (reviewed by (Carrard *et al.*, 2002; Keller *et al.*, 2002)). More specifically, an age-related decrease of tryptic, chymotryptic and PGPH proteolytic activities was found in aging rat spinal cord (Keller *et al.*, 2000), hippocampus and cortex (Keller *et al.*, 2000) and also in several brain regions in aged mice (Zeng *et al.*, 2005). We expected that this endogenous long-term decline in proteasome activity might be reflected in accumulation of UBB^{+1} in aged 6663 transgenic mice, as we found massive accumulation of the protein after short-term UPS inhibition with exogenous administered UPS inhibitors. However, we did not observe an alterations in UBB^{+1} immunoreactivity up to 18 months of age. This implicates that turnover of UBB^{+1} protein is unimpaired, and that a decline of proteasome activity might not be present. More likely, the levels of UBB^{+1} expression are at such a low level that a partial decrease of UPS activity (~40%, (Zeng *et al.*, 2005)) is not directly reflected in accumulation of UBB^{+1}. It must be noted that this decrease in proteolytic activity is measured in brain homogenates (Zeng *et al.*, 2005), including all brain-derived cell types, and might not accurately reflect the activity decline in individual neurons, the only cell type which expresses UBB^{+1}.

Our results show that in the 6663 transgenic mice, the UBB^{+1} protein is degraded by the neuronal UPS *in vivo*. This observation validates our previous results obtained in neuronal cell lines and primary cultures showing that UBB^{+1} is a ubiquitin fusion degradation substrate for proteasomal degradation at low expression levels (Lindsten *et al.*, 2002; van Tijn *et al.*, 2007). It also supports the hypothesis that in human, the UBB^{+1} protein is normally degraded and accumulates only when the UPS is compromised, as seen in the disease-specific hallmarks of tauopathies and polyglutamine diseases (Fischer *et al.*, 2003). This novel transgenic line expressing low-levels of UBB^{+1} can serve as a model system to further elucidate the properties of UBB^{+1} and to study its role in neurodegenerative disease.

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