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Aβ1-42-mediated down-regulation of Uch-L1 is dependent on NF-κB activation and impaired BACE1 lysosomal degradation

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Summary

Amyloid-β 1-42 accumulation is the major pathogenetic event in Alzheimer's disease (AD), believed to be responsible for synaptic dysfunction and neuronal cell death. However, the physiologic activity of Aβ peptides remains elusive: Aβ might not only play a toxic role, but also act as a functional signaling intermediate. We recently reported that Aβ1-42 promotes BACE1 transcription through the activation of the JNK-c-jun pathway. Here, we show that the Aβ1-42-mediated increase in BACE1 expression is accompanied by a decrease in ubiquitin C-terminal hydrolase L1 (Uch-L1) expression and activity in different cellular models such as neuroblastoma SH-SY5Y as well as NT_2 neuronal cells. We also found that the increase in BACE1 and the decrease in Uch-L1 are related events and depend on NF-κB pathway; thus, Aβ1-42 is able to activate NF-κB pathway and the pretreatment with a pharmacological inhibitor, able to block the nuclear translocation of the transactivating unit p65, almost completely prevents both the decrease in Uch-L1 and the increase in BACE1 expression. In addition, the decrease in Uch-L1 activity interferes with the lysosomal degradation of BACE1, as demonstrated by the decrease in Cathepsin D activity and the partial accumulation of BACE1 in lysosomes after Aβ1-42 treatment as well after Uch-L1 inhibition. In support of the *in vitro* data, we observed low protein levels of Uch-L1 associated with high protein levels of BACE1 in sporadic AD brains. Our data suggest that Uch-L1 could be an attractive target for the development of new therapeutic approaches for AD.

Key words: Azheimer's disease; Aβ1-42; BACE1; Uch-L1; NF-κB; lysosomes.

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Introduction

Alzheimer's disease (AD) is the most common age-related disorder that results in the loss of memory and cognitive functions. Accumulation in the brain of amyloid-β peptides (Aβ), the main components of senile plaques, represents the key pathological event of AD (Selkoe, 2001). Aβ results from two sequential endoproteolytic cleavages operated on the amyloid-β precursor protein (AβPP). First, β-secretase (BACE1) cleaves AβPP at the N-terminal end of the Aβ sequence to produce a secreted form of AβPP, named sAβPP, and a C-terminal membrane-bound 99-aminoacid fragment (C99). Then, γ-secretase cleaves C99 within the transmembrane domain to release the Aβ peptides (Selkoe, 2001). The altered activities of both secretases are involved in the pathogenesis of AD. Presenilin 1 (PS1) is the catalytic subunit of the γ-secretase. Mutations of PS1 gene, that are the major cause of early-onset familial AD, induce an altered γ-secretase cleavage, leading to a relatively increased production of Aβ1-42 species (Lemere *et al.*, 1996). BACE1 activity is increased in the brain of sporadic AD as well (Holsinger *et al.*, 2002; Yang *et al.*, 2003). Oxidative stress (Tamagno *et al.*, 2002, 2005), together with important oxidative stress-related risk factors related to AD such as hypoxia (Guglielmotto *et al.*, 2009), hyperglycemia (Guglielmotto *et al.*, 2012) and hypercholesterolemia (Gamba *et al.*, 2011; Mastrocola *et al.*, 2011), is the potential causes of the increased BACE1 activity. Studying the mechanism of oxidative stress-induced BACE1 up-regulation, we discovered that BACE1 activation is regulated by the γ-secretase activity and that Aβ1-42 is the product of the γ-secretase cleavage that up-regulates BACE1 expression (Tamagno *et al.*, 2008; Giliberto *et al.*, 2009). We also found that Aβ1-42 increases BACE1 gene transcription through the activation of JNK/c-jun signaling pathway (Guglielmotto *et al.*, 2011). Thus, Aβ1-42-induced gene expression may have important implications in the neuronal dysfunction and degeneration that occurs in AD.

Here, we show that Aβ1-42 down-regulates the activity of ubiquitin C-terminal hydrolase L1 (Uch-L1), through the activation of NF-κB pathway and that this event is associated with BACE1 up-regulation owing to a transcriptional effect and to an impairment of its lysosomal degradation. NF-κB has been recently identified as a molecular intermediate involved in the Aβ-mediated control of BACE1. Thus, the inhibitor of IκB kinase that blocks NF-κB transcriptional activity fully reverses the Aβ1-42-induced increase in BACE1 promoter transactivation (Buggia-Prevot *et al.*, 2008).

Lysosomal dysfunction has been linked to a spectrum of degenerative diseases (Cuervo, 2004), many of which involve the central nervous system. Alterations in the endosome/lysosome system have also been previously described in AD (Nixon & Cataldo, 2006). It is interesting to consider a potential role of the endosomal/lysosomal system in the increased BACE1 protein levels, because of defective degradation.

Ubiquitin C-terminal hydrolase L1 is an abundant neuronal enzyme, representing 1–2% of total soluble brain proteins (Wilkinson *et al.*, 1989). Ubiquitin C-terminal hydrolase L1 has two enzymatic activities. The first one, known as hydrolase, removes and recycles ubiquitin molecules from the degraded proteins. This recycling action is crucial for the degradation process as it generates free monomeric ubiquitin, which can be reused for further reactions of ubiquitination (Gong & Leznik, 2007). The second one, called ubiquityl ligase, links ubiquitin molecules, thus

generating polyubiquitin chains that tag proteins for disposal. To play this activity, the enzyme has to be dimerized *in vitro* and remains unclear whether this activity also occurs *in vivo* (Gong & Leznik, 2007). Several lines of evidence suggest that Uch-L1 function is impaired in AD and that this enzyme might be involved in the pathogenesis of AD (Pasinetti, 2001; Choi *et al.*, 2004).

Results

The Aβ1-42-mediated increase in BACE1 protein levels is accompanied by a decrease in ubiquitin C-terminal hydrolase L1 activity

Incubation of SH-SY5Y neuroblastoma cells with 1 μM Aβ1-42 was followed by a significant increase (approximately +100%) of BACE1 protein levels after 6, 12, and 24-h incubation, as shown in Fig. 1(A). Of note, incubation of cells with the scramble peptide or Aβ1-40 did not change the amount of BACE1 protein levels. We previously described that Aβ1-42 is able to increase BACE1 expression, resulting in a significant peak in the amount of nascent RNA after 1-h exposure to Aβ1-42 (Guglielmotto *et al.*, 2011).

Figure 1(B) shows the effect of Aβ1-42 treatment on the Uch-L1 protein levels in SH-SY5Y neuroblastoma cells. Incubation of SH-SY5Y neuroblastoma cells with 1 μM Aβ1-42 was followed by a significant ($P < 0.05$) decrease (approximately −60%) in Uch-L1 protein levels after 6, 12, and 24-h incubation. Incubation of cells with the scramble or Aβ1-40 did not change the amount of Uch-L1 protein levels. To establish that BACE1 is indeed active, the enzymatic activity was evaluated using a commercial kit in SH-SY5Y cells treated with 1 μM Aβ1-42 as well as with 5 μM LDN-57444 (LDN), a reversible Uch-L1 inhibitor up to 24 h. In our experiments (Fig. 1C), BACE1 activity was found to be significantly increased (+30–60%) in cells exposed to Aβ1-42 as well as to LDN compared to control cells.

Increase in BACE1 and decrease in ubiquitin C-terminal hydrolase L1 are related events and depend on NF-κB pathway

To investigate whether the Aβ1-42-mediated BACE1 up-regulation could be related to the concomitant inhibition of Uch-L1 activity, we used two different experimental approaches.

First, we preincubated neuroblastoma cells with the Uch-L1 inhibitor LDN before the treatment with Aβ1-42 peptide. As shown in Fig. 2(A), the pretreatment with LDN was followed, 6 h later, by a significant increase (approximately +150%) in basal BACE1 protein levels. The effectiveness of the LDN was tested measuring the Uch-L1 activity; as shown in Fig. 2(B), after 6-h treatment with LDN the Uch-L1 activity was decreased approximately by 60%. To confirm this result, we silenced Uch-L1 expression using a double-stranded RNA-mediated interference in SH-SY5Y cells. Accordingly, we observed that the inhibition of the production of Uch-L1 determined a strong and significant increase (approximately +100%) in basal BACE1 protein levels (Fig. 2C).

It remains to be investigated how Aβ1-42 mediates the effects observed. There are many signaling pathways that seem to be regulated or at least affected by Aβ peptides. Among these, Buggia-Prevot *et al.* (2008) found that Aβ1-42 was able to modulate BACE1 promoter transactivation and activity through a NF-κB-dependent pathway. Recently, a functional NF-κB response element was identified in the Uch-L1 promoter region, and it has been reported that the expression of NF-κB suppressed Uch-L1 gene transcription (Wang *et al.*, 2011).

Thus, we studied if pretreatment with Aβ1-42 peptides was able to activate the NF-κB pathway in our experimental model. We found that Aβ1-42 increased significantly nuclear translocation of p50 after 3- and 6-h incubation and p65 after 1 h and up to 8-h incubation (Fig. 3A) as well as nuclear activation of total NF- κB after 3 h ad up to 8-h incubation (Fig. 3B).

To confirm the role of NF-κB in the described events, we pretreated SH-SY5Y neuroblastoma cells with a pharmacological NF-κB inhibitor that blocks the nuclear translocation of p65, a NF-κB transactivating subunits. As shown in Fig. 3(C), the pretreatment with the inhibitor was followed by an almost completely protection of the Uch-L1 decrease as well as of the BACE1 increase mediated by Aβ1-42. The effectiveness of p65 inhibitor was tested and, as shown, the inhibitor blocked the nuclear translocation of the NF-κB subunit of approximately 60% (Fig. 3D).

Finally, to confirm the transcriptional role of NF-κB, we measured BACE1 mRNA levels after treatment with 1 μM Aβ1-42 or with 5 μM LDN up to 12 h. As shown in Fig. 3(E), cells treated with Aβ1-42 showed a significant increase in BACE1 mRNA expression after 3 h of incubation that persisted up to 12 h (Fig. 3E). As expected, LDN treatment did not modify BACE1 mRNA levels, confirming its influence at a posttranscriptional level only (Fig. 3E).

The Aβ1-42-mediated decrease in ubiquitin C-terminal hydrolase L1 interferes with the BACE1 lysosomal degradation

The literature data on the site of BACE1 degradation are somewhat discordant, as BACE1 has been shown to be degraded by the ubiquitin-proteasome pathway (Qing *et al.*, 2004) or via lysosomal pathway (Kang *et al.*, 2010). We therefore investigated the involvement of proteasome and lysosomal pathways in the degradation of BACE1.

We tested two lysosomal inhibitors such as chloroquine and NH_4Cl, which are known to inhibit lysosomal hydrolases by reducing the acidification of endosomal/lysosomal compartments (Koh *et al.*, 2005). We treated neuroblastoma cells with the lysosomal inhibitors up to 48 h. As shown in Fig. 4(A,B), endogenous BACE1 protein levels were markedly increased following treatment with either chloroquine or NH_4Cl; chloroquine induced an earlier increase in BACE 1 protein levels which is significant (approximately +100%) after 12-h treatment, whereas NH_4Cl induced a significant increase (approximately +100%) after 24 h. The involvement of lysosomal compartments was also confirmed by the additive increase in BACE1 protein levels observed in neuroblastoma cells pretreated with chloroquine or NH_4Cl and with Aβ1-42 (Fig. 4C,D).

Then, we investigated whether Aβ1-42, as well as the pharmacological Uch-L inhibitor, LDN, was able to interfere with the lysosomal pathway. We tested the activity of cathepsin D, a lysosomal protease that is expressed in all cells up to 48-h treatment with Aβ1-42 or LDN. We found that Aβ1-42 impaired (approximately −60%) the cathepsin D activity after 8 h and up to 48 h. Similarly, the treatment of neuroblastoma cells with LDN decreased cathepsin D activity significantly (−60%) at 3 h of incubation (Fig. 5A). Pepsatin, a cathepsin D inhibitor, used as positive control, induced a strong decrease (approximately −80%) in the enzymatic activity after 8-h incubation (Fig. 5A,B).

To determine the cellular compartment where BACE1 accumulates following treatment with Aβ1-42 or LDN, we carried out confocal laser scanning microscopy. Using various subcellular markers, we confirmed that after 6-h treatment with Aβ1-42 or LDN, a strong increase in

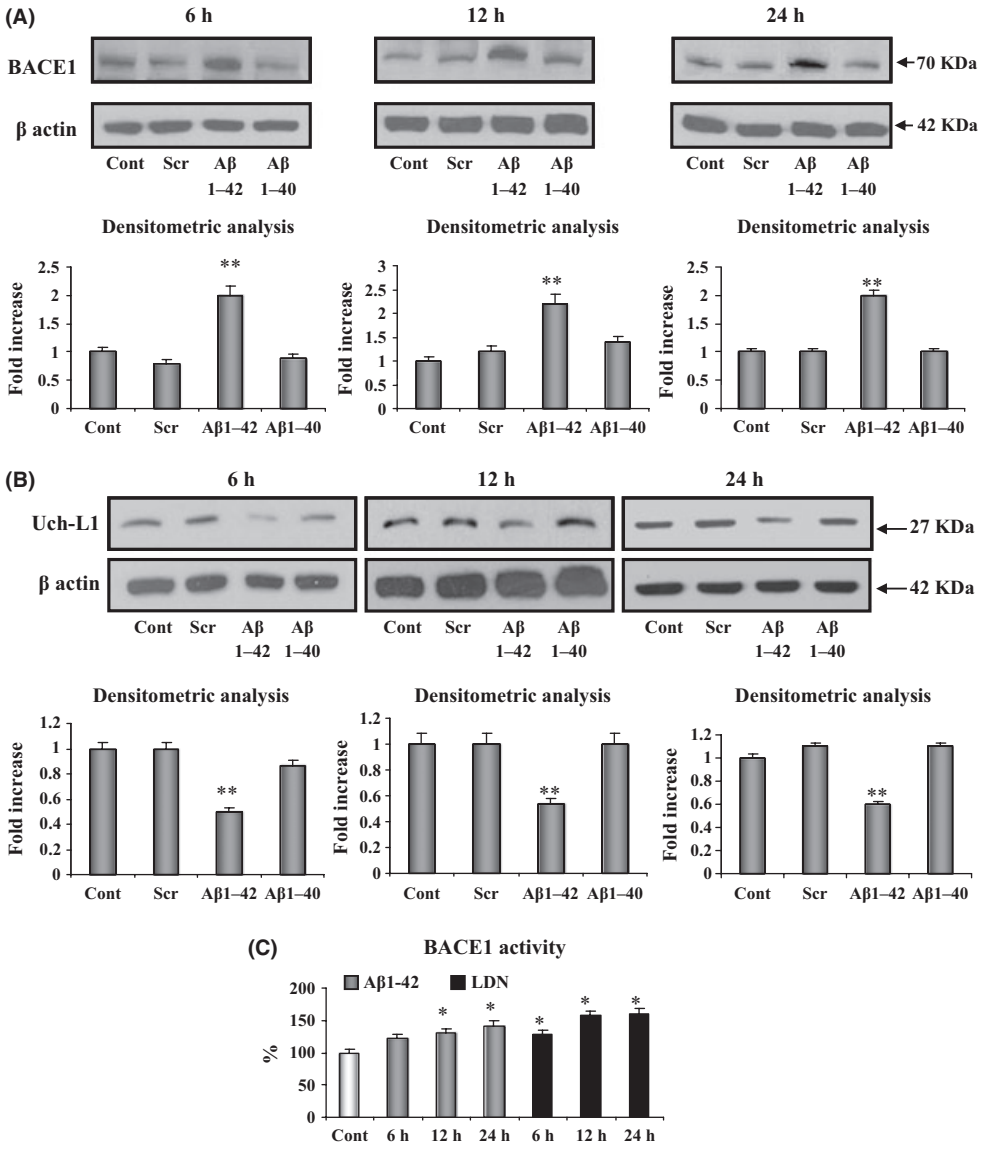

Fig. 1 Effect of Aβ1-42 on BACE1 and ubiquitin C-terminal hydrolase L1 (Uch-L1) expression. (A) Treatment of SH-SY5Y neuroblastoma cells with 1 μM Aβ1-42 doubles BACE1 protein levels after 6, 12, and 24-h incubation. Treatment with scramble peptide or Aβ1-40 did not change BACE1 protein levels. (B) Treatment of SH-SY5Y neuroblastoma cells with 1 μM Aβ1-42 significantly decreases Uch-L1 protein levels after 6, 12, and 24-h incubation. Treatment with scramble peptide or Aβ1-40 did not change Uch-L1 protein levels. (C) Treatment of SH-SY5Y cells with 1 μM Aβ1-42 as well as with 5 μM LDN-57444 (the pharmacological Uch-L1 inhibitor) significantly increases BACE1 activity after 6, 12, and 24-h incubation. The error bars represent standard deviations. Experiments were conducted in triplicate. **Significantly different from controls ($P < 0.02$).

BACE1 reactivity was observed in neuroblastoma cells and that BACE1 co-localized with TGN (anti-Golgi 58K) and partially with lysosomes (LAMP1) markers (Fig. 5C,D).

Finally, to confirm that Aβ1-42 and LDN induce lysosomal impairment, we analyzed the amount of ubiquitin-Lys-63 linked to BACE1. The conjugation of ubiquitin-Lys-63 can signal endocytosis of membrane proteins and protein sorting and trafficking, which promote recycling or degradation by the lysosomes (Hicke, 2001). BACE1 transiently transfected neuroblastoma cells were treated with Aβ1-42 and/or LDN for 8 h. Cell lysates were then immunoprecipitated with polyclonal BACE1 antibody and revealed with ubiquitin-Lys-63 antibody. We observed that BACE1 contained ubiquitin-Lys-63 linkages (Fig. 5E) and that Aβ1-42 and LDN treatments, together or alone, induced a slight but not significant accumulation of BACE1 ubiquitin-Lys-63 linkages (Fig. 5E). We also assessed the overall ubiquitination state of BACE1 using the panubiquitin antibody P4D1, no significant difference on this parameter has been shown after Aβ1-42 treatment or Uch-L1 inhibition (Fig. 5E).

Confirmation of the effect of the Aβ1-42-mediated decrease in ubiquitin C-terminal hydrolase L1 on the up-regulation of BACE1 in other cellular models

We confirmed the effect of Aβ1-42 on Uch-L1 and BACE1 in neuronal NT2 cells. We treated neuronal cells with 1 μM Aβ1-42 for 6, 12, and 24 h. As reported in Fig. 6(A), Aβ1-42 determined a threefold increase in

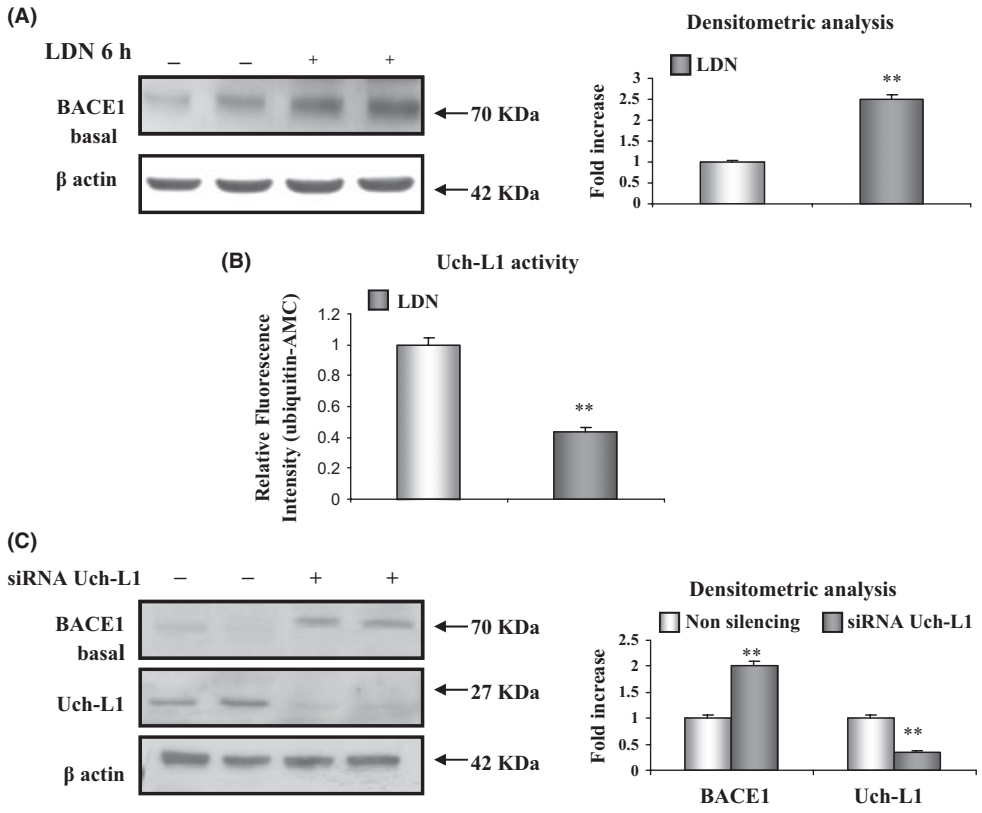

Fig. 2 Inhibition of ubiquitin C-terminal hydrolase L1 (Uch-L1) is related to BACE1 up-regulation. (A) Six-hour treatment of SH-SY5Y neuroblastoma cells with the pharmacological Uch-L1 inhibitor, LDN-57444, was followed by a significant increase in BACE1 basal protein levels. (B) Treatment of cells with LDN-57444 decreases the enzymatic activity approximately by 80% as compared to untreated cells. (C) RNAi silencing of Uch-L1 doubled the BACE1 basal protein levels. The error bars represent standard deviations. Experiments were conducted in triplicate. **Significantly different from controls ($P < 0.02$).

BACE1 protein levels after 6 h and up to 24 h of incubation. As expected, Uch-L1 levels displayed an opposite trend; the enzymatic levels were already halved after 6-h incubation with Aβ1-42 (Fig. 6A). We investigated the mechanism of the transcriptional activity operated by Aβ1-42. As found in SH-SY5Y, the treatment of neuronal NT_2 cells with Aβ1-42 was followed by significant nuclear activation of total NF-κB after 1 and up to 8-h incubation (Fig. 6B). Moreover, Aβ1-42, as well as LDN, impaired the cathepsin D activity (Fig. 6C) and determined, primarily, BACE1 localization in the TGN and in the endosomal compartment (Fig. 6D).

Ubiquitin C-terminal hydrolase L1 and free ubiquitin are decreased in the cerebral cortex of patients with Alzheimer's disease

We and others (Holsinger *et al.*, 2002; Yang *et al.*, 2003; Borghi *et al.*, 2007) have previously published that BACE1 protein levels and activity were significantly increased in AD cortex as compared to normal aging controls. Here, we show that the levels of Uch-L1 as well as those of free monoubiquitin, normalized with β-actin levels in controls as well as AD brains respectively, have an opposite trend. As shown in Fig. 6(E) and F BACE1 protein levels are significantly increased (approximately +60%), and Uch-L1 protein levels are significantly lower (approximately –65%) in AD cortex as compared to normal aging. The decrease in Uch-L1 was followed by a parallel decrease (approximately –50%) in free ubiquitin pool (Fig. 6G).

Discussion

We have found that Aβ1-42 down-regulates the activity of Uch-L1, through the activation of NF-κB pathway and that this event is associated to an up-regulation of BACE1.

Several lines of evidence suggest that the activity of Uch-L1 is down-regulated in AD brain (Pasinetti, 2001; Choi *et al.*, 2004). The level of Uch-L1 is inversely proportional to neurofibrillary tangles numbers in the brains of sporadic patients with AD (Choi *et al.*, 2004). Aβ increases in gracile axonal dystrophy mice that have no Uch-L1 expression (Ichihara *et al.*, 1995). Moreover, oxidation of Uch-L1, that impairs its enzymatic activity causing irreversible alteration in the enzyme conformation, are increased two- to ten-fold in AD brain with respect to normal aging (Castegna *et al.*, 2002). In a mouse model of AD, the double transgenic mouse overexpressing APP together with mutant presenilin 1 (APP/PS1 mice), the Uch-L1 protein expression and activity in brain are decreased (Gong *et al.*, 2006). Down-regulation of Uch-L1 seems to be at least in part responsible for the impairment of long-term potentiation (LTP) (Trinchese *et al.*, 2004). Molecular mechanisms that link Aβ accumulation, Uch-L1 down-regulation and dysfunction of LTP are not completely understood. The effect of Aβ on LTP is mediated by the inhibition of phosphorylation of the cAMP response element binding protein (CREB), a transcription factor activated by cAMP dependent protein kinase A (PKA) (Tong *et al.*, 2001). Protein kinase A activity is regulated by the degradation of its regulatory subunit. The inhibition of Uch-L1 activity would lead to impairment of the degradation of the PKA regulatory subunit, a

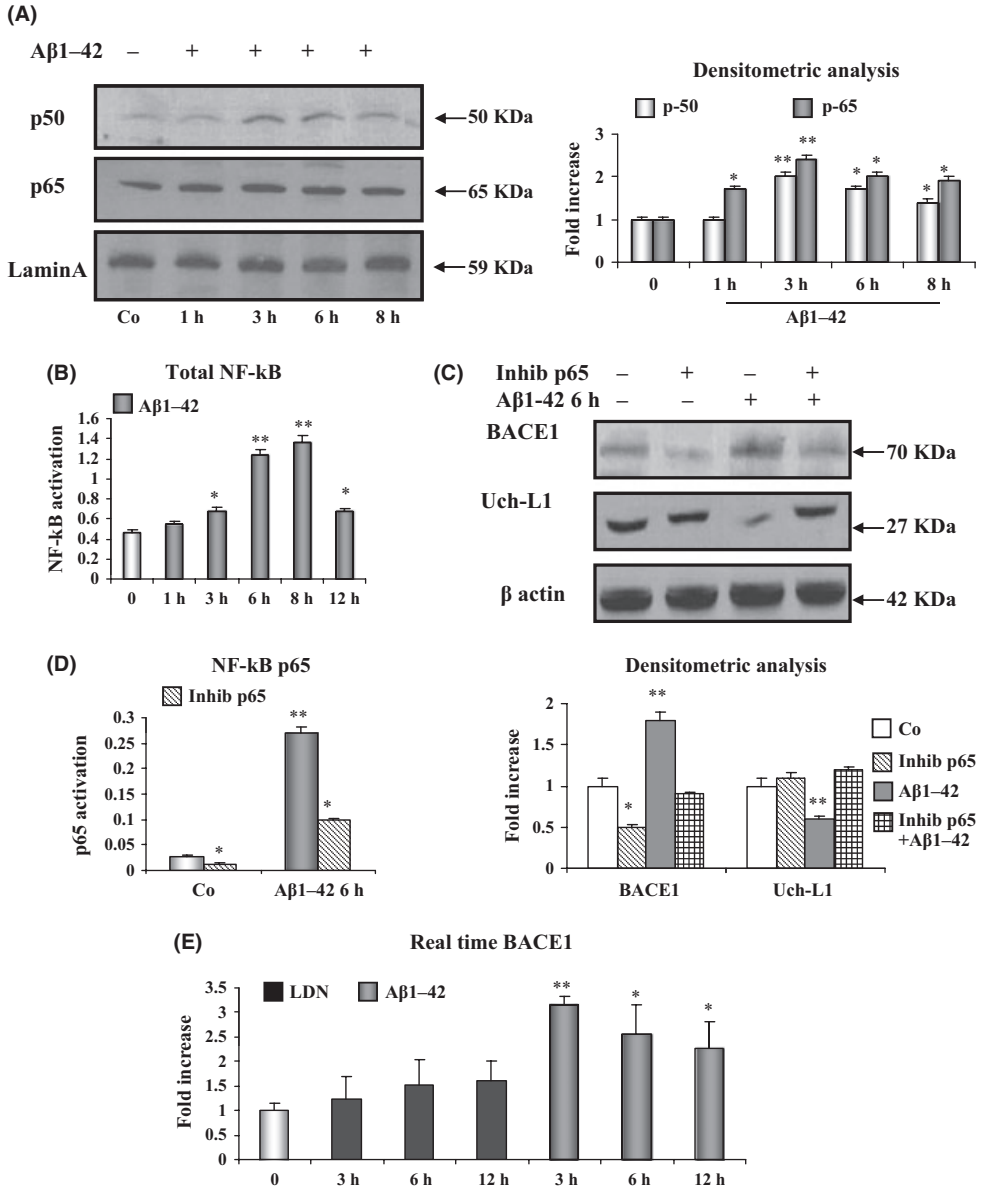

Fig. 3 NF-κB pathway activation mediates ubiquitin C-terminal hydrolase L1 (Uch-L1) decrease. (A) Treatment of SH-SY5Y neuroblastoma cells with 1 μM Aβ1-42 was followed by a significant translocation of NF-p65 as well as of p50 NF-κB subunits after 3–8-h incubation. (B) Aβ1-42 treatment was followed by a strong nuclear activation of total NF-κB that became significant after 3-h incubation. (C) Pretreatment of cells with inhibitor of p65 subunit protects cells by both the up-regulation of BACE1 and the decrease in Uch-L1 mediated by 6-h treatment with Aβ1-42. (D) Pretreatmet of cells with p65 pharmacological inhibitor blocks the nuclear activity of p65 NF-κB subunit by approximately 65%. (E) Pretreatment of SH-SY5Y with 1 μM Aβ1-42, but not with LDN-57444, is followed by a significant increase in BACE1 mRNA. The error bars represent standard deviations. Experiments were conducted in triplicate. **Significantly different from controls ($P < 0.02$).

decrease in its activity and subsequently down-regulation of CREB-dependent transcription (Gong *et al.*, 2006). Our results confirm and extend previous work of Zhang *et al.* (2012). In this work, it has been shown that inhibition of Uch-L1 significantly increases BACE1 protein levels in a time-dependent manner. Moreover, overexpression of Uch-L1 decreased APP c-terminal fragment C99 and Aβ in the Uch-L1-null AD mice (Zhang *et al.*, 2012).

Here, we show that the decrease in Uch-L1 activity is related with the Aβ1-42-mediated activation of NF-κB pathway. Recently, the UCH-L1 gene promoter region was cloned and functionally identified, and a NF-κB binding element within its promoter region has been identified. NF-kB signaling down-regulates UCH-L1 expression and mediates the inhibitory effect of lipolysaccharide and tumor necrosis factor-alpha (TNFa) on Uch-L1 expression (Wang *et al.*, 2011).

NF-κB pathway had a major role in the pathogenesis of AD: Buggia-Prevot *et al.* (2008) found that Aβ1-42 modulates BACE1 promoter transactivation and activity through a NF-κB-dependent pathway. Thus, the activation of NF-κB mediated by Aβ1-42 had two effects, the up-regulation of BACE1 and the inhibition of Uch-L1. Our data partially disagree a recent report showing that Aβ1-42 treatment does not induce increase in BACE1 mRNA in primary neurons and suggesting a posttranscriptional regulation of the enzyme (Sadleir & Vassar, 2012). This might be explained by the fact that Aβ1-42 preparation in the present work is not monomeric but oligomeric: We previously found that various aggregation

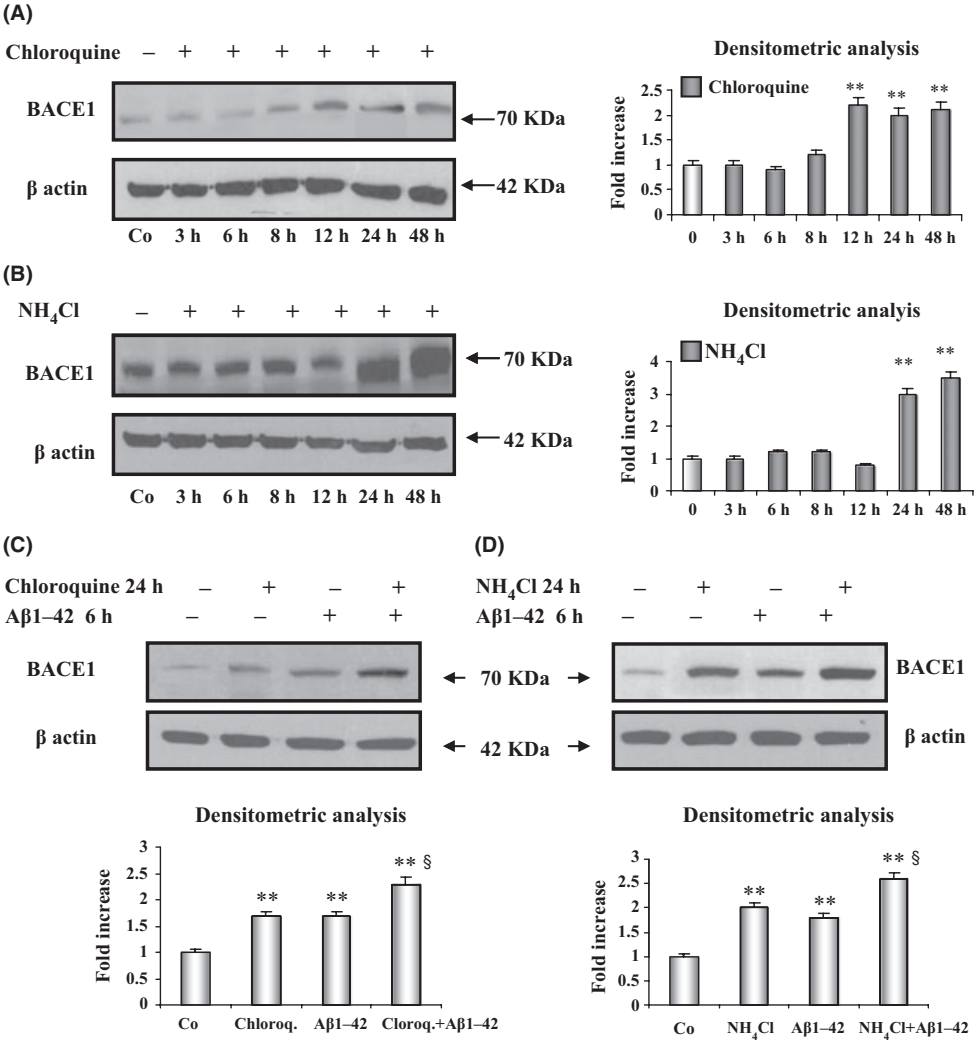

Fig. 4 BACE1 is degraded via the lysosomal pathway. (A and B) Treatment of SH-SY5Y neuroblastoma cells with 100 μM chloroquine (A) as well as 50 mM NH_4Cl (B) up to 48 h significantly increase BACE1 protein levels as compared to untreated cells. (C and D) Cells were treated for 24 h with 100 μM chloroquine in C or 50 mM NH_4Cl in D and then 1 μM Aβ1-42 (C and D) for 6 h. An additive increase in BACE1 protein levels in neuroblastoma cells was observed in these experimental conditions. The error bars represent standard deviations. Experiments were conducted in triplicate. **Significantly different from controls ($P < 0.02$).

states of Aβ1-42 mediate different effect on BACE1 expression (Tamagno *et al.*, 2006). Thus, we found that prefibrillar and oligomeric Aβ1-42 resulted in a dramatic increase in the oxidative stress markers 4-hydroxy-nonenal and hydrogen peroxide and in a rapid and significant induction of both apoptotic and necrotic neuronal cell death without affecting BACE-1 expression.

We next demonstrated that these effects were not only concomitant but also that the decrease in Uch-L1 rebounded on BACE1 degradation. We first examined the potential role of ubiquitin-proteasome system (UPS). In this scenario, it has been suggested that UPS dysfunction plays a crucial role in the pathogenesis of neurodegenerative diseases (Ciechanover & Brundin, 2003). Proteasome activity is inhibited by Aβ peptides and exposure to oxidative stress (Grune *et al.*, 1995; Almeida *et al.*, 2006). Ubiquitin C-terminal hydrolase L1 is believed to release ubiquitin from small adducts to generate ubiquitin monomers necessary to label abnormal proteins for 26S proteasomal degradation (Vigouroux *et al.*, 2004).

Moreover, there is evidence that the turnover of BACE1 protein is mediated by the UPS (Qing *et al.*, 2004). It has been found that BACE1 is ubiquitinated and that BACE1 degradation is inhibited by the proteasome inhibitor, lactacystin, in a time-dose-dependent manner (Qing *et al.*, 2004).

Differently from this finding, our data indicate that BACE1 is transported in the late endosomal/lysosomal compartment where it is degraded via the lysosomal pathway (Koh *et al.*, 2005), and not in the proteosome.

The involvement of lysosomal pathway in BACE1 degradation was confirmed by three findings. First, treatment of cells with lysosomal inhibitors was followed by a significant accumulation of BACE1. Second, confocal laser scanning microscope (CLSM) laser scanner demonstrated that BACE1 co-localizes with the lysosome marker LAMP-1. Third, we have determined that BACE1 is Lys-63-linked ubiquitinated.

Kang *et al.* (2010) previously reported a lys-63-linked ubiquitination of BACE1 showing that GGA3 regulates the BACE1 degradation via the

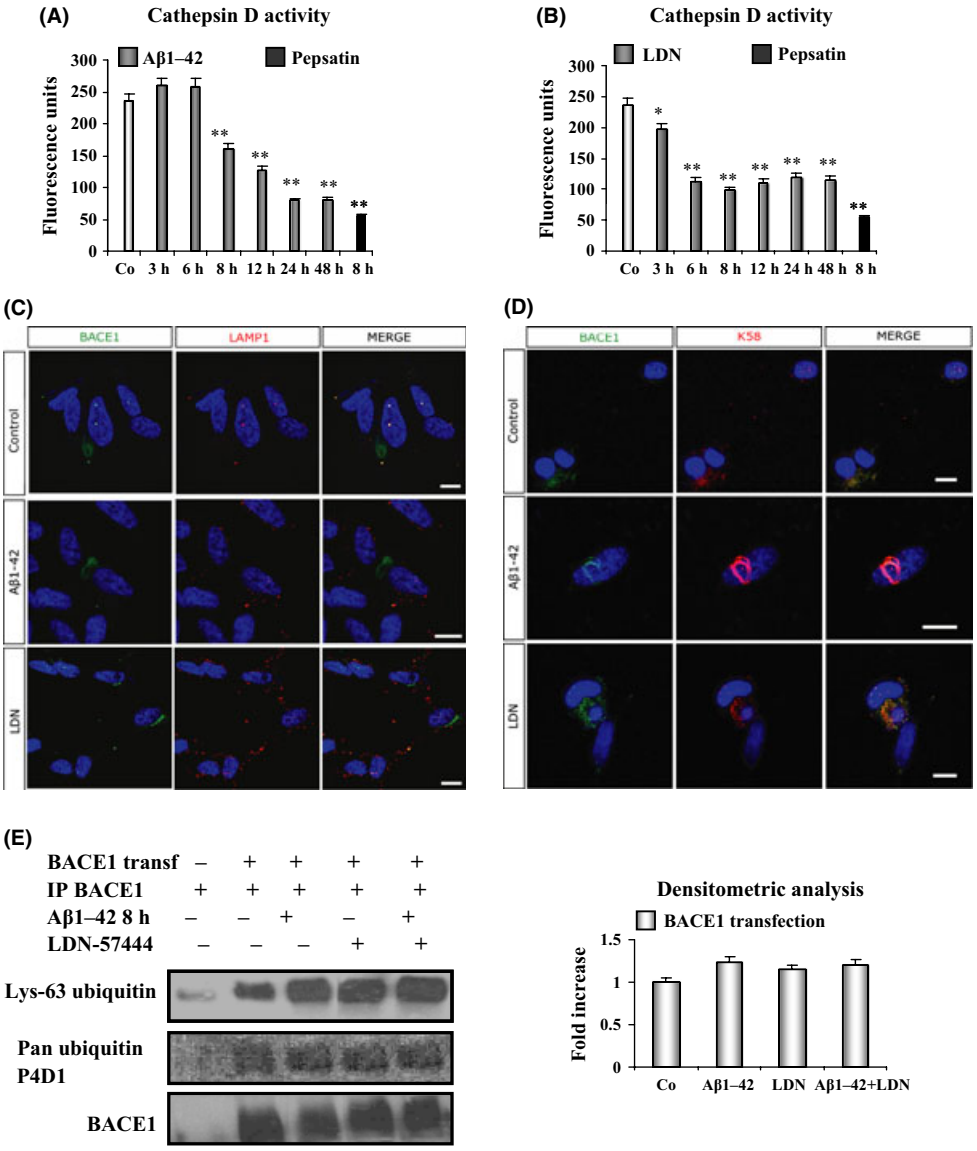

Fig. 5 Aβ1-42-mediated ubiquitin C-terminal hydrolase L1 (Uch-L1) inhibition interferes with the lysosomal pathway. (A and B) Treatment of SH-SY5Y cells with 1 μM Aβ1-42 (A) as well as with 5 μM LDN (B) was followed by a significant decrease in the Cathepsin D activity after 8 and 3 h, respectively. Pepsatin, used as positive control induced a 80% inhibition of cathepsin D activity after 8-h incubation. (C and D) Cells treated with Aβ1-42 for 6 h were stained, after fixation and permeabilization, with BACE1 polyclonal antibody and with antibodies for two cellular markers: K58 (TGN) in C and LAMP1 (late endosomes/lysosomes) in D. BACE1 resides primarily in TGN and to a lesser extent in the late endosomes. Bars: 10 μm. (E) Neuroblastoma cells transiently transfected with BACE1 construct were immunoprecipitated with polyclonal BACE1 antibody, treated for 8 h with Aβ1-42 and/or LDN treated cells and revealed with a Ubiquitin-Lys -63 antibody as well as with the panubiquitin P4D1. We found that BACE1 is linked to ubiquitin-Lys-63 to further confirm its preferential degradation via the lysosomal pathway. The error bars represent standard deviations. Experiments were conducted in triplicate. **Significantly different from controls ($P < 0.02$).

interaction with ubiquitin. In this scenario, loss of functional Uch-L1 could lead to inadequate ubiquitynation of BACE1 mediated by a decrease in free ubiquitin.

Of note, Lys-63-linked ubiquitin chains are a specific signal for protein sorting into multivesicular body pathway (Doss-Pepe *et al.*, 2005) and signaling functions in a proteasome-independent manner (Lim & Lim, 2011). Moreover, we found that the Uch-L1 inhibitor LDN-57444 impairs the activity of cathepsin D, considered a marker of lysosomal activity.

Lysosomal dysfunction has been linked to a spectrum of degenerative diseases (Cuervo, 2004), many of which involve the CNS (Lee, 2009). Alterations in the endosome/lysosome system have also been previously described in AD (Cardoso *et al.*, 2010). It has been shown that BACE1 and Aβ are enriched in lysosomes-related autophagic vesicles in APP transgenic mouse models (Yang *et al.*, 2011). The autophagic vesicles also accumulate in dystrophic neuritis in AD brains (Nixon *et al.*, 2005). Here for the first time, we found that Aβ1-42 determined a decrease in the activity of Uch-L1 by an induction of NF-kB pathway and this event determined an up-regulation of BACE1 by interfering with its lysosomal degradation. Finally, these data suggest that Uch-L1 could be an attractive target for the development of new therapeutic strategies to AD.

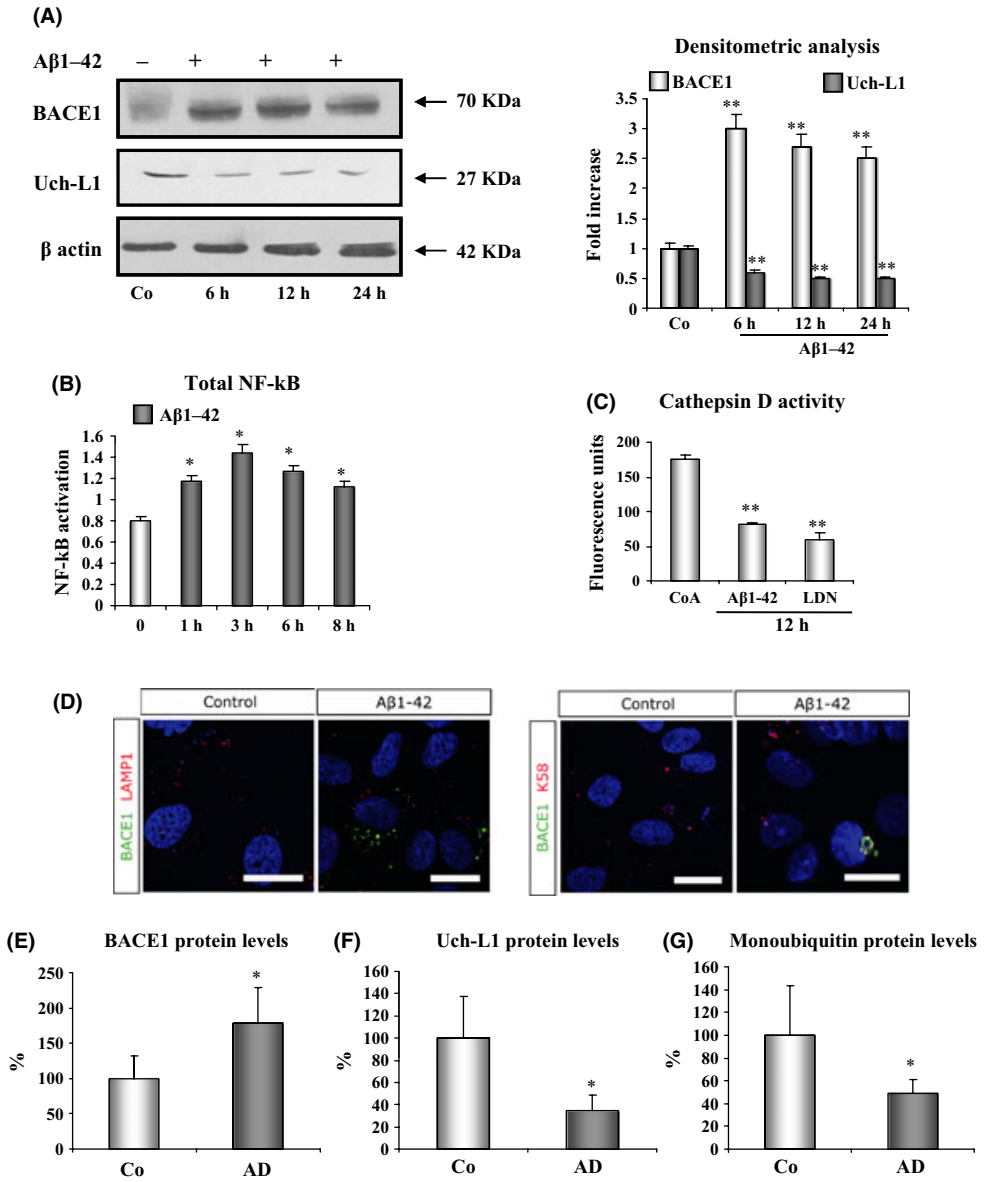

Fig. 6 Ubiquitin C-terminal hydrolase L1 (Uch-L1) decrease up-regulates BACE1 in NT2 neuronal cells as well as in cerebral cortex of patients with Alzheimer's disease (AD). (A) Treatment of NT_2 neuronal cells neuroblastoma cells with 1 μM Aβ1-42 significantly increases BACE1 and halves Uch-L1 protein levels after 6, 12, and 24-h incubation. (B) Aβ1-42 treatment was followed by a strong nuclear activation of total NF-κB that became significant after 1-h incubation. (C) Treatment of NT_2 neuronal cells with 1 μM Aβ1-42 as well as with 5 μM LDN was followed by a significant decrease in the Cathepsin D activity evaluated after 12-h incubations. (D) Confocal laser scanning microscope (CLSM) experiments confirm that BACE1 resides primarily in TGN and to a lesser extent in the late endosomes also in NT_2 neurons. Bars: 30 μm. (E–G) BACE1 (E) levels are significantly increased, while Uch-L1 (F) as well as free mono ubiquitin (G) levels is decreased in cortical brain samples of patients with AD. The error bars represent standard deviations. Experiments were conducted in triplicate. *Significantly different from controls ($P < 0.05$); **Significantly different from controls ($P < 0.02$).

Experimental procedures

Cell culture and treatments

SH-SY5Y neuroblastoma cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2 mM glutamine and supplemented with 10% fetal bovine serum (FBS), 1% antibiotic mixture (penicillin-streptomycin-amphotericin) (Sigma Chemical Company, St. Louis, MO, USA).

NT_2 teratocarcinoma cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS and 1% antibiotic mixture. Both cell lines were maintained in a humified atmosphere at 37 °C with 5% CO_2 (Sigma Chemical Company).

For differentiation, 2×10^6 NT_2 cells were plated in 75 cm^2 culture flasks (Costar, Lowell, MA, USA) and exposed to 10 μM retinoic acid (RA) (Sigma Chemical Company) for 5 weeks. Growth medium was changed three times a week. After 5 weeks, the mitotic inhibitors cytosine arabinoside (1 μM), fluorodeoxyuridine (10 μM), and uridine (10 μM) (Sigma Chemical Company) were added for 2 weeks to inhibit the division of non-neuronal cells.

Cells were incubated with 1 μM Aβ peptides (1–40, 1–42 scramble and 1–42; Anaspec, San Jose, CA, USA) up to 24 h. The lyophilized

commercial peptides were dissolved as a stock solution in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma Chemical Company) and stored at –80 °C in aliquots. The desired amount was left under the cell culture hood to allow for 1,1,1,3,3,3-hexafluoro-2-propanol to evaporate, brought to 1 μM, final concentration, with sterile double distilled water, centrifuged at 10 000 *g* for 10 min to avoid aggregation and immediately added to the cell medium. Twenty microliters of monomeric Aβ preparations, prepared as described previously, were added to a carbon-coated Formvar grid for 10 min, dried and stained with 2% fosfotungstic acid. Grids were examined by transmission electron microscopy, and no aggregation was observed. The state of aggregation of monomeric preparations was further evaluated by immunoblotting, employing the specific monoclonal antibody 4G8, a single 4.5 kDa band was revealed in our samples (data not showed).

The concentrations of protease inhibitors were as follows: Lactacystin 10 μM (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Bortezomib 2.6 μM (Millenium Phrmaceluticals Inc. Cambridge, MA, USA); chloroquine 100 μM (Sigma Chemical Company); NH_4Cl 50 mM (Sigma Chemical Company); cells were incubated with inhibitors up to 48 h.

LDN-57-444 (Calbiochem, Darmstadt, Germany), a competitive inhibitor for Uch-L1, was used at the final concentration of 5 μM and added to the cell medium up to 48 h. Pepsatin, a cathepsin D inhibitor was used at the concentration of 10 μg mL^{-1} medium and added to the cell medium for 8 h. Eighteen μM NF-κB p65 inhibitor (Santa Cruz Biotechnology) was added immediately before the incubation with Aβ1-42.

For immunoprecipitation experiments, SH-SY5Y cells were transfected with a construct coding BACE1 cloned into pcDNA3. BACE1 transient transfection into cells was performed using Lipofectamine 2000 (InVitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Transient transfections of empty vector, wild-type and mutant PS1 cDNA (S170F, L392V, M146V) in SH-SY5Y cells was carried out with Lipofectamine 2000 (InVitrogen) as previously described (Giliberto *et al.*, 2009).

Tissues

We used frozen cerebral cortex (superior frontal gyrus) from two groups of cases: (i) 26 control cases, free of amyloid plaques as determined by immunocytochemistry with the monoclonal antibody 4G8, able to recognize residues 17–24 in Aβ (data not shown). The mean age at death was 73 years ± 9 and the postmortem delay 9.2 h; and (ii) 32 cases with late onset sporadic AD with clinical history of disease and pathological diagnosis according to Consortium to establish a Registry for Alzheimer's Disease (CERAD) criteria, provided by the brain bank of the Case Western Reserve University, Cleveland, OH, USA. The mean age at death was 78 years ± 10 and postmortem delay 10.2 h.

Tissue and cell extracts

To obtain total tissue extracts for Western blot analysis, brains were homogenized in a hypotonic buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM ethylene glycol tetraacetic acid, and 1 mM EDTA (Sigma Chemical Company). To extract the dissolved proteins, samples were centrifuged at 12 000 *g* for 20 min, and supernatants were recovered (Borghi *et al.*, 2010).

Whole-cell extracts were prepared in ice-cold lysing buffer [1 mL of phosphate-buffered saline (PBS) was added with 10 μL Triton X100; 10 μL SDS 10%, 5 μL dithiothreitol (DTT) 1 M, 6 μL phenylmethylsulfonyl fluoride 0.1%, 10 μL aprotinin] for 20 min. The lysates were cleared by centrifugation at 14 000 *g* for 25 min. Cytosolic and nuclear cell fractions were obtained as previously described by the method of Andrew & Faller (1991).

Antibodies and immunoblot analysis

The following antibodies were used: polyclonal BACE1 antibody (dilution 1:400; Millipore, Temecula, CA, USA); monoclonal β-actin (dilution 1:1000; Sigma Chemical Company) and polyclonal lamin A (dilution 1:1000; Sigma, St. Louis, MO, USA); monoclonal Uch-L1 antibody (dilution 1:200; Santa Cruz Biotechnology); monoclonal ubiquitin Lys63-specific antibody (dilution 1:500; Chemicon-Millipore, Temecula, CA, USA); monoclonal antibodies NF-κBp65 and NF-κBp50 (dilution 1:500; Santa Cruz Biotechnology); monoclonal ubiquitin antibody (dilution 1:500; Chemicon, Millipore) and; monoclonal panubiquitin antibody P4D1 (Santa Cruz Biotechnology).

Total lysates, cytosolic and nuclear fractions were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis on 9.3% acrylamide gels using the mini-PROTEAN II electrophoresis cell (BioRad, Hercules, CA, USA) (See Methods in Data S1, Supporting information).

Gene expression analysis

For the quantitative SYBR Green (2× iQ YBR Green PCR Super Mix; BioRad, Hercules, CA, USA) real-time PCR, 40 ng of cDNA was used per reaction. RNA extraction was performed with the RNeasy kits, according to the manufacturer's protocol, for cell lines and brain tissue (Qiagen, Valencia, CA, USA), cDNA was obtained with the SuperScript® III CellsDirect cDNA Synthesis Kit (Invitrogen). For details see Methods in Data S1.

RNA interference

To knock down Uch-L1 expression, we used siRNA duplex (Dharmacon, Thermo Fisher Scientific, Loughborough, UK). The siRNA and related non-silencing (negative control) were transfected in neuroblastoma cells with lipofectamine 2000 (InVitrogen), according to the manufacturer's instructions for 48 h. To improve the silencing tecnique, we retransfected cells with siRNA or non-silencing control at the intermediate point of 36 h.

Confocal immunofluorescence

For immunofluorescence experiments, cells were plated in 12-well plates (BD Biosciences, San Jose, CA, USA) at a concentration of 40 000 cells cm^{-2}.

Cultured cells were fixed with 4% buffered paraformaldehyde for 15 min at room temperature. Samples were washed three times with phosphate buffer solution (PBS; pH 7.4). To permeabilize cells, samples were incubated for 10 min in PBS containing 0.3% Triton X-100 (PBST) followed by wash in PBS. After blocking unspecific binding sites with 1% BSA in PBST, cells were incubated in the same solution at 4 °C overnight with a mixture of primary antibodies, consisting in polyclonal BACE1 (dilution 1:200; Chemicon) alternatively mixed with either monoclonal anti-Lysosome-Associated membrane protein-1 (LAMP1) (dilution 1:200; Calbiochem, San Diego, CA, USA) or monoclonal anti-Golgi 58K protein/formiminotransferase cyclodeaminase (FTCD) (dilution 1:50; Sigma).

After washing in PBS, sections were incubated in a mixture of two secondary antibodies raised in different species: 1:200 cyanine 3-conjugated donkey anti-mouse secondary antibody and 1:100 cyanine 2-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories; West Grove, PA, USA) with 1% BSA in PBS. For counterstaining, cells were incubated 3 min with 4′,6-diamidino-2-phenylindole diluted 1:50 in

methanol 0.1 M and rinsed with PBS. Finally, coverslips were mounted with a drop of 1:1 phosphate buffer–glycerol solution. The samples were examined with the Leica TCS SP5 CLSM (Leica, Mannheim, Germany).

BACE-1 activity

The activity of BACE-1 was determined using a commercially available secretase kit from Calbiochem (Merck, Darmstadt, Germay), according to the manufacturer's protocol. Cells were lysed in cold 1× Extraction Buffer (ready for use in the kit) to yield a final protein concentration of 1 mg mL^{-1}. The method is based on the secretase-dependent cleavage of a secretase-specific peptide conjugated to the fluorescent reporter molecules EDANS and DABCYL, which results in the release of a fluorescent signal that can be detected on a fluorescence microplate reader (excitation wavelength of 355 nm and emission 510 nm). The level of secretase enzymatic activity is proportional to the fluorimetric reaction. Data were expressed as percentage change over activity level of control cells.

Ubiquitin C-terminal hydrolase L1 hydrolase activity assay

The hydrolase activity assay was performed using fluorogenic ubiquitin-7-amino-4-methylcoumarin (ubiquitin-AMC) (Boston Biochem, Cambridge, MA, USA) as a substrate. The substrate was diluted in an assay buffer (50 mM Tris–HCl pH 7.6, 0.5 mM EDTA, 5 mM DTT and 0.1 mg mL^{-1} ovalbumin) (Sigma). Reaction mixture containing 400 nM substrate and 100 μg protein samples was incubated for 5 min at room temperature. The reaction was monitored using a fluorescence spectrometer (LS55; Perkin Elmer Instruments, Waltham, MA, USA) at 25 °C. The free AMC fluorophore was excited at 380 nm and measured at 460 nm.

NF-κB activity

The activity of NF-κB was determined using a commercially available kit (Active Motif, Rixensart, Belgium), with a 96-well plate on which has been immobilized an oligonucleotide containing an NF-κB consensus binding site. The NF-κB contained in the nuclear extracts specifically binds to this oligonucleotide. The primary antibodies used in this kit recognize epitopes on p65, p50, p52, c-Rel, and Rel b proteins upon DNA binding.

Cathepsin D activity assay

The cathepsin D activity assay kit (Abcam, Cambridge, UK) is a fluorescence-based assay that utilizes the preferred cathepsin D substrate sequence GKPILFFRLK(Dnp)-DR-NH2 labeled with 7-methoxycoumarine-4-yl acetyl. Cell lysates that contain cathepsin D will cleave the synthetic substrate to release fluorescence, which can be easily be quantified using a fluorescence plate reader (model 680; BioRad Instruments, Hercules, CA, USA) at Ex/Em = 328/460.

Statistical analysis

Data in bar graphs represent mean ± SD and were obtained from average data of at least three independent experiments. Morphological images are representative of at least three experiments with similar results. Statistical analysis was performed by the Student's t-test or ANOVA, followed by Bonferroni *post hoc* test, when appropriate ($P < 0.05$ was considered significant).

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Author contribution

Guglielmotto M and Monteleone D performed cell cultures and Western blot analysis; Boido M and Piras A carried out confocal immunofluorescence experiments; Giliberto L involved in pulse chase experiments; Borghi R performed Western blot analysis of human AD and control brains; Vercelli A and Tabaton M carried out design and planning experiments; Fornaro M carried out statistical analysis; and Tamagno E involved in design and planning experiments and wrote the paper.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1 The online version of this article contains additional Methods, Results, References and Figs 1–3.

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