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

General Discussion

Partly based upon:

Frameshift proteins in autosomal dominant forms of Alzheimer disease and other tauopathies

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A decade of UBB+1

UBB+1 was discovered over a decade ago as an aberrant protein accumulating in the neuropathological hallmarks of Alzheimer’s disease (AD) and Down Syndrome (DS), including in neurofibrillary tangles, neuropil threads and dystrophic neurites (van Leeuwen et al., 1998). UBB+1 is formed by “molecular misreading”, a process giving rise to di-nucleotide deletions in messenger RNA (mRNA), preferably in or around GAGAG repetitive motifs (Evans et al., 1994; van Den Hurk et al., 2001). Subsequent translation of these aberrant mRNAs results in proteins showing a frameshift in the alternate +1 open reading frame. In the case of UBB+1, an mRNA ΔGT or ΔCT di-nucleotide deletion close to the C-terminus of the first or third ubiquitin repeat respectively results in translation of the UBB+1 protein, harboring a C-terminal extension of 19 amino acids (van Leeuwen et al., 1998). Molecular misreading has furthermore been shown to occur in amyloid-β precursor protein (APP) mRNA, resulting in a frameshifted APP protein (APP+1). APP+1 also accumulates in the neuropathological hallmarks of AD and DS and often co-localizes with UBB+1 (van Leeuwen et al., 1998). It was initially hypothesized that the rate of molecular misreading increased during aging; UBB+1 and APP+1 proteins were detected only in diseased subjects and aged controls. Hence, molecular misreading was hypothesized to contribute to the early pathology in AD and even be an underlying cause for AD pathology (van Leeuwen et al., 1998). However, later studies by our group and by others revealed that frameshifted UBB and APP mRNA transcripts are present at similar levels in diseased subjects as well as young control subjects (Fischer et al., 2003; Gerez et al., 2005), indicating that molecular misreading is a continuous process during life. The gradual accumulation of +1 proteins is thus more likely attributable to confounding factors allowing accumulation of aberrant proteins during aging or disease progression and not to an increased rate of molecular misreading.

The precise mechanism behind molecular misreading is as yet unknown. It has been suggested that the di-nucleotide deletions originate from RNA polymerase slippage, as well as from post-transcriptional RNA editing (van Leeuwen et al., 2000). Also a failure of RNA quality control could underlie the presence of the +1 transcripts. Normally, aberrant mRNAs are selectively targeted for degradation by the nonsense-mediated mRNA decay (NMD) pathway (reviewed by Chang et al., 2007). This pathway recognizes mRNAs which contain a (premature) termination codon located more than 50-55 nucleotides upstream of the 3’-outer exon-exon junction, marked by an exon-junction complex (Nagy and Maquat, 1998). In the UBB+1 transcript, no exon-exon boundary is present after the newly formed termination codon, and these transcripts are thus not likely to be targeted for NMD. On the other hand, APP+1 transcripts contain a premature termination codon in exon 9 or 10, far upstream of the outer 3’exon-exon junction at exon 17 and 18. Hypothetically, APP+1 transcripts should be subjected to NMD. The presence of APP+1
protein in diseased patients may thus point to a failing RNA quality control system. Recently, it was hypothesized that UBB\textsuperscript{+1} and APP\textsuperscript{+1} proteins could also result from ribosomal frameshifting during translation of intact UBB and APP mRNA. The outcome of this ribosomal frameshifting would be proteins with C-termini similar to UBB\textsuperscript{+1} and APP\textsuperscript{+1} (Wills and Atkins, 2006).

Degradation of proteins can be executed by various proteolytic systems, including lysosomal degradation, chaperone-mediated autophagy, and substrate-specific degradation by the ubiquitin-proteasome system (UPS) for protein quality control. The frequency of molecular misreading is very low, occurring in less than 1 in 10\textsuperscript{5} to 10\textsuperscript{6} transcripts (Gerez et al., 2005), and does not differ between AD and DS patients and non-demented control subjects (Fischer et al., 2003; Gerez et al., 2005). Therefore, it is likely that the accumulation of the aberrant proteins in diseased patients can be attributed to a failing protein quality control system. This hypothesis is further supported by the finding that the UBB\textsuperscript{+1} protein is a natural substrate for proteolytic degradation by the UPS (Lindsten et al., 2002). The decrease in proteasome function associated with aging (Keller et al., 2000) and neurodegenerative disease (reviewed by Ciechanover and Brundin, 2003) could underlie the accumulation of the UBB\textsuperscript{+1} protein. Accordingly, UBB\textsuperscript{+1} accumulation in disease is postulated to be an endogenous marker for general proteasome dysfunction (Fischer et al., 2003). In the case of APP\textsuperscript{+1}, it is not known if this aberrant protein is normally targeted for proteasomal degradation. However, similar to the wild-type APP cleavage products sAPP\textalpha and sAPP\beta, APP\textsuperscript{+1} is a secreted protein and accumulates in the cerebrospinal fluid in human (Hol et al., 2003), as well as in a transgenic mouse model neuronally expressing APP\textsuperscript{+1} (Fischer et al., 2006). Proteasomal degradation takes place in the cytosol as well as the nucleus, and proteasomes are occasionally localized on the cytosolic surface of the endoplasmatic reticulum membrane (Wojcik and DeMartino, 2003). APP\textsuperscript{+1} is normally trafficked through the endoplasmatic reticulum and Golgi complex (van Dijk et al., 2004) and could there be a target for the proteasomal degradation. However, if APP\textsuperscript{+1} accumulation is affected by a decrease in protein quality control has not been further studied yet.

Molecular misreading does not only occur in the nervous system; +1 frameshift proteins can also be formed in non-neuronal cells. This was first demonstrated in a transgenic mouse model for molecular misreading expressing multiple copies of the rat vasopressin gene. Frameshifted vasopressin protein was detected in tissues showing high transgene expression, including the epididymis and parotid gland (Van Leeuwen et al., 2000). More recently, UBB\textsuperscript{+1} accumulation was also detected in the pathological hallmarks of several non-neuronal diseases which show ubiquitin-positive pathology, including muscle disease (Fratta et al., 2004; Olive et al., 2008), alcoholic liver disease (McPhaul et al., 2002) and alpha(1)-antitrypsin deficiency (Wu et al., 2002). These results indicate that UPS dysfunction could be a general underlying cause for UBB\textsuperscript{+1} accumulation in a variety of diseases.
Processing of UBB+1, a dual proteasome substrate and inhibitor

The precise mechanism by which UBB+1 is processed remains elusive. Ubiquitin is translated as a fusion protein of ubiquitin and a ribosomal protein or as a polyubiquitin precursor protein of three head-to-tail ubiquitin repeats (UBB). To generate monomeric ubiquitin, these precursors are cleaved at the ubiquitin C-terminal glycine by ubiquitin C-terminal hydrolases (UCHs), belonging to the family of deubiquitinating enzymes (DUBs) (Larsen et al., 1998). Lam et al. were the first to show that UBB+1 is not proteolytically cleaved by the DUB UCH-D, due to the presence of a 19 amino acid C-terminal extension (Lam et al., 2000). In addition, this in vitro study showed that UBB+1 is not actively involved in the ubiquitination of substrate proteins, as it cannot conjugate to the ubiquitin-activating enzyme E1. Conversely, UBB+1 is ubiquitinated itself, and the resulting UBB+1 containing ubiquitin chains are refractory to deubiquitination by isopeptidaseT. This DUB requires an unconjugated C-terminal glycine for deubiquitination, which is not present in UBB+1. This study was also the first to show that ubiquitinated UBB+1 (Ub,-UBB+1) can inhibit proteasomal substrate degradation in a cell free system (Lam et al., 2000).

These results were corroborated in a neuroblastoma cell line, where high levels of UBB+1 expression resulted in stable levels of UBB+1 protein, ultimately leading to apoptotic-like cell death (De Vrij et al., 2001). UBB+1 also proved to hold seemingly opposing proteasome-related properties; UBB+1 is not only an inhibitor of the UPS, but also a ubiquitin-fusion degradation (UFD) substrate for the proteasomal degradation (Lindsten et al., 2002). Similar to other UFD substrates (Johnson et al., 1995), UBB+1 is ubiquitinated on internal lysine residues K29 and K48. Ubiquitination of UBB+1 at both lysine residues is essential for optimal processing by the proteasome. Intriguingly, enhancement of the UFD degradation signal further strengthens the proteasome inhibitory capacity of UBB+1 (Lindsten et al., 2002). Notably, UBB+1 is the first identified naturally occurring UFD substrate in human. The proteasome substrate properties of UBB+1 also offer an explanation for the resistance to deubiquitination by isopeptidaseT (Lam et al., 2000), as isopeptidaseT shows specific activity toward unanchored ubiquitin chains and does not cleave ubiquitin moieties from substrate proteins (Wilkinson et al., 1995). Confirming previous observations, Lindsten et al. also showed that UBB+1 acts as a UPS inhibitor, using a green fluorescent protein (GFP)-reporter cell line for proteasome activity expressing the artificial proteasome substrate UbG76V-GFP (Lindsten et al., 2002). UbG76V-GFP is normally degraded by the UPS, however, when the proteasome is inhibited, degradation of UbG76V-GFP is prevented resulting in green fluorescence (Dantuma et al., 2000). Overexpression of UBB+1 in this cell line resulted in GFP accumulation in the vast majority of transduced cells (Lindsten et al., 2002).
These dual properties of UBB$^+$ were further studied as described in Chapter 2. Here, we offer an explanation for the dual UPS substrate-inhibitory properties of UBB$^+$. We demonstrate in HeLa cells that UBB$^+$ shifts from UPS substrate to proteasome inhibitor depending on the level of expression. UBB$^+$ is a substrate for proteasomal degradation at low expression levels. Only at high expression levels the protein accumulates and subsequently induces dose-dependent proteasome inhibition. This observation also holds in mouse organotypic cortex slices, a system that reflects a multi-cellular environment wherein neuronal connectivity and neuron-glia interactions are considered to be well preserved (Sundstrom et al., 2005), and which closer resembles the human brain than the previously used HeLa and neuroblastoma cell lines. In these organotypic cultures, the UBB$^+$ protein is degraded when expressed at relatively low levels using a lentiviral vector (Chapter 2). Higher levels of expression, achieved with adenoviral transduction of UBB$^+$, lead to accumulation of UBB$^+$ and inhibition of the UPS.

The levels of expression required to initiate UBB$^+$ accumulation differ from one physiological setting to another. This is best exemplified by the differential effect of lentiviral UBB$^+$ transduction on accumulation of the protein in several (neuronal) model systems. Lentiviral UBB$^+$ transduction induced sufficient expression for UBB$^+$ to accumulate in the human neuroblastoma cell line SH-SY5Y, as reported in Chapter 2, as well as in mouse primary neuron cultures (Lindsten et al., 2003). On the other hand, in cortex slice cultures obtained from the same mouse strain, lentiviral expression did not give rise to UBB$^+$ accumulation, shown in Chapter 2. Also after stereotactical injection of lentiviral UBB$^+$ in the rat hippocampus, UBB$^+$ protein could not be detected (Fischer et al., 2003). These results indicate that not only the choice of expression vector, but also the physiological context wherein UBB$^+$ is expressed will influence the expression levels of the protein and thereby determine the UPS-related properties of the protein. Therefore, caution should be taken when these results obtained in vitro are translated to the human situation.

UBB$^+$ induced proteasome inhibition in vivo

To closer resemble the human UBB$^+$ neuropathology by studying the properties of UBB$^+$ in vivo, we developed three transgenic mouse lines expressing varying levels of neuronal UBB$^+$, as described in Chapters 3 and 4. Using these transgenic lines, the dual UPS substrate/inhibitor properties of UBB$^+$ as demonstrated in vitro could be corroborated in vivo. Indeed, when expressed at low levels (17% of endogenous UBB mRNA, transgenic line 6663), the UBB$^+$ protein is degraded by the proteasome. Only after hippocampal inhibition UPS of the using pharmacological proteasome inhibitors, UBB$^+$ protein accumulation can be observed. UBB$^+$ is expressed at high levels in the transgenic lines 3413 and 8630 (49% and 67% of endogenous UBB mRNA respectively). In these lines,
GENERAL DISCUSSION

UBB\(^{+1}\) accumulates in the neuronal population wherein the transgene is expressed. The relative UBB\(^{+1}\) mRNA expression level in lines 3413 and 8630 is increased by \(~3.5\)-fold compared to line 6663, whereas the difference on UBB\(^{+1}\) protein level is much greater: the expression of UBB\(^{+1}\) in line 8630 is \(~14\)-fold higher than in line 6663. These data also indicate that UBB\(^{+1}\) protein levels are regulated by a post-transcriptional mechanism and that UBB\(^{+1}\) expression must first surpass a threshold level before accumulation commences, similar to the data obtained \textit{in vitro} in Chapter 2. We also investigated if UBB\(^{+1}\) also acts as an inhibitor of the UPS \textit{in vivo} at high expression levels. The chymotryptic proteasome activity, measured in cortex homogenates of 3413 transgenic mice, indeed showed a modest significant decrease compared to wild-type mice. Also in the 8630 transgenic mice, the proteasome activity is decreased, as shown in Chapter 3.

To further explore these UPS inhibitory properties of UBB\(^{+1}\) \textit{in vivo}, we performed stereotactic injections of lentiviral UBB\(^{+1}\) into the hippocampus of mice expressing the Ub\(^{G76V}\)-GFP reporter (Lindsten \textit{et al.}, 2003). Conversely, we injected lentiviral UBB\(^{+1}\)K29,48R into line 3413 transgenic mice expressing neuronal UBB\(^{+1}\)Ub\(^{G76V}\)-GFP. As a control, we injected lentiviral UBB\(^{+1}\)K29,48R (Ub\(^{G76V}\)-GFP) with a double lysine mutation (UBB\(^{+1}\)K29,48R). UBB\(^{+1}\)K29,48R cannot be ubiquitinated and is thus not targeted to the proteasome to be degraded. The results of these experiments are summarized in Table 1.

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>Lentiviral Injection</th>
<th>Fluorescent GFP</th>
<th>DAB GFP</th>
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<tr>
<td>Ub(^{G76V})-GFP/2</td>
<td>UBB(^{+1})</td>
<td>-</td>
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<td></td>
<td>UBB(^{+1})K29,48R</td>
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<tr>
<td>UBB(^{-1}) line 3413</td>
<td>Ub(^{G76V})-GFP</td>
<td>-</td>
<td>+</td>
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<tr>
<td>wild-type control</td>
<td>UBB(^{-1})</td>
<td>NA</td>
<td>-</td>
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<td>UBB(^{+1})K29,48R</td>
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In the Ub\(^{G76V}\)-GFP transgenic mice, injection of lentiviral UBB\(^{-1}\) does not cause accumulation of the GFP reporter, suggesting that UBB\(^{-1}\) does not inhibit the proteasome in these mice. However, lentiviral UBB\(^{+1}\) expression is also not sufficient to cause accumulation of UBB\(^{+1}\) protein in these mice (data not shown), indicating that UBB\(^{-1}\) is expressed at low levels and degraded by the proteasome. When the Ub\(^{G76V}\)-GFP reporter is injected into UBB\(^{-1}\) transgenic mice, one would expect that the reporter accumulates due
to the UBB\(^{+1}\) induced proteasome inhibition. The results, as shown in Table 1, do not give conformation of decreased proteasome activity in the UBB\(^{+1}\) transgenic mice. Using fluorescent staining techniques, no GFP accumulation was detected. When GFP was assessed with a immuno-histochemical staining, only a few GFP positive cells were detected. These sparsely present cells showed an astrocytic morphology, suggesting that the GFP accumulation was not attributable to UBB\(^{+1}\), as UBB\(^{+1}\) is only expressed in neurons. When the Ub\(^{G76V}\)-GFP reporter was injected in wild-type mice, also a few GFP positive cells were detected. Therefore, the GFP accumulation in these mice is most likely caused by the stereotactic injections leading to cellular stress and subsequent accumulation of the reporter, and not to inhibition of the proteasome by UBB\(^{+1}\).

The absence of GFP reporter accumulation in the UBB\(^{+1}\) mice can have several underlying causes. As mentioned above, the expression levels of UBB\(^{+1}\) achieved with lentivirus could be insufficient to induce accumulation of the reporter. Also, the GFP reporter might not be sensitive enough to detect the modest decrease in UPS activity in the 3413 transgenic mice, as the reporter only accumulates at levels over 50% chymotryptic-like proteasome inhibition in cell lines (Dantuma et al., 2000), whereas in the 3413 transgenic line only a ~20% UPS inhibition is observed. A third possibility is that, despite the reported broad tropism of lentivirus and its ability to transduce neuronal cells (Ehrengruber et al., 2001), mostly astrocytes are transduced in the brain. This is further underscored by fact that the few GFP positive cells that were present showed an astrocytic morphology. Neuronal UPS inhibition induced by UBB\(^{+1}\) would then not affect the turnover of astrocytic-expressed GFP reporter.

We further analyzed the inhibitory properties of UBB\(^{+1}\) in vivo by crossing the 3413 transgenic line with GFP reporter line Ub\(^{G76V}\)-GFP/2 (Lindsten et al., 2003). However, GFP reporter accumulation could not be observed in the double transgenic mice up to 530 days of age, even though UBB\(^{+1}\) was expressed at high levels (fig. 1). One possible explanation could be that the reporter is not expressed in neuronal cells in this specific transgenic line (Ub\(^{G76V}\)-GFP/2), in spite of the CMV-β actin promoter, which should in principle ensure high levels of transgene expression in most cell types in the body, including neurons. In primary neurons of a comparable transgenic line (Ub\(^{G76V}\)-GFP/1), indeed GFP accumulation is present when proteasome activity is inhibited (Lindsten et al., 2003). In the line used for these experiments, a slight alteration in the transgene expression pattern, caused by differences in the transgenic integration site, could affect the neuronal expression levels of the reporter. Preliminary studies with primary cortical neuron cultures derived from Ub\(^{G76V}\)-GFP/2 mice did not give conclusive evidence for expression of the GFP reporter in neuronal cells. Also in cortex slices of these mice, the morphology of GFP accumulating cells responsive to UPS inhibition showed an astrocytic morphology (shown in Chapter 2 and (F.M. de Vrij, unpublished results)). Alternatively, the GFP reporter might not be sensitive enough to detect a modest neuronal UPS inhibition in vivo.
This hypothesis is partly underscored by the observation that intracerebral inoculation of Ub$_{G76V}$-GFP transgenic mice with mouse scrapie, an inhibitor of UPS activity in vitro, does lead to accumulation of the Ub$_{G76V}$-GFP reporter in the brains of these mice, accompanied by intraneuronal ubiquitin deposits (Kristiansen et al., 2007). However, the cell type wherein accumulation of the Ub$_{G76V}$-GFP reporter is present is not further specified in this study.

Figure 1   UPS inhibition is not detected in Ub$_{G76V}$-GFPxUBB$^{+1}$ transgenic mice. Vibratome sections of a 529 day-old female Ub$_{G76V}$-GFPxUBB$^{+1}$ line 3413 double transgenic mouse were immuno-histochemically stained using polyclonal antibodies against UBB$^{+1}$ (Ub3, (Fischer et al., 2003); left column) or GFP (Chemicon; right column). UBB$^{+1}$ is ubiquitously expressed in neurons in e.g. the cortex, striatum (A) and hippocampus (C, E). In the same animal, no GFP accumulation is present (B, D, F). Panels E and F are enlargements of panels C and D. These results indicate that a decrease in proteasome activity, as reflected by GFP accumulation, cannot be detected in the Ub$_{G76V}$-GFPxUBB$^{+1}$ double transgenic mice.
CHAPTER VII

Deubiquitination of UBB^+1

The UBB^+1 protein is ubiquitinated at two internal lysine residues, at amino acid positions 29 and 48. Ubiquitination at both these lysine residues is required to achieve maximal UBB^+1 induced proteasome inhibition, as the UBB^+1 double lysine mutant UBB^+1K29,48R has completely lost its UPS inhibitory properties (Lindsten et al., 2002). Expression of the single lysine mutants UBB^+1K29R or UBB^+1K48R also results in decreased proteasomal degradation of UFD substrates, although to a lesser extent than UBB^+1. The capacity of the proteasome to degrade N-end rule substrates is not compromised following expression of UBB^+1K29R or UBB^+1K48R. Therefore, ubiquitination of UBB^+1 at both lysines is a prerequisite for maximal UPS inhibition to take place (Lindsten et al., 2002).

As the ubiquitination state of UBB^+1 determines its UPS substrate as well as its UPS inhibitory properties, it is of importance to study the mechanisms by which Ub^x-UBB^+1 chains can be formed, e.g. by dissecting the nature of the ubiquitination enzymes involved in ubiquitinating UBB^+1. One of the E2 enzymes capable of ubiquitinating UBB^+1 is the ubiquitin-conjugating enzyme E2^25K/Hip-2. This E2 enzyme is capable of attaching preformed K48-linked ubiquitin chains to UBB^+1 in vitro (Lam et al., 2000) and can ubiquitinate UBB^+1 in a rat neuroblastoma cell line (Song et al., 2003). Possible additional involvement of specific ubiquitin E3 ligating enzymes or of the ubiquitin chain elongation factor E4 in the ubiquitination of UBB^+1 has so far not been studied.

However, the ubiquitination state of a substrate protein is governed by a tight balance between ubiquitination and deubiquitination. Deubiquitination is exerted by DUBs, a class of enzymes capable of cleaving ubiquitin moieties from a range of targets, including from ubiquitin chains and substrate proteins (reviewed by Nijman et al., 2005). Aside from deubiquitinating target substrates, DUBs can also be active at the proteasome, e.g. to edit the length of the ubiquitin chain of the proteasome bound substrate (Lam et al., 1997) or to delay substrate degradation (Hanna et al., 2006). Also several 19S subunits show deubiquitinating activity themselves, including the Rpn11 subunit mediating ubiquitin chain release from substrates (Verma et al., 2002; Yao and Cohen, 2002).

To further study the mechanisms behind the ubiquitination of UBB^+1, we aimed to identify the DUB involved in the deubiquitination of UBB^+1. We hypothesized that this DUB (or class of DUBs) would be able to cleave ubiquitin moieties from the ubiquitin chains attached to lysines K29 and K48 of UBB^+1, thereby decreasing the total amount of Ub^x-UBB^+1. Only when fully ubiquitinated, UBB^+1 is directed to the 26S proteasome and will subsequently cause UPS inhibition. By reducing the levels of Ub^x-UBB^+1, one could potentially alleviate the UPS inhibiting properties of UBB^+1 and possibly reverse the adverse affects of UBB^+1 expression. We anticipated to reveal the DUB(s) involved in this process using a DUB RNAi library screen (Brummelkamp et al., 2003). This library contains a total of 200 vectors expressing short hairpin RNAs (shRNAs) targeting a total of
50 human DUBs, with per DUB four pooled shRNA vectors targeting individual sequences (Brummelkamp et al., 2003; Nijman et al., 2005).

We used two complementary approaches to dissect the effect of knockdown of a DUB involved in deubiquitination of UBB+1. After co-transfection of UBB+1 and the knockdown library in a HeLa cell line expressing the UbG76V-GFP reporter, we measured Ub x-UBB+1 levels as well as UBB+1 induced UPS inhibition. In the first approach, we set out to analyze the levels of Ub x-UBB+1 by Western blot using an antibody directed against the +1 C-terminus of UBB+1 (*, right lane). All other staining appears to be non-specific as it is also present in the cell transduced with empty vector (left lane). B: Western blot analysis of cell lysates of HeLa UbG76V-GFP reporter cells or 293 cells transiently transfected with UBB+1 shows lower levels of UBB+1 expression in HeLa cells than in 293 cells. Similar protein amounts are loaded for the 293 cells and HeLa cells, confirmed by Western blot for β-actin (not shown). UBB+1 is detected with anti-UBB+1 Ubi3 antibody.
were observed in any DUB knockdown pool.

In addition to the previously described approach using Western blot, we also attempted to identify a UBB\(^{+1}\)-associated DUB by measuring UBB\(^{+1}\) induced UPS inhibition in the HeLa Ub\(^{G76V}\)-GFP reporter cell line. Theoretically, knockdown of a DUB which deubiquitinates UBB\(^{+1}\) gives rise to higher levels of Ub\(^{-}\)-UBB\(^{+1}\), which subsequently lead to increased UPS inhibition. This would be reflected in increased GFP accumulation in the Ub\(^{G76V}\)-GFP reporter cell line. We measured the percentage of GFP positive cells after co-transfection of UBB\(^{+1}\) and the DUB knockdown vectors using flow cytometry. In this setup, we could not find a DUB which significantly increased the amount of GFP positive cells. Surprisingly, knockdown of 5 DUBs, encoding UCH-25, USP-9X, IsopeptidaseT-T, UCH-26 and USP-45, showed a significant decrease in the percentage of GFP fluorescent cells, indicating that knockdown of these specific DUBs decreased the UPS inhibitory capacity of UBB\(^{+1}\) (fig. 4).

One of the underlying causes for this decrease in GFP fluorescence may be that these DUBs are involved in the deubiquitination of the Ub\(^{G76V}\)-GFP reporter, as Ub\(^{G76V}\)-GFP is ubiquitinated itself to be targeted to the proteasome (Dantuma et al., 2000). When expression of these specific DUBs is decreased, ubiquitinated Ub\(^{G76V}\)-GFP levels increase. The ubiquitinated Ub\(^{G76V}\)-GFP is subsequently transported to the proteasome and degraded. Consequently, the total levels of GFP reporter in the cell are decreased and UPS

![Figure 3](image.png)

**Figure 3** Effect of DUB knockdown on the levels of monomeric UBB\(^{+1}\). The effect of DUB knockdown on levels of monomeric UBB\(^{+1}\) was assessed by Western blot analysis. No significant effects of DUB knockdown on the levels of monomeric UBB\(^{+1}\) are present. Control levels of monomeric UBB\(^{+1}\) (measured by co-transduction of UBB\(^{+1}\) with empty knockdown vector) are set at 100%. Experiment was performed in biological duplicate.
inhibition by UBB\textsuperscript{+1} expression will result in lower levels of GFP fluorescence. In addition, Ub\textsuperscript{G76V}-GFP as well as UBB\textsuperscript{+1} are UFD substrates for proteasomal degradation (Dantuma et al., 2000; Lindsten et al., 2002). Therefore, these two substrates might compete for common DUBs, which makes it difficult to accurately determine a DUB specific for UBB\textsuperscript{+1}. Another disadvantage of this setup, using UPS activity as a readout, is that a possible effect of DUB knockdown on the ubiquitination state of UBB\textsuperscript{+1} first has to be translated to altered GFP fluorescence to be detected. This extra detection step might render this setup not sensitive enough to detect a target DUB for UBB\textsuperscript{+1}.

RNAi-mediated knockdown of a UBB\textsuperscript{+1}-associated DUB potentially enhances the toxicity of UBB\textsuperscript{+1} by increasing the levels of Ub\textsubscript{x}-UBB\textsuperscript{+1}. By decreasing the expression of one of the proteasome associated DUBs, also the turnover rate of UBB\textsuperscript{+1} could be modified. Therefore, the ultimate effect of knockdown of a UBB\textsuperscript{+1}-modifying DUB is difficult to predict. For example, knockdown of a DUB editing the ubiquitin chain at the proteasome could increase degradation of UBB\textsuperscript{+1} by reducing substrate rescue. On the other
hand, inhibiting release of the complete ubiquitin chain from UBB+1 could prevent degradation of UBB+1 and exert a detrimental effect by increasing the amount of Ubx-UBB+1 leading to proteasome inhibition and cellular toxicity. Further complicating the interpretation of the obtained results are the possible indirect effects of DUB knockdown on the levels of monomeric UBB+1 via Ubx-UBB+1 induced proteasome inhibition. The knockdown of a DUB which deubiquitinates UBB+1 can lead to a decrease of monomeric UBB+1 and an increase of Ubx-UBB+1. Subsequently, these high levels of Ubx-UBB+1 will increase proteasome inhibition. UPS inhibition then in turn leads to an increase of monomeric UBB+1 via a yet unknown mechanism (Lindsten et al., 2002; Fischer et al., 2003). Therefore, the overall effect of DUB knockdown on monomeric UBB+1 levels is hard to dissect, as it depends on the function of the DUB within the UPS and on the relative contribution of above described indirect effects.

With both readouts of this focused RNAi screen, we could not identify a target DUB which is involved in deubiquitination of UBB+1. The aforementioned drawbacks in the experimental setup could contribute to this result. Therefore, we cannot rule out the possibility that one of these 50 DUBs does affect UBB+1 ubiquitination. Another possibility is that the DUB responsible for deubiquitination of UBB+1 is not expressed in this library of 50 knockdown pools, as at the present moment approximately 100 human DUBs are known (Nijman et al., 2005).

Proteasome inhibition and cognitive function

Post-translational modification of substrate proteins by ubiquitination is essential for cell viability, and more specific, plays an important role in intact neuronal function. During neuronal development, ubiquitin-mediated proteolysis is involved in axonal growth and axon guidance, growth cone formation and regulation of synaptic number and size to ensure correct formation of CNS connectivity (Hegde and Upadhya, 2007). In the adult nervous system, neuronal homeostasis is sustained via ubiquitin-dependent turnover of a broad range of substrate proteins. In addition, the UPS plays an important role in maintenance of established synaptic connections and development of new synapses. Regulation of synaptic plasticity relies in part on proteasome-mediated degradation of substrate proteins in the pre-synapse as well as the post-synapse. Therefore, UPS activity directly influences synaptic strength and transmission, two processes underlying learning and memory formation (reviewed by (DiAntonio and Hicke, 2004; Yi and Ehlers, 2007)). It is thus believed that protein modification by the UPS is likely to be involved in cognitive function.

Our results, as described in Chapter 3 and Chapter 5, indeed confirm that intact forebrain proteasome activity is required for optimal cognitive performance; in 3413 UBB+1 transgenic mice, endogenous, life-long modest inhibition of the proteasome underlies a
decrease in cognitive ability. Notably, hippocampus-dependent spatial memory was affected in the Morris watermaze and in context-dependent Pavlovian fear conditioning, whereas spatial learning was unaffected. Also procedural (motor-) learning, as assessed in a rotarod paradigm, was not decreased due to UBB\textsuperscript{+1} induced proteasome inhibition, indicating that chronic low-level UPS inhibition in these mice only affects a specific subset of memory-related processes. In other mouse models, a variety of other UPS alterations also induce deficits in cognition, as reviewed in Chapter 1. The molecular mechanism by which proteasome dysfunction in the UBB\textsuperscript{+1} transgenic mice leads to spatial memory deficits is not fully understood. Our results suggest that high levels of UBB\textsuperscript{+1} expression in the hippocampus are causative for the cognitive dysfunction in hippocampus-related memory tasks. In line with these observations, cognitive processes involving brain regions in which UBB\textsuperscript{+1} expression is absent or only present at low levels, appear to be intact. These include motor learning and motor coordination, which are mainly mediated by the cerebellum (Hikosaka \textit{et al.}, 2002; Goddyn \textit{et al.}, 2006), an area devoid of UBB\textsuperscript{+1} expression. Cued fear conditioning is also unaffected in the UBB\textsuperscript{+1} transgenic mice. The amygdala, the brain region responsible for cued conditioning (Maren, 2001), indeed shows relatively low levels of UBB\textsuperscript{+1} expression.

A caveat when interpreting these behavioral data is the fact that proteasome inhibition as well as the concomitant accumulation of ubiquitinated proteins was measured in cortex homogenates. It is possible that this inhibition is absent in other brain regions in spite of high levels of UBB\textsuperscript{+1} expression, e.g. in the hippocampus. It is also conceivable that the extent of UPS inhibition is not directly correlated to the levels of UBB\textsuperscript{+1} protein expression and therefore varies between different regions in the brain. To directly correlate the UPS inhibition to cognitive function, it would be sensible to also determine UPS activity in the hippocampus. This could be performed by measuring the turnover of small fluorogenic proteasome substrates, in a similar fashion as the previous measurements on cortex samples, described in Chapter 3. These fluorogenic substrates have an disadvantage that they readily diffuse into the 20S proteasome core and therefore also do not require ubiquitination to be targeted to the 26S proteasome. An alternative method to measure UPS activity would be monitoring the turnover of well-defined endogenous substrates of the UPS. In this case, the proteasome has to be fully assembled to degrade these substrates. One of these substrates is Ub\textsubscript{5}DHFR, which can be ubiquitinated \textit{in vitro} with a defined K48-linked ubiquitin chain (Lam \textit{et al.}, 2005). Preliminary experiments measuring proteolytic activity in partially purified proteasomes of 3413 transgenic mice using Ub\textsubscript{5}DHFR however gave inconclusive results. Proteasome activity can also be measured using activity-based small molecule proteasome-probes which directly reflect the activity of all individual proteolytic subunits of the proteasome (Berkers \textit{et al.}, 2005) or by measuring the end-product of a dysfunctional UPS, i.e. the accumulation of K48-linked polyubiquitin chains (Bennett \textit{et al.}, 2007). The latter can be performed using a mass-spectrometry
based assay which renders it possible to define the amount of polyubiquitin in mouse brain samples, as shown in a HD transgenic mouse model (Bennett et al., 2007). As an alternative approach to correlate UPS inhibition to cognitive deficits in the 3413 transgenic mice, cortex-dependent memory could be examined by performing cortex-dependent behavioral tasks, e.g. examining fear extinction which depends on basal lateral amygdala function and medial pre-frontal cortex, although also hippocampal function might play a role in this process (reviewed by (Myers and Davis, 2007)).

One of the molecular mechanisms behind the UBB³⁺ induced memory deficit could be a decrease in synaptic plasticity. One of the best studied cellular mechanisms that may underlie learning and memory is long-term potentiation (LTP). LTP reflects activity-dependent strengthening of neuronal synaptic connections (synaptic plasticity) and can be evoked by high frequency stimulation of afferent fibres. Indeed, recent evidence strongly suggests that LTP may underlie hippocampal-dependent learning and memory processes (Martin and Morris, 2002; Pastalkova et al., 2006; Whitlock et al., 2006). We therefore investigated synaptic plasticity in male 3413 UBB⁺⁻ transgenic mice and wild-type littermates at the ages of 3, 6 and 9 months. The hippocampus is one of the main brain regions involved in memory formation (Martin and Morris, 2002) and hippocampus-dependent spatial memory is affected in UBB⁺⁻ transgenic mice. Therefore, we chose to specifically examine hippocampus-dependent LTP. Hippocampal slices were stimulated using electrodes placed in the Schaffer collaterals to induce LTP and extracellular field excitatory post-synaptic potentials (fEPSPs) were recorded at synapses in the CA1 region (fig. 5A). In this region, UBB⁺⁻ protein is highly expressed in the 3413 transgenic mice (fig. 5B).

![Figure 5](image)

**Figure 5**  
Schematic overview of electrophysiological measurements in UBB⁺⁻ transgenic mice. A: Schematic overview of the fEPSP recording-setup in the mouse hippocampus. The stimulation electrode is placed in the Schaffer collaterals to stimulate action potentials. The recording electrode is positioned in the CA1 region of the hippocampus to record extracellular field potentials. B: Expression pattern of UBB⁺⁻ protein in the hippocampus of a ~300 day old male 3413 UBB⁺⁻ transgenic mouse visualized with anti-UBB⁺⁻ antibody (Ubi3) immunostaining on a 50μm coronal vibratome section.
First, baseline synaptic properties were examined by determining the maximal slope and amplitude of the fEPSPs (fig. 6A), S value of the input-output graphs (fig. 6B) and the stimulation intensity needed to reach half maximal values for the slope as well as the amplitude (fig. 6C). Results show that the maximal slope and amplitude did not differ between wild-type and UBB^+1 transgenic mice at any age. The S value of the input-output curve was significantly altered for the slope in the 3 months old mice (p=0.046), this ef-

**Figure 6**  *Altered electrophysiological baseline properties in UBB^+1 transgenic mice.* Baseline electrophysiological properties in slices from the CA1 hippocampal area of 3413 UBB^+1 transgenic mice were examined at 3, 6 and 9 months of age. Input-output characteristics were examined by recording hippocampal field potentials (fEPSPs) A: Maximal output (slope and amplitude) after stimulation are plotted. No significant differences are observed. B: S-value of the input-output curve is determined. At 3 months of age, the S-value in the UBB^+1 transgenic mice is significantly decreased compared to wild-type mice C: half maximal stimulation input in UBB^+1 transgenic mice and wild-type littermates. At 6 months of age, UBB^+1 transgenic mice show a significant increase in half maximal stimulation intensity. This effect is not sustained at 9 months of age, *p<0.05.*
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Effect was not present at 6 and 9 months of age.

The half maximum stimulation intensities were comparable between wild-type and transgenic mice at the ages of 3 and 9 months. However, at 6 months of age, the stimula-

Figure 7  Hippocampal LTP is unaffected in UBB$^{+1}$ transgenic mice. LTP (measured by fEPSP recordings in the hippocampus) was measured following a single 100 Hz stimulation at time point 0 and expressed as percentage of pre-stimulation baseline values. Induction of LTP as well as average LTP levels at the end of the recording after 60 minutes are not significantly different in UBB$^{+1}$ 3413 transgenic mice (open circles) compared to wild-type mice (closed triangles) at 3, 6 and 9 months of age.
tion intensities needed to reach the half maximal values of the the slope and amplitude were significantly higher in the UBB\(^{+1}\) transgenic mice than in the wild-type mice (p=0.020 and p=0.006 respectively). In these mice, a higher stimulation intensity is needed in the transgenic mice to evoke a similar response as in the wild-type mice, indicating that the basal network connectivity is affected. However, this effect is not present at 9 months of age, therefore we cannot correlate this defect with the observed behavioral changes in the 3413 transgenic mice. Possible explanations for the changes in the basal synaptic responses observed at 6 months of age could include pre-synaptic deficits in neurotransmitter release, post-synaptic neurotransmitter uptake problems or other more basal structural changes in the neuronal network. However, additional studies on baseline synaptic transmission (mEPSCs, evoked AMPA receptor mediated synaptic responses) are required to fully investigate whether the mutation affects baseline synaptic transmission.

Then, baseline synaptic responses were recorded for 20 minutes to confirm stability of the recorded field potential. Next, high frequent stimulation (100 pulses @ 100 Hz) was delivered to induce LTP. When induction of hippocampal LTP was measured, results showed that LTP was not affected in the transgenic mice at the ages of 3, 6 and 9 months (fig. 7). Also the levels of LTP at 60 minutes after initial stimulation were comparable between transgenic and wild-type mice (fig. 7). Therefore, the spatial memory deficits in the 3413 transgenic mice at 9 months of age cannot be directly correlated to a decrease in LTP in the CA1 region of the hippocampus.

It could be that more subtle changes in synaptic plasticity underlie the observed memory deficits. In order to examine this, multiple stimulation paradigms should be applied to

Figure 8  Hippocampal PPF is unaffected in UBB\(^{+1}\) transgenic mice. Comparison of the PPF ratio (ratio of the second to the first fEPSP) at the Shaffer-CA1 pyramidal cell synapses between wild-type mice and UBB\(^{+1}\) transgenic mice is not significantly different between the wild-type mice and UBB\(^{+1}\) transgenic mice.
examine whether the synaptic response function (threshold to evoke LTP or long-term depression (LTD)) is altered as a consequence of UBB\textsuperscript{+1} expression (Mayford \textit{et al.}, 1995). It is also possible that alterations in synaptic plasticity in other regions of the brain, for example the hippocampal dentate gyrus or the cortex, underlie the observed memory deficits. Preliminary results indeed show decreased LTP in the dentate gyrus of 3413 transgenic mice (van Leeuwen, Dennissen \textit{et al.}, unpublished observations). An alternative approach would be to assess pre-synaptic function. Paired pulse facilitation (PPF) is a transient form of short term plasticity in which the evoked post-synaptic response to a second stimulation pulse is larger than the response to the shortly preceding pulse. PPF originates at the pre-synaptic side of the synaptic cleft (Zucker and Regehr, 2002). To examine pre-synaptic function in UBB\textsuperscript{+1} transgenic mice, we performed PPF in the hippocampus of wild-type and transgenic mice. Preliminary results on 9-months-old mice show that PPF, expressed by the PPF ratio, is enhanced in the UBB\textsuperscript{+1} transgenic mice (fig. 8). This effect is however not significant, which could in part be attributed to the low number of mice tested per genotype (n=3). Further experiments should be performed using a larger number of mice, to examine if these results are persistent. If so, the abnormal (enhanced) PPF ratio is indicative of defects in short-term synaptic plasticity in the UBB\textsuperscript{+1} transgenic mice. Increases in PPF point to an increased neurotransmitter release in response to the second stimulus, caused by abnormal presynaptic accumulation of intracellular Ca\textsuperscript{2+} (Zucker and Regehr, 2002).

Although no direct effect of UBB\textsuperscript{+1} induced UPS inhibition was detected on LTP in the CA1 region by means of electrophysiological measurements, UPS inhibition could still affect levels of substrate proteins at the synapse normally degraded by the proteasome. It has been shown that regulation of many synaptic proteins is dependent on proteasome activity levels in the post-synaptic density (e.g. (Ehlers, 2003; Yi and Ehlers, 2005)). No alterations were observed in levels of (ubiquitinated) synaptic proteins in cortex homogenates of UBB\textsuperscript{+1} transgenic mice using proteomic analysis in previous experiments, as shown in Chapter 3. However, it is conceivable that subtle changes in the levels of these relatively low-abundance proteins were not detected using this proteomics approach. Additional studies on the protein expression levels of these substrates in brain sections of 3413 transgenic mice could give further clues to the mechanism behind the memory deficits.

In several other mouse models with genetic manipulation of the UPS, cognitive deficits are found, as summarized in Chapter 1. These deficits are not always accompanied by LTP deficits, similar to the 3413 transgenic mice. Mice deficient for the DUB Uch13, exhibit decreased hippocampus dependent memory without decreases in LTP or PPF (Wood \textit{et al.}, 2005). In mice lacking the DUB Usp14, differences in short-term synaptic plasticity point to disturbed pre-synaptic function. These mice did not show differences in LTP compared to wild-type littermates, comparable to the 3413 transgenic mice (Wilson \textit{et al.},...
Also other groups have shown that lower short-term synaptic plasticity can lead to context-dependent memory defects, without the need for LTP deficits (e.g. (Silva et al., 1996)). The modest spatial memory deficits in the 3413 UBB⁺¹ transgenic mice, induced by inhibition of the UPS, could therefore have several underlying causes. With our current knowledge on the hippocampal electrophysiological properties of 3413 transgenic mice, deficiencies in basal synaptic transmission in combination with altered pre-synaptic function is one of the most promising candidates.

Cognitive decline is one of the most prominent and earliest clinical features of AD (Walsh and Selkoe, 2004). If UBB⁺¹ accumulation observed in the neuropathological hallmarks of AD patients can also directly affect cognitive function in human, analogous to the results we obtained in the UBB⁺¹ mouse model, is not known. However, inhibition of the UPS in general could be an underlying mechanism in AD contributing to the decline of cognitive functions. Indeed, changes in components of the UPS machinery are found in AD brain (Keller et al., 2000; Lopez Salon et al., 2000) and several AD-related proteins, including amyloid-β and tau, can diminish proteasomal activity (Gregori et al., 1995; Keck et al., 2003; Lopez Salon et al., 2003).

Role of UBB⁺¹ in human pathology

Accumulation of the UBB⁺¹ protein was first discovered to occur in the neuropathological hallmarks of AD and DS (van Leeuwen et al., 1998). Subsequently, UBB⁺¹ was found to be present in a broad selection of tauopathies, also including Pick disease (PiD) and fronto-temporal dementia (FTD) (Fischer et al., 2003). UBB⁺¹ also is present in the ubiquitin-positive neuronal intranuclear inclusions in polyglutamine disease, including Huntington’s Disease (HD) and spinocerebellar ataxia type-3 (SCA-3) (De Pril et al., 2004). As the UPS is compromised in many of these neurodegenerative disorders, as reviewed in Chapter 1 of this thesis, the co-localization of UBB⁺¹ with ubiquitin positive pathology in these tauopathies led to the hypothesis that UBB⁺¹ accumulation serves as an endogenous marker for a decreased UPS function (Fischer et al., 2003).

However, not all neurodegenerative diseases characterized by intracellular ubiquitin aggregates also show UBB⁺¹ accumulation. One of the most prominent examples hereof is Parkinson’s Disease (PD), a movement disorder neuropathologically characterized by α-synuclein containing Lewy bodies. In PD, as well as in other synucleinopathies, UBB⁺¹ accumulation is absent (van Leeuwen et al., 1998; Fischer et al., 2003; Zouambia et al., 2008). The differential presence of UBB⁺¹ in tauopathies and synucleinopathies might be attributed to the different roles the UPS plays in the aetiology of these diseases. First of all, ubiquitin-positive pathology in the intraneuronal tangles can be found in all cases of AD (Selkoe, 2001), in contrast to PD, where the ubiquitin containing Lewy bodies and Lewy neurites are present in the majority of cases, but are only sporadically found in cases
Figure 9  \textit{APP}^{+1} \textit{and UBB}^{+1} \textit{protein expression in various forms of AD}. Expression of APP^{+1} (left row, A-K) and UBB^{+1} (right row, B-M) in 50 \textmu m thick vibratome and 6 \textmu m thick paraffin (E-G) sections of the hippocampus in various forms of AD. A, B: Sporadic form of AD. Insert in (A) shows an APP^{+1} positive neuron with beaded neurites in a young DS patient. These neurites are also present in the absence of any form of AD type of neuropathology and are also present in autosomal dominant cases of AD. C, D: Flemish type FAD (APP A692G). Note the beaded neurite. E to G: HCHWA-D type FAD (APP E693Q). Insert shows APP^{+1} positive dystrophic neurites. H to M: PSEN1 forms of FAD (PSEN1 I143T in H, I, K, L and PSEN1 Y115C in J, M). L and M are enlargements of I and J respectively. K: APP^{+1} is present in dystrophic neurites forming a neuritic plaque. N, O: Beaded neurites in autosomal dominant cases of AD, composed of a stack of five images. N is an Enlargement of C, O is an enlargement of H. Bar = 50 \textmu m (except in I, J, N, and O, bar = 25 \textmu m). Figure adapted from (van Leeuwen \textit{et al.}, 2006).
of early-onset autosomal-recessive PD caused by a genetic mutation in the parkin gene (Savitt et al., 2006). This points to a general role for the UPS in the pathogenesis of AD, whereas PD can also develop without the UPS being compromised. As UBB^1 accumulation is an endogenous marker for decreased activity of the UPS, in AD it is conceivable that the general activity of the UPS is decreased and thus leads to UBB^1 accumulation (Fischer et al., 2003; Hol et al., 2005). In PD, a partial, transient decrease in UPS activity might underlie the disease phenotype (Fornai et al., 2005), although a general decrease in proteasome composition and activity has been shown in several studies using human PD patient material (McNaught et al., 2006). This transient decrease of UPS function might initiate the accumulation of several proteins, including α-synuclein, however might not be sufficient to cause UBB^1 to accumulate (Hol et al., 2005). Another possibility is that in PD, a specific subset of proteins is not targeted to the UPS correctly and thus accumulate in the ubiquitin-positive aggregates. In support of this hypothesis, two genes causative of familial PD encode enzymes involved in ubiquitination (parkin) (Kitada et al., 1998) and deubiquitination (UCHL1) (Leroy et al., 1998) of substrate proteins. These affected components of the ubiquitin-proteasome machinery may thus induce accumulation of specific -ubiquitinated- substrate proteins, but not affect the turnover of UBB^1 to such extent that UBB^1 will accumulate (Hol et al., 2005).

To find further evidence for a specific role of UBB^1 in tauopathies, we next investigated if +1 proteins were also present in the ubiquitin-positive neuropathological hallmarks in early onset familial Alzheimer’s disease (FAD) (van Leeuwen et al., 2006). Although the majority of the AD cases are sporadic, at least three genes are recognized in which inherited autosomal mutations are causative for FAD. These genes include the APP and presenilin 1 and 2 (PSEN1 and PSEN2) genes (Van Broeckhoven, 1998). FAD is neuropathologically indistinguishable from sporadic AD, both characterized by extracellular amyloid-β (Aβ) plaques and intraneuronal tangles. To identify +1 protein accumulation in FAD, we performed an immuno-histochesmical study on 50 μm vibratome and 6 μm paraffin sections of human brain material collected from FAD patients with a wide variety of APP and PS1 FAD mutations. APP^1 as well as UBB^1 could be clearly detected in a sporadic case of AD (fig. 9A-B), as reported previously (van Leeuwen et al., 1998). In FAD, APP^1 and UBB^1 proteins were detected in the neuropathological hallmarks (neurofibrillary tangles and neuritic plaques) of patients carrying Flemish (fig. 9C-D) and Dutch type APP mutations (fig. 9E-G) and in various PSEN1 mutations (fig. 9H-M). In addition to the presence of the +1 frameshift proteins in FAD, we also found UBB^1 and APP^1 in other tauopathies, such as Pick Disease (PiD) and progressive supranuclear palsy (PSP) (fig. 10). We hypothesized that, as the presence of +1 proteins coincides with neuritic pathology in FAD individuals and other tauopathies (e.g. PiD and PSP), +1 proteins might contribute to the pathogenesis of FAD in concert with other mechanisms of neurodegeneration (van Leeuwen et al., 2006).
Figure 10  APP$^+$ and UBB$^+$ protein expression in tauopathies. Immunocytochemical localization of APP$^+$ (left row) and UBB$^+$ (right row) in various tauopathies. A, B: FTD cases. C, D: PiD, pyramidal cells of the CA1 region; note discrete localization outside nucleus. E-I: PSP, note reaction in globose tangles (▲) and in tufted astrocytes (in G, at bottom, and I). J, K: Argyrophilic grain disease. Bar = 50 μm (A and B), 25 μm (C–I), and 60 μm (J and K). Figure adapted from (van Leeuwen et al., 2006).
We further studied the relation between FAD and UBB\(^{+1}\) using a mouse model for AD, described in Chapter 6 of this thesis. We double crossed the 3413 UBB\(^{+1}\) transgenic mouse line with an FAD mouse model expressing mutant APP (APPSwe) and mutant PS1 (PS1\^{dE9}) (Jankowsky \textit{et al.}, 2004) and analyzed the effect of UBB\(^{+1}\) on amyloid deposition as well as the effect of APPSwe and PS1\^{dE9} expression on the levels of UBB\(^{+1}\) accumulation. We demonstrated that amyloid-β deposition was significantly decreased in APPPS1\texttimes UBBS\(^{+1}\) triple transgenic mice compared to APPPS1 mice at 6 months of age, without alterations in UBB\(^{+1}\) protein levels or in the age of onset of pathology. Similar results were observed in mice aged 9 months, however, results did not reach significance at this age, possibly due to the low number of mice that were available for analysis per genotype.

The results obtained in this study using triple transgenic mice imply that neuronal UBB\(^{+1}\) expression and the consequent modest proteasome inhibition have a protective effect on the neuropathogenesis of AD. In this model, UBB\(^{+1}\) expression does not contribute to AD disease pathogenesis as was suggested for human FAD patients (van Leeuwen \textit{et al.}, 2006). Various different causes could underlie these seemingly opposing results. In the 3413 transgenic line, the level of UBB\(^{+1}\) mRNA and protein expression increases up to postnatal day 22 and is expressed at high levels onwards, as shown in Chapter 3. This in contrast to human, where UBB\(^{+1}\) mRNA is expressed at low levels during life and accumulation of the protein is only observed in aged or diseased subjects. In the 3413 transgenic mouse line, the highly expressed UBB\(^{+1}\) protein acts actively as an inhibitor of proteasome activity instead of being foremost a marker for UPS inhibition as observed in human (Fischer \textit{et al.}, 2003). The life-long modest inhibition of the UPS in the 3413 transgenic line could very well modify the effect of UBB\(^{+1}\) on plaque deposition as UPS inhibition is known to affect several steps of Aβ processing, as described in Chapter 6. In addition, the time course of AD neuropathogenesis differs between the APPPS1 mouse model and human FAD. In APPPS1 mice, the first neuropathology can already be detected by 3 months of age, increasing up to 12 months of age. Recently, it was reported that in this mouse model of AD, the formation of new plaques is a very rapid process, with amyloid plaque formation occurring within 24 hours (Meyer-Luehmann \textit{et al.}, 2008), whereas in human, the build up of neuropathology is believed to be a gradual process taking several years. In human FAD patients, the neuropathology is indistinguishable from sporadic AD pathology, including the presence of amyloid pathology and neurofibrillary tangles, consisting of intraneuronal paired helical filaments (PHF) composed of hyperphosphorylated tau. However, tau pathology is absent in the APPPS1 mouse model (Jankowsky \textit{et al.}, 2004), similar to other transgenic mouse models harboring FAD mutations in APP or APP and PS1 (McGowan \textit{et al.}, 2006; Eriksen and Janus, 2007). It could be conceivable that the lack of intraneuronal tau influences the effect of UBB\(^{+1}\) on amyloid deposition in the APPPS1 transgenic line. Further questions on the mechanism by which UBB\(^{+1}\) could alle-
violate amyloid pathology arise when the localization of the involved proteins is taken into account; UBB+1 and tau are intercellular proteins, whereas amyloid deposition in senile plaques is extracellular. In the UBB\(^{+1}\)xAPPPS1 triple transgenic mice (see Chapter 6), further studies on the precise localization of these proteins might further elucidate the mechanism by which UBB\(^{+1}\) can decrease plaque load. It could be possible that UPS inhibition by UBB\(^{+1}\) influences the levels of intracellular Aβ, which is believed to be an important mediator of AD pathogenesis (LaFerla \textit{et al.}, 2007). Furthermore, it has been reported that over-expression of DUBs (USP2 and USP21) results in increased amyloid beta secretion (patent application US26281699A1).

All the circumstances stated above could by themselves or combined underlie the differential effects of UBB\(^{+1}\) protein accumulation on Aβ deposition in human FAD and a murine FAD model. However, it is also very well possible that the presence of UBB\(^{+1}\) in human FAD patients is purely an effect of the disruption of intracellular homeostasis without directly contributing to disease pathogenesis. It could be that, once accumulated, UBB\(^{+1}\) does have an additive effect on already present UPS dysfunction in FAD as well as other neurodegenerative diseases.

**Concluding remarks**

The etiology of many neurodegenerative diseases is multifactorial and the (relative) contribution of the genetic, cellular and environmental factors influencing disease progression is still unclear. Over the last two decades, increasing empirical evidence has established a central role for the UPS in the maintenance of intracellular homeostasis (Goldberg, 2005). In parallel, a decline in UPS activity has been recognized as a pivotal factor involved in the pathogenesis of many neurodegenerative disorders, including AD and HD (Ciechanover and Brundin, 2003). The results as reported in this thesis support the observation that intracellular protein degradation, mediated by the UPS, is essential for intact cellular function. Impairment of this system can directly lead to accumulation of ubiquitinated substrate proteins and eventually cause a decline in cognitive function, thereby contributing to disease progression.

The intrinsic properties of the mutant ubiquitin UBB\(^{+1}\), accumulating in the ubiquitin-positive pathological hallmarks of a specific subset of neurodegenerative disorders, can provide us further insight in the role of UPS dysfunction in these diseases. As the UPS substrate UBB\(^{+1}\) accumulates only when proteasomal activity is compromised, its presence in the neuropathological hallmarks of e.g. AD presents additional evidence that decreased UPS function can be one of the underlying factors contributing to disease progression. In this respect, the newly developed transgenic mouse lines expressing high levels of UBB\(^{+1}\) provide us with useful models to study the effects of chronic low-level endogenous proteasome inhibition \textit{in vivo}. By employing these transgenic lines in future experiments,
we can obtain more understanding of the role of UPS function in a multitude of physiological parameters in health and disease.