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The hAPP-YAC transgenic model has elevated UPS activity in the frontal cortex similar to Alzheimer's disease and Down's syndrome

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Abstract

The ubiquitin-proteasome system (UPS) is critical for handling the intra-cellular load of abnormal and misfolded proteins in several neurodegenerative diseases. First, to determine the effects of the over-expression of human amyloid precursor protein (hAPP) on UPS, we measured proteasome activities using fluorescent substrates in the frontal cortex of hAPP-yeast artificial chromosome (YAC) transgenic (tg) mice (R1.40, hemizygous; Lamb, *Nat Genet*, **9**, 4; 1995). Chymotrypsin and PGPH-like activities of proteasome were increased in frontal cortex of hAPP-YAC tg mice. These proteasome activities (both chymotrypsin and PGPH-like) were further increased by cholinergic stimulation in littermate control mice, but not in hAPP-YAC tg mice. Nerve growth factor (NGF) levels were decreased by hAPP over-expression in the frontal cortex and hippocampus of hAPP-YAC tg mice, and further decreased by M1 agonist treatment in the hippocampus of littermate control and hAPP-YAC tg mice. Interestingly, the frontal cortex (BA9 area) of Alzheimer's disease (AD) patients (Stage 3, $n = 11$) and Down's syndrome (DS) patients ($n = 9$) showed similar up-regulation of the UPS activities to those seen in hAPP-YAC tg mice. M1 agonist stimulation increased the activities of α-secretase, which were down-regulated by hAPP over-expression in the frontal cortex of hAPP-YAC tg mice. These results demonstrate that (i) hAPP-YAC tg mice have an up-regulation in the frontal cortex of the UPS similar to AD and DS patients; (ii) muscarinic stimulation increase UPS activities, increase secreted APP (APPs) levels, and decrease amyloid beta 42/40 ratio only in littermate controls, but not in hAPP-YAC tg mice. Taken together, these results suggest that both the adaptive reactions in the proteostatic network and pathological changes in AD and DS need to be considered in the future potential therapeutics.

Keywords: amyloid precursor protein, cortex, patient, proteasome, transgenic mice, ubiquitin.

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Abnormal accumulation of amyloid peptide, abnormal protein degradation, cholinergic dysfunction, and malfunction of trophic factors may progressively lead to synaptic damage and neuronal cell loss relevant to memory function (Isacson *et al.* 2002). Alzheimer's disease (AD) is a neurodegenerative disease with progressive cognitive impairment and the accumulation of amyloid plaques and neurofibrillary tangles in the several regions of the brain. Amyloid plaque is constituted of a peptide of 39–42 amino acids derived from the amyloid precursor protein (APP). Since the over-expression of APP causes a progressive pathology in the patients of AD and Down syndrome (DS), several AD animal models have been developed by the over-expression of APP (Teller *et al.* 1996; Neve *et al.* 2000). AD model mice, including human APP (hAPP)-yeast artificial chromosome (YAC) transgenic (tg) mice (Lamb *et al.* 1993), Tg2576 tg mice (Sarsoza *et al.* 2009), and triple FAD tg mice (Kimura and Ohno 2009) with over-expression of APP, show accumulation of abnormal proteins forming plaques, and such animals also have cognitive dysfunction, and degeneration of cholinergic neurons by age (Haass *et al.* 1998; Harman 2006). To understand the direct and/or indirect interactions

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Abbreviations used: AD, Alzheimer's disease; AMC, 7-amido-4-methylcoumarin; Aβ, amyloid beta; DS, Down's syndrome; hAPP, human amyloid precursor protein; HD, Huntington's disease; NGF, nerve growth factor; PGPH, peptidyl-glutamyl peptide-hydrolyzing enzyme; tg, transgenic; UPS, ubiquitin-proteasome system; YAC, yeast artificial chromosome.

among these multiple AD and aging risk factors, pharmacologically induced cholinergic loss, or stimulation on the nicotinic or muscarinic receptors have been used as tools to reveal functional impairments (Fisher *et al.* 1993). In previous studies, muscarinic stimulation altered APP levels and trophic factors in the hippocampus of normal mice and DS model Ts65Dn mice (Seo and Isacson 2005). We also demonstrated that cholinergic stimulation by muscarinic M1 agonist decrease total APP and nerve growth factor (NGF) levels, and increase APPs levels in the hippocampus of normal BL6 mice (Seo *et al.* 2002). However, this dynamic regulation of APP and NGF by cholinergic stimulation was not seen in Ts65Dn mice, which have been used as an animal model of DS with consistent phenotypic abnormalities (Seo and Isacson 2005). These data suggest that a cholinergic pharmacological or related treatment must be evaluated on the basis of the age/or stage of disease, or as a responsiveness based on cholinergic synaptic function. Previously, we suggested that the comprehensive AD- and DS-like pathologies are produced by altered cholinergic and APP-related systems in DS mice model, which includes the over-expression of APP. To better understand the cellular mechanisms after cholinergic stimulation in AD model, we addressed to determine the functional levels of APP, APP-processing enzymes, ubiquitin-proteasome system (UPS), trophic regulation after M1 agonist stimulation in hAPP-YAC tg mice, which over-express human APP protein in a YAC system, where hAPP expression is regulated by its own promoter (R1.40, hemizygous; Lamb *et al.* 1993, 1995, 1997).

Materials and methods

M1 receptor agonist treatment

We administrated a selective muscarinic M1 receptor agonist, RS86 (gift from Novartis, Switzerland), to the hAPP-YAC tg or littermate mice. RS86 had been used for several previous pharmacological studies showing the central and peripheral cholinergic effects with the M1/M3 partial agonists (Palacios *et al.* 1986; Wanibuchi *et al.* 1990; Rupniak *et al.* 1992; Seo *et al.* 2002). All animals in the experiments were injected twice daily with the following dose: 1.5 mg/kg i.m. at approximately 9:00 AM and 2.0 mg/kg i.m. 8 h later, for 7 days. Control group animals received an equal volume of saline, i.m. at the same time points for 7 days.

Protein sample preparation

All animals in the experiments were anesthetized with sodium pentobarbital (50 mg/kg i.p.; Sigma Chemicals, St Louis, MO, USA). After CSF were collected from cisterna magna as previously described (Seo *et al.* 2002), the brains were removed and dissected on ice. Tissue samples from hippocampus and frontal cortex were used for Western blot analysis and ELISA.

Postmortem brain samples

Postmortem brain sample from AD (BA9 area, stage 3: $n = 11$) and normal control subjects ($n = 6$) were provided from Harvard Brain Bank at McLean Hospital and Postmortem brain sample from DS patients (BA9 area, stage 3, $n = 9$) and their normal control subjects ($n = 8$) were provided from Brain/Tissue Bank for Developmental Disorders in Maryland. Postmortem brains from patients and normal subjects were closely matched for age, sex, and postmortem interval. All brain samples were neuropathologically examined and provided as fresh frozen blocks, which were stored at −80°C before sample preparation.

Amyloid beta ELISA

Brain samples were analyzed for the peptide levels of amyloid beta (Aβ) 40 or 42 as previously described (Suzuki *et al.* 1994) using Aβ1–40 or Ab1–42 ELISA kit (Biosource International, Camarillo, CA, USA). The immunoreactivities of samples were read using fluorometric plate reader (Labsystem, Vienna, VA, USA). Aβ40 or 42 concentrations were determined based on sample values relative to the serially diluted standards representing a standard curve of known Aβ40 or 42 concentrations. Each sample was analyzed in triplicate.

Determination of α- and β- secretase activities

Proteolytic cleavages of the APP were detected by the determination of the activities of α- and β-secretases using commercially available enzyme activity detection kit (R&D systems, Minneapolis, MN, USA). Tissue samples with same protein amounts (50 μg of total protein) were incubated with proper substrates for each specific secretase for the enzymatic reaction for secretase activity. After 1 h incubation at 37°C, the plates were read on a fluorescent microplate reader (with the excitation between 335 and 355 nm, and the emission between 495 and 510 nm). All experiments were performed in triplicate for the comparison between different groups.

Determination of NGF protein expression levels

The protein levels of NGF in hippocampus and frontal cortex of the brain regions were determined using NGF Emax™ Immunoassay System as previously described (Promega, Madison, WI, USA; Zettler *et al.* 1996; Seo *et al.* 2002). Incubated ELISA plates with samples and antibodies were read using a Labsystem Multiscan Plus (Vienna, VA, USA) plate reader at 450 nm.

Determination of proteasomal function

Proteasome function was determined by continuously measuring the fluorescence of 7-amido-4-methylcoumarin (AMC; excitation 380 nm, emission 460 nm) generated from peptide-AMC linked substrates (Craiu *et al.* 1997). Reactions were conducted in a final volume of 200 μL containing 50 mM Tris-HCl buffer (pH 7.5) and 1 mM EDTA. After adding samples to the reaction mixtures, reactions were initiated by adding the following substrates, 50 mg/mL: Suc-Leu-Leu-Val-Try-AMC (65 μM) for chymotrypsin activity and Z-Leu-Leu-Glu-AMC (75 μM) for PGPH-like activity. Reactions were followed for 240 min at 25°C and enzymatic activities determined at linear rates and expressed as fluorescence units FU/min/mg of protein.

Statistical analysis

All statistical analyses were carried out using JMP (version 3.1.6; SAS Institute, Cary, NC, USA). Data were objectively compared between different groups at different stages of disease using

unpaired Student's *t*-test and two-way ANOVA followed by Turkey–Kramer *post hoc* analysis. Differences between groups were considered statistically significant when $p < 0.05$.

Results

Previously, we determined that activating muscarinic M1 receptors *in vivo* increase APPs levels in the CSF, and reduce APP protein levels in the several brain regions of normal BL6 mice (Seo *et al.* 2002). We also detected an altered pattern of APP processing by a muscarinic agonist in DS model mice (Ts65Dn) (Seo and Isacson 2005). This study determined if such muscarinic stimulation can influence several cellular functions considered relevant for AD, such as APP, NGF, secretases, and proteasome, in the hAPP-YAC tg mice.

Altered regulation of APP processing and NGF in hAPP-YAC tg mice

We detected higher Aβ42 levels in the hippocampus (approximately 2-fold) and frontal cortex (~1.8-fold) of hAPP-YAC mice (Fig. 1). The activities of APP processing enzymes, α- and β-secretases, were significantly down-regulated in the hAPP-YAC tg mice (Fig. 1). The Aβ42/40 ratio also increased in hAPP-YAC mice (Fig. 2). The secreted form of APP, APPs, which was detected in the CSF, also showed a tendency to increase, but this was not significant ($p > 0.05$; Fig. 2). This result indicates that over-expression of exogenous human APP protein in rodent system, reduce the activities of α- and β- secretases.

In previous studies, we determined that Ts65Dn, DS model mice have abnormal responses to cholinergic muscarinic M1 agonist treatment in terms of hippocampal APP, NGF levels (Seo and Isacson 2005). To determine whether the APP processing can be regulated by muscarinic stimulation also in this AD model mice (with the endogenous promoters), we administered M1 agonist (see 'Materials and methods') to hAPP-YAC tg mice. Interestingly, the Aβ42/40 ratio was further decreased by cholinergic M1 stimulation in littermate control mice, but paradoxically increased in hAPP-YAC tg mice. However, in our normal mice (littermate controls), in contrast to the AD model mice, cholinergic M1 agonist treatment significantly increased the levels of soluble secreted APPs in CSF.

In addition, NGF levels were significantly decreased by hAPP over-expression in the frontal cortex and hippocampus (Fig. 3). Interestingly, NGF was significantly decreased by M1 stimulation in the hippocampus of both littermate and hAPP-YAC tg mice. However, the NGF levels in the cerebral cortex in this model did not show significant change after M1 stimulation.

The regulation of protease activities in hAPP-YAC tg mice

Human APP over-expression decreased α- and β-secretase activities in hAPP-YAC tg mice (Fig. 4). hAPP over-expression also altered the effects of M1 agonist on α-secretase activities, which were increased by RS86 only in hAPP-YAC tg mice but not in littermate controls. These data suggest that APP expression can modify α-secretase response against cholinergic stimulation. These modifications by APP expression were not detected in the β-secretase activities against muscarinic stimulation.

To determine the effects of the over-expression of hAPP on UPS, we detected proteasome activities using fluorescent

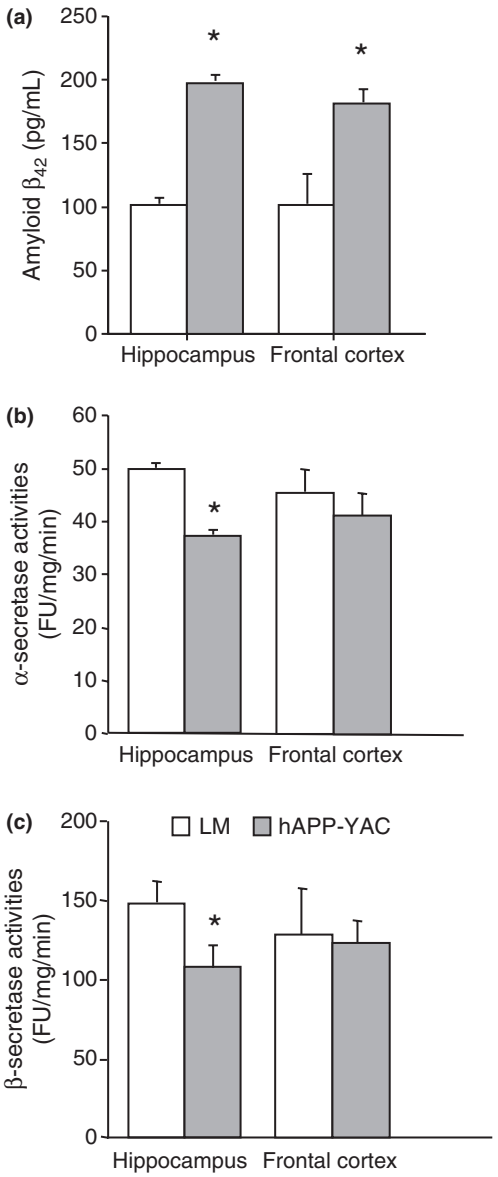

Fig. 1 Amyloid beta (Aβ) 42 levels were increased in human amyloid precursor protein (hAPP)-yeast artificial chromosome (YAC) transgenic mice (R 1.40, hemizygous) compared with their littermate control mice (LM) at 24 months of age (a, $*p < 0.05$). α-secretase (b) and β-secretase (c) enzyme activities were detected using florescent substrates. Both α-secretase and β-secretase enzyme activities were reduced in the frontal cortex of hAPP-YAC mice ($*p < 0.05$).

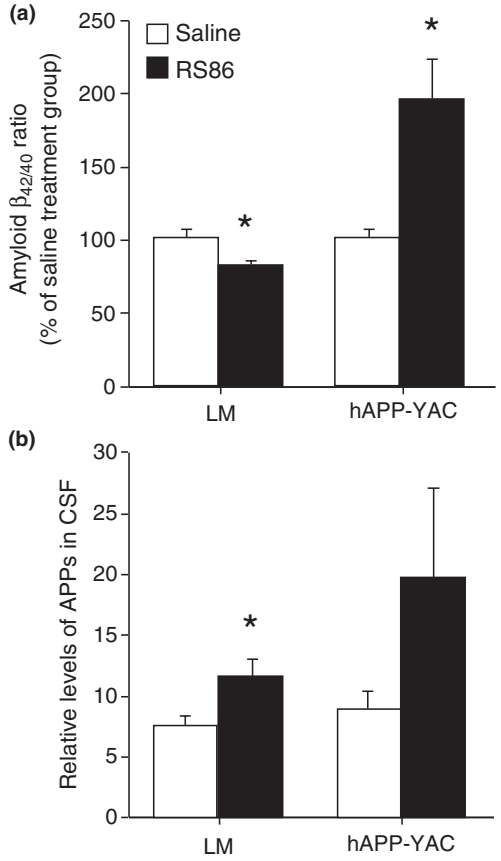

Fig. 2 The response to cholinergic M1 agonist on amyloid beta (Aβ) 42/40 ratio (a) and APPs (b) were determined in the frontal cortex of littermate control mice (LM) and human amyloid precursor protein (hAPP)-yeast artificial chromosome (YAC) transgenic mice. Cholinergic M1 agonist, RS86, decreased Aβ42/40 ratio in the frontal cortex of littermate control mice, but increased Aβ42/40 ratio in the frontal cortex of hAPP-YAC mice. RS86 increased APPs levels in CSF of littermate control mice while the average of increase level in the frontal cortex of hAPP-YAC transgenic mice did not reach to the significance (*$p < 0.05$).

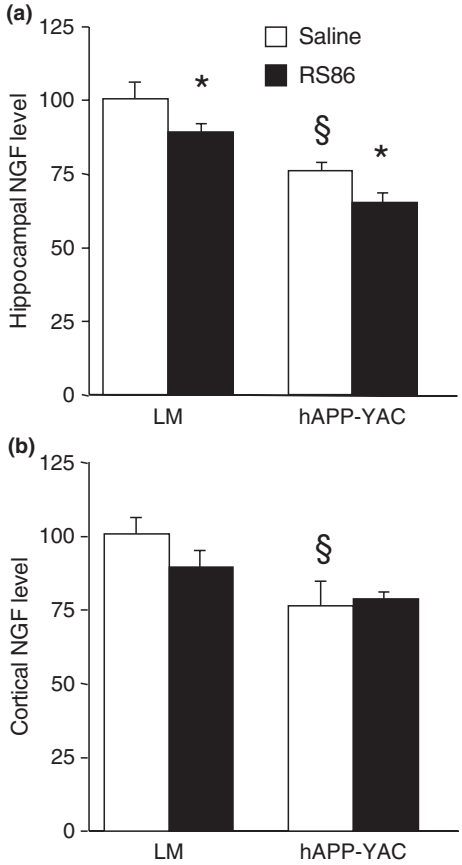

Fig. 3 Nerve growth factor (NGF) expression levels were significantly changed in the hippocampus (a) and frontal cortex (b) of human amyloid precursor protein (hAPP)-yeast artificial chromosome (YAC) transgenic mice (R 1.40, hemizygous) compared with wild-type littermate control mice (LM). Cholinergic M1 agonist decreased hippocampal NGF levels in hAPP-YAC transgenic mice (*$p < 0.05$) as previously shown in normal BL6 mice (Seo *et al.* 2002). However, cholinergic M1 agonist did not alter the NGF levels in the frontal cortex or hAPP-YAC transgenic mice.

substrates in the frontal cortex of hAPP-YAC tg mice (R1.40, hemizygous; Lamb *et al.* 1993, 1997). Chymotrypsin and peptidyl-glutamyl peptide-hydrolyzing enzyme (PGPH)-like activities of proteasome were increased in hAPP-YAC tg mice (Fig. 5). These proteasome activities (both chymotrypsin and PGPH-like) were further increased (52% and 63%) by cholinergic stimulation in littermate control mice, but not in hAPP-YAC tg mice. These results indicate that the basal level of proteasome activities in the frontal cortex of hAPP-YAC tg mice may already be saturated.

We also determined that the proteasome activities were changed in AD (grade 3) and DS patients. In the grade 3 AD patients, chymotrypsin and PGPH-like activities were significantly increased in the BA9 area (Fig. 6). We also detected a significant increase of chymotrypsin activity in the BA9 area of DS patients (Fig. 7).

Discussion

Amyloid precursor protein processing may be one of the critical factors in the progressive pathology of AD and DS. Abnormal products from APP processing may cause abnormal protein accumulation, dysfunction of trophic regulation, and synaptic connections (Isacson *et al.* 2002). In this study, we used the YAC-based hAPP over-expressing tg mice to study cholinergic stimulation, NGF, and proteasome activities under more normal gene regulatory conditions than seen with typical artificial promoters used in other tg mice.

Dysfunction of UPS regulation has been studied in several neurodegenerative diseases including AD and Huntington's disease (HD), to examine the contribution of UPS to abnormal accumulation of proteins (Seo *et al.* 2004, 2008;

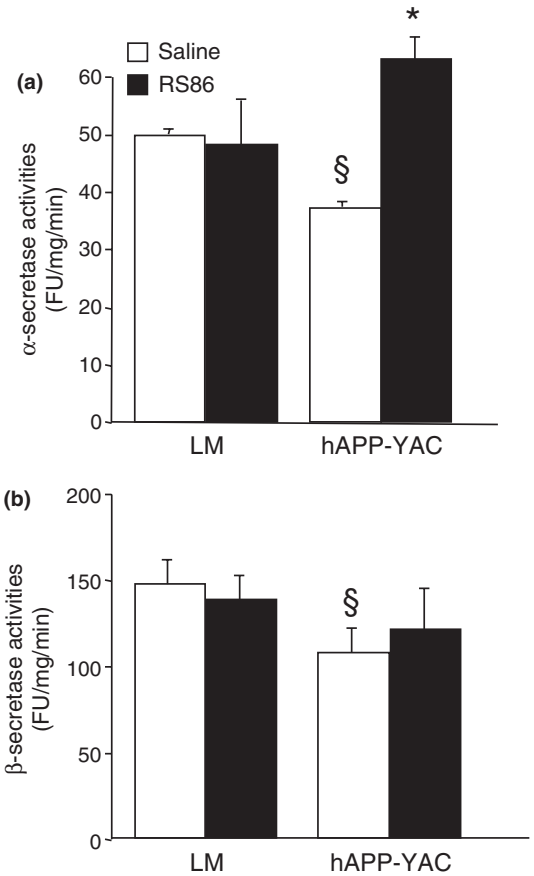

Fig. 4 Secretase activities in the frontal cortex of human amyloid precursor protein (hAPP)-yeast artificial chromosome (YAC) transgenic mice (R 1.40, hemizygous). α-secretase (a) and β-secretase (b) enzyme activities were detected using florescent substrates. Both α-secretase and β-secretase enzyme activities were reduced in the frontal cortex of hAPP-YAC mice. Cholinergic M1 agonist, RS86, did increase α-secretase activity but not β-secretase activity in hAPP-YAC transgenic mice (*$p < 0.05$); LM, littermate control.

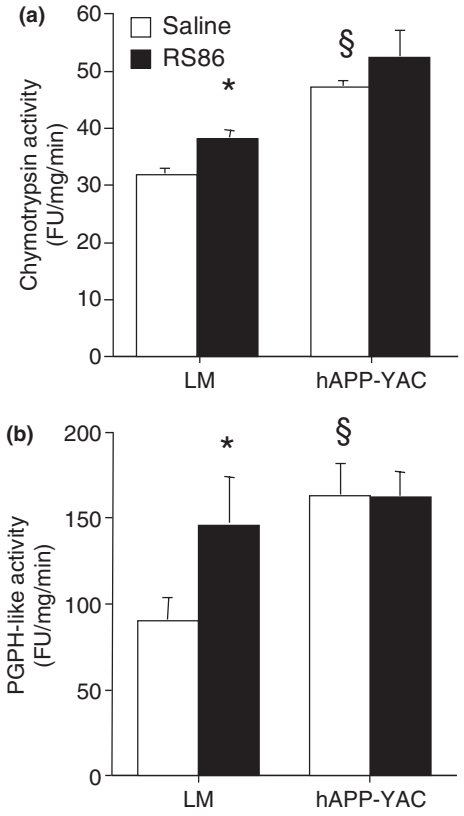

Fig. 5 Proteasome activities in the frontal cortex of yeast artificial chromosome (YAC)-human amyloid precursor protein (hAPP) transgenic mice (R 1.40, hemizygous). Both chymotrypsin activity (a) and PGPH-like activity (b) were significantly increased in hAPP-YAC transgenic mice compared with littermate control mice (LM). Administration of cholinergic M1 agonist, RS86, increased proteasome activities (both chymotrypsin and PGPH-like) in littermate control mice but not in hAPP-YAC transgenic mice (*$p < 0.05$).

Upadhya and Hegde 2007; Pan *et al.* 2008). Previous *in vitro* studies showed that the UPS activities were down-regulated by the Aβ peptide (Gregori *et al.* 1995; Cecarini *et al.* 2008). The proteasome degrades presenillin 1 and other APP-processing enzymes in *in vitro* cell culture system (Fraser *et al.* 1998; Steiner *et al.* 1998; Yamazaki and Ihara 1998; Honda *et al.* 1999) indicating that it has dual roles in the break-down and regulation of the APP-related proteins. The c-terminal fragment of APP is degraded by a proteasome dependent mechanism (Nunan *et al.* 2001). Interestingly, resveratrol-induced Aβ decrease was prevented by proteasome inhibitors in cultured cells (Marambaud *et al.* 2005). These data indicated that one of the pathways to reduce Aβ accumulation in pathological conditions is the UPS (Marambaud *et al.* 2005). In previous postmortem AD patient brain studies, proteasome activities were found to be reduced in the hippocampus, parahippocampal gyrus, and superior and middle temporal gyri, inferior parietal lobule, and the straight gyrus (Keller *et al.* 2000; Keck *et al.* 2003). However, we detected up-regulation of proteasome activities by APP over-expression in the frontal cortex of the hAPP-YAC AD model mice, and also in AD patients and DS patients. This could be owing to the differences of the brain regions or disease stages of samples used from different previous studies. The increase of proteasome activities seen in hAPP-YAC tg mice and patients could be caused by relative increases of enzymatic catalytic activity or absolute protein expression levels. Given that similar protein expression levels of proteasome core subunit were detected in hAPP-YAC tg mice and littermate control mice (data not shown), we believe that the increase of the proteasome activity is by increase of enzymatic/catalytic activities, and not by increase of protein expression levels or transcriptional control levels. Fundamentally, from data obtained in parallel

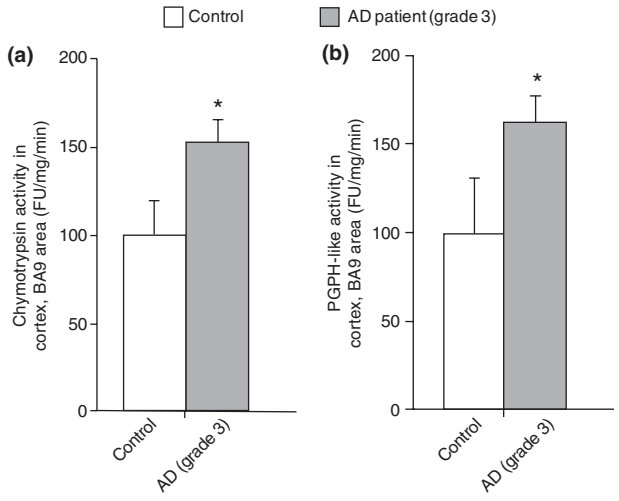

Fig. 6 Proteasome activities (both chymotrypsin and PGPH-like) are up-regulated in the frontal cortex (BA9 area) of Alzheimer's disease patients (stage 3, $n = 11$).

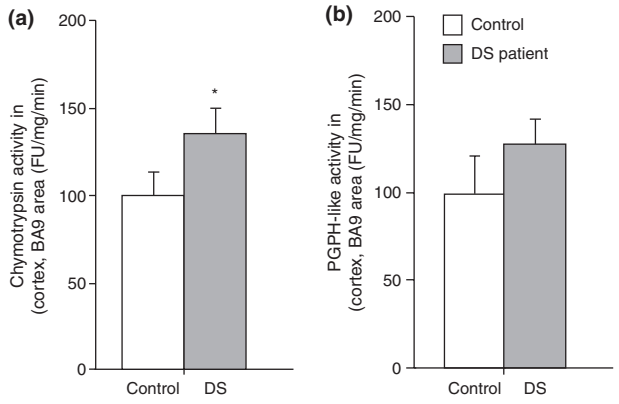

Fig. 7 Proteasome activities (both chymotrypsin and PGPH-like) are up-regulated in the frontal cortex (BA9 area) of Down's syndrome (DS) patients ($n = 9$).

studies of HD, it appears that UPS activity levels are up- or down-regulated depending on the disease stage (Seo *et al.* 2004, 2008). Previous data and models on HD (Seo *et al.* 2004, 2008) suggest that abnormal protein aggregation is gradually reducing (possibly blocking) UPS function. In early stages of disease, or in patients with very high huntingtin CAG repeat number and also in high CAG repeat tg animal models (R6/2), there is actually an up-regulation of the UPS activity. This up-regulation is presumably a cellular response to clear the cells of accumulating proteins. Interestingly, along this line of reasoning, even in HD skin-derived fibroblasts showing reduced UPS activities, there is an increased gene expression of the proteasome 20S core protein, which nonetheless does not manage to normalize UPS activities (Seo *et al.* 2004).

The over-expression of APP in AD and DS animal models causes decreased levels of NGF in cortex and hippocampus (Isacson *et al.* 2002; Seo and Isacson 2005; Cattaneo *et al.* 2008; Mufson *et al.* 2008). In this study, we detected down-regulation of NGF levels and APP-processing enzyme activities, including α- and β-secretases by APP over-expression in hAPP-YAC AD model mice. These data suggest that the increased level of APP protein itself may cause a failure of trophic molecular control and APP processing. Previously, cholinergic stimulation has been studied to improve the behavior and pathophysiological symptoms as therapeutic strategies for AD and DS. Stimulation with the M1/M3 selective agonist, talsaclidine, resulted in the up-regulation of β-secretase beta-site of APP cleaving enzyme (BACE1) expression (Zuchner *et al.* 2004). The stimulation of protein kinase C (PKC)-coupled M1/M3 muscarinic acetylcholine receptor increased secretory pathway activity of APP processing (Rossner *et al.* 1998). In this study, Aβ42/40 ratio levels were decreased, and APPs were increased by M1 agonist treatment in littermate control mice. These data suggest that muscarinic cholinergic stimulation can improve APP processing to become less amyloidogenic. However, these Aβ42/40 ratios or APPs levels were not properly regulated in hAPP-YAC tg mice, in contrast to what was seen in littermate control mice. In parallel, we found that M1 agonist stimulation increased α-secretase activities in hAPP-YAC mice, while it did not show significant changes on β-secretase activities in hAPP-YAC mice. Similar inverse changes caused by cholinergic stimulation have also been detected in Ts65Dn DS model mice (Seo and Isacson 2005).

Cholinergic neurons are dependent on NGF function for the maintenance. Steady-state number of cholinergic synapse is dependent on continuous tropic support, including NGF supply, receptor binding, and function (Isacson *et al.* 2002; Cattaneo *et al.* 2008; Mufson *et al.* 2008). Age-dependent degeneration of cholinergic neurons may be associated with reduced NGF function. M1 selective agonist has been reported to rescue cholinergic neurons in AD models (Fisher *et al.* 1993, 1996). In this study, we could not find any significant alterations of cortical NGF levels by M1 agonist stimulation. However, we detected the hippocampal NGF levels were down-regulated by M1 agonist in hAPP-YAC mice and its littermate controls.

Alzheimer's disease (AD) patients show comprehensive pathologies and altered cholinergic synaptic function, APP processing, and protein degradation system (Isacson *et al.* 2002; Mufson *et al.* 2008). These risk factors lead to the cholinergic imbalance, synaptic damage, and specific neuronal loss (Fisher *et al.* 1993). The muscarinic stimulation has been suggested as one of the potential therapeutic targets for AD. Our data suggest that these age-dependent progressive pathophysiological changes in AD need to be considered in the potential therapeutic treatment for AD.

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