UBIQUITIN CARBOXYL-TERMINAL HYDROLASE L1 IN ALZHEIMER'S DISEASE

by

Mingming Zhang

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Abstract

Alzheimer's disease (AD) is the most common form of neurodegenerative diseases. Its neuropathology is characterized by extracellular amyloid plaque deposition, intracellular neuritic fibrillary tangles and neuronal loss. The extracellular amyloid plaque consists of amyloid β (A β) protein, which is derived from β - and γ - cleavage of amyloid precursor protein (APP). The abnormal accumulation of A β initiates neuronal dysfunction and plays an important role in AD pathogenesis.

Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) is a de-ubiquitinating enzyme that cleaves ubiquitin at its carboxyl terminal. Dysfunction of UCHL1 has been implicated in various neurodegenerative diseases including childhood-onset progressive neurodegeneration, Alzheimer's disease and Parkinson's disease. UCHL1 protein level is reduced in AD and is inversely proportional to the number of neurofibrillary tangles in AD brains.Overexpression of UCHL1 could rescue learning and memory deficits in AD mouse model. However, whether UCHL1 affects APP processing, $A\beta$ production or AD pathogenesis remains unknown.

This thesis entails a thorough examination of the role of UCHL1 in AD pathogenesis. First, *UCHL1* gene transcriptional regulation was investigated. We identified a functional NF- κ B binding site within its 5'promoter region. We found that NF- κ B signaling down-regulated UCHL1 transcription. Next we demonstrated that UCHL1 affected APP processing and A β production by facilitating the degradation of APP, the precursor of A β , and BACE1, the β -secretase in vivo. The results were verified by transgene expression and pharmacological inhibition of UCHL1 in multiple cell lines. Moreover, we showed in neuroblastoma cell lines and primary neuronal culture that UCHL1 protected against oxidative stress- and A β -induced neuronal apoptosis by interfering with the caspase 8/caspase 3 pathway. Finally, we demonstrated that UCHL1 reduced A β production, inhibited amyloid plaque formation and rescued memory deficits in AD mouse models.

In summary, this study investigated the effect of UCHL1 on AD pathogenesis. It demonstrated for the first time that UCHL1 delays the development of AD pathology by regulating APP processing and reducing A β production. Furthermore, our findings indicated that transgene expression of UCHL1 is a disease-modifying strategy for AD therapeutic design.

Preface

After completing a Bachelor's of Science degree, I joined Dr. Weihong Song's research team to study the molecular mechanism that underlies Alzheimer's disease (AD) pathogenesis. Dr. Song introduced me to a project that focused on the transcriptional regulation of ubiquitin carboxyl-terminal hydrolase L1 (UCHL1), a deubiquitinating enzyme whose dysfunction is associated with neurodegenerative diseases. Chapter 2 is based on the findings from this project. I collaborated with Dr. Ruitao Wang, a PhD student in the laboratory, on this part of work. Dr. Wang and I cloned a series of deletion plasmids of the 5' flanking region of the human *UCHL1* gene. We performed the luciferase assay to measure the promoter activity. Dr. Wang performed the gel shifting assay to show the functional binding of NF-κB to UCHL1 promoter region. Dr. Wang also used semi-quantitative PCR and western blotting to demonstrate the down-regulation of UCHL1 by NF-κB. I confirmed the decreased UCHL1 protein level by NF-κB using western blot analysis. The RelA-KO cell line used in chapter 2 was generated by Gilmore laboratory (Gapuzan *et al.*, 2005).

As a collaborator of the project, I am a co-first author on a paper published in Journal of Neurochemistry in March 2011 (Wang, R., <u>Zhang, M.</u>, Zhou, W., Ly, P.T., Cai, F. & Song, W. (2011) NF-κB signaling inhibits ubiquitin carboxyl-terminal hydrolase L1 gene expression. <u>Journal of Neurochemistry</u>, **116**, 1160-1170). The journal granted permission for the author to include the published materials in this thesis.

In chapter 3, we investigated the effect of UCHL1 on the protein degradation of amyloid precursor protein (APP) and beta-site APP cleaving enzyme 1 (BACE1), two proteins that are critical to amyloid β protein (A β) production. I designed and carried

out the majority of the experiments. Ms. Yu Deng and Dr. Yawen Luo helped confirm using cycloheximide that UCHL1 accelerates BACE1 degradation. Heterozygous *gad* mice were originally generated by Dr. Keiji Wada's laboratory at the National Institute of Neuroscience of Japan (Yamazaki *et al.*, 1988). All animal studies were approved by the University of British Columbia Animal Care Committee (protocol number: A11-0025). Procedures for obtaining embryonic mouse primary neurons were approved by the University of British Columbia Animal Care Committee (protocol number: A09-0274).

Part of the discovery from chapter 3 has been published in Journal of Neurochemistry in March 2012 (Zhang, M., Deng, Y., Luo, Y., Zhang, S., Zou, H., Cai, F., Wada, K. & Song, W. (2012) Control of BACE1 degradation and APP processing by ubiquitin carboxyl-terminal hydrolase L1. *Journal of Neurochemistry*, **120**, 1129-1138). I am the first author of the paper. I wrote the manuscript and revised it with the help of my supervisor Dr. Weihong Song. The journal granted permission for the author to include the published materials in this thesis.

In chapter 4, we showed the effect of UCHL1 on APP processing and learning and memory in AD mouse models. I designed and conducted most of the experiments. Dr. Shuting Zhang, Dr. Fang Cai and Ms. Fiona Zhang helped me with some of the behavioral experiments. Dr. Shuting Zhang helped me confirm the effect of UCHL1 on CTF β production in *gad* mice. Haiyan Zou assisted me with the genotyping of APP23 and *gad* mice. Stereotaxic intracranial surgery procedures on adult mice were approved by the University of British Columbia Animal Care Committee (protocol number: A11-0025).

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List of Abbreviations

- 4-HNE 4-hydroxynonenal
- 5'UTR 5'-untranslated region
- **5'RACE** Rapid amplification of 5' complementary DNA ends
- AA amino acid
- AAV adeno-associated virus
- **ABC** avidin: biotinylated enzyme complex
- ADAM a disintegrin and metalloprotease
- **ADDL** amyloid β derived diffusible ligands
- AEBSF 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
- AICD APP intracellular domain
- ANOVA analysis of variance
- Aph-1 anterior pharynx factor-1
- **APLP** amyloid β precursor-like protein
- **ApoE** apolipoprotein E
- **APP** amyloid β precursor protein
- **Aβ** β-amyloid
- **BACE1** β -site APP cleaving enzyme 1
- **BACE2** β -site APP cleaving enzyme 2

bp base pair

- BSA bovine serum albumin
- CMA chaperone-mediated autophagy
- **CTF** C-terminal fragment

- **DS** Down Syndrome
- **DUB** de-ubiquitinating enzyme
- EGF epidermal growth factor
- ELISA enzyme-linked immunosorbent assay
- **ENaC** epithelial Na⁺ channel
- ER endoplasmic reticulum
- FAD familial Alzheimer's disease
- **FBL2** F-box and leucine rich repeat protein 2
- **FBS** fetal bovine serum
- FTDP-17 frontotemporal dementia with parkinsonism on chromosome 17
- GAPDH glyceraldehydes-3-phosphate dehydrogenase
- **GDP** gross domestic product
- GFAP glial fibrillary acidic protein
- **GH** growth hormone
- **GSA** gel shifting assay
- **GSI** γ -secretase inhibitor
- **GSK** glycogen synthase kinase
- **ΙκΒ -** α NF-κB inhibitor, α
- IL interleukin
- KO knockout
- LDH lactate dehydrogenase
- LRP1 low density lipoprotein receptor-related protein-1
- LTP long term potentiation
- MCI mild cognitive impairment
- **MEF** mouse embryonic fibroblasts

- **NFT** neurofibrillary tangles
- **NF-κB** nuclear factor kappa-light-chain-enhancer of activated B cells
- NICD Notch intracellular domain
- NRG1 neuregulin 1
- NRSE neuron restrictive silencer element
- NSAID nonsteroidal anti-inflammatory drug
- **ORF** open reading frame
- **OTU** ovarian tumour proteases
- **PBS** phosphate buffered saline
- **PCR** polymerase chain reaction
- PD Parkinson's disease
- PDGF platelet-derived growth factor
- **Pen-2** presenilin enhancer 2
- PFA paraformaldehyde
- PS1 Presenilin 1
- **PS2** Presenilin 2
- **PVDF-FL** polyvindylidine fluoride
- **RAGE** receptor for advanced glycation endproducts
- **RIPA** radio-immunoprecipitation assay deoxycholate
- **RP** regulatory particle
- **RT-PCR** reverse transcription polymerase chain reaction
- SAD sporadic Alzheimer's disease
- **SDS** sodium dodecyl sulfate
- **SDS-PAGE** SDS-polyacrylamide gel electrophoresis
- **TF** transcription factor

- TGN trans-Golgi network
- **TNF** α tumor necrosis factor α
- TSS transcription start site
- TUNEL terminal deoxynucleotidyl transferase dUTP nick end labeling
- UCH ubiquitin carboxyl-terminal hydrolase
- UCHL1 ubiquitin carboxyl-terminal hydrolase L1
- UPS ubiquitin proteasome system
- USP ubiquitin-specific protease
- WT wildtype

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To my family

Chapter 1

General introduction

1.1 Dementia and Alzheimer's disease

Dementia is a syndrome of serious global cognitive debilitation due to disease of the brain. It affects memory, thinking, behavior and the ability to perform everyday activities (ADI, 2010). Dementia is classified into many subtypes according to the pathophysiology of the disease. Some of the most common types are Alzheimer's disease (AD), vascular dementia, dementia with Lewy bodies and frontotemporal dementia.

AD is the most common form among all types of dementia. Approximately one of 10 individuals over the age of 65 years and nearly one-half of the people over 85 years are affected with AD (Thies & Bleiler, 2011). The disease is usually diagnosed in people over 65 years of age (Brookmeyer *et al.*, 1998), after which the risk of acquiring the disease doubles every five years (Di Carlo *et al.*, 2002; Bermejo-Pareja *et al.*, 2008). Early-onset Alzheimer's disease may occur much earlier, often with genetic mutations and in most cases is inherited in an autosomal dominant manner.

Socioeconomic burden of Alzheimer's disease. Alzheimer's disease has become a public health priority due to its global prevalence. Currently, more than 35 million people worldwide are suffering from dementia, among which two-thirds are AD cases. This number is expected to be doubled in 2020 and more than tripled in 2050 (ADI, 2013). The annual societal costs of AD worldwide in 2010 were US\$604 billion, or 1% of the aggregated worldwide Gross Domestic Product (GDP). In addition to the financial burden, the disease has imposed heavy social and psychological burden on patients as well as their families (ADI, 2010).

Clinical presentation of Alzheimer's disease. Clinical presentation of AD can be divided into four stages: the pre-dementia stage, mild stage, moderate stage and severe stage. Symptoms at the pre-dementia stage include mild impairment in acquiring new information and minor short-term memory loss, which may be mistakenly attributed to normal aging. The pre-clinical stage has also been termed "mild cognitive impairment" (MCI)(Arnaiz & Almkvist, 2003). MCI does not necessarily develop into AD. Currently no diagnostic method can distinguish with certainty between incipient AD and non-progressive memory impairment. At mild dementia stage, increasing impairment in learning and memory is the most outstanding clinical feature. The patient's reduced ability to plan, judge, and organize may show in relatively difficult household chores. At moderate stage of AD, logical reasoning, planning, and organizing significantly deteriorate. Speaking, reading and writing skills are progressively lost. At severe stage of AD, almost all cognitive functions are significantly impaired. Language is reduced to simple phrases. Patients need assistance with every simple task in their lives. Psychological symptoms include aggression, apathy and exhaustion. Life expectancy is reduced in AD patients. At the terminal stage, patients most often die of pneumonia, myocardial infarction and septicaemia, but not AD itself (Forstl & Kurz, 1999; Health et al., 2002).

Pathophysiological features of Alzheimer's disease. The main pathological hallmarks in AD brains are extracellular neuritic plaques, intracellular neurofibrillary tangles and neuronal loss. Extracellular neuritic plaques are mainly composed of dense and insoluble deposits of amyloid β protein (A β) surrounded by dystrophic neurites. Intracellular neurofibrillary tangles (NFTs) are aggregates of hyperphosphorylated microtubule-associated tau proteins. It should be noted that dense-cored A β plaque deposition is unique to AD cases whereas NFT is also detected in other forms of dementia and neurodegenerative diseases, including frontotemporal dementia with parkinsonism on chromosome 17 (FTDP-17), Pick's disease and progressive supranuclear palsy.

Neuronal loss is a prominent characteristic of AD. Interestingly, certain brain regions are more vulnerable to AD than other regions. Neuronal loss starts in the entorhinal cortex at very mild stage of AD, extends to hippocampus as the disease progresses, and is ultimately widespread in some areas of the neocortex (Van Hoesen *et al.*, 1991; Gomez-Isla *et al.*, 1996; Health *et al.*, 2002). Gross manifestation of the neuronal loss is the atrophy of the temporal and parietal lobes and restricted regions within the frontal cortex and cingulate gyrus (Wenk, 2003). While the temporal and spatial development of neuronal loss may provide a clue to the etiology of AD, no conclusion has been reached so far.

Genetic factors in Alzheimer's disease. While most AD cases are diagnosed in people over 65 years of age and are sporadic, approximately 3-5% are early-onset familial AD (FAD). FAD patients develop symptoms from as early as 40 years old (Campion *et al.*, 1999). The majority of early-onset FAD cases are caused by mutations in one of the three genes: β -amyloid precursor protein (APP), Presenilin 1 (PSEN1) and Presenilin 2 (PSEN2). To date, more than 30 pathogenic APP point mutations, more than 180 PSEN1 mutations and 14 PSEN2 mutations have been identified (http://www.molgen.ua.ac.be/ADMutations/). Most mutations increase A β production or alter the ratio of A β 42 over A β 40 (two A β proteins of different lengths), which suggest a central role of A β in AD pathogenesis. In sporadic AD (SAD), environmental and genetic differences may act as risk factors. The strongest genetic risk factor for SAD is apolipoprotein E (ApoE) genotype, with the ϵ 4 allele being an AD risk factor and the ϵ 2 being protective compared to the most common ϵ 3 allele (Corder *et al.*, 1993; Corder *et al.*, 1994).

1.2 APP processing pathway

The modern era of Alzheimer's research is marked by the milestone discovery of A β from β -sheet fibrils in cerebrovascular amyloidosis associated with AD (Glenner & Wong, 1984b) and from the amyloid plaque core in AD and aged individuals with Down syndrome (Glenner & Wong, 1984a; Masters *et al.*, 1985). The cDNA screening led to the discovery of its precursor, the amyloid β precursor protein (APP) (Goldgaber *et al.*, 1987; Kang *et al.*, 1987; Robakis *et al.*, 1987; Tanzi *et al.*, 1987). Since then, APP processing pathway has been well characterized.

APP is a type I transmembrane protein encoded by a single gene on chromosome 21. Multiple isoforms are produced through alternative splicing. Three major isoforms are: APP695 (Kang *et al.*, 1987), APP751 (Ponte *et al.*, 1988; Tanzi *et al.*, 1988) and APP770 (Kitaguchi *et al.*, 1988), with APP695 being the major isoform in neuronal cells (Kang *et al.*, 1987).

APP undergoes sequential proteolytic cleavages on its extracellular domain and transmembrane domain to produce multiple fragments (Figure 1.1). Under physiological conditions, the majority of APP processing is by α -cleavage, which is within the A β domain at Leu-17 and produces C-terminal fragement α (CTF α) C83. α -cleavage precludes A β production. Alternatively, APP is processed by β -cleavage at Asp-1 site to produce CTF β C99, or at Glu-11 site to produce CTF β C89. A small portion of APP may undergo θ -cleavage at Phe-20 site by BACE2, the θ -secretase, to yield CTF θ C80. γ -secretase subsequently cleaves CTF α (C83), CTF β (C89 and C99) and CTF θ (C80). Only the cleavage of C99 yields the amyloidogenic and pathogenic A β . C83, C89 and C80 are cleaved to generate the non-pathogenic fragments p3, truncated A β (tA β), and p3 θ , respectively.



Figure 1.1 APP processing pathways

APP is first cleaved by α - or β -secretase followed by γ -secretase complex. Under physiological conditions, the predominant APP processing is by α -cleavage within the A β domain at Leu-17 to produce sAPP α and membrane-bound CTF α C83. C83 is further cleaved by γ -secretase, producing extracellular fragment P3 and intracellular AICD C57/59 (middle blue arrow). Alternatively, APP is processed by β -cleavage (red arrow and left blue arrow). APP is cleaved at Asp-1 site to produce CTF β C99 and sAPP β , or at Glu-11 site to produce CTF β C89 and sAPP β . C99 is subsequently cleaved by γ -secretase to yield the amyloidogenic A β and intracellular AICD C57/59 (red arrow). C89 is cleaved by γ -secretase to yield the non-amyloidogenic truncated A β (tA β) and C57/59 (left blue arrow). β -secretase preferentially cleaves wildtype APP at Glu-11 site over Asp-1 site, whereas it cleaves APP Swedish mutant mainly at Asp-1 site. A small portion of APP may undergo θ -cleavage at Phe-20 site by BACE2 (right blue arrow). CTF θ C80 is subsequently cleaved by γ -secretase to produce P3 θ and C57/59.

1.2.1 α-secretase and the non-amyloidogenic α-cleavage of APP

Under normal conditions, the major APP proteolytic process prevents further A β generation. APP is cleaved by α -secretase between Lys-16 and Leu-17 within the A β domain to produce sAPP α and CTF α C83 (Esch *et al.*, 1990; Sisodia *et al.*, 1990). C83 is subsequently processed by γ -secretase to yield p3 and AICD C57/59 (Haass & Selkoe, 1993) (Figure 1.1). The α -secretase-mediated APP cleavage is non-amyloidogenic. Moreover, studies have shown that sAPP α bears important physiological functions such as maintaining synaptic integrity (see Section 1.3.3).

A few metalloproteases have been proposed to be the α -secretase, including several members of the "A disintegrin and metalloprotease" (ADAM) family: ADAM9, ADAM10 and ADAM17. Studies have provided strong support for the hypotheses: overexpression of ADAM9 has been shown to enhance α -cleavage (Koike *et al.*, 1999); overexpression of wildtype or dominant-negative form of ADAM10 increased or inhibited α -cleavage, respectively (Lammich *et al.*, 1999); ADAM inhibitor reduced sAPP α secretion in wildtype but not in ADAM17-knockout embryonic fibroblasts (Buxbaum *et al.*, 1998). The incomplete inhibition of sAPP α secretion by any one of the ADAMs suggested that more than one protease is responsible for the α -cleavage *in vivo*. On the other hand, the different phenotypes of ADAM9, ADAM10 and ADAM17 knockout mice indicated non-overlapping functions of those proteases (Black *et al.*, 1997; Hartmann *et al.*, 2002; Guaiquil *et al.*, 2009).

1.2.2 BACE1 and β-cleavage of APP

While the majority of full-length APP undergoes α -cleavage within the A β region, a minority of APP is cleaved by β -secretase. Beta-site APP cleaving enzyme 1 (BACE1) is the β -secretase *in vivo* (Hussain *et al.*, 1999; Sinha *et al.*, 1999; Vassar *et al.*, 1999; Yan *et al.*, 1999). BACE1 cleaves APP at two β -sites: at Asp-1 site to produce CTF β C99, which is subsequently cleaved by γ -secretase to yield the pathogenic A β , and at Glu-11 site to produce CTF β C89, the precursor of the non-pathogenic truncated A β (Figure 1.1). Notably, AD-associated APP mutations have different preferential β -cleavage sites compared to wild type APP (APP_{WT}). Studies by our lab showed that BACE1 mainly cleaves APP_{WT} at Glu-11 and produces non-pathogenic C89. By contrast, in APP_{SWE}, an APP mutation identified in a Swedish family with early-onset FAD, the preferential β -cleavage site shifts to Asp-1 site, resulting in more C99 and a much higher C99/C89 ratio compared to APP_{WT} (Deng *et al.*, 2013). The observation implicated a critical role of BACE1 and preferential β -cleavage site in AD pathogenesis.

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BACE1 as the major β-secretase *in vivo*. In 1999, several groups independently identified BACE1 as the β-secretase (Hussain *et al.*, 1999; Sinha *et al.*, 1999; Vassar *et al.*, 1999; Yan *et al.*, 1999) using different strategies. They validated the β-cleavage activity of BACE1 *in vitro* by its overexpression and antisense oligonucleotide inhibition. The expression pattern and localization of BACE1 were also confirmed by Northern analysis and *in situ* hybridization as previously predicted. BACE1 was further confirmed as the major β-secretase *in vivo* using BACE1-deficient embryonic cortical neurons (Cai *et al.*, 2001) and BACE1-knockout mice (Luo *et al.*, 2001; Roberds *et al.*, 2001). Consistently, crossing of BACE1-knockout mice and APP_{SWE} (Tg2576) transgenic mice resulted in dramatic reduction of Aβ and improved memory deficits (Ohno *et al.*, 2004).

BACE2, a homolog of BACE1, is not a β-secretase, but a θ-secretase. Shortly after the discovery of BACE1, a homologous novel aspartic protease, BACE2, was identified (Saunders *et al.*, 1999; Acquati *et al.*, 2000). BACE2 shares 52% amino acid sequence identity and 68% similarity with BACE1, and contains two aspartyl protease active sites at virtually identical positions to the two active sites in BACE1. Moreover, it resides in the obligate Down Syndrome (DS) region of chromosome 21 (Saunders *et al.*, 1999). Given all these features, BACE2 was at that time thought to be critical in the inevitable early-onset FAD syndrome in DS patients. Subsequent studies showed, however, that BACE2 cleaved APP at Phe-19 and Phe-20 sites much more efficiently than at Asp-1 site (Farzan *et al.*, 2000). Our lab further demonstrated that BACE2 mainly cleaved APP between Phe-19 and Phe-20, a novel θ-site that is downstream of α-site (Figure 1.1). Lentiviral infection of BACE2 to the primary neurons from APP transgenic mice markedly reduced Aβ production (Sun *et al.*, 2006b). Therefore, BACE2 is not a β-secretase *in vivo*, leaving BACE1 the only βsecretase identified so far.

1.2.3 **Presenilins and the γ-secretase complex**

C-terminal fragments (CTFs) of APP produced by α -, β -, or θ -cleavage are subsequently processed by γ -secretase (Figure 1.1). Specifically, γ -secretase cleaves C99 at the transmembrane Val-40 and Ala-42 sites to produce the amyloidogenic A β_{40} and A β_{42} . Notably, pathogenic mutations in APP, PS1 and PS2 genes from early-onset FAD result in either an increase in total A β level or a shift of γ -cleavage from Val-40 to Ala-42 (<u>http://www.molgen.ua.ac.be/ADMutations/</u>), which indicates the importance of both quantitative and qualitative changes of A β production. In addition to A β fragments, γ -cleavage also produces the intracellular AICD C57/59, which plays an important role in nuclear signaling (see Section 1.3.3).

PS1/PS2 as the catalytic core of γ -secretase complex. Presenilins were first linked to AD when mutations in presenilin 1 (PS1) and presenilin 2 (PS2) were identified from early-onset FAD patients (Group, 1995; Levy-Lahad *et al.*, 1995; Rogaev *et al.*, 1995; Sherrington *et al.*, 1995). De Strooper *et al.* defined PS1 as an important component of γ -secretase *in vivo* by showing that knockout of PS1 in primary neurons caused the accumulation of CTFs and a five-fold decrease of A β production (De Strooper *et al.*, 1998). One year later De Stooper *et al.* and Song *et al.* independently discovered that PS1 is essential for the γ -cleavage of Notch, further confirming its critical role as a γ -secretase (De Strooper *et al.*, 1999; Song *et al.*, 1999). Moreover, PS1 and PS2 were shown to be absolutely required for APP and Notch cleavage, as the production of A β and Notch intracellular domain (NICD) was completely abolished in PS1/2 double-knockout cells (Zhang *et al.*, 2000).

PS1 is a multi-transmembrane protein with nine-transmembrane (TM) topology (Thinakaran *et al.*, 1996; Spasic *et al.*, 2006). It is an aspartyl protease, and mutation of either of two conserved transmembrane aspartate residues in PS1, Asp 257 and Asp 385, substantially reduced A β production (Tong *et al.*, 2005). Moreover, the γ secretase activities of PS1 and PS2 require the cleavage of the 9-TMD holoprotein into the amino-terminal fragment (NTF) and the carboxyl-terminal fragment (CTF) during maturation (Thinakaran *et al.*, 1996). The endoproteolysis is considered autocatalytic (Zhang *et al.*, 2000; Tong *et al.*, 2005)

PS1/PS2, nicastrin, aph-1 and pen-2 as the components of γ-secretase complex. While PS1/PS2 was acknowledged as the catalytic core, other proteins were predicted in the γ -secretase complex, because (1) PS1/PS2 levels were regulated by other limiting cellular factors (Thinakaran et al., 1997), and (2) PS1 was co-eluted with a complex of high molecular weight (Li et al., 2000). Nicastrin was the first molecule co-immuno-extracted with PS1. Knockdown of nicastrin in C. elegan resulted in phenotypes similar in sel-12 and hop-1 (presenilins orthologue in *C. elegan*)-null worms, which indicated an important role of nicastrin in γ -cleavage (Yu *et al.*, 2000). Further genetic screening in C. elegan revealed another two components of γ secretase complex: aph-1 and pen-2 (Francis et al., 2002; Goutte et al., 2002). Coexpression of PS1, nicastrin, aph-1 and pen-2 was sufficient to increase γ -cleavage in mammalian cells (Kimberly *et al.*, 2003) and to reconstitute γ -secretase activity in yeast, a model organism that lacks endogenous γ -secretase (Edbauer *et al.*, 2003). Subsequent studies on individual components of the complex indicated that aph-1 stabilizes the presenilin holoprotein, whereas pen-2 is necessary for the endoproteolysis of presenilin (Takasugi et al., 2003).

Other substrates of γ -secretase complex. In addition to APP, γ -secretase complex has more than 80 substrates, including Notch, neuregulin, ErbB4, E-cadherins and Ncadherins, and growth hormone receptor (Song *et al.*, 1999; Zhang *et al.*, 2000; Ni *et al.*, 2001; Kim *et al.*, 2002; Marambaud *et al.*, 2002; May *et al.*, 2002; Haapasalo & Kovacs, 2011). Among them, the γ -cleavage in Notch signaling is of particular importance, because NICD cleaved by γ -secretase is essential for the transcriptional activation of genes that regulate cell proliferation and differentiation, which is critical to both development and in adult life (Maillard *et al.*, 2003; Maillard & Pear, 2003; Stanger *et al.*, 2005). The deficiency of PS1 led to Notch-knockout phenotype in mice, who died shortly after birth (Shen *et al.*, 1997; Wong *et al.*, 1997). Therefore, AD drug development that targets γ -secretase must work on substrate specificity to avoid mechanism-based side effects.

1.3 APP: its trafficking, degradation and functions

1.3.1 The intracellular trafficking of APP

APP is a type I transmembrane protein that undergoes classical secretory pathway. It is synthesized on the endoplasmic reticulum (ER), is N-glycosylated in the ER and is O-glycosylated in the Golgi apparatus before reaching the plasma membrane. It is then rapidly internalized to the endosome, after which the majority is sorted to lysosome for degradation while a small fraction is sent back to the trans-Golgi network (TGN). Part of the protein is directly sorted to endosome without reaching the plasma membrane (Figure 1.2) (Koo *et al.*, 1996; Small & Gandy, 2006). APP is continuously being cleaved by α -, β - or γ -secretase throughout the secretory pathway. Converging evidence has shown that the majority of α -cleavage is processed on the plasma membrane, while β -cleavage favors an acidic environment such as the late endosome (reviewed in (Small & Gandy, 2006)). Therefore, the trafficking and localization of APP affect the preferential cleavage between α -site and β -site, hence affecting CTF β and subsequent A β production.

The trafficking of APP is mainly regulated by its conservative C-terminal YENPTY motif. Specifically the YENP sequence regulates APP internalization from plasma membrane. Its mutants (YENP to AEAA or AENA) significantly impaired APP internalization and reduced A β production (Perez *et al.*, 1999). A few adaptor proteins have been identified to interact with the YENPTY motif. Fe65, a neuronal adaptor protein, bound to the YENPTY motif and regulated APP trafficking and metabolism

(Borg *et al.*, 1996). Overexpression of Fe65 accumulated APP at the plasma membrane and enhanced sAPP α secretion (Sabo *et al.*, 1999), although its effect on A β production remained inconclusive (Sabo *et al.*, 1999; Ando *et al.*, 2001). X11/mint, another neuronal adaptor protein, interacted with YENPTY motif, stabilized fulllength APP and inhibited A β production both *in vitro* (Borg *et al.*, 1998; Sastre *et al.*, 1998) and *in vivo* (Lee et al., 2003; Lee et al., 2004). JIP1b, a scaffolding protein involved in JNK pathway, also interacted with the YENPTY motif of APP. Overexpression of JIP1b stabilized immature form of APP, suppressed sAPP secretion and A β production (Taru *et al.*, 2002). To summarize, signals that stabilizes fulllength APP or prolongs the stay of APP at plasma membrane reduce β -site cleavage and subsequent A β production.



Figure 1.2 Intracellular trafficking of APP and BACE1

Both APP and BACE1 (red bar) are type-I transmembrane proteins that are sorted through multiple membranous compartments of the cell. The sorting triangle that interconnects the trans-Golgi network (TGN), cell surface, and the endosome is critically important for APP and BACE1 protein sorting. Both APP and BACE1 are N-glycosylated in the ER and O-glycosylated in the Golgi apparatus before reaching the plasma membrane. They are then rapidly internalized to the endosome, where some are sorted to lysosome for degradation while a small fraction is sent back to TGN. Part of the proteins are directly sorted to the endosome without reaching the plasma membrane.

1.3.2 Lysosomal and proteasomal degradation of APP

Among the two major intracellular degradation systems, i.e., the ubiquitin proteasome system (UPS) and lysosome system, APP is mainly degraded by the latter. The lysosome inhibitor chloroquine greatly inhibited the degradation of full-length APP (Caporaso et al., 1992). Furthermore, cell-surface biotinylation-labeled full-length APP was detected inside the cells, especially in the lysosome (Haass et al., 1992). Interestingly, the UPS has been recently shown to also take part in APP degradation. A few proteins have been identified to assist APP degradation through UPS. Synoviolin/Hrd1, a ubiquitin ligase for ER-associated protein degradation (ERAD), was shown to interact with and ubiquitinate APP, and accelerate its proteasomal degradation (Kaneko et al., 2010). Similarly, F-box and leucine rich repeat protein2 (FBL2), a component of the E3 ubiquitin ligase complex, enhanced APP degradation by ubiquitinating its C-terminal (Watanabe et al., 2012). Moreover, ubiquilin, a protein that physically associated with both ubiquitin E3 ligase and the proteasome, assisted the Lys-63-linked poly-ubiquitination of APP at Lys-688 site and inhibited its non-lysosomal degradation (El Ayadi et al., 2012). It is likely that while the majority of APP undergoes lysosomal degradation, a small proportion goes to proteasomal degradation, which was demonstrated in the above studies by the application of proteasome inhibitors. The report by Kaneko et al. also implicated that APP may shift to ERAD-proteasomal degradation when they cannot be properly folded under cellular stress (Kaneko et al., 2010).

1.3.3 Functions of full-length APP and its cleavage products

Researches on AD pathogenesis have focused on the proteolytic process of APP and the production of A β . In fact, full-length APP bears many important physiological roles. The overall effect from loss of APP is displayed on APP knockout mice, who showed body weight and brain weight deficits, alterations in locomotor activity and impaired spatial memory (Zheng *et al.*, 1995; Ring *et al.*, 2007). Moreover, sAPP and

AICD, cleavage products of full-length APP that have been largely overlooked in the AD field, also serve many important cellular functions.

Functions of full-length APP. One prominent role of full-length APP is to facilitate the axonal transport in neurons. Kamal *et al.* first discovered the interaction *in vivo* between APP and the KLC subunit of kinesin-I, a conventional microtubule motor protein. The axonal transport of APP was decreased in KLC mutant mice, which indicated the role of kinesin in APP transport (Kamal *et al.*, 2000). Subsequent studies revealed the colocalization of APP, BACE1 and PS1 in the same axonal membrane compartment. Surprisingly, the axonal transport of BACE1 and PS1 was diminished in APP^{-/-} mice, which implicated a role of APP itself in axonal transport (Kamal *et al.*, 2001). APP may also be involved in cell adhesion, since the extracellular sequence of APP interacted with various extracellular matrix components including laminin (Kibbey *et al.*, 1993), collagen type I (Beher *et al.*, 1996) and heparin (Clarris *et al.*, 1997). Indeed, heparin has been shown to bind to the E1 and E2 domain of APP and induce APP dimerization (Gralle *et al.*, 2006), which promote intercellular adhesion of mouse embryonic fibroblasts (MEFs) (Sabo *et al.*, 1999).

Functions of APP cleavage products. sAPP α , the secreted N-terminal fragment of APP, plays important physiological roles. The first indication came from the rescue experiments in APP knockout animals, as the knockin of sAPP α rescued almost all of the phenotypes displayed in APP^{-/-} mice (Ring *et al.*, 2007). Moreover, endogenous sAPP α was required for hippocampal long-term potentiation (LTP) and spatial memory (Taylor *et al.*, 2008). Exogenous sAPP α or overexpression of ADAM10 in transgenic mice significantly increased synaptic density (Roch *et al.*, 1994; Meziane *et al.*, 1998; Bell *et al.*, 2008).

As is described in Section 1.3.1, Fe65 binds to the YENPTY motif of APP C-terminal and regulates the trafficking of APP. Studies have further revealed that AICD, the intracellular APP C-terminal fragment that is released after γ -cleavage, formed a multimeric complex with Fe65 and the hitone acetyltransferase TIP60. The complex potently stimulated gene transcription (Cao & Sudhof, 2001), indicating a role of AICD in gene expression regulation. Interestingly, one of its target genes was neprilysin, a metalloprotease that is partly responsible for the degradation of Aβ (Pardossi-Piquard *et al.*, 2005).

1.4 BACE1: expression regulation, trafficking, degradation and functions

BACE1 is the β -secretase *in vivo*, which is required for A β generation. A slight increase of BACE1 expression is sufficient to promote C99 and A β production (Li *et al.*, 2006). Moreover, elevated C99 is not only associated with the overall production of heterogeneous A β , but is directly and positively correlated with the production of A β_{42} , the major toxic and a hydrophobic species of A β (Yin *et al.*, 2007). Notably, increased BACE1 level and β -secretase activity are detected in AD brains (Holsinger *et al.*, 2002; Yang *et al.*, 2003; Chen *et al.*, 2011), indicating that dysregulation of BACE1 may contribute to AD pathogenesis. Endogenous BACE1 expression is tightly regulated at transcriptional and translational level. BACE1 activity is also regulated by its trafficking between cellular compartments and by its intracellular degradation.

1.4.1 Expression regulation of BACE1

BACE1 is tightly regulated at transcriptional level. Our lab first cloned the human BACE1 promoter region, which contains many putative transcription factor (TF) binding sites such as Sp1, NF- κ B, hypoxia-inducible factor 1- α (Hif1 α) and activating protein 2 (AP2) (Christensen *et al.*, 2004). We identified Sp1 as an essential TF for BACE1 transcription (Christensen *et al.*, 2004) and NF- κ B as a TF that upregulates BACE1 expression (Chen *et al.*, 2011). Recently we discovered that an isoform of glycogen synthase kinase (GSK), GSK3 β , regulates BACE1 expression via NF- κ B signaling (Ly *et al.*, 2013). BACE1 expression is also regulated post-transcriptionally. The GC-rich 5'-untranslated region (5'UTR) was shown to be a constitutive translation barrier (Lammich *et al.*, 2004). Moreover, upstream ATGs inhibits the translation of the main open reading frame (ORF) (De Pietri Tonelli *et al.*, 2004), which is controlled by leaky scanning and reinitiation (Zhou & Song, 2006).

Interestingly, BACE1 expression is regulated in response to cellular stress, including oxidative stress (Tamagno *et al.*, 2002; Tong *et al.*, 2005), hypoxia (Sun *et al.*, 2006a; Zhang *et al.*, 2007) and ischemia (Wen *et al.*, 2004). Specifically, our lab demonstrated that hypoxia upregulated BACE1 transcription through binding to the hypoxia responsive element, which further increased CTF β production, A β plaque deposition and impaired memory functions in AD mouse model (Sun *et al.*, 2006a). The observation provided a molecular mechanism for the higher incidence of AD in people who have previously experienced stroke attack.

1.4.2 The trafficking of BACE1

As a type I transmembrane protein, BACE1 undergoes classical secretory pathway as APP does (Figure 1.2). It is synthesized in the ER, undergoes maturation and modification in the ER and Golgi, and traffics from TGN to either the plasma membrane or to the endosome. Cell surface BACE1 is internalized to early endosome, after which they either retrograde to TGN or reach lysosome for degradation (reviewed in (Tan & Evin, 2012)). As described above, β -secretase activity is maximized in an acidic environment such as within the endosome.

The trafficking of BACE1 is regulated through its C-terminal di-leucine motif (Leu499-Leu500), a signal for sorting protein from TGN and plasma membrane to endosome (Sandoval & Bakke, 1994). Mutation of the LL motif prevents the

internalization of BACE1 from plasma membrane to endosome (Huse *et al.*, 2000; Pastorino *et al.*, 2002). The γ -adaptin ear-containing ADP ribosylation factor-binding protein (GGA) is a clathrin adaptor protein that mediates the intracellular trafficking of transmembrane protins by interacting with their DXXLL sorting signals. The VHS domain of GGA binds to the LL motif on BACE1 and regulates its recycling (He *et al.*, 2002; He *et al.*, 2005). The binding is further potentiated by the phosphorylation of Ser498 (Shiba *et al.*, 2004).

1.4.3 The degradation of BACE1

BACE1 is degraded by both the ubiquitin-proteasome pathway (Qing *et al.*, 2004) and lysosome pathway (Koh *et al.*, 2005). Our lab demonstrated that BACE1 is ubiquitinated. Treatment of lactacystin, a specific proteasome inhibitor, results in the accumulation of BACE1, C99 and A β levels (Qing *et al.*, 2004). Gong *et al.* further discovered that BACE1 can be ubiquitinated by the Skp1-Cullin1-Fbx2-Roc1 (SCF (Fbx2)) -E3 ligase (Gong *et al.*, 2010). On the other hand, Koh *et al.* showed that lysosomal inhibitor chloroquine and NH₄Cl lead to the accumulation of BACE1. Moreover, the C-terminal di-leucine sequence of BACE1 is responsible for sorting BACE1 to endosome and lysosome (Koh *et al.*, 2005). Interestingly, ubiquitination may regulate the lysosomal degradation of BACE1. BACE1 was shown to be mono-ubiquitinated and Lys-63-linked polyubiquitinated at C-terminal Lys-501 (Kang *et al.*, 2010). GGA3 binds to ubiquitinated BACE1 and targets it to lysosome for degradation (Tesco *et al.*, 2007; Kang *et al.*, 2010).

1.4.4 Other substrates of BACE1 and its normal functions

Apart from APP, BACE1 also cleaves other substrates. Alleged substrates include neuregulin 1(NRG1) (Willem *et al.*, 2006), voltage-gated sodium channel (Na_V1) β 2 subunit (Kim *et al.*, 2007), low density lipoprotein receptor-related protein-1 (LRP1) (von Arnim *et al.*, 2005), amyloid β precursor-like protein 1 (APLP1) (Li & Sudhof, 2004) and APLP2 (Pastorino *et al.*, 2004). Prominently, BACE1 cleaves NRG1 and releases its secreted fragment, whose interaction with ErbB receptor is required for axonal myelination and remyelination (Hu *et al.*, 2006; Willem *et al.*, 2006). BACE1 also cleaves $\beta 2$ subunit of Na_V1, the sodium channel that carries out the rising phase of action potentials (Catterall, 2000). The accessory $\beta 2$ subunit regulates the expression of the pore-forming α -subunit, and the density and function of Na_V1 (Chen *et al.*, 2002). BACE1 overexpression results in increased Na_V1.1 mRNA and protein levels intracellularly, but reduces its cell surface expression (Kim *et al.*, 2007). When designing BACE1 inhibitor for AD therapeutics, it is important to take into account physiological functions of BACE1 to avoid mechanism-based side effects.

1.5 The amyloid hypothesis of Alzheimer's disease

1.5.1 The amyloid plaque hypothesis

To explain the cause of Alzheimer's disease, Hardy & Higgins proposed the "amyloid cascade hypothesis" in 1992. They proposed that the deposition of A β , the main component of the plaque, is the causative agent of Alzheimer's pathology. The neurofibrillary tangles, cell loss, vascular damage, and dementia follow as a direct result of this deposition (Hardy & Higgins, 1992). To distinguish this hypothesis from the revised amyloid oligomer hypothesis, we refer to it as the amyloid plaque hypothesis in this dissertation.

The amyloid plaque hypothesis is based on the breakthrough discoveries in the 1980s and 1990s. Glenner and Wong first purified A β from β -sheet fibrils in cerebrovascular amyloidosis associated with AD (Glenner & Wong, 1984b). One year later Masters *et al.* discovered that the amyloid plaque core protein in AD and aged individuals with Down syndrome was the same molecule (Masters *et al.*, 1985). Subsequent cDNA library screening with probes from A β led to the discovery of the 695-amino acid APP on chromosome 21, of which DS patients have a third copy (Goldgaber *et al.*, 1987; Kang *et al.*, 1987; Robakis *et al.*, 1987; Tanzi *et al.*, 1987). The findings revealed a
genetic linkage between DS and the inevitable AD pathology. The identification of the first few mutations in APP (Goate *et al.*, 1991; Hendriks *et al.*, 1992; Mullan *et al.*, 1992) in early-onset FAD and cerebral hemorrhage due to cerebral amyloid angiopathy further supported the A β hypothesis for AD pathogenesis.



Dementia with plaque and tangle pathology

Figure 1.3 The amyloid hypothesis of Alzheimer's disease

The amyloid cascade hypothesis stated that the deposition of $A\beta$ plaques is the causative agent of Alzheimer's pathology (the black arrow). The neurofibrillary tangles, cell loss, vascular damage, and dementia follow as a direct result of this deposition. The revised amyloid oligomer hypothesis blamed $A\beta$ oligomers to be the culprit of neuronal dysfunction and neuronal death that lead to AD (the orange arrow). A β plaques, although widely observed in AD brains, may not be the direct cause of AD.

In the last 20 years, $A\beta$ hypothesis has received the most popularity in the AD field, although some researchers are advocates of the newly-raised "tau hypothesis", or the oldest "cholinergic hypothesis". $A\beta$ hypothesis is strongly supported by scientific observations in a few aspects. The first and strongest support comes from early-onset FAD. Most of the FADs are caused by one of the three genes: APP, PS1 and PS2. So far more than 30 FAD-associated mutations in APP, more than 180 mutations in PS1 and 14 mutations in PS2 gene have been identified, all of which lead to elevated total $A\beta$ production or increased $A\beta_{42}/A\beta_{40}$ ratio

(http://www.molgen.ua.ac.be/ADMutations/). Those missense mutations are sufficient to cause AD in their carriers, who display initial symptoms as early as in their 40s. This is potent evidence for the causal role of A β in AD pathogenesis, which advocates of other hypotheses have to explain or incorporate into their theories. Secondly, tau mutation has not been discovered in AD. In fact, tauopathies caused by tau mutation, such as frontotemporal dementia with parkinsonism (FTDP-17), display distinct clinical manifestation from AD with no A β plaque deposition as in AD (Hutton *et al.*, 1998; Spillantini *et al.*, 1998). Thirdly, ApoE, the most significant genetic risk factor for late-onset AD (Corder *et al.*, 1993; Corder *et al.*, 1994), plays an important role in A β production, clearance and aggregation (Bu, 2009), again indicating a central role of A β in AD pathogenesis.

Despite the mounting support, there are some observations that A β plaque hypothesis fails to explain. The strongest objection comes from the poor correlation between the number of amyloid plaques in the brain and the degree of cognitive impairment in AD patients. Rather, NFT pathology, as described in Braak staging of AD (Braak & Braak, 1991), correlates better with the severity of AD (Arriagada *et al.*, 1992). Another objection comes from the lack of amyloid-only dementia. While tau-only pathology may cause some forms of frontotemporal dementia, A β pathology is always accompanied by tauopathy in AD, suggesting the involvement of tauopathy in AD development.

1.5.2 The revised amyloid oligomer hypothesis

In 2002, Hardy and Selkoe proposed the revised amyloid oligomer hypothesis that better accommodates the discrepancy between some of the observations and the A β plaque hypothesis. The revised A β hypothesis blamed A β oligomers to be the culprit of neuronal dysfunction and neuronal death that lead to AD (Hardy & Selkoe, 2002). A β plaques, although widely observed in AD brains, may not be the direct cause of AD.

The revised amyloid hypothesis has been embraced by the AD society, for a few reasons. First, it provided explanations for the lack of association between plaque deposition and cognitive impairment stage in AD. In fact, levels of soluble A β oligomers are well correlated with clinical status in AD (McLean *et al.*, 1999; Mc Donald *et al.*, 2010). Secondly, the hypothesis reflected the A β oligomer toxicity to synaptic plasticity and learning and memory in AD mouse models. Studies have shown that both synthetic small diffusible A β oligomers (ADDLs) and naturally-secreted A β oligomers inhibited hippocampal LTP (Lambert *et al.*, 1998; Walsh *et al.*, 2002). A 56-kD soluble A β assembly (A β *56) from APP transgenic mice disrupted memory in young rats (Lesne *et al.*, 2006). Strikingly, soluble A β oligomer extracts from brains of AD patients impaired synaptic plasticity and memory in normal rodents, which was prevented by antibodies to the N-terminus of A β (Shankar *et al.*, 2008). These studies demonstrated the toxicity of endogenous A β oligomers and strongly supported the A β oligomer hypothesis.

It is not conclusive yet whether the $A\beta$ plaque hypothesis or the revised $A\beta$ oligomer hypothesis is a better model for AD pathogenesis. Whichever is true, limiting $A\beta$ production and accelerating $A\beta$ clearance are the primary goals of AD therapeutics. The main focus of this thesis is on strategies that reduce $A\beta$ production. While the experiments were not designed to test the validity of the two hypotheses, they may provide support for one hypothesis over the other.

1.6 Inflammation, NF-kB and Alzheimer's disease

AD is a chronic disease accompanied by prolonged inflammation. In the brains of AD patients, prominent activation of the inflammatory processes and the innate immune responses is observed, which is probably stimulated by damaged neurons and insoluble A β plaque deposition (reviewed in (Akiyama *et al.*, 2000)). Abnormal expression of cytokines, chemokines, compliments and other proteins related to inflammatory responses has been confirmed by immunohistochemistry, western blot and mRNA measurements (Akiyama *et al.*, 2000). Among them, NF- κ B, interleukin (IL)-1 α , the chemokine CCL20 and tumor necrosis factor (TFN)- α -induced protein 2 have been reported to increase in AD in more than one study (Wyss-Coray, 2006).

While beneficial immune responses such as the phagocytosis of damaged cells may inhibit AD, it is believed that chronic inflammation induces detrimental changes in the brain (Wyss-Coray, 2006). Early support for this notion came from epidemiological studies that discovered a lower incidence of AD in people with rheumatoid arthritis, who generally received nonsteroidal anti-inflammatory drugs (NSAIDs) (McGeer et al., 1990). Subsequent postmortem studies confirmed the reduction of activated microglia by three folds in the brains of those patients. Interestingly, NSAID use did not alter the number of amyloid plaques and tangles, indicating an inflammationdependent and plaques and tangles-independent mechanism in AD pathogenesis (Mackenzie & Munoz, 1998). Further studies revealed the regulation of A β production and plaque formation by COX, the main target of NSAIDs. Lim et al. demonstrated the effect of ibuprofen, a common NSAID, on the reduction of microglial activation and amyloid plaque numbers in Tg2576 AD mouse model (Lim et al., 2000). In accordance, overexpressing human COX-2 in APP_{SWE}/PS1_{A246E} transgenic mice exacerbated Aβ plaque formation (Xiang *et al.*, 2002). These reports suggested a role of inflammation in AD pathogenesis in both plaque-dependent and -independent ways. The transcription factor NF- κ B is an important part of immune responses. While normal NF-kB activity is required for neuronal integrity and learning and memory (Kaltschmidt & Kaltschmidt, 2009), its dysregulation may induce chronic inflammation and contribute to neurodegeneration in AD (reviewed in (Granic et al., 2009)). NF- κ B and A β have been shown to regulate each other through a positive feedback loop. Extracellular Aß activates NF-kB via Receptor for Advanced Glycation Endproducts (RAGE), an A β receptor, in APP/RAGE transgenic mice (Arancio et al., 2004). On the other hand, NF-kB regulates AB production and amyloid pathology in APP transgenic mice (Sung et al., 2004). Bourne et al. looked into the molecular mechanism and identified an NF-kB binding site on rat BACE1 promoter, through which NF-kB upregulated BACE1 transcription in activated astrocytes (Bourne et al., 2007). Recently, Chen et al. identified four NF-kB binding sites on human BACE1 promoter, and demonstrated that overexpression of NF-KB p65 enhanced BACE1 transcription (Chen et al., 2011). Ly et al. confirmed the effect of NF-κB on BACE1 transcription expression and further identified GSK3β as one of the upstream regulators of NF-KB activation in this pathway (Ly et al., 2013). Studies to identify other AD-associated molecules that are regulated by NF-kB signaling will provide more information for the role of NF-κB and inflammation in AD pathogenesis.

1.7 Ubiquitin signaling

Ubiquitination is a type of post-translational modification, where ubiquitin, a 76amino-acid protein, is covalently attached to its substrate by an isopeptide bond. The addition of ubiquitin marks the substrate for a variety of cellular events, and is one of the important strategies recruited by cells to regulate protein metabolism and functions.

Ubiquitin is best known for its role in proteasome degradation. When it was first isolated in the 1970s, however, it was thought to have lymphocyte-differentiating

properties (Goldstein, 1974; Goldstein *et al.*, 1975). The name ubiquitin indicates its ubiquitous expression in almost all living cells (Schlesinger *et al.*, 1975). In 1980, Ciechanover *et al.* identified a polypeptide component of an ATP-dependent and lysosome-independent proteolytic system (Ciechanover *et al.*, 1980a; Ciechanover *et al.*, 1980b; Hershko *et al.*, 1980), which turned out to be ubiquitin (Wilkinson *et al.*, 1980). Since then, ubiquitin has been shown to direct the ubiquitin-proteasome protein degradation as well as many other cellular processes, including protein localization, histone regulation, viral infection, to list a few.

Ubiquitin is encoded by four genes in the human genome: *UBB, UBC, UBA52* and *UBA80. UBB* and *UBC* are translated into linear fusions of three and nine copies of ubiquitin respectively, while *UBA52* and *UBA80* each contains one copy of ubiquitin that is fused to ribosomal protein L40 and S27a respectively (summarized in (Komander *et al.*, 2009)). After the precursor is processed into mono-ubiquitin, the C-terminal glycine 76 is conjugated to a lysine residue of its substrate with the help of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3) (Figure 1.4). In the ubiquitination cascade, the E1 is able to bind dozens of E2s, each of which recognizes many E3s, in a hierarchical way. The substrate-specific E3s ensure selective protein ubiquitination for specific cellular events.



Figure 1.4 The ubiquitination process

The activation of ubiquitin is initiated by the ubiquitin-activating enzyme (E1) via binding to its Cterminus. The reaction requires ATP. The activated ubiquitin on E1 is then transferred to the cysteine residues of a ubiquitin-conjugating enzyme (E2). The E2 protein acts in conjunction with ubiquitin ligases (E3). E2-E3 complexes bind to protein substrates and catalyse the transfer of ubiquitin to a lysine on the substrate protein. The substrate is thus mono-ubiquitinated. The process can be repeated to form polyubiquitin chains.

The ubiquitination process can be repeated to form polyubiquitin chains. The glycine-76 of a second ubiquitin is conjugated to one of the seven lysines of the ubiquitin that is already bound to the substrate. Among the seven lysine residues, Lys-48 and Lys-63 are the ones most commonly recruited to form the isopeptide bonds. The type of substrate ubiquitination (mono-ubiquitination, Lys-48 polyubiquitination, Lys-63 polyubiquitination, etc.) provides another level of specificity for downstream cellular processes.

1.7.1 Ubiquitin in ubiquitin-proteasome system

1.7.1.1 The ubiquitin proteasome system

The most prominent role of ubiquitin is mediating protein degradation by the UPS. The UPS and the autophagy-lysosome system are two major intracellular degradation systems. The UPS rapidly degrades short-lived proteins to regulate many cellular processes, while autophagy is mainly responsible for the degradation of long-lived proteins and large organelles (Kraft et al., 2010). The UPS degrades proteins that are polyubiquitinated. When chains composed of at least four Lys-48-linked ubiquitins are formed, the target protein is then recognized and transported to the 26S proteasome for degradation into small peptides (Weissman, 2001). The 26S proteasome is a large multisubunit protease complex that consists of a 20S catalytic core complex and two 19S regulatory complexes at both ends. While the proteolytic activities are conferred by three subunits in the interior of the 20S chamber, the 19S complex serves as the gate of the proteasome. It recognizes and removes the Lys-48 polyubiquitin from the substrate, and unfolds the substrate for translocation into the 20S chamber (Dahlmann, 2005). Three proteasome-associated de-ubiquitinating enzymes (DUBs), RPN11, UPS14 and UCH37, regulate the de-ubiquitination process of the substrates. They either remove ubiquitin from the substrates and accelerate their degradation (as in RPN11), or reverse the ubiquitination and rescue proteins from being degraded (as in USP14 and UCH37) (see Section 1.8.1).

1.7.1.2 The UPS and AD

The UPS regulates the turnover of a wide variety of intracellular proteins. Notably, many of the AD-associated proteins, including β -secretase BACE1 and components of γ -secretase complex are all at least partially degraded via the UPS. Our lab demonstrated that treatment with specific proteasome inhibitor led to the accumulation of BACE1, C99 and A β protein, suggesting UPS-degradation of BACE1 (Qing *et al.*, 2004). Similarly, we and others have confirmed the turnover of PS1, aph-1 and nicastrin by the UPS (Fraser *et al.*, 1998; He *et al.*, 2006; He *et al.*, 2007). Interestingly, although the majority of APP undergoes lysosome degradation (Caporaso *et al.*, 1992; Haass *et al.*, 1992), at least a fraction of it can be ubiquitinated and degraded via the UPS (Kaneko *et al.*, 2010; El Ayadi *et al.*, 2012; Watanabe *et al.*, 2012) (described in Section 1.3.2). These findings suggested that the UPS is vital in APP processing and A β production.

Dysfunctional UPS is associated with AD. Keller *et al.* demonstrated that proteasome activities decreased in AD brains, especially in hippocampus, perihippocampal gyrus, superior and middle temporal gyri, and the inferior parietal lobule (Keller *et al.*, 2000). Lopez Salon *et al.* further showed that E1 and E2 activities were significantly reduced in AD samples (Lopez Salon *et al.*, 2000). Moreover, UBB⁺¹, a mutant form of ubiquitin generated from a di-nucleotide deletion of the ubiquitin mRNA, was accumulated in brains affected by AD (van Leeuwen *et al.*, 1998). Overexpression of UBB⁺¹, which lacked the ability to ubiquitinate, significantly induced nuclear fragmentation and cell death, indicating a causal role of abnormal ubiquitin in neuronal death (De Vrij *et al.*, 2001).

1.7.2 Ubiquitin as a UPS-independent signal

In addition to its role in the UPS, ubiquitin also serves as a signal for many other cellular events. In those events, substrates are usually not Lys-48-linked polyubiquitinated. Instead, they are most often mono-ubiquitinated or Lys-63-linked polyubiquitinated. Important functions of UPS-independent ubiquitination include protein endocytosis, autophagy-lysosome degradation, viral infection, and histone regulation. Here we briefly introduce ubiquitin signaling in protein trafficking and degradation-related pathways.

1.7.2.1 Protein endocytosis by ubiquitin signaling

Ubiquitination marks cell-surface proteins for internalization from the plasma membrane. Identified substrates in mammalian cells include growth hormone (GH)

receptor, platelet-derived growth factor (PDGF) receptor, epidermal growth factor (EGF) receptor and epithelial Na⁺ channel (ENaC) (Mori *et al.*, 1992; Galcheva-Gargova *et al.*, 1995; Strous *et al.*, 1996; Staub *et al.*, 1997). Most ubiquitinated proteins are internalized and targeted for lysosome degradation, although a few require the assistance of proteasome to be fully degraded (reviewed in (Hicke, 1999)). Mono-ubiquitination is generally sufficient for proteins to be internalized into primary endosomes, and Lys-63-linked polyubiquitination enhances internalization rates (Galan & Haguenauer-Tsapis, 1997; Springael *et al.*, 1999).

1.7.2.2 Autophagy-lysosome degradation by ubiquitin signaling

Autophagy mainly degrades long-lived proteins and organelles. Protein degradation by autophagy is largely non-selective. However, lines of evidence have suggested the existence of selective autophagy, which recruits ubiquitin as one of the signals (Kraft et al., 2010). Studies have identified several proteins that transport specifically ubiquitinated proteins to autophagosome for degradation, including p62, NBR1 and NDP52 (Pankiv et al., 2007; Kirkin et al., 2009; Thurston et al., 2009). All of them contain a ubiquitin-binding domain and one or two recognition domains for LC3/Atg8, a critical component of autophagosome. In addition to selective autophagy, the common ubiquitination signal hosts the crosstalk between proteasomal and autophagic degradation. When proteasome is inhibited, autophagic activity is enhanced (Ding et al., 2007; Pandey et al., 2007). In this process, histone deacetylase 6 (HDAC6) promotes aggresome formation of already ubiquitinated protein, which is then transported on microtubule to be degraded by autophagy (Iwata et al., 2005; Pandey et al., 2007). Other proposed mechanisms for the compensatory autophagic degradation include the upregulation of Atg5/Atg7 and LC3 expression upon proteasome inhibition, probably by transcription factor ATF4 (Milani et al., 2009; Zhu et al., 2010).

1.8 De-ubiquitinating enzymes (DUBs)

Deubiquitination is the reverse process of ubiquitination, which removes ubiquitin from its substrates. DUBs are a large group of enzymes that carry out this process. They specifically cleave ubiquitinated substrates after the terminal carbonyl of ubiquitin Gly-76 (Amerik & Hochstrasser, 2004).

There are approximately 80 DUBs identified so far, which can be classified into five distinct subgroups based on their sequence similarities and likely mechanisms of action: ubiquitin carboxyl-teminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumour proteases (OTUs), Josephins, and JAB1/MPN/MOV34 metalloenzymes (JAMM or MPN+). The first four subgroups belong to cysteine proteases, whose enzymatic activities rely on a Cys-His catalytic diad or a Cys-His-Asp/Asn triad. The fifth subgroup (JAMM/MPN+) is a type of zinc-dependent metalloprotease (reviewed in (Komander *et al.*, 2009)).

1.8.1 Physiological roles of DUBs

The large group of DUBs serve a variety of functions, which include processing ubiquitin precursors, rescuing substrate from degradation, removing non-degradative ubiquitin signal, removing ubiquitin immediately before protein degradation, editing and recycling free ubiquitin and disassembly of unanchored ubiquitin oligomers (Komander *et al.*, 2009) (Figure 1.5).



Figure 1.5 Physiological roles of DUBs

(A) Ubiquitin is encoded by four genes (*UBB*, *UBC*, *UBA52* and *UBA80*). Ubiquitin precursors are linear fusions of multiple copies of ubiquitin (as of *UBB* and *UBC*) or ubiquitin fused to the amino terminus of ribosomal proteins (as of *UBA52* and *UBA80*). Generation of free ubiquitin from these precursors is a key function of deubiquitinases (DUBs). (B) Deubiquitylation can reverse both degradative and non-degradative ubiquitin signal. (C) Proteasome-associated DUBs can remove ubiquitin from the substrate immediately before it enters the 20S core of proteasome for degradation. (D) DUBs have a crucial role in maintaining ubiquitin homeostasis. They are responsible for editing and recycling used ubiquitin, and the disassembly of polyubiquitin chains.

Processing ubiquitin precursors. Human ubiquitin is encoded by four genes: *UBB*, *UBC*, *UBA52* and *UBA80*. It is transcribed and translated into a linear fusion of multiple copies of ubiquitin (as in *UBB* and *UBC*) or fusion of a ubiquitin and a ribosomal protein (as in *UBA52* and *UBA80*). Proper processing of these precursors is essential for the generation and function of ubiquitin. DUBs are able to co-translationally cleave ubiquitin sandwich fusions and provide highly coordinated and efficient production of ubiquitin (Turner & Varshavsky, 2000). Among all DUBs, UCHL1 and UCHL3, two DUBs of the UCH family, are likely to carry out this function, as they were able to cleave translational products of *UBA52* and *UBA80* efficiently *in vitro* (Larsen *et al.*, 1998).

Regulation of protein stability. Some DUBs remove the polyubiquitin chains from the substrates and rescue them from proteasomal degradation. For example, USP28 stabilizes MYC, a proto-oncogene in many human tumors, by interacting with its E3 ligase (Popov *et al.*, 2007). USP2a deubiquitinates fatty acid synthase and protects it from proteasomal degradation (Graner *et al.*, 2004). Other prominent examples include USP7 and its specific substrate MDM2, an E3 ubiquitin ligase for p53 (Cummins & Vogelstein, 2004). Protein stabilization by DUBs is also exemplified in endosomal-lysosomal degradation. For example, AMSH is an endosome-associated DUB in that it disassembles Lys-63-linked polyubiquitination of EGFR and rescues it from lysosomal degradation (Clague & Urbe, 2006). Similarly, UCHL3 deubiquitinates ENaC and brings it back to the apical surface, which will otherwise be endocytosed and degraded with the ubiquitin tag (Butterworth *et al.*, 2007)

Negative regulation of ubiquitin signals. DUBs also reverse non-degradative ubiquitin signalings. One example is the dynamic ubiquitination/deubiquitination of histones. Four DUBs (MYSM1, USP3, USP16 and USP22) have been shown to directly deubiquitinate histone H2A or H2B and regulate gene transcription (Joo *et al.*, 2007; Nicassio *et al.*, 2007; Zhu *et al.*, 2007; Zhang *et al.*, 2008). Another wellcharacterized reversible ubiquitination is exemplified in the NF- κ B signaling. The activation of NF- κ B requires the activation of IKK (inhibitor of NF- κ B kinase), which is signaled by ubiquitination of TRAF6 or RIP1. A20, a DUB that belongs to the OTU family, reverses the ubiquitination of TRAF6 and RIP1, and therefore negatively regulates the activation of NF- κ B signaling (Boone *et al.*, 2004; Wertz *et al.*, 2004).

Disassembly of ubiquitin oligomers. Unanchored polyubiquitin chains are produced in most eukaryotes (Pickart, 2000), possibly from endo-deubiquitination of polyubiquitinated proteins by specific DUBs. The further disassembly of polyubiquitin chains is critical in that (1) excessive polyubiquitin chains inhibit proteasomal degradation and (2) recycled ubiquitin is an important source of the free ubiquitin pool. Studies show that one particular DUB, isopeptidase T in mammals and Ubp14 in yeast, is responsible for cleaving unanchored polyubiquitin chain into free ubiquitin (Hadari *et al.*, 1992; Wilkinson *et al.*, 1995; Amerik *et al.*, 1997).

Proteasome-associated DUBs. After proteins are marked by Lys-48-linked polyubiquitin chains, DUBs can deubiquitinate proteins and rescue them from degradation. Alternatively, they can remove ubiquitin chains and promote protein degradation. This concept is exemplified by three distinct DUBs that are associated with the 26S proteasome: RPN11 (also known as POH1), USP14 and UCH37 (also known as UCHL5) (reviewed in (Lee *et al.*, 2011)). All three DUBs are associated with the 19S regulatory particle. Interestingly, the three DUBs affect the UPS in different ways. RPN11 is responsible for removing ubiquitin chains from substrates to facilitate their translocation to the narrow cylindrical 20S proteasome core (Yao & Cohen, 2002). In contrast, inhibition of USP14 and UCH37 promotes the proteasomal degradation in the cell (Koulich *et al.*, 2008; Lee *et al.*, 2010).

1.9 Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1)

UCHL1 belongs to the UCH family of the DUBs. It is encoded by a single gene located on chromosome 4. It is a 223-amino acid cytosolic cysteine protease, whose catalytic mechanism involves a nucleophilic cysteine thiol in a catalytic triad. UCHL1 is able to cleave ubiquitin precursors as well as ubiquitinated proteins such as α synuclein and β -catenin. In addition to its hydrolase activity, UCHL1 has also been found to have ubiquitin ligase activity at higher concentrations, transforming monoubiquitinated substrates into di-ubiquitinated proteins. UCHL1 is essential for neuronal integrity, especially for neurons in the gracile tracts. Moreover, dysfunction of UCHL1 has been identified in neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease, and in many types of cancer.

1.9.1 Distribution of UCHL1

UCHL1 was originally detected by two-dimensional polyacrylamide gel electrophoresis in an effort to discover brain-specific proteins in humans (Jackson & Thompson, 1981). It is expressed predominantly in the brain and neuroendocrine systems, with detectable expression in kidneys and reproductive system (Jackson & Thompson, 1981; Doran *et al.*, 1983; Wilson *et al.*, 1988). UCHL1 accounts for 1-2% of total brain soluble proteins. Immunolabeling showed that UCHL1 is localized to neurons in the cerebral cortex but not to glial cells (Doran *et al.*, 1983). Further immunostaining studies showed that UCHL1 is expressed strongly and uniformly in almost all neurons, especially in large neurons such as Purkinje cells and cortical neurons of the cerebrum (Wilson *et al.*, 1988). Because of its rich expression in large neurons throughout the axons to the smallest terminal branches, UCHL1 has been used as a neuronal marker to visualize periphery nerve network (Nemeth & Puri, 2000; Pintelon *et al.*, 2007) and to detect re-innervation of the nervous system (Gray *et al.*, 1992; Grisk *et al.*, 2001).

Apart from its specific and strong expression in neurons, UCHL1 is also a highly conserved protein. Using a polyclonal rabbit antiserum raised against intact human UCHL1, Jackson *et al.* showed that UCHL1 is not only expressed in mammals such as sheep, rat and guinea-pig, but also in species evolutionarily far from humans such as chicken, trout, and frog, which indicated its existence from at least 400 million years ago (Jackson *et al.*, 1985).

1.9.2 Biochemical and structural properties of UCHL1

UCHL1 is a cysteine protease, whose catalytic cysteine thiol resides in its catalytic triad. Site-directed mutagenesis revealed that Cys90 and His161 are the two active site residues (Larsen *et al.*, 1996). Among a number of ubiquitin derivatives, UCHL1 preferentially cleaves small substrate such as amino acid and oligopeptide from C-terminus of ubiquitin, but not large leaving groups such as proteins (Larsen *et al.*,

1998). UCHL1 is also able to cleave proubiquitin gene products such as UBA52 (Larsen *et al.*, 1998). Structural studies confirmed the above discoveries from biochemistry experiments. Using X-ray crystallography, Das *et al.* demonstrated that the putative catalytic triad C90, H161, and D176 reside on three secondary structure elements: a helix (α 3), a strand (β 3), and a loop (L9), which together form the active site cleft. Moreover, ligand-induced conformational change is required for the activation of UCHL1, implicating a mechanism for substrate specificity of UCHL1 (Das *et al.*, 2006).

1.9.3 Enzymatic activities of UCHL1

As described above, UCHL1 serves as a ubiquitin hydrolase and specifically cleaves ubiquitin after the C-terminal glycine. Apart from its de-ubiquitinating activities, UCHL1 also has other important functions. Liu *et al.* demonstrated that UCHL1 exhibits ubiquityl ligase activity. In a crude cell-free system, incubation of UCHL1 with free ubiquitin and ubiquitin AMC, a fluorogenic substrate for DUBs, led to the production of di-ubiquitins with the Lys-63 linkage (Liu *et al.*, 2002). This poly-ubiquitin chain extension, if proved to be true *in vivo*, may be a signal for directing substrates away from ubiquitin proteasome degradation. UCHL1 also serves as a mono-ubiquitin stabilizer. Osaka *et al.* demonstrated decreased free ubiquitin pool in *gad* mice, an equivalent of UCHL1 knock-out mouse model. In accordance, overexpression of UCHL1 prolongs the half-life of free ubiquitin (Osaka *et al.*, 2003). While the substrates of UCHL1 may be limited due to its structural and biochemical characteristics (Larsen *et al.*, 1998; Das *et al.*, 2006), it is possible that UCHL1 regulates protein metabolism in an indirect way by recycling ready-to-use free ubiquitins.

1.9.4 Substrates of UCHL1

UCHL1 has been shown to process ubiquitin precursors UBA52 and UBA80 (Larsen *et al.*, 1998). Biochemical and structural studies predict that it is probably not efficient in cleaving ubiquitins from large proteins (Larsen *et al.*, 1998). Nevertheless, a few substrates of UCHL1 have been reported. UCHL1 is able to directly cleave ubiquitin from β-catenin (Bheda *et al.*, 2009) and α-synuclein (Liu *et al.*, 2002) and rescue them from degradation. UCHL1 also reduces levels of ubiquitinated inhibitor of kappa B- α (IkB- α) and attenuates NF- κ B activity (Takami *et al.*, 2007). Moreover, physical interactions have been detected between UCHL1 and JAB1-p27 complex (Caballero *et al.*, 2002), and UCHL1 and lysosome-associated membrane protein 2-A (LAMP-2A) (Kabuta *et al.*, 2008a), although it has not been defined whether any of them are its substrates.

1.9.5 Gene regulation of UCHL1

Shortly after the cloning of human *UCHL1* cDNA (Day & Thompson, 1987), its proximal promoter region was characterized. A classic TATA box was identified 20 to 30 bp upstream of the transcription start site (TSS) (Day *et al.*, 1990). Furthermore, a fragment within the region -182 to -123 relative to the TSS was identified as an essential positive cis-acting regulatory sequence (Mann *et al.*, 1996). This 59-bp fragment turned out to be a bi-directional regulator, which activates the transcription of UCHL1 in human neuroblastoma SH-SY5Y cells and represses it in Hela cells (Trowern & Mann, 1999). Subsequent researches revealed the mechanism of bidirectional regulation. It was discovered that a 20-bp sequence within this 59-bp fragment is a functional neuron restrictive silencer element (NRSE). In non-neural cells, repressor element 1 silencing transcription factor/neuron restrictive silencer factor (REST/NRSF) binds to NRSE and represses the transcription of UCHL1 (Barrachina *et al.*, 2007). The transcriptional regulation by REST/NRSF may explain its neuronal-specific expression. The promoter activity of *UCHL1* is also regulated by the β -catenin/TCF transcriptional complex (Bheda *et al.*, 2009). Bheda *et al.* identified two putative TCF4 binding sites on *UCHL1* promoter. Overexpression of TCF4 together with β -catenin activator LiCl significantly increases the activity of *UCHL1* promoter, but not that of mutants whose putative TCF4 binding sites are mutated (Bheda *et al.*, 2009). The discovery suggested a mechanism for elevated UCHL1 expression in cancerous tissues via the oncogenic β -catenin/TCF pathway.

1.9.6 Post-translational modification of UCHL1

Post-translational modification of UCHL1 is another way by which the activity of UCHL1 is regulated. UCHL1 can be covalently modified by 4-hydroxynonenal (4-HNE), an endogenous product in cells under oxidative stress. The oxidation of UCHL1 by 4-HNE reduces its hydrolase activity (Nishikawa *et al.*, 2003) and ubiquitin-binding ability (Kabuta *et al.*, 2008b). Notably, the oxidative modification of UCHL1 is associated with idiopathic AD and PD. By using 2D gel electrophoresis and mass spectrometry, Choi *et al.* identified UCHL1 as a major target of oxidative damage. The level of carbonyl-modified and therefore partially dysfunctional UCHL1 is significantly higher in AD and PD brains than in controls (Choi *et al.*, 2004).

Another important post-translational modification of UCHL1 is its own monoubiquitination. UCHL1 can be ubiquitinated at lysine residues near its catalytic site. Mono-ubiquitination inhibits the ubiquitin-binding ability of UCHL1. The time span of this modification is regulated by UCHL1 itself, as UCHL1 catalyzes its own deubiquitination intramolecularly (Meray & Lansbury, 2007).

1.10 UCHL1 and diseases

Dysfunction of UCHL1 has been identified in neurodegenerative diseases such as AD and PD, and in many types of cancer. Researches on its role in neurodegenerative diseases have obtained information mainly from its missense mutations in patients, and from its spontaneous deletions in laboratory mouse strains. In cancer research, upregulation or down-regulation of UCHL1 has been identified in different types and at different stages of cancerous tissues. However, whether dysfunctional UCHL1 plays a causal role in cancer development remains elusive.

1.10.1 UCHL1 and cancer

UCHL1 has caught the eye of cancer researchers, for its abnormal expression in many types of cancerous tissues. While under normal condition UCHL1 is mainly expressed in the brain (Doran *et al.*, 1983; Wilson *et al.*, 1988), its abnormal expression was first detected in non-small cell lung cancer (Hibi *et al.*, 1998; Hibi *et al.*, 1999). Further studies revealed the dysregulation of UCHL1expression in many types of cancers. However, the results were conflicting and far from conclusive. Up-regulation of UCHL1 has been found in pancreatic cancer (Tezel *et al.*, 2000), invasive colorectal cancer (Yamazaki *et al.*, 2002), esophageal squamous cell carcinoma (Takase *et al.*, 2003), gallbladder cancer (Lee *et al.*, 2006), and osteosarcoma (Liu *et al.*, 2009), whereas lower expression of UCHL1 has been detected in prostate cancer (Ummanni *et al.*, 2011) and cervical carcinoma cell lines (Rolen *et al.*, 2006).

The controversy of UCHL1's role in cancer lies in whether it serves as an oncogene or a tumor suppressor gene, two essentially opposite functions. Advocates for its oncogene function demonstrated that increased UCHL1 was associated with advanced pathological stage in non-small cell carcinoma (Hibi *et al.*, 1999), and with tumor progression in colorectal cancer (Yamazaki *et al.*, 2002). Supporters for its tumor suppressor role claimed that hypomethylation and increased expression of UCHL1

was associated with better prognosis in breast cancer (Trifa *et al.*, 2013), decreased cell proliferation in prostate cancer (Ummanni *et al.*, 2011) and reduced metastasis in colorectal cancer (Mizukami *et al.*, 2008). It should be noted, however, that most of the reports were correlative analysis without experimental manipulations, and therefore were not able to establish a causal relationship between UCHL1 and cancer development.

Recently studies have revealed the molecular mechanism underlying the oncogenic property of UCHL1. Using co-immunoprecipitation, Bheda *et al.* identified physical interaction of endogenous UCHL1 and β -catenin. UCHL1 decelerated the degradation of β -catenin and activated the oncogenic β -catenin/TCF signaling (Bheda *et al.*, 2009). Zhong *et al.* confirmed in both *in vitro* and *in vivo* colorectal cancer model that UCHL1 activated β -catenin/TCF pathway, which in turn increased cell proliferation and migration (Zhong *et al.*, 2012). Kim *et al.* proposed another mechanism that supported UCHL1's oncogenic role. In a non-small lung cancer model, UCHL1 promoted tumor cell invasion and metastasis through Akt signaling pathway (Kim *et al.*, 2009). Consistently, UCHL1 overexpression strongly accelerated lymphomagenesis in Emu-myc transgenic mice through Akt pathway by downregulating the antagonistic phosphatase PHLPP1 (Hussain *et al.*, 2010). On the other hand, studies supporting UCHL1 as a tumor suppressor gene are lacking.

1.10.2 UCHL1 in neurodegenerative diseases

UCHL1 first raised the attention in the field of neurodegenerative disease when it was found co-localized with cortical Lewy bodies in PD (Lowe *et al.*, 1990). Not long after, an I93M missense mutation of UCHL1 was identified in two siblings with earlyonset familial PD in a German pedigree, further indicating its potential roles in PD pathogenesis (Leroy *et al.*, 1998). Moreover, genetic deletions of UCHL1 in mouse strains (Yamazaki *et al.*, 1988; Walters *et al.*, 2008) and a missense mutation in a consanguineous union family in Turkey (Bilguvar *et al.*, 2013) resulted in phenotypes of severe progressive neurodegeneration. The evidence strongly supported a causal relationship between UCHL1 and neurodegenerative diseases.

1.10.2.1 The I93M mutation of UCHL1 in PD

In 1998, an I93M missense mutation in UCHL1 was identified in two siblings in a German family with early-onset familial PD (Leroy *et al.*, 1998). The mutation in one chromosome was sufficient to cause the disease. Both patients displayed typical PD symptoms, which began with resting tremor at age 49 and 51, and progressed to rigidity, bradykinesia and postural instability (Leroy *et al.*, 1998). Combined with previous discovery that UCHL1 was co-immunostained with Lewy bodies in the brains of PD patients (Lowe *et al.*, 1990), UCHL1 became a promising candidate for PD pathogenesis. Subsequent studies using transgenic mice confirmed the effect of I93M mutation in PD development. In a transgenic mouse strain expressing human UCHL1_{193M} to the level of approximately 1% of endogenous mouse UCHL1, less dopaminergic neurons in the substantia nigra were detected at the age of 20 weeks old. Moreover, these mice displayed PD-like neuropathologies (Setsuie *et al.*, 2007).

Mounting researches have tried to explain why this single missense mutation leads to early-onset familial PD. One hypothesis is its loss of hydrolase activity to ~50% of UCHL1_{WT} (Leroy *et al.*, 1998). However, this notion has been challenged. In UCHL1_{193M} transgenic mouse, extra human UCHL1_{193M} is expressed in addition to endogenous mouse UCHL1, which meant that these mice exhibited as much endogenous UCHL1 hydrolase activity as wildtype mice. Therefore, PD-like neuropathologies in these mice indicated a gain-of-toxic function of this mutation. Other hypotheses addressing the toxicity of UCHL1_{193M} include its insolubility and its effect on the aggregation of other proteins. Kabuta *et al.* demonstrated that, similar to carbonyl-modified UCHL1, UCHL1_{193M} contained less α -helix content but more β sheet content, and therefore was prone to form insoluble proteins (Kabuta *et al.*, 2008b). The same group also discovered that compared to UCHL1_{WT}, UCHL1_{193M} abnormally interacted with LAMP-2A, the lysosome receptor for chaperone-mediated autophagy (CMA). The aberrant interaction inhibited CMA and subsequently increased the protein level of α -synuclein (Kabuta *et al.*, 2008a). Using a system biology approach, Proctor *et al.* combined mathematical modeling with actual experiments, and discovered that UCHL1_{193M} led to protein aggregation even in the absence of proteasome inhibition (Proctor *et al.*, 2010). The increased vulnerability by this mutation, even when cellular stress (eg. proteasome inhibition) was not present, may account for its toxicity to neurons. Biophysical studies on I93M revealed the structure basis of its biochemical characteristics. By intrinsic fluorescence and far-UV CD, UCHL1_{193M} was shown to have more exposure of hydrophobic surface area, which may explain its increased insolubility and aberrant interaction with LAMP-2A (Andersson *et al.*, 2011).

1.10.2.2 The S18Y polymorphism of UCHL1 in PD

A case-control study was conducted in PD patients in order to investigate the association of PD with the UCHL1 I93M mutation. The mutation was not identified in any of the PD patients or controls. Instead, a common S18Y polymorphism of UCHL1(Lincoln *et al.*, 1999) was found to have a protective role against PD (Maraganore *et al.*, 1999). Since the initial report, at least 15 studies have been carried out to examine the association of the S18Y variant with PD, which reached mixed positive and negative results (meta-analysized in (Maraganore *et al.*, 2004; Healy *et al.*, 2006)). The apparently conflicting data may be due to population discrepancy and the relatively small sample sizes in individual studies. While the association with PD may appear inconclusive, the frequency of the S18Y variant indeed varies across geographic regions. The frequency of the Y allele is much higher among people of Asian descent (~50%) than among European descent (~20%). Further analysis separated subjects of Asian origin from those of European origin, and confirmed the reduced risk of PD by the Y allele in both subpopulations (Ragland *et al.*, 2009).

Several research groups have been investigating the structural and enzymatic characteristics of UCHL1_{S18Y}. Liu *et al.* discovered that, apart from the de-ubiquitination activity, UCHL1 also serves as a ubiquitin ligase and produces α -synuclein-(ubiquitin)₂ through Lys-63 linkage. Since UCHL1_{S18Y} has comparable hydrolase activity but lower ligase activity than UCHL1_{WT}, Liu *et al.* proposed that the S18Y variant reduces the risk of PD by decreasing level of α -synuclein-(ubiquitin)₂, thus preventing α -synuclein from forming protofibril and from depositing in Lewy bodies in brains (Liu *et al.*, 2002). Alternatively, Kyrati *et al.* suggested that the protective role of S18Y is conferred by its antioxidant function, as it reduces the generation of reactive oxygen species upon various insults (Kyratzi *et al.*, 2008).

1.10.2.3 The E7A mutation of UCHL1 in childhood-onset neurodegeneration

While the I93M mutation and S18Y polymorphism in UCHL1 may cause some controversies over whether they are truly pathogenic or protective, the recently identified homozygous missense mutation E7A induced striking phenotype of early-onset neurodegeneration. Three siblings born of consanguineous parents from a Turkish family developed progressive optic atrophy at age 5, followed by spasticity, cerebellar ataxia, peripheral neuropathy and myokymia (Bilguvar *et al.*, 2013). The genetic abnormality was pinpointed to the E7A mutation in UCHL1 using homozygosity mapping and whole-exome sequencing. The mutation in the ubiquitin binding region of UCHL1 led to at least sevenfold reduction in its affinity to ubiquitin, and a near complete loss of its hydrolase activity (Bilguvar *et al.*, 2013). The parents of the siblings were both heterozygous for the mutation but did not develop any phenotypes of neurodegeneration, which indicated the redundant expression of UCHL1 in the nervous system.

1.10.2.4 Gad mice, an equivalent of UCHL1 knockout model

In addition to point missense mutations in human beings, spontaneous deletions of UCHL1 have been discovered in laboratory mouse strains. The loss-of-function models provide invaluable information for studying the function of UCHL1. Among them the best-characterized model is the *gad* mice, whose name was derived from its prominent phenotype of gracile axonal dystrophy.

As early as in 1984, a few mice in an inbred colony maintained in Nagaya University in Japan began to exhibit hindlimb paralysis followed by early death at the age of 5 or 6 months old (Yamazaki et al., 1988). The traits were inherited in an autosomal recessive manner and were pinpointed to a deletion of exon 7 and 8 of the UCHL1 gene. A shorter fragment of UCHL1 cDNA was amplified in gad mice using RT-PCR, whereas no full-length or truncated UCHL1 protein was detected (Saigoh et al., 1999). Therefore, the gad mouse is an equivalent of UCHL1 knockout model. Its major neurological phenotype is the 'dying back' axonal dystrophy starting from the axons in the gracile nucleus all the way to the cell bodies in the dorsal root ganglia (Mukoyama et al., 1989; Kikuchi et al., 1990). Other degenerative processes are observed in the spinocerebellar tracts (Kikuchi et al., 1990) and motor neurons (Miura et al., 1993). In accordance to the 'dying back' style of axonal dystrophy, accumulated APP and A β were detected in the gracile tract in the same temporal and spatial pattern (Ichihara et al., 1995). Since APP plays an important role in axonal transport and is a central molecule in AD pathogenesis, this observation confirmed the axonal degeneration process and indicated a possible role of UCHL1 in AD. Notably, gad mice displayed impaired memory maintenance in passive avoidance test and impaired theta-burst stimulation-induced LTP at as early as 6 weeks old, when no visible atrophy or abnormal A β accumulation was detected in the hippocampus or cortex (Sakurai et al., 2008). This finding suggested that loss of UCHL1 also affects daily neuronal activity in addition to axonal degeneration.

By comparing *gad* and WT mice, researchers have been looking for proteins that are regulated by UCHL1. Using 2D gel electrophoresis, Goto *et al.* identified glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and 14-3-3 as two most prominently accumulated proteins in *gad* mice. Further histochemical analysis revealed the increase of sulfonated GAPDH and 4-HNE, two markers of oxidative stress, in the axons of *gad* mice (Goto *et al.*, 2009), indicating possible involvement of oxidative stress in axonal degeneration.

Other spontaneous mutations of UCHL1 in mouse strains include the UCHL1^{gad-J} (nm3419) strains. The nm3419 mutation removes the final 24-bp of exon 6 and the first 771-bp of intron 6 of *UCHL1*, resulting in the complete loss of protein expression (Walters *et al.*, 2008). The homozygous mice started to display neuromuscular defects at 4 weeks of age, followed by severe weight loss and premature death (Walters *et al.*, 2008). The similar phenotypes in the two mouse strains confirmed the indispensible role of UCHL1 in neuronal integrity.

1.10.2.5 UCHL1 and AD

Although mutations in UCHL1 have not yet been identified in familial AD, its dysregulation has been demonstrated in sporadic AD cases. A correlative study performed by Choi *et al.* showed the downregulation of UCHL1 protein level in sporadic AD and PD brains. In particular, soluble UCHL1 level was inversely proportional to the number of neurofibrillary tangles in AD brains (Choi *et al.*, 2004). Interestingly, the levels of carbonyl-modified UCHL1 in AD and PD brains were significantly higher than those in controls, leaving even less functional UCHL1 (Choi *et al.*, 2004).

In *gad* mice, loss of UCHL1 led to impaired LTP and memory maintenance in passive avoidance test at as early as 6 weeks of age (Sakurai *et al.*, 2008), which indicated a role of UCHL1 in synaptic plasticity and learning and memory. Inspired by this

observation, Gong *et al.* performed rescue experiments of UCHL1 in APP/PS AD mouse model. They showed that UCH activity was reduced in APP/PS mice compared to WT mice. Application of UCHL1 protein rescued UCH activity as well as the LTP impairment in hippocampal slices. Moreover, intraperitoneal injection of UCHL1 TAT-fusion protein improved the retention of contextual learning in fear conditioning test in APP/PS mice (Gong *et al.*, 2006).

It is intriguing that single injection of UCHL1 protein could acutely affect synaptic plasticity and improve learning and memory deficits. This function probably is not closely related to its role in long-term neuronal integrity of the gracile tract. As AD-associated proteins including APP, BACE1 and components of γ -secretase complex can all at least partly be degraded through ubiquitination signaling, we proposed that UCHL1 is able to affect AD pathogenesis by regulating the degradation of those AD-associated proteins. This effect, if true, may explain the observations made by Gong et al., and indicate a disease-modifying strategy in AD therapeutics.

1.11 Rationale of this study

Pathogenic mutations in UCHL1 have been identified in a variety of neurodegenerative diseases, including early-onset familial PD (Leroy *et al.*, 1998), childhood-onset progressive neurodegeneration (Bilguvar *et al.*, 2013), and gracile axonal dystrophy (Yamazaki *et al.*, 1988). Although no mutation in UCHL1 has been identified in AD, its protein level has been found to be reduced and inversely proportional to the number of neurofibrillary tangles in AD brains (Choi *et al.*, 2004). Despite the extensive effort made by the researchers, the molecular mechanisms underlying the role of UCHL1 in neurodegenerative diseases remain elusive.

Recently, Gong *et al.* proposed a mechanism by which UCHL1 affects the memory ability in AD. They demonstrated that overexpression of UCHL1 in APP/PS double

transgenic mice promoted the PKA/CREB signaling pathway, restored LTP in the hippocampus and thereby rescued the learning and memory deficits (Gong *et al.*, 2006).

We hypothesized from another point of view, that UCHL1 affects AD progression by regulating APP processing, A β production and neuronal death. We brought forward the hypothesis for the following reasons. Firstly, in *gad* mice, the UCHL1 knockout model, APP and A β accumulated in the gracile tract concomitantly with the 'dying back' style of axonal dystrophy (Ichihara *et al.*, 1995). Secondly, many of the AD-associated proteins, including β -secretase BACE1 and components of γ -secretase complex could all be degraded via the UPS (Fraser *et al.*, 1998; Qing *et al.*, 2004; He *et al.*, 2006; He *et al.*, 2007). As a DUB, UCHL1 may regulate the ubiquitination/deubiquitination process and affect the degradation of those A β -producing molecules. Thirdly, the striking neurodegenerative phenotypes caused by UCHL1 loss-offunction mutations strongly implicate its role in neuronal loss in AD (Yamazaki *et al.*, 1988; Bilguvar *et al.*, 2013). Specifically, we proposed:

(1) To examine the transcriptional regulation of UCHL1. Previous findings suggested that UCHL1 may play an important role in AD pathogenesis. Therefore the lower expression of UCHL1 in AD brains (Choi *et al.*, 2004) may have pathogenic significance. Thus we were interested in revealing the underlying mechanism that leads to UCHL1 expression dysregulation. Specifically we looked into the transcriptional regulation of UCHL1. If we could identify some transcription factors that regulate UCHL1 transcription, we would have a better understanding of why UCHL1 level is chronically decreased in AD brains. The results may also indicate the signaling pathways that contribute to AD pathogenesis through the regulation of UCHL1. In this chapter, we cloned the 5' promoter region of *UCHL1* and identified functional NF- κ B binding site on it. The expression regulation of UCHL1 was examined at mRNA level and protein level.

(2) To investigate the role of UCHL1 in A β production and neuronal death. We hypothesized that UCHL1 affects APP processing, A β production and neurodegeneration. From our preliminary experiment, we observed the increased BACE1 protein level by UCHL1 inhibition (Figure 3.1B). Therefore we started with determining the role of UCHL1 in BACE1 degradation. We would also explore its effect on the degradation of other molecules that are crucial for A β production. Since UCHL1 is a DUB that regulates ubiquitin signalings, we would focus on its effect on the ubiquitination and de-ubiquitination of these molecules. In addition to A β plaque deposition, neuronal loss is a prominent feature of AD pathology. In particular, apoptosis and activated caspases were detected in AD brains (Su *et al.*, 1994) and in cultured neurons treated with A β (Loo *et al.*, 1993). We looked into whether UCHL1 protects against neuronal death, especially that from A β -induced apoptosis. In this thesis we examined *in vitro* the effect of UCHL1 on APP processing, A β production and neuronal death by transgene expression and knockdown of UCHL1.

(3) To explore the potential of UCHL1 as a treatment strategy for AD. If UCHL1 does alter A β production or A β -induced toxicity *in vitro*, we would explore whether the hypotheses are applicable *in vivo*. We would examine the long-term effect of UCHL1 on the development of AD pathology and its potential as a disease-modifying strategy for AD therapeutics. In this thesis we examined the effect of UCHL1 on A β production, amyloid plaque formation and learning and memory abilities in AD mouse models.

The overall goal of this thesis was to investigate the transcriptional regulation of UCHL1, and the role of UCHL1 in APP processing, A β production and neuronal loss. Moreover, its effect on AD pathogenesis *in vivo* and its potential as a treatment strategy for AD were evaluated.

Chapter 2

NF-κB signaling inhibits UCHL1 gene expression

2.1 Introduction

UCHL1 is one of the most abundant proteins in the brain, accounting for approximately 1-2% of total soluble brain proteins. It is expressed predominantly in the central nervous system and neuroendocrine systems (Jackson & Thompson, 1981; Doran *et al.*, 1983; Wilson *et al.*, 1988). Interestingly, its expression is dysregulated in sporadic AD and PD brains (Choi *et al.*, 2004) and in various types of cancers (eg.(Hibi *et al.*, 1998; Tezel *et al.*, 2000; Yamazaki *et al.*, 2002)). Expression regulation of UCHL1 is worth exploring for a few reasons. Firstly, UCHL1 is highly expressed in neurons but not in other non-neuronal cells such as glials, indicating an important role in the nervous system and a cell type-specific expression regulation mechanism. Secondly, experimental studies have implicated potential causal relationships between UCHL1 and neurodegenerative diseases as well as cancers. Therefore, it is important to investigate why UCHL1 is dysregulated in these diseases for the better design of therapeutic interventions.

Researches have revealed a few strategies by which the transcriptional expression of UCHL1 is regulated. For example, a 59-bp fragment on the *UCHL1* promoter region was identified as a bi-directional regulator, which activates UCHL1 transcription in human neuroblastoma SH-SY5Y cells and represses it in Hela cells (Trowern & Mann, 1999). Further studies discovered a 20-bp functional NRSE within this region, to which REST/NRSF binds and represses UCHL1 transcription in non-neuronal cells (Barrachina *et al.*, 2007). The studies provided a mechanism for the inhibition of UCHL1 expression in non-neuronal cells. UCHL1 transcriptional expression is also regulated by the β -catenin/TCF complex. Two TCF4 binding sites were identified on *UCHL1* promoter, to which β -catenin/TCF binds and increases the promoter activity

of UCHL1 (Bheda *et al.*, 2009). The study suggested a role of the oncogenic β catenin/TCF pathway in the elevated UCHL1 expression in cancerous tissues. To date, however, no reports have indicated potential mechanisms that lead to UCHL1 dysregulation in neurodegenerative diseases. Here we proposed to study the transcriptional regulation of UCHL1 and look for answers to this question.

In this chapter, we cloned and analyzed the *UCHL1* gene promoter region. We identified a functional NF- κ B binding site within the promoter region. NF- κ B signaling was found to down-regulate UCHL1 expression and to mediate the inhibitory effect of lipopolysaccharide (LPS) and tumor necrosis factor- α (TNF α) on UCHL1 expression. Our study suggested that chronic inflammation in AD brains might compromise neuronal functions via the interaction of NF- κ B and UCHL1.

2.2 Methods

2.2.1 Primers and plasmids

The 5'-flanking regions of the human *UCHL1* gene were amplified by PCR from human BAC DNA clone RP11-397A2 (BACPAC Resources Center, CHORI). Primers were designed to include restriction enzyme sites so that PCR products could be easily cloned into the multi-cloning sites of pGL3-Basic (Promega, Madison, WI, USA). Ten fragments covering the 5' flanking region of the *UCHL1* gene from -1746 bp upstream to +36 bp downstream of the transcription start site at +1 (guanine) were amplified by PCR and inserted in front of the luciferase reporter gene (Luc) in the pGL3-basic expression vector. To construct the longest promoter plasmid pUCHL1-A, the primers -1746fXhoI (5'-ccgctcgaggagcgagactccgtctcaaaac) and +36rNcoI (5'catgccatgggcgcccggcagaaatagcctaggg) were used. This fragment was cloned into pGL3-Basic at the XhoI and NcoI sites. To construct pUCHL1-D, pUCHL1-A was cut with XhoI and NcoI. The fragment from the Xho I site on the insert (-336 bp) to the vector NcoI site was cloned into pGL3-Basic. To construct plasmids pUCHL1-B, pUCHL1-C, pUCHL1-E, pUCHL1-F, pUCHL1-G and pUCHL1-H, the following pairs of primers were used: -1746fXhoI and -746rHindIII (5'-gtatgattttgagcgagaggctg); -751fHindIII (5'-cettggttgccattccctttette) and +36rNcoI;-210fXhoI (5'caactcgaggacaaccaccagattatet) and +36rNcoI; -129fXhoI (5'caactcgagggttegtacccatetgg) and +36rNcoI; -129fXhoI and -18rHindIII (5'caacaggettggcagetataaage); -93fXhoI (5'- caactegaggettegtetegee) and +36rNcoI. A plasmid with the *UCHL1* promoter fragment from -129 bp to +36 bp in the reverse direction was constructed to generate pUCHL1-Fr with primers +36fXhoI (5'-caactegaggggeceggcagaaatage) and -129rNcoI(5'-catgccatggggttegtacccatetgge). To generate pUCHL1-I, pUCHL1-A was cut with Nhe I and NcoI. The fragment from the Nhe I site on the insert (-7 bp) to the vector NcoI site was cloned into pGL3-Basic at the NheI and Nco I sites. The human *UCHL1* promoter region and all inserts of constructed plasmids were sequenced by an automatic fluorescence-based DNA Sequencer (ABI PRISM DNA analyzer; Applied Biosystems). Computer-aided sequence analysis was performed with the SeqMan software (DNASTAR, Inc.).

2.2.2 Cell culture and transfection

HEK293, N2a cells, and SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mmol/L of sodium pyruvate, 2 mmol/L of L-glutamine and 1% penicillin/streptomycin (Invitrogen). RelA knockout (RelA-KO) fibroblast cell line, derived from E12.5–E14.5 mouse embryo fibroblasts, was cultured in DMEM supplemented with 15% FBS, β -mercaptoethanol, and ESGRO® (LIF) (Chemicon ESG1106) (Gapuzan *et al.*, 2005). All cells were cultured in a 37°C incubator regulated at 5% CO₂. Cells were plated and cultured to approximately 50% confluence before transfection. Cells were transfected using calcium phosphate or Lipofectamine 2000 as per manufacturer's instructions.

2.2.3 Luciferase assay

N2a cells, ReIA-KO cells or the wildtype control MEFs were plated onto 24-well plates 24 h prior to transfection. Cells were transfected with 0.5 μ g of DNA per well using Lipofectamine 2000 system. The pCMV-Luc plasmid of 1 ng was co-transfected to normalize the transfection efficiency of various luciferase reporter constructs. Forty eight hours after transfection, cells were harvested and lysed with 100 μ L 1X passive lysis buffer (Promega) per well. Firefly luciferase activities and *Renilla* luciferase activities were measured using the dual-luciferase reporter assay system (Promega). The firefly luciferase activity was normalized to the *Renilla* luciferase activity and expressed as relative luciferase units to reflect the promoter activity.

2.2.4 Rapid amplification of 5' complementary DNA ends assay

5'-RACE-PCR was performed to determine the transcription initiation site of *UCHL1*. Total RNA was extracted from SH-SY5Y cells with TRI reagent following the manufacturer's protocol (Sigma). Two reverse primers, 5'-ggaattctctcccgggtagcggtgca and 5'-ggaattccgcccggcagaaatagccta, corresponding to bp +90 to +110 and to bp +17 to + 36 of the 5' untranslated region, were synthesized. The 5'-RACE-PCR was carried out according to the FirstChoice[®]RLM-RACE Instruction Manual.

2.2.5 Gel shift assay

Gel shift assay was performed as previously described (Christensen *et al.*, 2004). To make NF- κ B-enriched nuclear extract, HEK293 cells were transfected with p65 expression vector and lysed in hypotonic buffer for subcellular fractionation. Probe oligonucleotides were labeled with IR700 Dye (LI-COR Biosciences) and annealed to generate double-stranded probes at a final concentration of 0.1 pmol/ μ L. For competition studies, nuclear extract was incubated with 0.1 pmol/ μ L labeled probes and 10× or 100× of unlabelled competition oligonucleotides for 20 min at room

temperature. For the super-shift assay, mouse anti-NF- κ B p65 monoclonal antibody (Sigma) was added to the gel shift reaction. The sequences of the oligonucleotides were: consensus NF- κ B: 5'-agttgaggggactttcccaggc; mutant NF- κ B: 5'- agttgagggccactttcccaggc; UCHL1-NF- κ B-Wt: 5'-ctcaaggaacacccacaca. The samples were analyzed on a 4% non-denaturing polyacrylamide gel and the gel was scanned using the Odyssey scanner (LI-COR Biosciences) at a wavelength of 700nm.

2.2.6 LPS and TNFα treatment

LPS (MILLIPORE) and TNF α (UPSTATE) were reconstituted in sterile water. For luciferase assay, N2a cells were co-transfected with pUCHL1-D/pUCHL1-E and pCMV-Luc. Twenty four hours after transfection, cells were treated with LPS at 30 ng/ml and TNF α at 10 ng/ml for 24 h. Cell lysates were analyzed for luciferase activity. For immunoblotting and semi-RT-PCR, N2a and SH-SY5Y cells were exposed to LPS at 30 ng/ml and TNF α at 10 ng/ml for 24 h and then lysed for protein and RNA extraction.

2.2.7 Semi-quantitative RT-PCR

Total RNA was extracted from cells using TRI reagent (Sigma). ThermoScript reverse transcriptase (Invitrogen) was used to synthesize the first-strand cDNA from an equal amount of the RNA sample according to the manufacturer's instructions. The synthesized cDNA templates were further amplified by Platinum *Taq* DNA polymerase (Invitrogen) in a 20 µl reaction volume. Thirty to 40 cycles of PCR were performed to cover the linear range of the PCR amplification. The *UCHL1* gene-specific primers 5'- agcgtgagcaaggagaagtc and 5'- gcgtgtctgcagaacagaag were used to amplify a 290-bp fragment of the *UCHL1* gene coding region. *β-actin* mRNA level served as internal control. Gene-specific primers 5'-ggacttcgagcaaggagatg and 5'- gaagcatttgcggtggag were applied to amplify a 462-bp fragment of the *β-actin* gene. The products were run on a 1% agarose gel. Gel images were captured using the GelDoc-It^{IM} imaging System and quantified with the Kodak Image Analysis.

2.2.8 Immunoblot analysis

Cells were lysed with RIPA buffer containing: 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulphate and protease inhibitor cocktail Complete (Roche Molecular Biochemicals). Cell lysates were briefly sonicated and centrifuged at 14,000 rpm for 10 min. For western blot analysis, samples were diluted in 4X SDS-sample buffer and loaded onto 12% tris-glycine or 16% Tris-Tricine SDS-PAGE and transferred to polyvindylidine fluoride (PVDF-FL) membranes. Membranes were blocked for 1 h in phosphate-buffered saline (PBS) containing 5% non-fat dried milk followed by overnight incubation at 4°C in primary antibodies diluted in the blocking medium. UCHL1 was detected using the anti-UCHL1 antibody BH7 (Novus Biologicals) (1:10 000). β-actin was detected using monoclonal antibody AC-15 (Sigma) (1:20 000). The membranes were rinsed in PBS with 0.1% Tween-20 and incubated with IRDye 800CW-labelled goat antimouse or IRDye 680CW-labelled goat anti-rabbit antibodies in PBS with 0.1% Tween-20 at 22°C for 1 h, and visualized on the Odyssey system (LI-COR Biosciences).

2.2.9 Statistical analysis

All results were presented as mean \pm SEM and analyzed by 2-tailed Student's *t*-test, one-way ANOVA or two-way ANOVA. Statistical significance is accepted when p<0.05 (*p<0.05, **p<0.01, ***p<0.001).

2.3 Results

2.3.1 Characterization of the human *UCHL1* gene promoter and its transcription start site

The human UCHL1 gene contains nine exons spanning 11 519 bp on chromosome 4 (4p14). It encodes a 24.824-kDa protein of 223 amino acids (Figure 2.1A). To study the transcriptional regulation of the human UCHL1 gene expression, the 5' flanking region of the human UCHL1 gene was cloned from human BAC genomic DNA clone RP11-397A2 (CHORI). The region spanning 1782 bp was sequenced (Figure 2.1B). 5'-RACE-PCR was performed to identify the transcription start site of the human UCHL1 gene. Two reverse primers, an outer primer located from +90 bp to +110 bp and an inner primer from +17 bp to +36 bp downstream of the translation start site ATG, were used for 5'-RACE assay. The RACE-PCR assay yielded a 90 bp DNA product (Figure 2.1C). DNA sequencing indicated that the major transcription start site was located at 45 bp upstream of the translation start site ATG (Figure 2.1D). This transcription initiation site was designated as +1 for the subsequent experiments. Sequence analysis showed that the human UCHL1 gene has a complex transcriptional unit. Computational analysis of the promoter region using MatInspector2.2 software (Genomatrix) revealed that the 5' flanking region contains several putative regulatory elements, such as NF-kB, GATA, NFAT, CREB, NRSF, YY1, AP1, and STAT (Figure 2.1B).



Figure 2.1 Sequence features of the human UCHL1 gene promoter

(A) The genomic organization of human *UCHL1* gene on chromosome 4. E represents exons. *UCHL1* gene consists of 9 exons. (B) The nucleotide sequence of the human *UCHL1* gene promoter. A 1782 bp fragment of the 5' flanking region of the human *UCHL1* gene was cloned from the human BAC genomic DNA by the primer walking strategy. The guanine +1 represents the transcription start site. The putative transcription factor binding sites are underlined in bold face. (C) The 90-bp product from 5'-RACE-PCR assay. (D) The 90-bp 5'RACE product was sequenced. The arrow points to the 5' end of the 5'RACE product, which corresponds to the transcription start site.

2.3.2 Functional analysis of the human UCHL1 gene promoter

To investigate the transcriptional regulation of the human *UCHL1* gene, a series of nested deletions of the 5' flanking fragments were subcloned into a promoterless luciferase reporter plasmid pGL3-basic. Expression of luciferase relies on the insertion and proper orientation of a functional promoter upstream of the luciferase gene. The pGL3-basic vector, which lacks eukaryotic promoter and enhancer
sequences, has little luciferase expression. Ten fragments covering different lengths from -1746 to +36 bp of the 5' flanking region of the human *UCHL1* gene were amplified by PCR and subcloned into pGL3-Basic vector according to restriction enzyme cutting sites as described in Section 2.1.1 (Figure 2.2A). The constructs were verified by gel analysis and sequencing (Figure 2.2B).



Figure 2.2 Deletion analysis of the human UCHL1 gene promoter

(A) Schematic diagram of a series of the human *UCHL1* deletion promoter constructs in pGL3basic vector. Arrows show the direction of transcription. The numbers represent the end points of the human *UCHL1* inserts relative to the TSS site. (B) The deletion plasmids were confirmed by restriction enzyme digestion. The vector size is 4.8 kb. *UCHL1* promoter fragment sizes range from 0.043 to 1.78 kb. The sequences of the inserts were further confirmed by sequencing. (C) The plasmid constructs were co-transfected with pCMV-Luc into N2a cells. Forty eight hours after transfection, the cells were harvested and luciferase activity was measured and expressed in relative luciferase units (RLU). The pCMV-Luc luciferase activity was used to normalize the transfection efficiency. The values represent means \pm SEM. N = 3, **p < 0.01, *p < 0.05, by oneway ANOVA with the *post hoc* Newman–Keuls test. Asterisks on individual columns stand for comparisons between that column and the control V column. To investigate whether the 5' flanking region contains the promoter of the human *UCHL1* gene, the deletion plasmids were co-transfected into N2a cells along with plasmid pCMV-Luc, which served as an internal transfection control. Plasmid pGL3-basic served as negative control (Figure 2.2C). Plasmid pUCHL1-A contained a 1.78-kb segment of the 5' flanking from -1746 to +36 of the *UCHL1* gene upstream of the luciferase reporter gene. Luciferase assay indicated that pUCHL1-A had significant promoter activities, which was valued 90.4 ± 2.1 relative luciferase unit (RLU) relative to pGL3-Basic (p < 0.01). The data indicated that the 1.78 kb fragment contained the functional promoter region of the human *UCHL1* gene.

Among all the deletion fragments, it was notable from the data that deletion from -336 bp to -210 bp resulted in a significant increase of promoter activity from 57.8 ± 0.3 RLU in pUCHL1-D (-336bp to +36bp) to 224.7 ± 9.6 RLU in pUCHL1-E (-210bp to +36bp) (p < 0.01), suggesting that this 126 bp fragment negatively affected the human *UCHL1* promoter activity. Moreover, deletion of the promoter region spanning -210 bp to -129 bp resulted in a significant decrease in promoter activities (24.6 ± 0.8 RLU in pUCHL1-F compared to 224.7 ± 9.6 RLU in pUCHL1-E, p<0.01). This indicated that there are potential positive-regulatory *cis*-acting elements in this region.

2.3.3 Identification of a functional NF-κB binding site on the human *UCHL1* gene promoter

The search for transcriptional factor binding sites revealed that the 1790 bp 5' flanking region of the human *UCHL1* gene contained one putative NF- κ B binding site at around -235 bp (Figure 2.1B). To determine whether the element is a functional NF- κ B binding site, gel shifting assay (GSA) was carried out. UCHL1-NF- κ B, a 20-bp double-stranded oligonucleotide probe corresponding to the *UCHL1* promoter region -243 to -224 bp, was synthesized and end labeled for GSA. A shifted protein-DNA complex band was observed after incubating the UCHL1-NF- κ B probe with HEK293

nuclear extract (Figure 2.3, lane 2). The intensity of this retarded band was reduced by adding $10 \times$ and $100 \times$ unlabeled NF- κ B consensus competition oligonucleotides (Figure 2.3, lanes 3 and 4). Consistently, the addition of the $10 \times$ and $100 \times$ unlabeled UCHL1-NF- κ B homologous probe also markedly decreased the signal of the shifted band (Figure 2.3, lanes 7 and 8). As expected, excessive mutant NF- κ B oligonucleotides with the binding site mutations had no competitive effect on the UCHL1-NF- κ B shifted band (Figure 2.3, lanes 5 and 6).



Figure 2.3 Identification of NF-kB binding element in the *UCHL1* gene promoter by gel shifting assay

GSA was performed as described in Methods. In lane 1 only free probe was detected. Lane 2 was a shifted DNA-protein complex formed between labeled UCHL1-NF-κB with nuclear extracts. Competition assays were carried out by further applying different concentrations of unlabeled competition oligonucleotides, including consensus NF-κB (lanes 3 and 4), mutant consensus NF- κ B (lanes 5 and 6), and homologous UCHL1-NF-κB (lanes 7 and 8). Lane 9 showed supershifted band with the anti-NF-κB antibody. The supershifted bands were eliminated by further addition of unlabeled competition NF-κB consensus oligonucleotides (lane 10).

A supershift analysis was conducted to further confirm the binding of NF- κ B element to the UCHL1-NF- κ B probe. A super-shift band was detected after the incubation of the anti-NF- κ B antibody with UCHL1-NF- κ B probe and nuclear extracts (Figure 2.3, lane 9). The supershifted band was competed away by further addition of a 100× unlabeled NF- κ B consensus oligonucleotides (Figure 2.3, lane 10). Taken together, the results clearly demonstrated that the human *UCHL1* promoter contained functional NF- κ B binding site in the region of -243 to -224 bp.

2.3.4 NF-κB down-regulates the human *UCHL1* gene promoter activity and inhibits UCHL1 expression

To determine whether NF- κ B affects *UCHL1* gene transcription, NF- κ B p65 expression plasmid or empty vector was co-transfected with pUCHL1-D into N2a cells. Overexpression of p65 markedly reduced *UCHL1* promoter luciferase activity to 4.8 ± 0.3% compared to controls (p < 0.01) (Figure 2.4A). To further examine whether NF- κ B affects the endogenous gene expression, semi-quantitative RT-PCR was conducted. Expression of NF- κ B p65 significantly reduced the endogenous mRNA level of the *UCHL1* gene to 59.1 ± 2.1% in N2a cells (p < 0.01) (Figure 2.4B and C), and to 52.5 ± 1.9% in SH-SY5Y cells (p < 0.01) (Figure 2.4D and E) compared to controls. Consistently, western blot analysis confirmed the decrease in UCHL1 expression following NF- κ B p65 overexpression. The protein levels of UCHL1 in N2a (Figure 2.4F and G) and SH-SY5Y (Figure 2.4H and I) cells were reduced by NF- κ B p65 expression to 49.0 ± 4.0% and 29.9 ± 3.2% (p < 0.01), respectively. Taken together, the data demonstrated that NF- κ B inhibited *UCHL1* gene expression via its transcriptional regulatory effect on the *UCHL1* gene promoter.



Figure 2.4 NF-KB inhibits UCHL1 transcriptional activation

(A) Repressed transcriptional activation of the *UCHL1* promoter by NF- κ B. The *UCHL1* promoter plasmid pUCHL1-D containing the NF- κ B binding site was co-transfected with NF- κ B expression plasmid pNF- κ B p65 into N2a cells. Expression of p65 markedly decreased the pUCHL1-D promoter activity. The values represent means \pm SEM. N= 3, *p < 0.01 by Student's *t*-test. (**B-E**) Decrease of endogenous UCHL1 mRNA levels in N2a cells (**B**) and SH-SY5Y cells (**D**) with transfected pNF- κ B p65. The mRNA levels of UCHL1 were determined by semi-quantitative RT-PCR and normalized against the levels of β -actin. (**C**)(**E**) Quantification of UCHL1 mRNA level by Kodak Image Analysis in N2a (**C**) and SH-SY5Y cells (**E**). Data are presented as mean \pm SEM. N = 3, *p < 0.01 by Student's *t*-test. (**F**)(**H**) Transfection of pNF- κ B p65 in N2a (**F**) and SH-SY5Y cells (**H**) resulted in reduction in UCHL1 protein expression. Cell lysates were analyzed by western blotting using mouse anti-UCHL1 monoclonal antibody and β -actin was used to control for protein loading. (**G**)(**I**) Quantification of UCHL1 protein levels in N2a (**F**) and SH-SY5Y cells (**H**) by Li-COR Imager. Data are presented as mean \pm SEM. N = 3, *p < 0.01 by Student's *t*-test.

2.3.5 UCHL1 gene transcription is up-regulated in RelA-KO cells

To further confirm the suppression of UCHL1 gene expression by NF- κ B, *UCHL1* promoter activity was examined in RelA-KO fibroblasts derived from the embryos of NF- κ B p65 knockout mice. pUCHL1-D was transfected into RelA-KO and wildtype controls. There was significant increase of luciferase activity in RelA-KO cells (239.4 ± 11.1%, *p* < 0.01) compared to control (Figure 2.5A), which confirmed the inhibitory effects by NF- κ B on UCHL1 transcriptional activation. We further

examined the endogenous mRNA level by semi-quantitative RT-PCR. UCHL1 mRNA level was increased by $346.3 \pm 5.8\%$ in RelA-KO cells relative to WT (p < 0.01) (Figure 2.5B and C). Consistent with the elevation of UCHL1 mRNA, western blot analysis showed that the UCHL1 protein level increased by $616.0 \pm 51.4\%$ fold in RelA-KO cells compared to WT (p < 0.01) (Figure 2.5D and E). The results further demonstrated that NF- κ B negatively regulated the transcription of the human *UCHL1* gene.





(A) The human *UCHL1* promoter activity was significantly increased in RelA-KO cells. RelA-KO and wildtype MEFs were transfected with pUCHL1-D. Values represent the percentages of normalized luciferase activity in the mean \pm SEM. N = 3 and *p < 0.01 by Student's *t*-test. (B) Endogenous UCHL1 mRNA level in RelA-KO and wildtype MEFs. Total RNA was isolated from RelA-KO and wildtype cells. Semi-quantitative RT-PCR was conducted to measure the endogenous level of the UCHL1 mRNA. (C) The ratio of UCHL1 to β -actin mRNA level was quantified by Kodak Image Analysis. The endogenous UCHL1 mRNA level was markedly increased in RelA-KO cells relative to control cells. Values represent mean \pm SEM. N = 3, *p < 0.01 by Student's *t*-test. (D) Immunoblot of endogenous UCHL1 mRNA-KO and control cells. UCHL1 protein band was detected by mouse anti-UCHL1 monoclonal antibody. (E) Quantification of (D) by Li-COR Imager. The endogenous UCHL1 protein level was significantly elevated in RelA-KO compared to control cells. Data are presented as mean \pm SEM. N = 3, *p < 0.01 by Student's *t*-test.

2.3.6 LPS and TNFa inhibits UCHL1 gene expression

NF-κB signaling plays an important role in gene regulation. LPS and TNFα are strong activators of NF-κB signaling pathway in inflammation. To investigate whether the NF-κB binding element in *UCHL1* promoter mediates the inflammatory effect on UCHL1 expression, we investigated the effect of LPS and TNFα on *UCHL1* promoter activity. N2a cells transfected with pUCHL1-D or pUCHL1-E were treated with LPS at 30 ng/mL or TNFα at 10 ng/mL for 24 h. Stimulation of LPS and TNFα led to a significant inhibition of *UCHL1* promoter activity (57.3 ± 0.9% and 54.9 ± 1.1%, respectively) in cells transfected with pUCHL1-D (p < 0.01) (Figure 2.6A, left). On the contrary, LPS and TNFα treatment had no effect on the promoter activity of pUCHL1-E, in which NF-κB response element mediated the inhibitory effect of LPS and TNFα on human *UCHL1* promoter activity.

Next semi-quantitative RT-PCR and western blot analysis were carried out to detect the endogenous UCHL1 mRNA and protein levels. N2a and SH-SY5Y cells were treated with LPS (30 ng/mL) or TNF α (10 ng/mL) for 24 h before RNA and protein extractions. β -actin expression served as protein loading control. Stimulation with LPS and TNF α resulted in marked decrease in endogenous UCHL1 mRNA level in N2a (77.8 ± 2.3% and 73.2 ± 1.5%, respectively, p < 0.01) (Figure 2.6B and C), and in SH-SY5Y cells (42.9 ± 1.3% and 32.4 ± 2.3%, respectively, p < 0.01) (Figure 2.6D and E). Consistently, UCHL1 protein levels were also significantly reduced by LPS to 44.9 ± 0.6%, and by TNF α to 33.7 ± 1.5% in N2a cells (p < 0.01) (Figure 2.6F and G), and 42.0 ± 2.9% and 21.9 ± 4.7% in SH-SY5Y cells (p < 0.01) (Figure 2.6H and I), respectively. The data suggested that activation of NF- κ B signaling by LPS and TNF α negatively regulated *UCHL1* gene transcription and expression.



Figure 2.6 Repression of UCHL1 transcription in response to LPS and TNFa

(A) Inhibition of human *UCHL1* promoter activity by LPS and TNF α . pUCHL1-D/pUCHL1-E and pCMV-Luc were co-transfected into N2a cells. Cells were then stimulated by LPS at 30 ng/mL, TNF α at 10 ng/mL, or vehicle solution for 24 h. Luciferase activities were determined by dual luciferase assay. pCMV-Luc luciferase activity was used for transfection efficiency normalization. The values represent mean ± SEM. N = 3, *p < 0.01 by two-way ANOVA with *post hoc* Bonferroni test. (B) N2a and (D) SH-SY5Y cells were exposed to LPS at 30 ng/mL or TNF α at 10 ng/mL for 24 h. Total RNA was extracted. The mRNA levels of UCHL1 were determined by semi-quantitative RT-PCR and normalized against the levels of β -actin. Quantification of UCHL1 mRNA levels in N2a (C) and SH-SY5Y cells (E) by Kodak Image Analysis. Data are presented as mean ± SEM. n = 3, *p < 0.01 by one-way ANOVA with the *post hoc* Newman–Keuls test. (F)(H) Lysates from LPS- or TNF α -treated N2a (F) and SH-SY5Y (H) cells were analyzed by immunoblotting with mouse anti-UCHL1 monoclonal antibody. (G)(I) Quantification of UCHL1 protein levels in N2a (G) and SH-SY5Y (I) cells by Li-COR Imager. The values represent mean ± SEM. N = 3, *p < 0.01 by one-way ANOVA with the *post hoc* Newman–Keuls test.

2.4 Discussion

UCHL1 is highly expressed in neurons and is essential for maintaining neuronal integrity. Complete loss of UCHL1 leads to progressive neurodegeneration at very early stage of life, for example, starting at 5 years of age in humans with its E7A missense mutation (Bilguvar et al., 2013) and at 12 weeks of age in UCHL1 knockout mice (Yamazaki et al., 1988) followed by premature death before AD or PD has a chance to develop. Partial loss of UCHL1, on the other hand, does not cause prominent phenotypes in people with heterozygous E7A mutant (Bilguvar *et al.*, 2013). In other words, they may survive to the late stage of life when AD or PD starts to develop. Notably, UCHL1 expression was indeed partially reduced in sporadic AD and PD brains (Choi et al., 2004). Moreover, rescue experiments have shown restored spine density (Smith et al., 2009) and improved learning and memory deficits by UCHL1 in AD mouse models (Gong et al., 2006). These findings strongly suggested that reduced UCHL1 level in the brain may play a role in the development of AD and PD. However, the mechanisms leading to the reduced UCHL1 expression in AD and PD brains remain unknown. To study the regulation of UCHL1 gene expression, we cloned and characterized the human UCHL1 gene promoter. A functional NF- κ B binding site was identified in the promoter region at around -235 bp. Moreover, NF- κ B physically bound to this region and suppressed *UCHL1* gene transcription. Inflammatory stimulators, LPS and TNFa, also inhibited UCHL1 expression, further confirming the role of NF-κB signaling and inflammation in UCHL1 gene regulation.

It should be noted that previous reports have revealed some forms of regulation on *UCHL1* promoter activity. A REST/NRSF binding site was identified on *UCHL1* promoter (Barrachina et al., 2007), which may be one of the mechanisms for the inhibition of UCHL1 expression in non-neuronal cells. Besides, the β -catenin/TCF complex has been identified to bind to two TCF4 binding sites on *UCHL1* promoter and activate its transcription (Bheda *et al.*, 2009), suggesting a mechanism for elevated UCHL1 expression in cancerous tissues via the oncogenic β -catenin/TCF

pathway. So far, however, no studies have focused on UCHL1 expression alteration in neurodegenerative diseases. Our study highlighted NF- κ B, an important molecule involved in inflammation, in UCHL1 transcriptional regulation. Interestingly, NF- κ B is upregulated in AD brains (Wyss-Coray, 2006; Chen *et al.*, 2011). The increased NF- κ B activity may account for the concomitantly decreased UCHL1 expression in AD.

There is accumulating evidence indicating an important role of inflammation in the pathogenesis of neurodegenerative diseases including AD and PD (Rogers, 2008). While immune responses such as phagocytosis of damaged cells may be beneficial to the brain, it is believed that chronic inflammation induces detrimental changes in the brain. Abnormal expression of inflammatory cytokines and other proteins, including NF- κ B, was detected in AD brains (Akiyama *et al.*, 2000; Wyss-Coray, 2006). Thus, NF- κ B may be an underlying link between inflammation and AD. Indeed, our lab has discovered that NF- κ B p65 level is significantly increased in AD brains. Moreover, increased NF- κ B signaling promotes BACE1 expression and A β production (Chen *et al.*, 2011). Ly *et al.* confirmed the BACE1 transcriptional activation by NF- κ B and further identified GSK3 β as one of the upstream regulators of this NF- κ B pathway (Ly *et al.*, 2013). Conversely, A β activates NF- κ B signaling, probably via RAGE, the cell surface receptor of A β (Arancio *et al.*, 2004). Therefore NF- κ B and A β form a positive feedback loop and potentiate the activities of each other.

Here we revealed another pathway by which NF- κ B may affect AD pathogenesis. NF- κ B signaling may down-regulate UCHL1 expression, which results in endangered neuronal survival. It is noteworthy that UCHL1 conversely regulates NF- κ B activity. UCHL1 attenuates NF- κ B activity by de-ubiquitinating and stabilizing I κ B- α (Takami *et al.*, 2007), an inhibitor of NF- κ B. Thus, NF- κ B and UCHL1 forms a vicious cycle, in which abnormal activation of NF- κ B, such as in chronic neuroinflammation, leads to reduced UCHL1 expression, which in turn disinhibits NF- κ B activity. The ultimate

outcome is that disturbance of NF- κ B may trigger the continuous decrease of UCHL1 expression and increase of NF- κ B activity, which push the system away from equilibrium.

The work in this chapter and previous findings by our lab demonstrate two possible pathways by which NF- κ B signaling affects AD. Abnormal increase of NF- κ B signaling may (1) facilitate A β production by promoting BACE1 cleavage of APP, and (2) do harm to neuronal integrity by inhibiting UCHL1 gene expression. Interestingly, BACE1 is partly degraded by the UPS following ubiquitination, the process in which UCHL1 may be involved as a DUB. We were eager to find out whether UCHL1 regulates the degradation of BACE1, and that was part of our work in chapter 3.

2.5 Conclusion

In conclusion, we demonstrated that *UCHL1* gene transcription was regulated by NF- κ B signaling. In particular, we identified a functional NF- κ B response element on the 5' flanking region of the human *UCHL1* gene. Expression of NF- κ B suppressed *UCHL1* gene transcription. Accordingly, activation of NF- κ B signaling by the inflammatory stimulator LPS and TNF α resulted in decreased UCHL1 mRNA and protein levels. Future studies will reveal the consequence of the interaction between NF- κ B and UCHL1 in AD pathogenesis.

Chapter 3

The role of UCHL1 in A β production and neuronal death

3.1 Introduction

The UPS and autophagy lysosomal pathway are two important pathways by which intracellular proteins are degraded. Proteasomal degradation requires Lys-48-linked polyubiquitination. Autophagy-lysosomal degradation may also require ubiquitin signaling, especially when the proteasome is dysfunctional (Kraft *et al.*, 2010). Our lab and others have discovered that many AD-associated proteins, including BACE1 and various subunits of the γ -secretase complex, could be degraded by the UPS (Fraser *et al.*, 1998; Qing *et al.*, 2004; He *et al.*, 2006; He *et al.*, 2007). Furthermore, lysosomal degradation of BACE1 can be facilitated by the mono-ubiquitination or Lys-63-linked polyubiquitination at its C-terminal Lys501 (Kang *et al.*, 2010). Fulllength APP, the precursor of A β , can also be ubiquitinated and directed to the proteasome for degradation (Kaneko *et al.*, 2010), although it is mainly degraded by the autophagy lysosomal pathway. These findings suggested that ubiquitin signaling is important for APP processing and A β production.

DUBs cleave ubiquitin after its terminal carbonyl Gly-76, and serve a variety of functions, including processing ubiquitin precursors, reversing degradative and nondegradative ubiquitin signalings and recycling free ubiquitins. UCHL1 belongs to the UCH family of DUB. It has been shown to effectively hydrolyze amino acids from ubiquitin and cleave di-ubiquitins (Larsen *et al.*, 1998). It may also serve as a ubiquitin ligase at concentrations higher than 1 μ M, further adding ubiquitin to already mono-ubiquitinated proteins (Liu *et al.*, 2002). In addition, it acts as a free ubiquitin stabilizer, providing ready-to-use ubiquitin for various cellular events (Osaka *et al.*, 2003). Its multiple enzymatic activities and its abundance in neurons indicate its important role in regulating protein degradation and homeostasis in the nervous system.

Dysfunction of UCHL1 has been reported in many neurodegenerative diseases. For example, genetic deletions of UCHL1 in mouse strains (Yamazaki et al., 1988; Walters et al., 2008) led to gracile axonal dystrophy, neuromuscular defects, and premature death. The missense recessive mutation E7A (Bilguvar et al., 2013) resulted in childhood-onset progressive neurodegeneration. Furthermore, the I93M missense mutation was identified in early-onset familial PD cases (Leroy et al., 1998). As of AD, UCHL1 protein level was reduced in sporadic AD brains and was inversely proportional to the number of neurofibrillary tangles (Choi et al., 2004). Notably, overexpression of UCHL1 in the AD model APP/PS double transgenic mice elevated the otherwise reduced UCH activity in the brain and rescued learning and memory deficits in these mice (Gong et al., 2006). The authors implied that UCHL1 facilitated the degradation of PKA regulatory subunit IIa, thereby exerting a positive effect on the PKA/CREB pathway that is involved with learning and memory (Gong et al., 2006). We proposed from another point of view that UCHL1 affects AD progression by regulating APP processing, A β production, and neuronal death, because (1) in gad mice, APP and A β were accumulated in the gracile tract together with the 'dying back' style of axonal dystrophy (Ichihara et al., 1995), and (2) many of the AD-associated proteins, including BACE1 and components of γ -secretase complex could all be degraded via the UPS (Fraser et al., 1998; Qing et al., 2004; He et al., 2006; He et al., 2007). As a DUB, UCHL1 may regulate the ubiquitination/de-ubiquitination process and affect the degradation of those A β -producing molecules.

Neuronal loss is one of the key pathology features in AD brains. Apoptosis is associated with AD and may partly be responsible for the neuronal loss in AD in two aspects. First, apoptosis and activated caspase 3, 8 and 9 are detected in hippocampal neurons of AD brains (Su *et al.*, 1994; Stadelmann *et al.*, 1999; Rohn *et al.*, 2001;

Rohn *et al.*, 2002). Second, Aβ directly induces apoptosis of cultured neurons (Loo *et al.*, 1993), which may be mediated by caspase 2, 8 or 12 (Ivins *et al.*, 1999; Nakagawa *et al.*, 2000; Troy *et al.*, 2000).

UCHL1 has been shown to be involved in apoptosis, although its actual role remains controversial. Some studies suggested that UCHL1 exerts an anti-apoptotic role. For example, genetic ablation of UCHL1 increased the cell's sensitivity to oxygen-glucose deprivation treatment (Shen *et al.*, 2006). Moreover, pharmacological inhibition of UCHL1 and UCHL1 deficiency in β -cells of patients with type 2 diabetes induced ER stress, which led to cell apoptosis (Tan *et al.*, 2008; Costes *et al.*, 2011). On the other hand, UCHL1 has pro-apoptotic properties, as it induced G0/G1 cell cycle arrest and apoptosis through stabilizing p53 (Xiang *et al.*, 2012). It also potentiated cancer chemosensitivity by stabilizing NOXA, a BH3-only pro-apoptotc protein (Brinkmann *et al.*, 2013). The discrepancy in those reports is probably due to UCHL1's temporally and spatially specific functions. We were interested in the role UCHL1 plays in regulating neuronal apoptosis, especially in A β -induced apoptosis.

In this chapter, we examined *in vitro* the effect of UCHL1 on APP and BACE1 degradation. By the overexpression and inhibition of UCHL1, we showed in multiple cell lines that UCHL1 facilitated the degradation of both full-length APP and BACE1. We proposed differential molecular mechanisms for APP and BACE1 regulation based on our further observations. Moreover, we demonstrated that UCHL1 reduced CTF and Aβ production in cells. We also examined the role of UCHL1 in neuronal apoptosis by using various toxin-mediated neurodegenerative cell culture models.

3.2 Methods

3.2.1 cDNA constructs

Human cDNA library was generated from mRNA extracted from HEK293 cells by RT-PCR. Human UCHL1 cDNA was amplified using the forward primer 5'- cgcggatccgccaccatgcagctcaagccgatg and reverse primer 5'- ccgctcgagttaggctgccttgcagagagc. The PCR product was cloned into mammalian expression vector pcDNA4-mycHisA (Invitrogen) at the BamHI and XhoI sites to generate the mammalian expression plasmid pZ-UCHL1-st. The same forward primer and another reverse primer 5'-ccgctcgagggctgccttgcagag were used to clone myc-tagged UCHL1 plasmid pZ-UCHL1-myc.

To make the adeno-associated virus (AAV) expression plasmid that contained human *UCHL1* gene, the pAAV-GFP-cDNA6 vector was used (from Vector Biolabs). pAAV-UCHL1-GFP was generated by cutting the human *UCHL1* cDNA sequence from pcDNA4-UCHL1-st and inserting it into pAAV-GFP-cDNA6 at BamHI and XhoI sites. The pAAV-GFP-cDNA6 vector without the inserted transgene was used as the GFP control plasmid. Separate CMV promoters allowed the bi-*cis*tronic expression of the inserted transgene and enhanced GFP, resulting in the expression of UCHL1 and GFP as individual proteins in transduced cells. The recombinant AAV1-UCHL1-GFP and AAV1-GFP were made by combining AAV2-based genomic constructs with cap genes derived from AAV serotype 1 (Vector Biolabs).

3.2.2 Cell cultures, transfection and viral infection

HEK293 cells were cultured in DMEM supplemented with 10% FBS, 1% sodium pyruvate, 1%L-glutamine and 1% penicillin/streptomycin (Invitrogen). All cells were maintained at 37°C in an incubator containing 5% CO₂. Transient transfections of plasmids were performed using the calcium phosphate method or Lipofectamine 2000. To generate the stable cell lines HUCH-st and HUCH-myc, HEK293 cells were

transfected with pZ-UCHL1-st and pZ-UCHL1-myc respectively, using calcium phosphate method. To generate the stable cell lines SUCH-st and SUCH-myc, SHSY5Y cells were transfected with pZ-UCHL1-st and pZ-UCHL1-myc, respectively, by Lipofectamine 2000 system. The transfected cells were selected using 800 µg/mL zeocin and positive clones were selected for several generations. Stably transfected colonies were confirmed by western blotting using anti-UCHL1 antibody and antimyc antibody 9E10 and maintained in media containing 50 µg/mL zeocin.

For primary neuron cultures, neocortical tissues were removed from C57BL/6 mice at embryonic day 16 (E16), and digested with 0.025% trypsin. The cells were suspended in neurobasal medium supplemented with B27 and plated at a density of 2×10^6 cells per 35mm plate coated with PDL, or 2×10^5 cells per well for 96-well plate. For the experiment on primary cortical neurons from homozygous *gad*, heterozygous *gad* and WT mice, we treated neurons with 100 μ M H₂O₂ for 18 h at DIV14. Fibroblasts from the embryos were used to isolate genomic DNA for genotyping. For the experiment to infect primary neurons with AAVs, AAV1-UCHL1-GFP or AAV1-GFP (10⁵ genomic copies/neuron) were added to the media at DIV 3. Ten days post-infection, neurons were treated with 200 nM aged A β_{42} for 18h.

3.2.3 Reagents

DMEM was purchased from Invitrogen Life Technologies. Cycloheximide (CHX) and anti- β -actin mAb AC-15 were obtained from Sigma-Aldrich. Anti-UCHL1 mouse mAb BH7 was purchased from Novus Biologicals. MG132, chloroquine, UCHL1 inhibitor LDN-57444 and anti-ubiquitin rabbit pAb (1:1000) were obtained from Calbiochem. Anti-BACE1 rabbit mAb D10E5 (1:1000), anti-cleaved caspase 3 rabbit pAb (#9661) (1:1000) and anti-caspase 8 mouse mAb (#9746) (1:1000) were from Cell Signaling Technologies. Synthetic A β_{42} was synthesized by EZBiolab. Rabbit C20 that recognized last 20 C-terminal amino acids of APP was generated previously by our laboratory (Li *et al.*, 2006). IRDyeTM 680-labelled goat anti-rabbit and IRDyeTM 800CW-labelled goat anti-mouse antibodies were from LI-COR Biosciences.

3.2.4 Pharmacological treatment

Protein half-life was determined by using a 100 µg/mL CHX chase for 0, 6, 12, 24 or 36 h as previously described (Liu *et al.*, 2008). A dose of 10 µM LDN-57444 was applied to cells to specifically inhibit UCHL1 hydrolase activity. Chloroquine (100 µM) was used to inhibit lysosomal protein degradation. MG132 (5 µM) was used to inhibit proteasomal protein degradation. H₂O₂ (100 µM) was applied to SH-SY5Y/SUCH cells or primary neurons for 18 h to induce cell death. Aged A β_{42} at a concentration of 10 µM for SH-SY5Y/SUCH cells or 200 nM for primary neurons were applied for 18 h to induce cell death.

3.2.5 Immunoprecipitation

Transfected Haw cells or 20E2 cells with or without treatments were lysed in NP-40 buffer (10 mM Hepes, pH 7.5, 142.4 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1% NP-40, and Roche protease inhibitor cocktail Complete), sonicated and centrifuged at 14,000g for 20 min. The supernatant was pre-cleared with Sepharose CL-4B for 1 h. Primary capturing antibodies were incubated with protein A/G sepharose beads (Santa Cruz) for 1 h before incubation with the protein lysates at 4°C overnight. The following day the immunoprecipitates were washed three times with NP-40 buffer and one time with PBS on ice. Sample loading buffer was added to the pellets and samples were boiled for 5 min before they were subjected to western blot analysis.

3.2.6 Co-immunoprecipitation

Co-immunoprecipitation was performed using Pierce Crosslink IP Kit (MF157118). Briefly, primary capturing antibodies (C20 antibody made in-house, or anti-BACE1 antibody from Cell Signaling) were incubated with Pierce Protein A/G Plus Agarose at room temperature for 1 h in Coupling Buffer. The bound antibodies were then crosslinked with Pierce Protein A/G Plus Agarose by DSS for 1h at room temperature to prevent antibodies from co-eluting with the antigens later. Haw cells or 293B2 cells that had undergone transfections or treatments were harvested and lysed in IP Lysis/Wash Buffer (0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP-40, 5% glycerol; pH 7.4). Lysates were pre-cleared by Control Agarose Resin slurry at 4°C for 1 h and added to the antibody-crosslinked resin for incubation overnight at 4°C. The following day samples were eluted using Elusion Buffer. 5X Lane Marker Sample Buffer with 100mM DTT were added to samples to make a 1X final solution. Samples were heated at 95-100°C for 5 min before western blot analysis.

3.2.7 Immunoblotting

Cells were lysed with RIPA buffer containing: 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulphate and protease inhibitor cocktail Complete (Roche Molecular Biochemicals). Cell lysates were briefly sonicated and centrifuged at 14,000 rpm for 10 min. For the western blotting to detect caspases, cells were lysed with Chaps Cell Extract Buffer (Cell Signaling) with 5mM DTT and 1mM PMSF. Lysates were freezed and thawed three times, and centrifuged at 14,000 rpm for 10 min. For western blot analysis, samples were diluted in 4X SDS-sample buffer and loaded onto 8% tris-glycine, 12% tris-glycine or 16% Tris-Tricine SDS-PAGE and transferred to PVDF-FL membranes. Immunoblot procedure was carried out as indicated in Section 2.2.8.

3.2.8 Aβ40/42 ELISA

2EB2 cells were transfected with pZ-UCHL1-st or control plasmid for 48 h. Conditioned culture media from 24 h to 48 h post-transfection were collected. Protease inhibitors and AEBSF were added to prevent degradation of A β proteins. The concentrations of A β_{40} and A β_{42} were detected by β -amyloid 1–40 and β -amyloid 1–42 Colorimetric ELISA kit (Covance, SIG-38954, SIG-38956, Princeton, NJ, USA) according to manufacturer's instructions.

3.2.9 Animals

The gracile axonal dystrophy (*gad*) mouse is a spontaneous mutant with an in-frame deletion in exons 7 and 8 of *UCHL1* (Saigoh *et al.*, 1999), and is equivalent to a UCHL1 knockout mouse model. Heterozygous *gad* mice were originally generated and kindly provided by Dr. Keiji Wada at the National Institute of Neuroscience of Japan.

3.2.10 Genotyping

All transgenic mice were genotyped at one month of age. Mice were anesthetized with isoflurane and earmarked. The tissues were digested in 300 µL of lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 150 mM NaCl, 0.5% SDS) with Proteinase K (100 ng/mL) (New England Biolabs) at 55°C overnight. The next day, samples were centrifuged at 16,000g for 10 min and supernatants were transferred to new tubes. Genomic DNA was extracted with phenol-chloroform, precipitated with 0.7X volume of isopropanol, and pelleted by centrifugation at 16,000g for 15 min. DNA was then washed twice with 70% ethanol, air dried and re-suspended in sterile de-ionized water. For the genotying of UCHL1 expression, genomic DNA was subjected to PCR to amplify mouse UCHL1 using the forward primer UCHL1-F4 (AGCCAAGTGTTTCGAGAAGAACGAG), and reverse primers UCHL1-R3(TGGCAGCATCCTGAAAAGGAGAGAGGTG) and UCHL1-R5(TACAGATGGCCGTCCACGTTGTTGA).

3.2.11 Preparation of Aβ fibrils

Synthetic A β_{42} (EZBiolab) was dissolved in sterile deionized water to a concentration of 1 mM and allowed to incubate at 37°C for 1 hour. The dissolved A β was then aliquot and stored at -80°C until use. To age the A β_{42} fibrils, A β_{42} was diluted with equal volumes of sterile PBS to 0.5 mM, and placed in the 37°C incubator for 4 days. The fibrils were dissociated by vigorously triturating and were further diluted with complete DMEM to the appropriate concentration. In this chapter, SH-SY5Y or SUCH cells were treated with 10 μ M A β for 18 h. Primary neurons were treated with 200 nM A β for 18 h.

3.2.12 MTS, LDH and TUNEL assays

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was performed following manufacturer's instruction (Promega) to measure cell viability after H_2O_2 or A β treatment. The absorbance of the formazan product at 490nm was measured. Cell media were collected and LDH (lactate dehydrogenase) assay was performed (Clontech) to measure cell toxicity. A red, formazan-class dye, which was converted by LDH that was leaked to cell media, was measured by absorbance at 492 nm. For the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, cells were fixed by 4% formaldehyde and the assay was performed following manufacturer's instruction (Promega). DNA fragmentation was detected using green fluorescence under fluorescent microscopy.

3.2.13 Caspase-8 and Caspase-9 activity assays

After Aβ treatment, caspase 8 and caspase 9 activities in the primary neurons were measured with Caspase-Glo® 8 (Promega) and Caspase-Glo® 9 (Promega) assay systems following manufacturer's instructions. Luminescence was measured with a TD-20/20 luminometer (Turner Designs).

3.2.14 Statistical analysis

All results were presented as mean \pm SEM and analyzed by 2-tailed Student's *t*-test, one-way ANOVA or two-way ANOVA. Statistical significance is accepted when p<0.05 (*p<0.05, **p<0.01, ***p<0.001).

3.3 Results

3.3.1 Inhibition of UCHL1 promotes the accumulation of BACE1

To investigate the effect of UCHL1 on APP processing, we first established cell lines stably over-expressing UCHL1. HUCH-st and HUCH-myc cells were established by introducing UCHL1 cDNA plasmids (pZ-UCHL1-st or pZ-UCHL1-mycHis) into HEK293 cells and positively selected transfected cells with zeocin. Western blot analysis showed that HUCH-myc cells had robust expression of exogenous mycHis-tagged UCHL1, which was detected by both anti-myc antibody 9E10 and anti-UCHL1 antibody, while over-expression of UCHL1 in HUCH-st cells was detected by anti-UCHL1 antibody. Non-transfected HEK293 cells had a lower level of endogenous UCHL1 (Figure 3.1A). These data indicated that we have established two UCHL1-stably expressing cell lines, HUCH-st and HUCH-myc, which are valuable tools for studying UCHL1 in mammalian cells.

Our previous studies suggested that BACE1 degradation is mediated by ubiquitin proteasome pathway (Qing *et al.*, 2004). Since UCHL1 is a DUB, it may be involved in the de-ubiquitination and degradation of BACE1. To investigate whether UCHL1 affects AD pathogenesis by regulating BACE1 degradation, we first examined whether inhibition of UCHL1 would affect BACE1 protein level. LDN-57444 (LDN) is a competitive and active site-directed inhibitor of UCHL1 and is ~28-fold more selective for UCHL1 than for other UCH members such as UCHL3. HUCH-st cells were treated with 10 μ M LDN, a concentration that maximally inhibited UCH activity (Gong *et al.*, 2006). HUCH-st cells were transiently transfected with pZ-BACE1-myc for 48 h before the treatment of LDN. Cells were lysed after 0, 12, 24, 36 h and western blot analysis was performed (Figure 3.1B). BACE1 level was significantly increased with the duration of LDN treatment, to 130.7 ± 7.3% after 12 h (*p* < 0.01), 136.8 ± 2.4% after 24 h (*p* < 0.01), and up to 205.0 ± 2.3% after 36 h (*p* < 0.01) (Figure 3.1C). The results demonstrated that BACE1 protein level was significantly elevated in the presence of UCHL1 inhibitor LDN.



Figure 3.1 Inhibition of UCHL1 elevates BACE1 protein level

(A) Stable cell line characterization. The UCHL1-stably transfected cell lines HUCH-st and HUCH-myc were established by transfecting UCHL1 cDNA plasmid constructs pZ-UCHL1-st and pZ-UCHL1-mycHis into HEK293 cells and selected with 800 µg/mL zeocin. 9E10 was used to detect myc-tagged UCHL1 protein (left panel). Anti-UCHL1 antibody BH7 (Novus Bio) detected both myc-tagged and endogenous UCHL1 proteins (right panel). β -actin was used as protein loading control. (B) HUCH-st cells transiently transfected with pZ-BACE1-myc were treated with 10 µM UCHL1 inhibitor LDN-57444 (LDN) for 0 (vehicle control), 12, 24 or 36 h. Cells were harvested at the same time. BACE1 level was detected by 9E10 antibody. (C) Quantification of (B). LDN significantly increased BACE1 protein level after 12, 24 and 36 h. BACE1 level was normalized by β -actin and plotted as a percentage compared with the amount at 0 h. The values represent mean ± SEM. N = 3, **p < 0.01 by one-way ANOVA with the *post hoc* Newman–Keuls test.

3.3.2 UCHL1 accelerates the degradation of BACE1

The accumulation of BACE1 by UCHL1 inhibitor suggested the disequilibrium between BACE1 synthesis and degradation. To eliminate the possible involvement of UCHL1 in BACE1 synthesis and to confirm its role in the degradation pathway, we examined how UCHL1 affects the half-life of BACE1. Cycloheximide (CHX) is a protein synthesis inhibitor produced by the *Streptomyces griseus bacterium* (Kerridge, 1958; Kay & Korner, 1966). By stopping protein synthesis with CHX, the amount of protein remaining after a certain time interval can be used to calculate the protein halflife (Schoenfeld *et al.*, 2000; Touitou *et al.*, 2001). We first compared the half-life of BACE1 in HEK293 and in HUCH-st cells. Over-expression of UCHL1 significantly facilitated the degradation rate of BACE1 (p < 0.01) (Figure 3.2A, B). To further confirm the results, we treated HUCH-st cells with UCHL1 inhibitor LDN. The half-life of BACE1 significantly increased by LDN treatment (p < 0.01) (Figure 3C, D). Taken together, the data indicated that UCHL1 accelerates the degradation of BACE1.



Figure 3.2 UCHL1 accelerates BACE1 degradation

(A)HEK and HUCH-st cells were transiently transfected with pZ-BACE1-myc for 48 h before treated with 100 µg/mL CHX at the same time for 0, 6, 12 or 24 h. Cells were lysed in equal volumes of RIPA for protein detection. BACE1 was detected by 9E10. (B) Quantification of the CHX assay from (A). UCHL1 overexpression promoted BACE1 degradation. BACE1 level was plotted as a percentage of the amount at 0 h. N=4, **p < 0.01, by two-way ANOVA group comparison. (C) HUCH-st cells were transfected with pZ-BACE1-myc for 48 h, and then treated with 100 µg/mL CHX together with LDN (or DMSO) for 0, 6 or 12 h. (D) Quantification from (C). UCHL1 inhibition decelerated BACE1 degradation. Values represent mean ± SEM. N = 4, **p < 0.01, by two-way ANOVA with *post hoc* Bonferroni test.

3.3.3 UCHL1 does not physically interact with BACE1

UCHL1 was reported to interact and cleave ubiquitin from β-catenin (Bheda *et al.*, 2009) and α-synuclein (Liu *et al.*, 2002), or act as a ligase to poly-ubiquitinate α-synuclein (Liu *et al.*, 2002). On the other hand, it is able to recycle and stabilize free ubiquitin (Osaka *et al.*, 2003), thereby indirectly regulating the ubiquitination of a variety of proteins. To investigate whether UCHL1 physically interacts with BACE1, we transfected UCHL1 or control plasmid into 293B2 cells (HEK cell stably overexpressing BACE1) for 48 h, before treating the cells with 100 μ M chloroquine, 5 μ M MG132 or control solution for 6 h. Cells were harvested and co-immunoprecipitated with anti-BACE1 antibody using Pierce Crosslink IP Kit. Samples were then subjected to western blot analysis with anti-UCHL1 antibody (Figure 3.3). Interaction between UCHL1 and BACE1 was not detected under either treatment or normal conditions. The data implicated that UCHL1 may indirectly regulate BACE1 degradation by providing more ready-to-use ubiquitin in the cell. Alternatively, there may be adaptor proteins or intermediate players that await identification.



Figure 3.3 UCHL1 does not physically interact with BACE1

293B2 cells (HEK stably overexpressing BACE1) were transfected with pZ-UCHL1-st or control plasmid for 48 h and then treated with 100 μ M chloroquine, 5 μ M MG132 or control solution for another 6 h. Cells were harvested and co-immunoprecipitated with anti-BACE1 antibody D10E5 (Cell Signaling) using Pierce Crosslink IP Kit. Samples were then immunoblotted with anti-UCHL1 antibody BH7 (Novus Bio). No physical interaction between UCHL1 and BACE1 was detected under any of the treatments.

3.3.4 UCHL1 reduces protein level of mature APP

APP is mainly degraded via lysosomal pathway (Caporaso *et al.*, 1992; Haass *et al.*, 1992). Interestingly, APP can also be ubiquitinated and sorted to proteasome for degradation (Kaneko *et al.*, 2010; El Ayadi *et al.*, 2012). When we used multiple cell lines to confirm the accumulation of BACE1 by UCHL1 inhibitor LDN (Section 3.3.1), we also observed enhanced mature full-length APP level (Figure 3.4A). In this experiment LDN was applied to 20E2 cells, the HEK cell line overexpressing APP_{SWE}, for 0, 3 or 6 h. UCHL1 inhibition led to a marked accumulation of mature APP, to $134.3 \pm 6.7\%$ (p < 0.01) at 3 h and $169.3 \pm 6.4\%$ at 6 h (p < 0.01) (Figure 3.4B).We reasoned that UCHL1 also regulates APP degradation, probably by affecting its ubiquitination followed by one of its degradation pathways.

To investigate whether UCHL1 affects APP degradation, we first confirmed the inverse regulation of APP protein level by UCHL1 overexpression. Transfection of UCHL1 plasmid in 20E2 cells for 48h reduced mature APP level to $59.2 \pm 5.1\%$ compared to control (p < 0.05) (Figure 3.4C, D). While APP Swedish mutation causes early onset familial AD, most AD cases are sporadic, and patients have the WT *APP* gene. To confirm the effect of UCHL1 on APP_{WT}, UCHL1 was transfected into Haw cells, the HEK cell line overexpressing APP_{WT} (Figure 3.4E). Overexpression of UCHL1 reduced mature APP_{wT} level 48 h post-transfection, to $70.0 \pm 4.3\%$ compared to control (Figure 3.4F).



Figure 3.4 UCHL1 reduces protein level of mature APP

(A) 20E2 cells (HEK stably overexpressing APP_{SWE}) were transfected with pz-UCHL1-st for 48 h and then treated with LDN for 0, 3 or 6 h. Cell lysates were subjected to western blot analysis. Full-length APP was detected by C20 antibody. β -actin served as protein loading control. (B) Quantification of mature APP level in (A). Inhibition of UCHL1 significantly elevated mature APP level. N=3, **p<0.01 by one-way ANOVA followed by *post-hoc* Newman-Keuls test. (C) 20E2 cells were transfected with pZ-UCHL1 or control plasmid for 48h. Cell lysates were detected for full-length APP. (D) Quantification of mature APP_{SWE} level in (C). Overexpression of UCHL1 markedly reduced mature APP_{SWE} level. N=3, *p<0.05, by Student's *t*-test. (E) Haw cells (HEK stably overexpressing APP_{WT}) were transfected with pZ-UCHL1 or control plasmid for 48h. Cell lysates to the transfected for full-length APP. (F) Quantification of mature APP_{WT} level in (E). Overexpression of UCHL1 markedly reduced mature APP_{WT} level mature APP_{WT} level in (E). Overexpression of UCHL1 markedly reduced mature APP. (F) Quantification of mature APP_{WT} level in (E). Overexpression of UCHL1 markedly reduced mature APP_{WT} level. N=3, *p<0.05, by Student's *t*-test. Values represent mean ± SEM.

3.3.5 UCHL1 promotes the ubiquitination of APP

UCHL1 is a de-ubiquitinating enzyme (Larsen *et al.*, 1998). It is able to cleave ubiquitin from substrates such as β -catenin (Bheda *et al.*, 2009) and α -synuclein (Liu *et al.*, 2002). Besides, it may serve as a ligase at concentrations higher than 1 μ M (Liu *et al.*, 2002). Since UCHL1 reduced mature APP (Figure 3.4), we were interested in exploring whether UCHL1 exerted its effect by regulating APP ubiquitination /deunbiquitination process. For this purpose, 20E2 cells were transfected with pZ-UCHL1 for 48 h before being treated with LDN or control solution for 6 h. Cells were lysed in NP-40 buffer and were immunoprecipitated by C20 antibody. Samples were then subjected to western blot analysis using anti-ubiquitin antibody. High-molecularweight (HMW) APP-ubiquitin conjugates were detected (Figure 3.5A), which were, in other words, ubiquitinated APP. LDN treatment reduced the level of ubiquitinated APP (Figure 3.5A). Conversely, overexpression of UCHL1 in 20E2 cells elevated the level of APP-ubiquitin conjugates (Figure 3.5B). We then repeated the experiment in Haw cells to confirm the same effect by UCHL1 on wildtype APP. Overexpression of UCHL1 increased the level of ubiquitinated APP_{wT} as well (Figure 3.5C).

Taken together, we demonstrated that UCHL1 inhibition led to decreased ubiquitinated APP and increased mature APP level (Figure 3.5A and 3.4A); in contrast, overexpression of UCHL1 resulted in elevated ubiquitinated APP and reduced mature APP level (Figure 3.5B and 3.4C; figure 3.5C and 3.4E). The results indicated that UCHL1 accelerated APP degradation by enhancing its ubiquitination. In addition, it exerted similar effect on APP_{SWE} and APP_{WT}.





(A) 20E2 cells (HEK stably overexpressing APP_{SWE} mutant) were transfected with pz-UCHL1-st for 48 h and then treated with 10 µM LDN or DMSO for 6 h. Cell lysates in NP-40 lysis buffer were immunoprecipitated with C20 antibody followed by western blotting with anti-ubiquitin antibody. High-molecular-weight (HMW) APP complex was detected. LDN reduced HMW APP level. Negative control lysate was immunoprecipitated with pre-bleed of C20 antibody serum. (B) 20E2 cells were transfected with pz-UCHL1-st or control plasmid for 48 h before lysed in NP-40 buffer. Cell lysates were immunoprecipitated with C20 antibody followed by western blotting with anti-ubiquitin antibody. Overexpression of UCHL1 enhanced HMW APP level. (C) The experiment in (B) was repeated in Haw cells (HEK stably overexpressing APP_{WT}). Overexpression of UCHL1 elevated HMW APP_{WT} level.

3.3.6 Lysosomal inhibition prevents UCHL1-mediated APP degradation

APP is mainly degraded via lysosomal pathway (Caporaso et al., 1992; Haass et al., 1992), while it can also be ubiquitinated and sorted to proteasome for degradation (Kaneko et al., 2010; El Ayadi et al., 2012). We have demonstrated that UCHL1 accelerated APP degradation, probably by promoting its ubiquitination. We were then interested in finding out whether UCHL1 assists APP degradation by the lysosomal or proteasomal pathway. To this end, we first investigated the APP protein level when proteasome or lysosome function was inhibited. MG132, a proteasomal inhibitor, or chloroquine, a lysosomal inhibitor, was applied to Haw cells for 6 h. Chloroquine significantly accumulated mature APP in the cell, to $166.1 \pm 4.4\%$ compared to controls (p<0.01) (Figure 3.6A lane 5 and 6, figure 3.6B). Surprisingly, MG132 did not lead to accumulation of APP. Instead, it decreased mature APP level to 65.6 ± 10.9 % compared to controls (p<0.05) (Figure 3.6A, lane 3 and 4, figure 3.6B). The experiments were replicated in 20E2 cells using the same strategies. Similarly, chloroquine treatment led to marked APP accumulation to 134.6 ± 7.0 % compared to controls (p<0.05) (Figure 3.6C, lane 5 and 6, figure 3.6D), while MG132 treatment reduced mature APP level to 72.4 ± 6.3 % compared to controls (p<0.05) (Figure 3.6C lane 3 and 4, figure 3.6D).

While it was not clear why proteasomal inhibition decreased mature APP level, it was obvious that APP mainly underwent lysosomal degradation. To investigate whether UCHL1 reduced APP protein level by promoting its lysosomal degradation, Haw cells were transfected with UCHL1 for 48 h and then treated with 100 µM chloroquine for 6 h (Figure 3.6E). Lysosomal inhibition attenuated the effect of UCHL1 on APP degradation (p>0.05) (Figure 3.6F, lane 3 compared to lane 4). We replicated the experiments in 20E2 cells (Figure 3.6G). Similarly, chloroquine treatment attenuated the UCHL1-regulated reduction of mature APP level (p>0.05) (Figure 3.6H, lane 3 compared to lane 4). Our results indicated that UCHL1 may promote the degradation of APP via the lysosomal pathway.



Figure 3.6 Lysosomal inhibition attenuates the effect of UCHL1 on APP protein level

(A)Haw cells were treated with 5 μ M MG132, 100 μ M chloroquine, or control solutions for 6 h. Full-length APP_{WT} was detected by C20 antibody. β -actin served as protein loading control. (B) Quantification of (A). MG132 treatment reduced mature APP_{WT} level, whereas chloroquine treatment enhanced it. N=3, *p<0.05, **p<0.01 by one-way ANOVA followed by *post-hoc* Newman-Keuls test. Values represent mean ± SEM. (C)(D) Experiments in (A) were repeated in 20E2 cells. N=3, *p<0.05 by one-way ANOVA followed by *post-hoc* Newman-Keuls test. (E) Haw cells were transfected with pz-UCHL1-st or control plasmid for 48 h and then treated with chloroquine or control solution for 6h. Full-length APP_{WT} were detected by C20 antibody. (F) Quantification of (E). Overexpression of UCHL1 reduced mature APP_{WT} level. Chloroquine treatment attenuated the difference in APP level between UCHL1 and control groups. N=3, *p<0.05, **p<0.01 by two-way ANOVA followed by *post-hoc* Bonferroni test. Values represent mean ± SEM. (G)(H) Experiments in (E) were repeated in 20E2 cells. N=3, *p<0.05, **p<0.01 by two-way ANOVA followed by *post-hoc* Bonferroni test.

3.3.7 UCHL1 physically interacts with APP

To investigate whether UCHL1 regulates APP degradation by physically interacting with APP, we transfected UCHL1 or control plasmid into Haw cells for 48 h, before treating the cells with chloroquine or control solution for 6 h. Cells were harvested and co-immunoprecipitated with C20 antibody using Pierce Crosslink IP Kit. Samples were then subjected to western blot analysis using anti-UCHL1 antibody (Figure 3.7). Interaction between UCHL1 and APP was detected (lane 2). Furthermore, the interaction was potentiated with chloroquine treatment (lane 4). Taken together, our results suggested that UCHL1 might directly bind to and ubiquitinate APP, thereby promoting its degradation.



Figure 3.7 UCHL1 physically interacts with APP

Haw cells were transfected wth pZ-UCHL1-st or control plasmid for 48 h and then treated with 100 μ M chloroquine or control solution for another 6 h. Cells were harvested and coimmunoprecipitated with C20 antibody using Pierce Crosslink IP Kit. Samples were then immunoblotted with anti-UCHL1 antibody BH7 (Novus Bio). Interaction between UCHL1 and APP was detected (lane 2). The interaction was potentiated with chloroquine treatment (lane 4).

3.3.8 Increased free ubiquitin level may be partly responsible for reduced mature APP level by UCHL1

UCHL1 was reported to prolong the half-life of ubiquitin and provide cells with ready-to-use free ubiquitin. To investigate whether UCHL1 regulates the free ubiquitin pool and whether this is one of the mechanisms UCHL1 recruits to promote the ubiquitination and degradation of APP, we overexpressed ubiquitin and/or UCHL1 in Haw cells. Haw cells were co-transfected with pZ-UCHL1-st and PCW7 (a plasmid expressing myc-tagged ubiquitin) or their control plasmids for 48 h. Full-length APP was detected by C20 antibody. Endogenous ubiquitin and exogenous myc-ubiquitin were detected by anti-ubiquitin antibody (Figure 3.8A). Overexpression of UCHL1 significantly elevated free ubiquitin level to 175.0 ± 22.3 % compared to controls (p<0.05) (Figure 3.8A lane 1 and 2, figure 3.8C). PCW7 further increased free ubiquitin levels (p<0.01) (Figure 3.8A lane 3 and 4 compared to lane 1 and 2, figure 3.8C). Mature APP level was reduced by UCHL1 as expected (p<0.05) (Figure 3.8B) lane 1 and 2). Moreover, the overexpressed free ubiquitin by PCW7 further reduced mature APP levels (p<0.01) (Figure 3.8A lane 3 and 4 compared to lane 1 and 2, figure 3.8B). The data indicated that the increased free ubiquitin level by UCHL1 (Figure 3.8A lane 1 and 2) may be responsible for the increased ubiquitinated APP and decreased mature APP (Figure 3.5B, figure 3.4B and figure 3.8A lane 1 and 2).



Figure 3.8 Increased free ubiquitin level reduces protein level of mature APP

(A) Haw cells were co-transfected with pZ-UCHL1-st and PCW7 (a plasmid containing myctagged ubiquitin) or their controls for 48 h. Cell lysates were subjected to western blot analysis. Full-length APP was detected by C20 antibody. Ubiquitin was detected by rabbit anti-ubiquitin antibody (Calbiochem). β -actin served as protein loading control. (B) Quantification of mature APP level in (A). Overexpression of UCHL1 significantly reduced mature APP level. Overexpression of ubiquitin further decreased mature APP level. N=3, *p<0.05, **p<0.01 by twoway ANOVA followed by *post-hoc* Bonferroni test. Values represent mean \pm SEM. (C) Quantification of ubiquitin level in (A). Overexpression of UCHL1 upregulated ubiquitin level. N=3, *p<0.05, **p<0.01 by two-way ANOVA followed by *post-hoc* Bonferroni test. Values represent mean \pm SEM.

3.3.9 UCHL1 reduces APP CTF levels and Aβ levels *in vitro*

Since UCHL1 inversely regulated APP and BACE1 levels, we then asked whether it would affect the production of APP CTFs and A β . For this purpose we first examined CTF levels produced from APP_{SWE}. HEK and HUCH-st cells were transfected with pZ-APPswe for 48 h. In cells with endogenous BACE1, the majority of APP was cleaved by α -secretase to produce CTF α while the minority of APP was cleaved by BACE1 to produce CTF β . For APP Swedish mutant, BACE1 favored the Asp-1 cleavage site over the Glu-11 site and therefore the major CTF β was C99 (Figure 3.9A, lane 1 and 2). In HUCH cells, C99 level was decreased to 71.5 ± 2.3% relative to HEK cells (p < 0.01) (Figure 3.9B). Moreover, C99/C83 ratio was also decreased by UCHL1 (p < 0.05) (Figure 3.9C). The results were confirmed in 20E2 cells (Figure 3.9A, lane 3 and 4). In 20E2 cells transfected with UCHL1 plasmid, C99 level was reduced to 42.2 ± 9.5% compared to control (p < 0.05) (Figure 3.9E). The reduction in C99 level may result from either the reduction of full-length APP, or the reduction of BACE1

mediated by UCHL1. On the other hand, the reduced C99/C83 ratio indicated decreased β -cleavage/ α -cleavage ratio, which was probably due to UCHL1-induced down-regulation of BACE1.

We next confirmed the effect of UCHL1 on CTF and A β production from wildtype APP. Haw cells were transfected with UCHL1 plasmid or control for 48 h (Figure 3.9F). For wildtype APP, the majority of β -cleavage is at Glu-11 site to produce CTF β C89. With endogenous BACE1, the majority of APP still undergoes α -cleavage and mainly produces CTF α C83. Since CTF β levels were very low, we quantified and compared total CTF amounts. Overexpression of UCHL1 significantly decreased total CTF amount to 71.5 ± 2.3% relative to control (p<0.05) (Figure 3.9G).

The results were further confirmed with UCHL1 inhibition in cells overexpressing BACE1 (Figure 3.9H). HUCH-st cells were co-transfected with BACE1 and APP_{SWE} plasmids for 24 h and treated with LDN for another 24 h. With exogenous BACE1 expression, APP was mainly cleaved at β -site to produce CTF β , while CTF α C83 could barely be detected (Figure 3.9H). Thus we quantified and compared the amount of total CTF β . UCHL1 inhibition led to a marked accumulation of CTF β to 138.9 ± 5.8% compared to control (p<0.01) (Figure 3.9I). Taken together, we used multiply strategies to demonstrate that, in addition to full-length APP and BACE1, UCHL1 also regulates the downstream APP processing and CTF production.

To further determine whether UCHL1-regulated APP processing also affects A β production, we performed the ELISA assay to detect A β levels in our cell culture systems. 2EB2 cells, a HEK293 cell line stably expressing both APP_{SWE} and BACE1, were transfected with pZ-UCHL1-st or control plasmid for 48 h. Conditioned media from 24 h to 48 h were collected for A β ELISA. Overexpression of UCHL1 markedly reduced A β 40 level to 83.0 ± 5.9% (p < 0.05, figure 3.9J) and A β 42 level to 87.1 ± 1.9% (p < 0.01, figure 3.9K).



Figure 3.9 UCHL1 reduces APP CTF levels and Aβ levels in vitro

(A) HEK and HUCH-st cells were transfected with pZ-AppSwe (lanes 1 and 2) for 48 h. 20E2 cells were transfected with pZ-UCHL1-st or control plasmid (lanes 3 and 4) for 48 h. APP CTFs were detected by C20 antibody. β -actin served as protein loading control. Quantification of APP C99 level (B)(D) and C99/C83 ratio (C)(E) from (A). Overexpression of UCHL1 markedly down-regulated C99 levels as well as C99/C83 ratios. N=3, *p<0.05, **p<0.01 by Student's *t*-test. (F) Haw cells were transfected with pZ-UCHL1-st or control plasmid for 48 h. (G) Quantification of APP CTF level. Overexpression of UCHL1 significantly reduced total CTF level. N=3, *p<0.05 by Student's *t*-test. (H) HUCH-st cells were co-transfected with pZ-AppSwe and pZ-BACE1-myc for 24 h and then treated with 10 μ M LDN or vehicle solution for additional 24 h. (I) Quantification of APP CTF β level from (H). Inhibition of UCHL1 elevated total CTF β level. N=3, **p<0.01 by Student's *t*-test. (J)(K) 2EB2 cells (HEK stably overexpressing both Swedish APP and BACE1) were transfected with pZ-UCHL1-st or control for 48 h. Conditioned media from 24 h to 48 h were collected and A β ELISA was performed following manufacturer's instruction . A β_{40} and A β_{42} levels were plotted as the percentage of controls. N = 3, *p < 0.05, **p < 0.01 by Student's *t*-test. All the values represent mean \pm SEM.

3.3.10 UCHL1 protects against oxidative stress-induced neuronal apoptosis

Dysfunction of UCHL1 is associated with neurodegeneration. Knockout equivalent of UCHL1(Yamazaki et al., 1988) or its loss of function mutation E7A (Bilguvar et al., 2013) causes early-onset progressive neurodegeneration. Neuronal loss is one of the key pathology features in AD brains. We proposed that UCHL1 protects against neuronal loss in AD. It is notably that the brain is under increased oxidative stress with aging. Considerable data have accrued indicating that oxidative stress may have a role in neurodegeneration in AD. Therefore we first looked at the effect of UCHL1 in oxidative stress-induced neuronal death. To this end, we challenged the neuroblastoma cell line SH-SY5Y and SUCH cells (SH-SY5Y stably overexpressing UCHL1) with H_2O_2 . SH-SY5Y and SUCH cells were treated with 100 μ M H_2O_2 for 18 h, after which the MTS assay and LDH assay were performed (Figure 3.10A and B). The MTS assay measures mitochondrial reductase activity, which reflects the number of viable cells present. The LDH assay measures the leakage of the lactate dehydrogenase to the media as an indirect way of assessing cell membrane integrity. While cell viability measured by MTS decreased in both SH-SY5Y and SUCH cells, UCHL1 overexpression significantly improved cell survival from $43.1 \pm 1.9\%$ living cells to $62.8 \pm 3.0\%$ living cells (p<0.01) (Figure 3.10A). Consistently, UCHL1 markedly reduced cytotoxicity measured by LDH assay from $32.8 \pm 3.5\%$ cell death to $22.3 \pm 1.7\%$ (p<0.01) (Figure 3.10 B).

To investigate if UCHL1 deficiency exacerbates neuronal death upon oxidative stress, we extracted primary cortical neurons from E16 embryos of homozygous *gad*, heterozygous *gad* and WT littermates and treated them with 100 μ M H₂O₂ for 18 h at DIV 14. MTS assay showed that neurons from homozygous *gad* were the most vulnerable to H₂O₂ challenge while neurons from WT embryos were the most resistant (16.6 ± 1.1% living cells in homozygous *gad*, 43.0 ± 3.8% in heterozygous *gad*, 60.7 ± 8.6% in WT) (p<0.01) (Figure 3.10C). Taken together, the results indicated that UCHL1 protects against oxidative stress-induced neuronal death.

Research has shown that apoptosis is associated with AD and may be partly responsible for the neuronal loss in AD brains. Since UCHL1 rescued cells from oxidative stress-induced death, we would like to find out whether the protective effect is through the inhibition of apoptosis. SH-SY5Y and SUCH cells were treated with 100μ M H₂O₂ for 18 h and TUNEL assay was performed (Figure 3.10 D). TUNEL assay is a method to detect DNA fragmentation resulting from apoptotic signaling cascade. UCHL1 significantly inhibited DNA fragmentation induced by H₂O₂ treatment (5.4 ± 0.5% TUNEL positive cells in SUCH compared to 13.2 ± 1.3% in SH-SY5Y cells, p<0.01) (Figure 3.10 E), indicating that UCHL1 protected against H₂O₂-induced apoptosis.

Apoptosis can be activated through extrinsic or intrinsic pathways. It is initiated extrinsically by death receptors such as Fas and TNFR, and activated through caspase 8/caspase 3 pathway (Ashkenazi & Dixit, 1998). Alternatively, it is initiated intrinsically by the release of cytochrome c from mitochondria to cytosol, followed by apoptosome formation with apaf-1 and caspase 9, and the activation of caspase 3 (Jiang & Wang, 2004). We were interested in finding out which pathway UCHL1 interferes with to exert its anti-apoptotic effect. Protein lysates from H₂O₂-treated SH-SY5Y and SUCH cells were detected for cleaved caspases 3, 8 and 9 (Figure 3.10 F).




(A)(B) SH-SY5Y and SUCH cells (SH-SY5Y cells stably overexpressing UCHL1) were treated with 100μ M H₂O₂ for 18 h before (A) MTS assay and (B) LDH assay were performed. Overexpression of UCHL1 in SUCH cells significantly protected against H₂O₂-induced cell death in both MTS assay (A) and LDH assay (B) compared to SH-SY5Y cells. N=3, **p<0.01 by two-way ANOVA followed by *post-hoc* Bonferroni test. (C) Primary cortical neurons from

homozygous *gad*, heterozygous *gad* and WT E16 embryos were treated 100 μ M H₂O₂ for 18 h at DIV 14. MTS assay was performed to evaluate cell viability. Cell viability for each genotype was plotted as the percentage of their controls. Loss of UCHL1 markedly exacerbated H₂O₂-induced cell death. N=6 for WT, n=12 for heterozygous *gad*, n=8 for homozygous *gad*, **p<0.01, *p<0.05 by two-way ANOVA followed by *post-hoc* Bonferroni test. (**D**) SH-SY5Y and SUCH cells were treated with 100 μ M H₂O₂ for 18 h before being fixed for TUNEL assay. Cells were stained with TUNEL, and nuclei were counterstained with DAPI. TUNEL staining was indicated by green color, DAPI staining by blue. (**E**) Quantification from (D). Overexpression of UCHL1 in SUCH cells markedly reduced H₂O₂-induced DNA fragmentation as indicated by TUNEL staining. Numbers of DAPI-positive and TUNEL-positive cells were counted by Image J. N=3, **p<0.01 by two-way ANOVA followed by *post-hoc* Bonferroni test. (**F**) SH-SY5Y and SUCH cells were treated with 100 μ M H₂O₂ for 18 h before they were harvested for cleaved caspases 3, 8 and 9 detection. Quantification of cleaved caspase 3 (**G**) and cleaved caspase 8 (**H**) from (F). UCHL1 protected cells against caspase 3 and caspase 8 activation upon H₂O₂ treatment. N=3, **p<0.01 by two-way ANOVA followed by *post-hoc* Bonferroni test. Values represent mean ± SEM.

UCHL1 overexpression significantly reduced H_2O_2 -induced caspase 3 activation (114.4 ± 4.6% in SUCH cells compared to 192.4 ± 4.9% in SH-SY5Y cells, p<0.01) (Figure 3.10 G). Moreover, UCHL1 markedly repressed H_2O_2 -triggered caspase 8 activation (98.6 ± 14.6% in SUCH cells compared to 173.1 ± 4.4% in SH-SY5Y cells, p<0.01) (Figure 3.10 H). Western blot analysis did not detect cleaved caspase 9 band in control cells or in H_2O_2 -treated cells (data not shown), possibly because H_2O_2 -induced cell apoptosis was activated mainly by the extrinsic pathway. Taken together, the data suggested that UCHL1 protects against H_2O_2 -induced neuronal apoptosis through the caspase 8/caspase 3 pathway.

3.3.11 UCHL1 protects against Aβ-induced neuronal apoptosis

Extracellular A β is toxic to neurons and is thought to be the culprit of AD. Specifically, A β induces apoptosis in cultured neurons (Loo *et al.*, 1993), which may be mediated by activated caspase 2, 8 or 12 (Ivins *et al.*, 1999; Nakagawa *et al.*, 2000; Troy *et al.*, 2000). We have shown the neuroprotective effects of UCHL1 against oxidative stress-induced apoptosis. Next we would like to explore whether UCHL1 also protects against A β -induced apoptosis, which is closely related to conditions in AD brains. For this purpose, we first treated SH-SY5Y or SUCH cells with 10 μ M aged fibrillar A β and measured A β -induced cytotoxicity by LDH assay (Figure 3.11A). UCHL1 overexpression significantly reduced cytotoxicity from $48.8 \pm 2.0\%$ cell death in SH-SY5Y cells to $28.6 \pm 3.3\%$ in SUCH cells (p<0.01). We then examined whether UCHL1 intervened with A β -induced apoptosis and determined the specific apoptotic pathway involved. After A β treatment, cell lysates were detected for cleaved caspase 3, 8 and 9 (Figure 3.11B). Similar to the effects in H₂O₂-induced apoptosis, UCHL1 inhibited the activation of caspase 3 (p<0.01) (Figure 3.11C) and caspase 8 (p<0.01) (Figure 3.11D) induced by aged A β . Cleaved caspase 9 was not detected, possible due to weak signals (data not shown).

To confirm the blockage of A β -induced caspase 8 activation by UCHL1 and to reexamine the caspase 9 activation using a more sensitive strategy, we used the Caspase-Glo® 8 Assay and Caspase-Glo® 9 Assay kits (Promega). The cleaved caspase 8 and caspase 9 were transformed to luminescence signals, which were then measured with a TD-20/20 luminometer (Turner Designs). Primary cortical neurons from C57BL/6 E16 mice were extracted and at DIV 3 infected with AAV1-UCHL1-GFP, an AAV that carried UCHL1 gene. AAV1-UCHL1-GFP was prepared as described in Section 3.2.1. AAV1-GFP was used as control. Transgene expression efficiency was indicated by GFP expression in the neurons (Figure 3.11E). Eight days post-infection GFP was moderately expressed; by day 10, GFP was robustly expressed in the soma as well as in neurites. Therefore we waited 10 days before $A\beta$ treatment. 10 days post-infection, neurons were treated with 200 nM aged A β for 18 h, after which caspase 8 and caspase 9 activities were measured (Figure 3.11F and G). Consistent with earlier findings, UCHL1 markedly repressed Aβ-induced caspase 8 activation by ~40% (p<0.01) (Figure 3.11F). On the other hand, A β also induced caspase 9 activation (Figure 3.11G, lane 1 compared to lane 3), but UCHL1 were not able to inhibit it (p>0.05) (Figure 3.11G, lane 3 compared to 4).





(A) (B) SH-SY5Y and SUCH cells were treated with 10 μ M aged A β for 18 h. (A) LDH assay was performed. Overexpression of UCHL1 in SUCH cells significantly protected against A β -induced lactate dehydrogenase leakage compared to SH-SY5Y cells. N=3, **p<0.01 by two-way ANOVA followed by *post-hoc* Bonferroni test. (B) Cell lysates from (A) were detected for cleaved caspases 3, 8 and 9. Quantification of cleaved caspase 3 (C) and cleaved caspase 8 (D) from (B). UCHL1

protected cells against caspase 3 and caspase 8 activation upon A β treatment. N=3, **p<0.01 by two-way ANOVA followed by *post-hoc* Bonferroni test. (E) Primary cortical neurons from C57BL/6 E16 embryos were infected with AAV1-UCHL1-GFP or AAV1-GFP at DIV 3 (10⁵ genomic copies /neuron). Infection efficiency was indicated by GFP expression. At 10 days postinfection, GFP was robustly expressed in the soma as well as in neurites. (F)(G) Ten days postinfection, neurons were treated with 200 nM A β for 18h. Activities of caspase 8 and caspase 9 were measured by Caspase-Glo® 8 Assay (Promega) and Caspase-Glo® 9 Assay (Promega). Aβinduced caspase 8 activity was significantly lower in UCHL1-overexpressing neurons compared with controls (F). A β -induced caspase 9 activities were similar between the two groups (G). N = 3, **p < 0.01 by two-way ANOVA followed by *post-hoc* Bonferroni test. Values represent mean \pm SEM. (H) Ten days post-infection, neurons were treated with 200 nM A β for 18h together with z-DEVD-FMK (caspase 3 inhibitor), z-IETD-FMK (caspase 8 inhibitor), z-LEHD-FMK (caspase 9 inhibitor) or controls. Neurons were lysed for cleaved caspase 3 detection. UCHL1-overexpressing neurons reduced A β -induced caspase 3 activation (lane 1 and 2). Caspase 8 inhibitor abolished the Aβ-induced caspase 3 activation (lane 5 and 6). Caspase 9 inhibition partially reduced caspase 3 activation, with UCHL1 further inhibiting caspase 3 cleavage. There was no statistic calculation because the experiment has been done for only once.

In the extrinsic apoptotic pathway, the apoptosis executor caspase 3 is activated following the cleavage of caspase 8. To further confirm the effect of UCHL1 on the caspase 8-caspase 3 axis, various caspase inhibitors were applied together with Aβ treatment. Primary neurons were infected with AAV1-UCHL1-GFP or control plasmid at DIV 3. Ten days post-infection, neurons were treated with 200 nM aged Aβ and caspase inhibitors (z-DEVD-FMK for caspase 3 inhibition, z-IETD-FMK for caspase 8 inhibition, z-LEHD-FMK for caspase 9 inhibition). Cell lysates were then detected for cleaved caspase 3. As expected, UCHL1 overexpression repressed Aβ-induced caspase 3 activation (Figure 3.11 H, lane 1 and 2). Caspase 8 inhibitor blocked caspase 3 cleavage in both UCHL1-overexpressing neurons and controls (Figure 3.11H, lane 5 and 6). Caspase 9 inhibitor, on the other hand, partially reduced caspase 3 activation but did not inhibit the rescue effect by UCHL1 (Figure 3.11H, lane 7 and 8). This experiment has been done for only once and needs further replication. Taken together, our data suggested that UCHL1 protects against Aβ-induced apoptosis through the caspase 8/caspase 3 pathway.

3.4 Discussion

As a DUB enzyme, UCHL1 has been shown to regulate UPS-mediated protein degradation. For example, it promotes β -catenin/TCF pathway by inhibiting UPS-mediated β -catenin degradation (Bheda *et al.*, 2009). It also attenuates NF- κ B activation by inhibiting I- κ B degradation (Takami *et al.*, 2007). Here we identified two additional proteins, APP and BACE1, whose expression levels are regulated by UCHL1. Our work demonstrated for the first time that UCHL1 facilitated the degradation of BACE1 and APP, both of which are clearly associated with AD pathogenesis. Therefore, we propose that APP and BACE1 degradation is dependent on UCHL1 activity and this may be one of the mechanisms by which UCHL1 affects AD pathogenesis.

We first demonstrated the accumulation of BACE1 protein level by the UCHL1 inhibitor LDN (Figure 3.1), which indicated the disequilibrium between BACE1 protein synthesis and degradation. We predicted that UCHL1 functions as an accelerator for BACE1 degradation and confirmed the hypothesis using cycloheximide chase assay (Figure 3.2). Previously our lab reported the ubiquitination of BACE1 (Qing et al., 2004). Moreover, GGA3, a clathrin adaptor protein, was identified to bind to ubiquitinated BACE1 and accelerate BACE1 degradation (Kang et al., 2010). Here we showed that UCHL1 also facilitated BACE1 degradation. However, the molecular mechanisms may be different, because we did not detect direct interaction between UCHL1 and BACE1 under any of the conditions (Figure 3.3). The lack of interaction was not likely due to deficiency in protein amount, since we had overexpressed both UCHL1 and BACE1 in the cell. On the other hand, we detected increased free ubiquitin by UCHL1 overexpression (Figure 3.8), which supported the notion that UCHL1 recycles mono-ubiquitin and enriches the free ubiquitin pool (Osaka et al., 2003). Taken together, we propose that UCHL1 indirectly regulates BACE1 degradation, probably by providing more ready-to-use ubiquitin for the cell. We do not exclude the possibility that there are intermediate

players between UCHL1 and BACE1/ubiquitinated BACE1. Further experiments are warranted to explore this possibility.

APP is mainly degraded via lysosomal pathway (Figure 3.6A,C) (Caporaso *et al.*, 1992; Haass *et al.*, 1992). Interestingly, several reports indicated that APP can also be ubiquitinated and sorted to proteasome for degradation (Kaneko *et al.*, 2010; El Ayadi *et al.*, 2012). Here we confirmed the ubiquitination of APP (Figure 3.4). Moreover, UCHL1, the DUB of our interest, inversely regulated APP protein levels (Figure 3.3) and the reduced APP level was concomitant with elevated ubiquituinated APP (Figure 3.3 and 3.4). The results indicated that UCHL1 facilitates APP degradation by promoting its ubiquitination. We further observed that chloroquine, a lysosomal inhibitor, prevented UCHL1-mediated degradation of APP (Figure 3.6), suggesting that UCHL1-facilitated APP degradation is mainly via lysosomal pathway. This is not too surprising, as ubiquitination has been shown to act as a signal for lysosomal degradation for many proteins (Kraft *et al.*, 2010).

Next we tried to reveal the underlying mechanisms for UCHL1-regulated APP degradation. UCHL1 is one of the DUBs, which may reverse the ubiquitination in UPS and rescue proteins from being degraded (Koulich *et al.*, 2008; Lee *et al.*, 2010). On the other hand, proteasome-associated DUB may accelerate protein degradation by cleaving the polyubiquitin chain from the substrate and allowing it to reach the catalytic core of the proteasome (Yao & Cohen, 2002). Things are even more complicated when it comes to UCHL1, as it has been reported to have two apparent opposite enzymatic activities: it may serve as a ubiquitin hydrolase as well as a ligase (Liu *et al.*, 2002). To investigate how UCHL1 upregulated ubiquitin-APP conjugates and facilitated APP degradation, we explored whether UCHL1 physically interacts with APP. We identified that UCHL1 bound to APP, and the binding was potentiated by chloroquine (Figure 3.7). This result was different from our observation with UCHL1 and BACE1.

degradation regulation by UCHL1. It is likely that UCHL1 physically binds to APP and directly exerts its hydrolase or ligase activity.

We demonstrated the enriched free ubiquitin pool by UCHL1 overexpression. Moreover, exogenous ubiquitin resulted in decreased APP protein level in both UCHL1 transfected and non-transfected cells (Figure 3.8). This observation implied that (1) ubiquitin is not yet saturated in the cell, (2) an abundant free ubiquitin pool assists APP degradation and (3) the elevated free ubiquitin level may be one of the mechanisms for UCHL1-regulated facilitation of APP degradation.

Taken together, we propose three possible mechanisms by which UCHL1 reduces APP protein level, in addition to its role in providing ready-to-use ubiquitin. Firstly, UCHL1 may serve as a ubiquitin ligase and assist the poly-ubiquitination of APP. This function has been demonstrated in the ubiquitination of α -synuclein (Liu *et al.*, 2002). A cell-free system with ubiquitin, APP, UCHL1 and other necessary reagents can be employed to test this hypothesis. Secondly, UCHL1 may act as an adaptor protein that escorts ubiquitinated APP to lysosome for degradation. This explains why UCHL1-APP binding was potentiated upon lysosome inhibition. Further investigation into the co-localization of UCHL1 and APP in lysosome will provide more information for this hypothesis. Thirdly, UCHL1 may facilitate APP degradation by cleaving ubiquitin from APP immediately before it is degraded, a function similar to that of RPN11, the proteasome-associated DUB. The three hypotheses are not mutually exclusive. Therefore UCHL1 may implement more than one of them to regulate protein degradation.

Since UCHL1 inversely regulated APP and BACE1 levels, we went on to explore its effect on the downstream products of APP processing, i.e., the APP CTF and A β production. As expected, UCHL1 inhibited APP CTF and A β production (Figure 3.9). Notably, the effects were significant in both APP_{SWE}-overexpressing cell lines and in

cells that overexpressed wildtype APP, which indicated that the regulation of APP processing by UCHL1 may be applicable to both FAD and SAD. Meanwhile, it should be noted that whether UCHL1 regulates APP CTF or A β degradation is not included in this dissertation. Transfection of plasmids that contain cDNA sequences of APP CTFs or A β can be employed to address this question. For a comprehensive study of UCHL1's role on APP processing, its regulation on the degradation of α -secretase or γ -secretase complex should also be explored in future experiments.

Apoptosis has been observed in AD brains (Su *et al.*, 1994). In particular, activated caspase 3, 8 and 9 have been detected in hippocampal neurons of the AD brains (Stadelmann *et al.*, 1999; Rohn *et al.*, 2001; Rohn *et al.*, 2002). Apoptosis may be activated by extracellular A β in the brain, or by oxidative stress that increases with aging, or in response to other challenges. Experiments have shown that A β indeed induces apoptosis in cultured neurons (Loo *et al.*, 1993). Caspase 2, 8 and 12 have been individually observed to mediate A β -induced cell death in each of the studies (Ivins *et al.*, 1999; Nakagawa *et al.*, 2000; Troy *et al.*, 2000). In this chapter we demonstrated that H₂O₂ induced neuronal apoptosis by caspase 8/caspase 3 pathway. Furthermore, aged A β was able to induce neuronal apoptosis through the activation of caspase 3, 8 and 9.

The role of UCHL1 in apoptosis remains controversial. Studies have revealed both anti-apoptotic (Shen *et al.*, 2006; Tan *et al.*, 2008; Costes *et al.*, 2011) and proapoptotic functions of UCHL1 (Kwon *et al.*, 2005; Xiang *et al.*, 2012; Brinkmann *et al.*, 2013). The discrepancy is probably due to its temporally and spatially specific activities. Here we showed that in neuroblastoma cell lines and primary neuronal culture, overexpression of UCHL1 protected against both H_2O_2 - and A β -induced neuronal apoptosis. Specifically, UCHL1 attenuated the activation of caspase 8 and caspase 3 under both challenges (Figure 3.10F, figure 3.11B and F) but not that of

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caspase 9 induced by A β (Figure 3.11G). Taken together, UCHL1 inhibited neuronal apoptosis by interfering with the caspase 8/caspase 3 pathway.

Unlike homozygous *gad* mice, heterozygous *gad* mice do not experience axonal atrophy or display neuromuscular phenotypes. However, it does not necessarily mean that one allele of functional UCHL1 is always sufficient for neuronal survival. Neurons with less UCHL1 expression may be more vulnerable under challenging situations such as increased oxidative stress. Indeed, primary cortical neurons from WT, heterozygous, and homozygous *gad* embryos reacted differently against H₂O₂-induced oxidative stress (Figure 3.10C). The results implied that reduced level of UCHL1 in the brain, although may not affect neuronal integrity at younger age or in a healthy environment, may jeopardize neuronal survival when the environment is challenged. In other words, the decreased level of UCHL1 in sporadic AD brains (Choi *et al.*, 2004) may be partly responsible for the neuronal loss in AD.

3.5 Conclusion

In summary, in this chapter we investigated the role of UCHL1 in APP processing, $A\beta$ production, and neuronal death *in vitro*. We revealed that UCHL1 reduced CTF and A β production by facilitating the degradation of APP and BACE1. Moreover, UCHL1 protected neurons against H₂O₂- and A β -induced apoptosis by inhibiting the caspase 8/caspase 3 signaling pathway.

Chapter 4

Overexpression of UCHL1 as a therapeutic strategy for treating Alzheimer's disease

4.1 Introduction

Despite the extensive effort to find means to treat AD, currently there are no effective medications. Five drugs have been approved by FDA to treat the cognitive problems of AD. Among them four are acetylcholinesterase inhibitors and another is an NMDA receptor antagonist. Unfortunately clinical effectiveness of those drugs is only marginal (Raina *et al.*, 2008). Besides, they only treat the symptoms of AD but do not target the pathogenic steps. Medications that could attenuate or even reverse the neurodegenerative process are urgently needed. In recent years, drugs that aim at reducing A β production and attenuating amyloid plaque deposition are being developed, some of which have reached clinical trials.

Dysfunction of UCHL1 plays an important role in various neurodegenerative diseases. However, the underlying mechanisms remain unknown. Recently, Gong *et al.* proposed a mechanism by which UCHL1 may rescue the memory deficit in AD. They demonstrated in AD mouse model that UCHL1 promoted the PKA/CREB signaling pathway, restored LTP in the hippocampus and thereby rescued the learning and memory deficits (Gong *et al.*, 2006). We hypothesized from another point of view that UCHL1 affects AD progression in a long-term fashion by regulating APP processing and A β production. In chapter 3, we demonstrated that UCHL1 altered APP processing and reduced A β production *in vitro*. We would like to explore the longterm effect of UCHL1 on AD pathogenesis *in vivo*, and evaluate its potential as a disease-modifying strategy for AD therapeutics. In this chapter, we overexpressed and knocked down UCHL1 in AD mouse models and examined their effects on APP processing and A β production *in vivo*. We performed immunohistochemical staining to detect amyloid plaque formation. In addition, a batch of behavioral tests were performed to assess muscle strength, locomotor activity, as well as learning and memory abilities in these mice.

4.2 Methods

4.2.1 Transgenic animals

APP23 transgenic mice carry human APP751 cDNA with the Swedish mutation at positions 670/671 (KM/NL) under control of the murine Thy-1.2 promoter (Sturchler-Pierrat *et al.*, 1997; Sturchler-Pierrat & Staufenbiel, 2000). PS45 transgenic mice carry human PS1 cDNA with the G384A mutation identified in a family with early-onset FAD under control of the murine Thy-1 promoter (De Jonghe *et al.*, 1999; Herzig *et al.*, 2004; Qing *et al.*, 2008). The gracile axonal dystrophy (*gad*) mouse carries a spontaneous mutant with an in-frame deletion in exons 7 and 8 of *UCHL1* (Saigoh *et al.*, 1999), and is equivalent to a UCHL1 knockout mouse model. Heterozygous *gad* mice were originally generated and kindly provided by Dr. Keiji Wada at the National Institute of Neuroscience of Japan. The APP23/PS45 double transgenic mice were generated by breeding the APP23 hemizygous strain with the *gad* heterozygous strain. The genotypes of the mice were confirmed by PCR from DNA extracted from ear tissue.

4.2.2 Genotyping

All transgenic mice were genotyped at one month of age. The genotyping protocol was described in Section 3.2.10. For the genotyping of APP23, genomic DNA was subjected to PCR to amplify human APP using the primers Thy1E2F (CACCACAGAATCCAAGTCGG) and APP1082R (CTTGACGTTCTGGCCTCTTCC). For the genotying of UCHL1 expression,

genomic DNA was subjected to PCR to amplify mouse UCHL1 using the forward primer UCHL1-F4 (AGCCAAGTGTTTCGAGAAGAACGAG), and reverse primers UCHL1-R3(TGGCAGCATCCTGAAAAGGAGAGAGGTG) and UCHL1-R5(TACAGATGGCCGTCCACGTTGTTGA).

4.2.3 Virus injection

Seven-week-old APP23/PS45 mice were anesthetized by isoflurane (1-2%) and secured on the stereotaxic frame (Kopf Instruments). Meloxicam (2mg/kg) was injected subcutaneously before the skull was exposed. A small section of the skull was removed using a micro drill (Fine Science Tools, 0.5mm tip diameter) at the coordinates of 2.0 mm rostral and 2.0 mm lateral to the bregma. One microliter of AAV1-UCHL1-GFP or AAV1-GFP (10^{13} GC/ml) was injected bilaterally or unilaterally (1.8 mm ventral from the dura) at a rate of 0.2 µl/min. After each injection, the needle was left in place for an additional 2 min before withdrawal.

4.2.4 Rotarod test

Mice were placed on a single station, standard mouse rotarod (ENV-576M, Med Associates Inc., USA) with a shaft diameter of 3.2 cm, lane width of 5.7 cm, fall height of 16.5cm, and divider diameter of 24.8 cm. The rod was accelerated from 0 to 20 rpm over a time period of 300 seconds. Latency to fall was measured. The test was stopped at the end of 300 seconds if the mouse was still riding the rod. Mice were returned to their home cages for 2 hours, after which they were retested. The average of the two trials was taken as a measure of balance and motor coordination.

4.2.5 Hanging wire test

The hanging platform for hanging wire tests consisted of a section of 1 cm by 1 cm wire mesh, with a 9 cm by 12 cm area isolated by clear plastic tape. Mice were allowed to grip the center of the mesh and were swiftly inverted over a black container 50 cm in height. A black, cushioned pad was placed at the bottom of the container to

ensure that falling mice would not be injured. Latency to fall was measured, with a maximum hanging time of 60 sec. Mice were returned to their home cages for approximately 2 hours, after which they were re-tested. The average of the two trials was taken as a measure of hang strength.

4.2.6 Morris water maze test

The Morris Water Maze test was performed as previously described (Qing et al., 2008). Briefly, the test was performed in a 1.5-meter diameter pool with a 10-cm diameter platform placed in the southwest quadrant of the pool. The procedure consisted of one day of visible platform tests and 4 days of hidden platform tests, plus a probe trial 24 h after the last hidden platform test. In the visible platform test, the platform was placed 1 cm above the clear water surface. The position of the platform and the starting direction varied across trials. Mice were tested for 5 continuous trials with an inter-trial interval of 75 min. Mice were allowed to swim for at most 60 sec before climbing onto the platform. Failed mice were gently guided to the platform and remained there for 20 sec before they were sent back to their home cages. In the hidden platform tests, the platform was submerged 1 cm below an opaque water surface in a fixed position in the southwest/third quadrant. Mice were trained for 5 trials with an inter-trial interval of 75 min. Mice were allowed to swim for at most 60 sec before climbing onto the platform, and again, failed mice were gently guided to the platform and remained there for 20 sec. In the probe trial, the platform was removed and mice were allowed to swim for 60 sec. Mouse behavior including speed and escape latency was automatically video-recorded by automated video tracking (ANY-maze, Stoelting).

4.2.7 Immunohistochemistry

The immunohistochemical staining was performed as previously described (Ly *et al.*, 2011). Briefly, mice were sacrificed and half of the brains were fixed in 4% paraformaldehyde, followed by 30% sucrose solution, and sectioned with a Leica

Cryostat to 30 µm thickness after embedding in O.C.T. solution. Every 12th slice with the same reference position was mounted onto the slides for staining. The slices were stained with biotinylated monoclonal 4G8 antibody. Plaques were visualized by the ABC and DAB method and photos were taken under microscopy at 40X magnification. Plaques were quantified and the average plaque counts per slice were recorded for each mouse. Thioflavin-S staining of plaques was performed with 1% thioflavin-S. The green fluorescence-stained plaques were visualized using fluorescence microscopy.

4.2.8 Immunoblotting

Brain tissues were lysed in RIPA lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 0.15M NaCl, 0.05M Tris-HCl, pH 7.2) supplemented with 200 mM sodium orthovanadate, 25 mM β -glycerophosphate, 20 mM sodium pyrophosphate, 30 mM sodium fluoride, 1 mM PMSF, and a complete mini protease inhibitor cocktail tablet (Roche Diagnostics). The samples were diluted in 4X SDS-sample buffer, boiled, and resolved on 8% tris-glycine, 12% tris-glycine or 16% tristricine SDS-PAGE followed by transferring to PVDF-FL membranes. Immunoblotting procedure was carried out as indicated in Section 2.2.8. Rabbit anti-C20 antibody and rabbit anti-GFP antibody were made in-house.

4.2.9 Human Aβ ELISA

Tissue extracts from transgenic mouse hippocampal regions were collected and prepared according to manufacturer's instructions. Briefly, tissues were homogenized in 5 M guanidine HCl/50 mM Tris HCl (pH 8.0) and allowed to mix at room temperature for 4 h. The samples were then diluted in ice-cold reaction buffer (PBS with 5% BSA, 0.03% Tween-20, and supplemented with AEBSF and Roche mini protease inhibitor cocktail tablet). The final guanidine HCl concentration was less than 0.1M. The concentration of A β_{40} was detected using A β 40 Human ELISA Kit (Invitrogen).

4.2.10 Statistical analysis

All results were presented as mean \pm SEM and analyzed by 2-tailed Student's *t*-test, one-way ANOVA or two-way ANOVA. Statistical significance is accepted when p<0.05 (*p<0.05, **p<0.01, ***p<0.001). Outliers were determined by Tukey's boxplot.

4.3 Results

4.3.1 Knockdown and overexpression of UCHL1 in AD mouse models

To examine the effect of UCHL1 on APP processing and AD pathogenesis, we manipulated UCHL1 expression in AD model mice in two ways. We knocked down UCHL1 expression by breeding APP23 mice with *gad* mice to generate APP23/*gad*. The APP23 AD mouse model carries human APP751 cDNA with the Swedish mutation at positions 670/671 (KM/NL) under control of the murine Thy-1.2 promoter. The gracile axonal dystrophy (*gad*) mouse carries a spontaneous mutation with an inframe deletion in exons 7 and 8 of *UCHL1* (Saigoh *et al.*, 1999). Since no full-length or truncated UCHL1 is detected, *gad* mouse is equivalent to a UCHL1 knockout mouse model. APP23/*gad* contains one allele of functional *UCHL1* and expresses approximately 50% of UCHL1 protein compared to APP23 or wildtype C57BL/6 mice.

We overexpressed UCHL1 by intracranially injecting AAV1 that contains human *UCHL1* gene to the hippocampal regions of APP23/PS45 mice (Figure 4.1A). APP23/PS45 was derived from the crossing of APP23 and PS45 mice. PS45 is a mouse strain that carries the human familial AD-associated G384A mutant PS1. The introduction of PS45 mutation greatly facilitates A β plaque deposition and learning and memory deficits in APP23 mice, which shortened the breeding time prior to the experiments. To make the AAV that contained human *UCHL1* gene, human *UCHL1* cDNA sequence was cloned into the pAAV-GFP-cDNA6 vector as described in

Section 3.2.1. AAV1-UCHL1-GFP and AAV1-GFP were made by Vector Biolabs. Separate CMV promoters on AAV1-UCHL1-GFP allowed bi-cistronic expression of UCHL1 and eGFP in individual transduced neurons. Eight weeks after viral injection, the mice underwent a batch of behavioral tests, after which they were sacrificed and the brains were processed for histochemical analysis, western blot analysis and A β ELISA assay.

To verify the gene expression, hippocampal tissues from saline-, AAV1-GFP- and AAV1-UCHL1-GFP-injected mice were lysed for protein detection 8 weeks after viral injection (Figure 4.1B). EGFP was detected in both AAV1-GFP- and AAV1-UCHL1-GFP-injected hippocampi but not in saline-injected ones. UCHL1 level was increased in AAV1-UCHL1-GFP-injected hippocampi compared to those treated with AAV1-GFP.

To further verify the viral transduction, another two mice at 7 weeks of age were unilaterally injected with AAV1-UCHL1-GFP or AAV1-GFP to the left hippocampi and saline to the right hippocampi. Eight weeks post-injection, eGFP was robustly expressed in the left hippocampi of both mice. Only trace amount of eGFP was detected in the right hippocampi, which was probably due to the virus leakage to the other hemisphere through cerebrospinal fluid (CSF). UCHL1 was increased in the left hippocampus of AAV1-UCHL1-GFP-injected mouse but not in AAV1-GFP-injected mouse (Figure 4.1C).





APP23/PS45 mice

(A) APP23/PS45 mice at the age of seven weeks old were intracranially injected with AAV1-UCHL1-GFP or AAV1-GFP control to the hippocampal region. One microliter of rAAV (10^{13} GC/ml) was injected to each hippocampus (2.0 mm rostral to bregma, 2.0 mm lateral to bregma, 1.8 mm ventral from the dura) (Keith B. J. Franklin, 2001) at a rate of 0.2 µl/min. Eight weeks post-injection, the mice underwent behavioral tests and were then sacrificed. (B) Hippocampal tissues from saline-, AAV1-GFP- and AAV1-UCHL1-GFP-injected APP23/PS45 mice were lysed in RIPA buffer for protein detection 8 weeks after the injection. EGFP was detected by rabbit anti-GFP antibody made in-house. UCHL1 was detected by anti-UCHL1 antibody BH7 (Novus). EGFP was detected in both AAV1-GFP- and AAV1-UCHL1-GFP-treated mice but not in saline-treated ones. UCHL1 level was increased in AAV1-UCHL1-GFP-treated hippocampi compared to those treated with AAV1-GFP. β -actin served as protein loading control. (C) Mice were unilaterally injected with AAV1-UCHL1-GFP or AAV1-GFP to the left hippocampi and saline to the right hippocampi at 7 weeks of age. Eight weeks post-injection, eGFP was robustly expressed in the left hippocampi of both mice whereas only trace amount was detected in the right hippocampi. UCHL1 was increased in the left hippocampus of AAV1-UCHL1-GFP-injected mouse but not AAV1-GFP-injected mouse.

4.3.2 UCHL1 overexpression reduces Aβ plaque deposition in APP23/PS45 transgenic mice

To investigate the effect of UCHL1 overexpression on AB plaque deposition, APP23/PS45 mice were bilaterally injected with AAV1-UCHL1-GFP or its control AAV1-GFP at seven weeks of age. Eight weeks later the mice underwent a batch of behavioral tests before they were sacrificed. Aß specific 4G8 immunostaining and thioflavin-S staining for β -sheet were used to detect A β -containing neuritic plaques in the brain. UCHL1 overexpression significantly reduced the number of Aβ plaques in the hippocampal region of APP23/PS45 mice compared to controls (Figure 4.2Aa and b). Quantification showed that UCHL1 overexpression decreased plaque number by approximately 50% (54.0 \pm 4.3 Vs. 104.3 \pm 6.5 per slice, p<0.01) (Figure 4.2B). Thioflavin-S staining verified the reduction of β -sheet formation by UCHL1 overexpression (Figure 4.2Ac and d). Strikingly, viral injection of UCHL1 to the hippocampi also significantly affected the A β deposition in the neocortex area (Figure 4.2 Ca and b). The cortex of UCHL1-treated mice contained approximately 45% Aβ plaque numbers compared to controls $(44.9 \pm 4.6 \text{ Vs}. 98.1 \pm 5.5 \text{ per slice}, p < 0.01)$ (Figure 4.2D). Thioflavin S staining further confirmed this effect (Figure 4.2 Cc and d).

In APP23 and APP23/gad mice, behavioral experiments were carried out at six months of age, after which mice were sacrificed for histochemical and biochemical analysis. At six months old, the single transgenic APP23 mice merely started to develop A β plaques, with at most 1 to 2 plaques per slice (data not shown). While plaque numbers increased in APP23/gad mice compared to APP23 (~1.5 plaques per slice in APP23/gad compared to ~0.5 plaque per slice in APP23), the number was too small to implicate any biological significance.



Figure 4.2 Overexpression of UCHL1 significantly decreases amyloid plaque formation in

APP23/PS45 double transgenic mice

(A) (B)APP23/PS45 mice at the age of seven weeks old were bilaterally injected with AAV1-UCHL1-GFP or AAV1-GFP control to the hippocampal region. Eight weeks post-injection the mice underwent behavioral tests, after which they were sacrificed. The brains were dissected, fixed and sectioned to 30 µm thickness. Amyloid β plaques were detected by Aβ-specific monoclonal antibody 4G8 and DAB method. The plaques were visualized by microscopy with 40X magnification. The number of $A\beta$ plaques in the hippocampal region was significantly decreased in APP23/PS45 mice with AAV1-UCHL1-GFP injection (A,b) compared to controls (A,a). The results were confirmed by thioflavin-S staining. Thioflavin-S-positive amyloid structures in the hippocampal region were greatly reduced in AAV1-UCHL1-GFP-treated mice (A.d) compared to controls (A,c). (B) Quantification of A^β plaques in (A, a-b) by Image J. The number stands for the average plaque number on each photo taken by microscopy under 40X magnification. The value represents mean \pm SEM. N = 8 for each group. **p < 0.01 by Student's *t*-test. (C) Detection of amyloid plaques in the cortical region in the same group of mice as in (A). The number of A β plaques detected by 4G8 in the cortical region was significantly decreased in AAV1-UCHL1-GFP -injected APP23/PS45 mice (C,b) compared to controls (C,a). Thioflavin-S-positive amyloid structures in the cortical region were greatly reduced in UCHL1-overexpressed mice (C,d) compared to controls (C,c). Bar: 500 μ m. (D) Quantification of A β plaques in (C, a-b). The value represents mean \pm SEM. N = 8 for each group. **p < 0.01 by Student's *t*-test.

4.3.3 Overexpression or partial loss of UCHL1 does not affect motor coordination or muscle strength in AD mouse models

Homozygous *gad* mice started to show the phenotypes of gracile axonal dystrophy at 12 weeks of age, including sensory ataxia followed by hindlimb paralysis. While such phenotypes were not reported in heterozygous *gad* mice, it was important to exclude any neuromuscular defects in APP23/*gad* mice before they underwent further behavior tests to examine their learning and memory ability. Otherwise the neuromuscular defects would become confounding factors when interpreting those tests. We also checked whether injection of AAV-UCHL1-GFP or AAV-GFP to APP23/PS45 affects their motor function and muscle strength or not.

Here we present the results in APP23/PS45 mice first, followed by APP23 and APP23/gad mice, to be consistent with the data presentation throughout the chapter. The rotarod test was carried out to access the motor coordination of the mice. There was no difference of the latency to fall between AAV1-UCHL1-GFP-injected and AAV-GFP-injected APP23/PS45 mice (p>0.05) (Figure 4.3A). The hanging wire test was to measure the muscle strength of the mice. There was no difference of the hanging time between the two groups either (p>0.05) (Figure 4.3B). However, there was one mouse in the AAV-UCHL1-GFP-injected group whose latency to fall in the rotarod test and hanging time in the hanging wire test were both outliers and significantly lower than rest of the mice in the group (by Tukey's boxplots). There was also one mouse in the AAV-GFP-treated group who was the outlier of its group. Both mice were excluded from future watermaze test. For APP23 and APP23/gad mice, both the latency to fall (Figure 4.3C) and the hanging time (Figure 4.3D) were similar between the two groups (p>0.05). There were no outliers in either group in either of the tests. Therefore the APP23 Vs. APP23/gad mice was a valid model for future behavioral experiments to assess the effect of UCHL1 deficiency on learning and memory ability.



Figure 4.3 Overexpression or partial loss of UCHL1 does not affect motor coordination or

muscle strength in AD mouse models

(A)(B) Eight weeks post-injection of AAV-UCHL1-GFP or control AAV, APP23/PS45 mice were examined for motor coordination ability and muscle strength. Motor coordination was assessed by the rotarod test. Muscle strength was measured by the hanging wire test. N=17 for AAV1-GFP group including 10 males and 7 females, n=14 for AAV1-UCHL1-GFP group including 8 males and 6 females. (A) There were no differences in the latency to fall in the rotarod test between treatment and control group (p>0.05). (B) There were no differences in the hanging time in the hanging wire test between the two groups (p>0.05). (C)(D) Six-month-old APP23 or APP23/gad mice were examined in the rotarod and the hanging wire test. N=12 for APP23 group including 5 males and 7 females, n=10 for APP23/gad group including 4 males and 6 females. (C) There were no differences in the latency to fall in the rotarod test between the two groups (p>0.05). (D) There were no differences in the hanging wire test between the two groups (p>0.05). (D) There were no differences in the hanging wire test between the two groups (p>0.05). (D) There were no differences in the hanging wire test between the two groups (p>0.05). (D) There were no differences in the hanging wire test between the two groups (p>0.05).

4.3.4 Overexpression of UCHL1 rescues memory deficits in APP23/PS45

transgenic mice

To investigate whether UCHL1 overexpression affects the memory deficit in AD, the Morris watermaze was carried out in AAV-UCHL1-GFP-injected APP23/PS45 mice and the control group. In the visible platform test on day 1, there was no difference of the escape latency (p>0.05) (Figure 4.4A) or swimming speed (p>0.05) (Figure 4.4B) between the two groups. There were no outliers of the escape latency or swimming speed in either group (Figure 4.4A and B, by Tukey's boxplots). The results indicated that UCHL1 overexpression did not affect the swimming ability or vision. In the hidden platform test from day 2 to day 5, UCHL1-overexpressing APP23/PS45 mice

showed significantly shortened escape latency compared to the control group (p<0.05, group comparison by two-way ANOVA) (Figure 4.4C), indicating improved learning and memory ability. In the probe trial test on day 6, UCHL1-overexpressing mice spent markedly increased time in the quadrant where the hidden platform was placed from day 2 to day 5 (20.6 ± 1.6 s Vs. 15.0 ± 1.9 s, p<0.05) (Figure 4.4D), suggesting improved memory abilities. The data demonstrated the rescued memory deficits by UCHL1 in AD mouse model.





APP23/PS45 double transgenic mice

Morris watermaze test consists of one day of visible platform trials, 4 days of hidden platform trials and a probe trial 24 h after the last hidden platform trial. Eight weeks after AAV1-UCHL1-GFP or AAV1-GFP injection, APP23/PS45 mice were subjected to Morris watermaze test at the age of 15 weeks old. N=16 for AAV1-GFP group (9 males and 7 females), n=13 for AAV1-UCHL1-GFP group (7 males and 6 females). (A)(B) On the first day of visible platform test, AAV-UCHL1-GFP-injected mice displayed similar escape latency (A) and swimming speed (B) as control mice. p>0.05 by Student's *t*-test. There were no outliers in either mouse group for either test (A)(B) by Tukey's schematic boxplots. (C) During day 2 to day 5 of hidden platform test, AAV1-UCHL1-GFP mice exhibited shorter escape latency compared to control mice. *p<0.05 by two-way ANOVA group comparison. (D) On day 6 in the probe trial, AAV1-UCHL1-GFP-injected mice in the target quadrant than controls. *p<0.05 by Student's *t*-test. The values are expressed as mean±SEM.

4.3.5 Partial loss of UCHL1 exacerbates memory deficits in APP23 transgenic mice

To examine if loss of UCHL1 exacerbates memory deficits in AD, Morris watermaze was carried out in APP23 and APP23/*gad* mice. We chose APP23 single transgenic AD model over APP23/PS45 double transgenic mice because APP23/PS45 mice develop severe A β plaque deposition and memory deficits that may not leave room for further deterioration with the knockdown of UCHL1.

In the visible platform test on day 1, there was no difference in the escape latency (p>0.05) (Figure 4.5A) or swimming speed (p>0.05) (Figure 4.5B) between APP23 and APP23/gad mice. Neither were there any outliers in these tests (Figure 4.5A and B, by Tukey's boxplots). The data indicated that partial loss of UCHL1 did not affect the swimming ability or vision. In the hidden platform test from day 2 to day 5, APP23/gad mice showed significantly prolonged escape latency compared to APP23. On day 4 and day 5, APP23/gad mice spent more time looking for the hidden platform compared to APP23 $(34.8 \pm 3.8 \text{ Vs}. 24.8 \pm 2.5 \text{ for day } 4, 31.6 \pm 2.8 \text{ Vs}. 21.3 \pm 2.1 \text{ s}$ for day 5, p<0.05 by two-way ANOVA *post-hoc* test) (Figure 4.4C). In the probe trial test on day 6, there was a trend for less time in the target quadrant in APP23/gad mice, although the difference was not yet statistically significant (18.5 ± 1.5 s Vs. 14.8 ± 2.2 s, p>0.05). The times that mice passed the virtual platform, the exact area where the platform was put from day 2 to day5, on the other hand, was statistically different between the two groups. APP23/gad mice passed the virtual platform less frequently than APP23 mice $(0.8 \pm 0.4 \text{ Vs. } 2.7 \pm 0.7, \text{ p} < 0.05)$. Taken together, the data demonstrated that partial loss of UCHL1 further exacerbated the memory deficits in AD mouse model.



Figure 4.5 Partial loss of UCHL1 exacerbates memory deficits in APP23 transgenic mice

Six-month-old APP23 or APP23/gad mice were subjected to Morris water maze test. N=12 for APP23 group (5 males and 7 females), n=10 for APP23/gad group (4 males and 6 females). (A)(B) On the first day of visible platform test, APP23 and APP23/gad mice displayed similar escape latency (A) and swimming speed (B). P>0.05 by Student's *t*-test. There were no outliers in APP23 or APP23/gad group for either escape latency (A) or swimming speed (B) by Tukey's schematic boxplots. (C) During day 2 to day 5 of hidden platform test, APP23/gad mice exhibited significantly longer escape latency compared to APP23 mice on the 4th and 5th day. *p<0.05 by two-way ANOVA *post-hoc* Bonferroni test. (D) On day 6 in the probe trial, APP23 and APP23/gad mice spent similar time in the target quadrant compared to controls. P>0.05 by Student's *t*-test. (E) On day 6 in the probe trial, APP23/gad mice awhere the hidden platform was previously placed, for significantly fewer times than APP23 mice. *p<0.05 by Student's *t*-test. The values are expressed as mean±SEM.

4.3.6 UCHL1 reduces APP CTF and Aβ production *in vivo*

In chapter 3 we demonstrated clearly that UCHL1 inversely regulated APP CTF and Aβ production *in vitro*. Here we confirmed the effect of UCHL1 on APP processing *in* vivo. APP23/PS45 mice injected with AAV1-UCHL1-GFP or control virus were sacrificed after behavioral tests. The hippocampi were dissected and lysed in RIPA buffer for western blot analysis. APP CTFs were detected by C20 antibody. The CTFs from the hippocampi of the UCHL1-overexpressing mice were significantly reduced to $64.3 \pm 6.6\%$ of those in control mice (p<0.05) (Figure 4.6A,B). Another batch of hippocampi were homogenized in 5 M guanidine HCl/50 mM Tris HCl (pH 8.0) for Aβ ELISA assay following manufacturer's instruction (Invitrogen). Overexpression of UCHL1 markedly lowered the A β_{40} level in the hippocampi to $68.8 \pm 7.6\%$ of that in control mice (p<0.05) (Figure 4.6C). Notably, viral transduction of UCHL1 to the hippocampi also reduced the CTF levels in neocortex, including the frontal lobe, occipital lobe, and temporal lobe (Figure 4.6D). Quantification of the CTFs in neocortex as a whole showed statistically lowered CTF levels in UCHL1-treated mice $(60.7 \pm 7.7\%)$ compared to controls (p<0.05) (Figure 4.6E). Next we examined whether UCHL1 knockdown affected APP processing in vivo. Six-month-old APP23/gad and APP23 mice were sacrificed after behavioral tests. As expected, APP CTFs in the hippocampi of APP23/gad mice were significantly higher $(155.9 \pm 11.4\%)$ than those in APP23 mice (Figure 4.6F,G). Taken together, the data suggested that UCHL1 inversely regulates APP CTFs and Aβ production in AD mouse models.



Figure 4.6 UCHL1 inversely regulates APP CTFs and Aβ production in vivo

After behavioral tests, mice were sacrificed and the brains were homogenized for protein detection. (A) Hippocampi of AAV1-UCHL1-GFP- or AAV1-GFP-treated APP23/PS45 mice were lyzed in RIPA buffer. APP CTFs were detected by C20 antibody. β -actin served as protein loading control. (B) Quantification of (A). Total CTFs in AAV1-UCHL1-GFP-treated mice were lower than in controls. N=8 for each group. **p<0.01 by Student's *t*-test. (C) Hippocampi of AAV1-UCHL1-GFP- or AAV1-GFP-treated APP23/PS45 mice were lysed in 5 M guanidine HCl/50 mM Tris HCl (pH 8.0) for A β detection by ELISA (Invitrogen). A β_{40} levels of AAV1-UCHL1-GFP-treated mice were significantly lower than control mice. N=5 for each group. *p<0.05 by Student's *t*-test. (D) Immunoblotting of APP CTFs from cortical tissues of frontal lobe, temporal lobe and occipital lobe from AAV1-UCHL1-GFP- or control APP23/PS45 mice. (E) Quantification of (D). CTFs from the cerebral cortex were quantified as a whole. N=8 for each group. *p<0.05 by Student's *t*-test. (F) Immunoblotting of APP CTFs from hippocampal tissues of APP23 and APP23/gad mice. (G) Quantification of (F). Total CTFs in APP23/gad mice were higher than in APP23 mice. N=8 for each group. *p<0.05 by Student's *t*-test. (F) Immunoblotting of APP CTFs from hippocampal tissues of APP23 and APP23/gad mice.

4.3.7 UCHL1 reduces APP and BACE1 protein levels in vivo

We have demonstrated *in vitro* the facilitated degradation of APP and BACE1 by UCHL1. To verify this effect in vivo, hippocampi of AAV1-UCHL1-GFP- and control-injected APP23/PS45 mice were detected for APP and BACE1 (Figure 4.7A). Viral overexpression of UCHL1 significantly lowered the full-length APP level $(39.5 \pm 5.5\%)$ compared to control (p<0.05) (Figure 4.7B). There was a trend of decreased BACE1 level in UCHL1-overexpressing mice ($84.7 \pm 9.7\%$ compared to control), although the difference was not yet statistically significant (p>0.05) (Figure 4.7C). Similarly, when comparing APP23/gad and APP23 mice (Figure 4.7D), partial loss of UCHL1 in APP23/gad mice markedly increased the full-length APP level $(201.2 \pm 35.2\%)$ compared to APP23 mice (p<0.05) (Figure 4.7E). There was a trend of elevated BACE1 level in APP23/gad mice ($120.0 \pm 13.2\%$), but it was not statistically significant (p>0.05) (Figure 4.7F). The lack of statistical significance in Figure 4.7C and F may be due to a milder effect of UCHL1 on BACE1 degradation combined with a relative small sample size. To further examine the regulation of BACE1 protein level by UCHL1 in vivo, we extracted the hippocampi from 5-monthold homozygous gad mice and wildtype littermates. No UCHL1 protein was detected in homozygous gad mice (Figure 4.7G). BACE1 level in gad mice was increased to $212.5 \pm 17.6\%$ of that in WT mice (p<0.05) (Figure 4.7G,H), indicating that complete loss of UCHL1 led to marked BACE1 accumulation in vivo.



Figure 4.7 UCHL1 inversely regulates full-length APP and BACE1 protein levels in vivo

(A) Immunoblot of full-length APP and BACE1 from hippocampal tissues of AAV1-UCHL1-GFP-injected or control APP23/PS45 mice. (B) Quantification of full-length APP levels in (A). APP levels in AAV1-UCHL1-GFP-injected mice were lower than in controls. N=8 for each group. *p<0.05 by Student's *t*-test. (C) Quantification of BACE1 levels in (A). BACE1 levels were similar in two groups. N=8 for each group. p>0.05 by Student's *t*-test. (D) Immunoblot of fulllength APP and BACE1 from hippocampal tissues of APP23 and APP23/gad mice. (E) Quantification of APP levels in (D). APP levels in APP23/gad were enhanced than in APP23 mice. N=8 for each group. *p<0.05 by Student's *t*-test. (F) Quantification of BACE1 levels in (D). BACE1 levels were statistically the same in two groups. N=8 for each group. p>0.05 by Student's *t*-test. (G) Hippocampal tissues from 5-month-old *gad* and wildtype (WT) littermates were lysed. Endogenous BACE1 was detected by anti-BACE1 antibody (Cell Signaling). (H) Quantification of (G). BACE1 level in *gad* mice was markedly enhanced than in WT mice. N=8 for each group. *p<0.05 by Student's *t*-test. The values represent as mean±SEM.

4.4 Discussion

Currently, there are no medications that can delay or halt the progression of AD. Medications that are being used to treat AD are acetylcholinesterase inhibitors (tacrine, rivastigmine, galantamine and donepezil) and an NMDA receptor antagonist (memantine), all of which are symptom-relieving but not disease-modifying (Raina et al., 2008). Drugs that interfere with the pathogenic steps of AD are absolutely needed. APP processing and A β production play a central role in AD pathogenesis, and therefore have become promising biological targets for AD therapeutic design. In chapter 3 we showed that UCHL1 decreased A^β production *in vitro*. In this chapter we further demonstrated its Aβ-reducing effect *in vivo*. We used rAAV to intracranially deliver UCHL1 to the hippocampal area of the mice. This method ensured the longterm overexpression of UCHL1 and the temporal and spatial specificity. We showed that UCHL1 indeed altered APP processing, reduced Aβ production, inhibited plaque formation and rescued learning and memory deficits in AD mouse models. Thus, in addition to the temporary LTP-restoring function demonstrated by Gong et al. (Gong et al., 2006), UCHL1 may also improve learning and memory in AD by reducing AB production and delaying the development of AD pathology. Taken together, the data implicated that transgene expression of UCHL1 is a disease-modifying strategy for AD therapeutic design.

In this chapter we also confirmed the inverse regulation of APP and BACE1 by UCHL1 *in vivo*. Interestingly UCHL1 seems to have a moderate inhibitory effect on full-length APP and a mild effect on BACE1 *in vivo*, as (1) decrease of UCHL1 by half led to ~100% increase in APP, while the increase in BACE1 was statistically significant only by complete loss of UCHL1 (Figure 4.7), and (2) CTF β and CTF α levels in mouse brains were proportionally reduced by UCHL1 without the expected significant CTF β /CTF α ratio change if BACE1 activity was markedly altered (Figure 4.6). This is to some extent good news for UCHL1 as a drug candidate, for it avoids the possible side effects from potent inhibition of BACE1, such as the deficits in synaptic plasticity and changed behavior in open field test observed in BACE1 knockout mice (Laird *et al.*, 2005). On the other hand, the reduction on APP should not cause too serious problems, as the APP family proteins in mammals (APP, APLP1 and APLP2) have redundant and partly overlapping functions (further discussed in Section 5.5).

The expression of UCHL1 in the nervous system is thought to be redundant, as its pathogenic mutations cause neurodegenerative phenotypes in a recessive manner (Yamazaki *et al.*, 1988; Walters *et al.*, 2008; Bilguvar *et al.*, 2013). In accordance, in our lab the heterozygous *gad* mice did not display any neuromuscular phenotype. However, this does not necessarily mean that partial loss of UCHL1 does no harm to neuronal integrity. In fact, we have shown in chapter 3 (Figure 3.3.10) that primary neurons from heterozygous *gad* mice were more vulnerable upon oxidative stress challenge. In humans, one functional allele of UCHL1 may allow the survival to later stage of life when diseases in the elderly such as AD and PD start to develop. At that stage, partial loss of UCHL1 may increase the odds of getting AD or accelerate its progression. To explore the possibility of this theory, we compared the heterozygous APP23/*gad* with APP23 mice. Indeed reduction of UCHL1 by ~50% exacerbated AD-like pathology and behavioral performance. The data suggested that the lower expression of UCHL1 in AD brains may be partly responsible for the pathophysiology and memory impairment in patients.

4.5 Conclusion

In conclusion, we demonstrated that UCHL1 reduced A β production *in vivo* by moderately decreasing full-length APP level and mildly down-regulating BACE1 level. Moreover, UCHL1 inhibited A β plaque deposition and rescued memory deficits in AD mouse models. Our results indicated that overexpression of UCHL1 is a candidate therapeutic strategy to treat AD.

Chapter 5

Conclusions and discussions

5.1 Conclusions

5.1.1 Chapter 2: NF-κB signaling inhibits UCHL1 gene expression

Previous findings suggested that reduced UCHL1 level in the brain may play a role in the development of AD and PD. However, the mechanisms leading to its reduced expression remain unknown. To address this question, we studied the transcriptional regulation of UCHL1. We cloned the human *UCHL1* gene promoter region and identified a functional NF- κ B binding site on it. We demonstrated that NF- κ B suppressed *UCHL1* gene transcription. Moreover, activation of NF- κ B signaling by the inflammatory stimulator LPS and TNF α resulted in decreased *UCHL1* mRNA and protein levels. Taken together, we demonstrated that NF- κ B signaling inhibited UCHL1 gene expression. The discovery implicated that the chronic inflammation and NF- κ B up-regulation in AD brains may account for the concomitantly lowered UCHL1 expression.

5.1.2 Chapter 3: UCHL1's effect on Aβ production and neuronal death

UCHL1 has been shown to process ubiquitin precursors and regulate the degradation of a few proteins, including α -synuclein, β -catenin and I κ B- α . In this chapter we identified two additional proteins, APP and BACE1, whose expression levels are regulated by UCHL1. We demonstrated that UCHL1 facilitated the degradation of APP and BACE1, both of which are critical to APP processing. Overexpression of UCHL1 further reduced CTF production, C99/C83 ratio, and A β production, which indicated its potential role in delaying AD pathogenesis. Moreover, we revealed that UCHL1 may regulate APP and BACE1 degradation by divergent mechanisms. While the degradation of both APP and BACE1 may be facilitated with enhanced free ubiquitin level by UCHL1, UCHL1 physically interacts with APP and therefore may also directly regulate APP degradation. Neuronal loss is a prominent feature of AD pathology. In particular, apoptosis and activated caspases have been detected in AD brains and in cultured neurons treated with A β . In this chapter we demonstrated that UCHL1 protected against oxidative stress- and A β -induced neuronal apoptosis. Furthermore, UCHL1 exerted its anti-apoptotic effect by inhibiting the caspase 8/caspase 3 pathway, but it had no effect on caspase 9 activation. This discovery provided a mechanism for the neuroprotective role of UCHL1 in AD brains.

Future experiments. In this chapter we examined the effect of UCHL1 on APP processing and A β production *in vitro*, which laid the foundation for further experiments *in vivo* as shown in chapter 4. In addition, we tried to explore the underlying molecular mechanisms, for example, of how UCHL1 facilitates the lysosomal degradation of APP. There are some aspects that we would like to investigate but have not had the chance yet. Here we present future experiments that we have planned.

We have detected the physical binding of APP and UCHL1 by coimmunoprecipitation. However, we do not know yet what enzymatic activity UCHL1 exerts on APP, that is, whether it deubiquitinates or attaches ubiquitin to APP as an E3 ligase, or both (Liu *et al.*, 2002). Since overexpression of UCHL1 led to increased ubiquitinated APP, UCHL1 may serve as a ligase on APP. This hypothesis needs verification in a cell-free system, where other DUBs and ubiquitin ligases are excluded. If the incubation of purified ubiquitin, APP, and UCHL1, together with necessary reagents results in the production of ubiquitinated APP, it strongly supports the above hypothesis. However, it should be noted that UCHL1 might not be an ideal ligase to mono-ubiquitinate substrates (Liu *et al.*, 2002); instead, it is more efficient in attaching a second ubiquitin to already mono-ubiquitinated protein. In this case the cell-free system should include purified ubiquitin, mono-ubiquitinated APP and

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UCHL1. Poly-ubiquitinated APP is expected if UCHL1 is able to further ligate ubiquitin to mono-ubiquitinated APP.

The type of poly-ubiquitination of APP also needs further exploring. The fate of ubiquitinated protein is largely dependent on the type of its ubiquitination. Generally the proteasomal protein degradation requires Lys-48-linked polyubiquitination, while mono-ubiquitinated or Lys-63-linked polyubiquitination of cell surface protein triggers its internalization and trafficking to lysosome for degradation. Since our data indicated the facilitated lysosomal degradation of APP by UCHL1, the type of ubiquitination that UCHL1 regulates is probably Lys-63-linked. As we have shown the increased ubiquitinated APP by UCHL1 (Figure 3.5), the type of polyubiquitination can be examined by introducing K48R or K63R mutant ubiquitin in which the Lys-48 or Lys-63 was replaced by arginine, and evaluating their effects on ubiquitinated APP level. The lysosomal degradation pathway can be confirmed by lysosomal inhibitors.

Another area that we would like to investigate in the future is UCHL1's effect on APP trafficking. APP can be transported from the plasma membrane to endosome or directly from TGN to endosome for lysosomal degradation. Its internalization can be regulated by its C-terminal motif of YENPTY and by ubiquitin signaling. Specifically, the ubiquitination of Lys-688 has been found to affect the trafficking and degradation of APP (El Ayadi *et al.*, 2012). We are intrigued to find out whether UCHL1 regulates the Lys-688 ubiquitination and APP trafficking. To this end, APP_{K688R} and APP_{WT} will be employed and UCHL1's effect on the ubiquitination will be examined. Cell-surface biotylation assay can be used to detect the retrieval of APP from the plasma membrane.

5.1.3 Chapter 4: Overexpression of UCHL1 as a therapeutic strategy for treating Alzheimer's disease

In chapter 3 we showed that UCHL1 decreased A β production *in vitro*. In this chapter we further examined its A β -reducing effect *in vivo*. By intracranial AAV-mediated delivery of UCHL1 to the mouse brain, we demonstrated that UCHL1 modestly decreased APP protein level and mildly decreased BACE1 level. Moreover, UCHL1 reduced A β production, inhibited plaque formation and rescued learning and memory deficits in AD mouse models. Our results indicated that overexpression of UCHL1 is a candidate therapeutic strategy to treat AD.

5.2 Significance of the research

Previous reports have suggested a link between UCHL1 and AD. Correlational study showed that UCHL1 is expressed at a low level and erroneously modified in AD brains. Furthermore, overexpression of UCHL1 rescued memory deficits in AD mouse model. However, whether a causal relationship exists between UCHL1 and AD development remains unknown. Our study is the first to investigate the role of UCHL1 in AD pathogenesis *in vitro* and *in vivo*.

First of all we discovered that UCHL1 transcriptional expression is inhibited by NF- κ B, an important molecule involved in neuroinflammation. The finding indicated that the chronic inflammation and NF- κ B up-regulation in AD brains may account for the concomitantly lowered UCHL1 expression. Moreover, it implied a potential pathway by which inflammation and NF- κ B affects AD pathogenesis.

This is the first report to thoroughly examine the role of UCHL1 in APP processing. We showed that UCHL1 regulates APP processing by facilitating the degradation of both full-length APP and BACE1, which results in reduced CTF and Aβ production. Moreover, we discovered that UCHL1 protects against oxidative stress- and Aβinduced neuronal apoptosis, which provided a mechanism for the neuroprotective role of UCHL1 in AD brains. It also indicated the detrimental consequence of decreased UCHL1 level in AD brains.

Thirdly we demonstrated that overexpression of UCHL1 reduced AD-like pathology and rescued memory deficits in AD model mice. We showed that viral delivery of UCHL1 ensured its long-term expression, and that UCHL1 altered APP processing and A β plaque deposition *in vivo*. Therefore UCHL1 may improve learning and memory ability in AD in a disease-modifying way. **The comprehensive approach used in this project demonstrated the role of UCHL1 in AD pathogenesis, and provided fundamental information on the pharmaceutical potential of UCHL1based AD treatment strategies.**

5.3 Further discussions on Aβ hypothesis

In the last ten years, the $A\beta$ plaque hypothesis has evolved to $A\beta$ oligomer hypothesis and received most popularity. A critical revision from the $A\beta$ plaque hypothesis to the $A\beta$ oligomer hypothesis is to re-define the role of $A\beta$ plaque deposition. In $A\beta$ oligomer hypothesis, amyloid plaque is no longer the culprit of AD. Rather, $A\beta$ plaque is considered a biologically inert reservoir which can be reverted to toxic oligomer species (Martins *et al.*, 2008), or is even protective towards functional deficits (Cheng *et al.*, 2007). On the other hand, $A\beta$ oligomers have been shown to induce neuronal toxicity and memory impairment *in vivo* (Lesne *et al.*, 2006; Shankar *et al.*, 2008). Moreover, $A\beta$ oligomer levels correlate with the clinical stage of AD better than $A\beta$ plaques (McLean *et al.*, 1999; Mc Donald *et al.*, 2010).

Despite the mounting evidences supporting $A\beta$ oligomer hypothesis, there are limitations in the current researches on $A\beta$ oligomer toxicity, which are mainly from the inevitable discrepancy between the actual cellular events in the AD brain and what is simulated in the laboratory setting. We will discuss the issue in this section. The
recently developed PET tracer ¹¹C-labeled Pittsburgh Compound-B (¹¹C-PIB) has allowed fibrillar A β plaque detection in living AD patients. We will look at the clues it has provided to the "plaque Vs. oligomer" debate. Finally, we will include results from our experiments in this section.

5.3.1 Limitations in the current researches

The spectrum of A β peptides. The cleavage of APP generates A β peptides of various lengths, ranging from 37 to 43 amino acids, with A β_{40} and A β_{42} being the main components. In experiments using synthetic A β peptides, A β_{42} is usually employed, because it is the most aggregate-prone form and the major component in A β plaques in AD brains. However, A β_{42} does not necessarily dominate in the soluble fraction of the A β pool. Moreover, A β_{42} /A β_{40} ratio may be critical for the initiation of A β fibrillization and AD pathogenesis, as is shown in many PS1/PS2 mutations that lead to early-onset FAD. Therefore, A β_{42} alone is not an ideal model to mimic the A β composition *in vivo*.

Equilibrium between A β monomer, oligomer and plaque formation. Studies on A β oligomer toxicity have recruited A β oligomers of various sizes, spanning from as small as dimers to as large as annular protofibrils (APF, 36-mers). Most reports have focused on one type of purified oligomers, for example, A β dimers in (Shankar *et al.*, 2008), ADDLs in (Lambert *et al.*, 1998) and A β *56 in (Lesne *et al.*, 2006). However, in the brains of AD patients, A β assemblies are in a dynamic equilibrium from monomers to fibril plaques. The composition of the A β oligomers may be crucial to neuronal toxicity. Therefore, results obtained from A β oligomer of one single type may not be applicable to what is happening in AD brains.

The pharmacological concentration of $A\beta$ in toxicity assays. $A\beta$ oligomers have been shown to be toxic to neurons in a plethora of measurements, including apoptosis activation, dendritic spine morphology, and synaptic plasticity (Walsh *et al.*, 2002; Wogulis *et al.*, 2005; Kuperstein *et al.*, 2010; Wu *et al.*, 2010). Many of the experiments, however, applied a much higher concentration of A β than in normal physiological conditions. While the pharmacological concentration of A β is necessary in order to induce neuronal damage in a few hours or a few days, it should be noted that the acute toxicity stimulated in the laboratory may be mechanistically different from the development of AD, a chronic process that takes years before symptoms appear.

5.3.2 Implications from recent researches on AD biomarkers

The challenge for $A\beta$ oligomer hypothesis. Apart from the limitations in $A\beta$ oligomer research, the $A\beta$ oligomer hypothesis cannot explain very well some of the biochemical data from AD patients. Firstly, $A\beta$ levels in the CSF of AD patients are much lower than in healthy controls. While discrepancy in $A\beta$ detection techniques across the labs led to huge variations in the absolute value of human CSF $A\beta$ across different studies, in each individual report, the CSF $A\beta$ in AD patients is almost always lower, to ~50% of that in healthy controls (Vanderstichele *et al.*, 2000; Strozyk *et al.*, 2003). It is hard to explain why neurons incubated with less soluble $A\beta$ will degenerate, if the $A\beta$ oligomer hypothesis is true. Secondly, studies of people with FAD mutations showed that CSF $A\beta$ in mutation carriers started to decline 25 years before the onset of AD and remained lower than that in healthy controls for 20 years before the disease onset (Bateman *et al.*, 2012). If soluble $A\beta$ oligomers were the culprit of AD development, why in those people who were destined to develop early-onset FAD, soluble $A\beta$ levels were constantly lower than healthy controls at the pre-symptomatic stage for two decades?

New clues from the PiB-PET scan. A β plaque hypothesis has received some supports from the newly developed PiB-PET scan that visualizes fibrillar A β plaques in living AD patients. Using PiB-PET, both cross-sectional and longitudinal studies have been done to track the development of fibrillar A β plaque in FAD, SAD and normal healthy people. By comparing people with probable AD, of MCI and healthy controls, it was found that people with probable AD had the highest PiB score (most fibrillar Aß plaques) while healthy controls had the lowest. More importantly, MCI patients with high PiB score had a much higher chance to develop AD than those who had low PiB score. Healthy controls who showed more fibrillar Aß plaque also were more likely to develop MCI (Villemagne *et al.*, 2011). Therefore, fibrillar Aβ level is a good predictor of the risk to develop AD. In a study on people with the highly penetrable pathogenic PS1 E280A mutation, it was found that A^β plaque started to deposit about 21 years before dementia and 16 years before MCI. In addition, fibrillar A β levels continued rising for 9 years and then plateaued for another 6 years before symptoms of MCI appeared (Fleisher et al., 2012). It seems that in AD patients AB plaques increase steadily at pre-symptomatic stage and has reached the plateau by the time of mild stage AD. On the other hand, CSF A β level drops pre-clinically but gradually increases at later stages. It is possible that sometime at the pre-symptomatic stage, soluble A β starts to fibril and deposit as plaques. By the time of MCI, A β plaque accumulation has reached plateau and therefore newly secreted AB will not increase plaque numbers. However, it may be the A β plaques that exert chronic toxic effects on neurons, which ultimately transform into clinical symptoms of AD. In other words, the weak association between A β plaque numbers and AD progression does not necessarily exclude the A β plaque hypothesis.

5.3.3 Implications from this project

In this thesis, our experiments were not specifically designed to look for evidence to support one $A\beta$ hypothesis over the other, but at least two experiments provided some information in this respect.

We showed in chapter 3 that aged fibrillar $A\beta_{42}$ induced cell toxicity in SH-SY5Y neuroblastoma cells and primary cortical neurons (Figure 3.11). Moreover, the Aβ-induced neuronal toxicity is mediated by caspase 8/caspase 3 apoptotic pathway. Our

results neither supported nor challenged the A β oligomer hypothesis, since we did not treat the cells with A β oligomers. Rather, we provided some supports for the toxicity of insoluble A β . Moreover, the aged fibrillar A β used in our experiments had its clinical relevance. The aged fibrillar A β was prepared by incubation in PBS at 37 °C for 4 days, a condition that is similar to normal physiological condition in the brain. Although the concentration of A β in the brain is lower than what we used, it will produce some aged fibrillar A β given enough time. The aged A β induced cell toxicity as measured by LDH assay (Figure 3.11A) and caspases activation (Figure 3.11B,F,G). The neuronal apoptosis caused by fibrillar A β will very likely happen in AD brains, and therefore partly accounts for the brain atrophy and cognitive dysfunctions in AD.

We also showed the positive association between A β plaques and memory deficits in AD mouse models (Figure 4.2 and 4.4). In APP23/PS45 double transgenic mice, UCHL1 treatment improved the memory deficits and reduced the number of A β plaques compared to control mice, a positive correlation between memory deficits and plaque number. Notably, the reduction of total A β level by UCHL1 was also observed. A β_{40} levels from the mouse hippocampi were measured by ELISA assay (Invitrogen). UCHL1 treatment decreased A β_{40} level by ~30%. While the reduction in soluble A β may be the cause of memory improvement, our data implied that decreased A β plaques may be responsible as well. Further studies will provide more information to confirm the positive correlation between memory deficits and A β plaque deposition. For example, APP23/PS45 mice can be intracranially injected with AAV-UCHL1 at different time point, and their behavior test and histochemical analysis be carried out later at the same time point. Alternatively, UCHL1 does not have to be involved. Conditional APP transgenic mice can be used to manipulate A β production and plaque formation in the brain.

To summarize, our experiments demonstrated the neuronal toxicity induced by fibrillar A β , and the positive correlation between plaque deposition and learning and

memory deficits. Our results indicated that fibrillar A β plaque may play an important role in AD pathogenesis.

5.4 A closer look at UCHL1 in neurodegenerative diseases

A few UCHL1 mutations have been discovered to be related to neurodegenerative diseases, including the I93M dominant missense mutation and the S18Y polymorphism in PD, the E7A recessive missense mutation in childhood-onset progressive neurodegeneration and exonal deletion in gracile axonal dystrophy in mice (Yamazaki *et al.*, 1988; Leroy *et al.*, 1998; Maraganore *et al.*, 1999; Bilguvar *et al.*, 2013). However, whether they are true pathogenic mutations (or protective variants) is to some extent controversial. Moreover, whether it is its hydrolase activity, ligase activity or ubiquitin-recycling ability that accounts for the neurodegeneration remains elusive. It also awaits exploration whether UCHL1's effect on protein degradation is through proteasomal or lysosomal pathway. Here we take a close look at research reports and our findings in this thesis for the role of UCHL1 in neurodegeneration.

5.4.1 Mutations of UCHL1 in neurodegenerative diseases

Is I93M a true PD-causing mutation? The dominant UCHL1_{193M} mutation was identified in two siblings from a German family who both developed early-onset PD (Leroy *et al.*, 1998), indicating a role of UCHL1 in PD pathogenesis. Researchers have established transgenic mouse line carrying this mutation and looked for structural and enzymatic alterations that accounted for its toxicity (described in Section 1.10.2.1). However, if we scrutinize researches on UCHL1_{193M}, it appears that its pathogenic role in PD still needs further confirmation. Firstly, the mutation has not been identified in any PD patients or healthy controls other than this German family. Thus the PD development might be due to other genetic or environmental factors and coincide with this mutation. Secondly, the father who transmitted the mutation to the siblings did not develop PD-like symptoms, indicating either an incomplete

penetrance or again a coincidence. Thirdly, the transgenic mouse model, which expressed human UCHL1_{193M} to the level of approximately 1% of mouse UCHL1, was a gain-of-function model, and therefore did not fully mimic the dominant mutation in humans. Nevertheless, since the I93M mutation reduced the hydrolase activity of UCHL1 to ~50%, it is highly possible that the mutation is indeed pathogenic. Further confirmation, for example, from UCHL1_{193M} knockin mice, is absolutely necessary.

Is S18Y polymorphism truly protective against PD? Whether $UCHL1_{S18Y}$ is a true protective variant is largely controversial. The group that first identified the variant demonstrated in multiple reports its protective role, while some other studies did not support this hypothesis. The contradictory results may be due to racial differences in subject selection and relatively small sample sizes in individual studies. Meta-analysis implicated that the significance of this polymorphism, if any, is not as prominent as ApoE4 polymorphism in AD (Maraganore *et al.*, 2004). Interestingly, if the data were analyzed within each ethnical group, the results were more consistent with a statistically significant difference in each group, indicating a similar effect of the S18Y variant across different populations and a hidden factor that stratified the ethnical populations.

Is E7A or *gad* true mutation for neurodegeneration? Compared to the controversial I93M and S18Y variants, the pathogenic role of the E7A recessive mutation in humans and *gad* mutations in mice are truly convincing. Three siblings born of consanguineous parents displayed striking early-onset neurodegeneration while the other three siblings or their parents did not display any phenotypes. Homozygosity mapping and whole-exome sequencing pinpointed the genetic abnormality to the UCHL1_{E7A} mutant. Genotyping of the other siblings and the parents proved the recessive nature of this mutation. Moreover, the development of the disease was similar to that of the well-characterized *gad* mice, an equivalent of

UCHL1 knockout mouse model. The neurodegenerative phenotypes by complete loss of UCHL1 have been confirmed by generations of crossings. Finally, more than one spontaneous UCHL1 mutations have been identified in laboratory mouse strains that develop similar neuromuscular defects and neurodegeneration. Two strains are equivalent of UCHL1 knockout (namely *gad* and *gad-J*) and another strain is a spontaneous C to A mutation on exon 4 of *UCHL1* (namely *gad-2J*). The convergent phenotypes confirmed the critical role of UCHL1 in neuronal survival ((Yamazaki *et al.*, 1988; Walters *et al.*, 2008) and Jackson Lab).

5.4.2 Hydrolase, ligase or ubiquitin recycler?

Studies have identified three enzymatic activities of UCHL1, that is, hydrolase, ligase and ubiquitin recycler. The ubiquitin recycling function is possibly served by removing protein remnants from ubiquitin and making it ready-to-use. It is a strategy employed by UCHL1 to indirectly regulate the degradation of a variety of proteins. UCHL1 is crucial for neuronal survival. Moreover, its mutations are the cause of some of the neurodegenerative diseases. Therefore it would provide valuable information for the drug design to sort out what enzymatic activity is most important in those diseases.

The most controversial enzymatic activity of UCHL1 is its ligase function. So far only one study has reported its ligase activity, in which the authors demonstrated in a cell-free system the ligation of free ubiquitin to form di-ubiquitin, and the ligation of ubiquitin to already mono-ubiquitinated α -synuclein (Liu *et al.*, 2002). Moreover, they attributed the alleged protective role of UCHL1_{S18Y} against PD to its decreased ligase activity compared to UCHL1_{WT}. While the experiments were done clearly and beautifully, the conclusion still needs confirmation from future studies. Interestingly, results from our experiments in chapter 3 supported the ligase activity of UCHL1. We showed that overexpression of UCHL1 led to an increased level of ubiquitinated APP,

although further cell-free experiments are required to demonstrate direct ligase activity of UCHL1 on APP.

It is difficult to differentiate the hydrolase activity and ubiquitin recycling function of UCHL1, since the two functions are largely overlapping and the substrates of UCHL1 are largely unknown. So far only two substrates have been identified, i.e., α -synuclein (Liu *et al.*, 2002) and β -catenin (Bheda *et al.*, 2009). UCHL1 was shown to exert dual hydrolase and ligase activities on α -synuclein, while it only de-ubiquitinates β -catenin. As its substrates are largely unknown, many of the effects of UCHL1 can only be attributed to its ubiquitin-recycling function. Future studies should focus on the identification of the substrates of UCHL1, so that its enzymatic activities and the mechanisms by which it affects neurodegeneration can be better explored.

5.4.3 Proteasomal degradation Vs. lysosomal degradation

The UPS and lysosome system are the two major degradation systems for intracellular proteins. UPS is characterized by the polyubiquitination of the proteins to be degraded. Therefore we originally expected that UCHL1, as a DUB, may affect the proteasomal degradation of A β -producing molecules by regulating their ubiquitination/de-ubiquitination process. Interestingly, we found that it is the lysosomal degradation of APP that UCHL1 accelerates. This is reasonable on second thought, because the lysosmal protein degradation could also be regulated by ubiquitination. For example, BACE1 could be ubiquitinated at Lys-501 and be targeted for lysosomal degradation by GGA3, a clathrin adaptor protein (Kang *et al.*, 2010).

Previous studies have reported the effect of UCHL1 on both of the degradation pathways. For example, UCHL1 rescued β -catenin and I κ B- α (Takami *et al.*, 2007; Bheda *et al.*, 2009) from proteasomal degradation by inhibiting their ubiquitination. On the other hand, UCHL1 has been shown to interact with LAMP-2A, the lysosome receptor for CMA (Kabuta *et al.*, 2008a). Moreover, UCHL1 deficiency resulted in up-regulation of lysosomal components (Walters *et al.*, 2008). It is possible that UCHL1 is involved in both degradation systems. Future experiments revealing the substrates of UCHL1 and the type of polyubiquitination that UCHL1 regulates will provide more information for this issue.

5.5 Potential applications of UCHL1 overexpression in AD therapeutics

We have demonstrated both *in vitro* and *in vivo* that UCHL1 reduced protein levels of full-length APP, BACE1, APP CTFs, and A β . In AD mouse models, UCHL1 further decreased A β plaque formation in the brain and rescued learning and memory deficits. Moreover, UCHL1 protects against A β -induced neuronal apoptosis, which may be partly responsible for AD pathogenesis. The data strongly indicated that UCHL1 is a promising drug candidate for AD therapeutics.

Advantages of UCHL1 as a drug candidate. As a potential disease-modifying drug for AD, UCHL1 may have fewer side effects as a moderate modulater compared to other A β -targeting drugs, such as the γ -secretase inhibitors (GSI) and BACE1 inhibitors. Since γ -secretase has dozens of substrates, GSIs usually affect the cleavage of substrates other than APP, including the essential Notch signaling pathway, which may lead to undesired effects such as gastrointestinal toxicity (Searfoss *et al.*, 2003). Second generation GSIs are 'Notch-sparing', but they still face challenges from the inhibition of other γ -secretase substrates as well as from increased CTF β level associated with decreased A β level. BACE1 inhibitors, if they can ever be developed for clinical use, may have to deal with the possible side effects of neuronal remyelination defects and Nav1 Na⁺ channel dysfunction. By contrast, UCHL1 may have fewer side effects as it inhibits APP processing by exerting a moderate reduction effect on full-length APP and a mild effect on BACE1 degradation. In this way, it circumvents the problem of substrate specificity in GSIs, and is not likely to cause the side effects which arise from complete BACE1 inhibition. The reduction of full-length APP by UCHL1 and the probable subsequent decrease in sAPPα and AICD production, however, may cause some issues, as they all have important physiological roles. While it requires further testing, serious problems are not expected, for (1) full-length APP is only partially reduced by UCHL1 and (2) the APP family (APP, APLP1 and APLP2 in mammals) proteins have redundant and partly overlapping functions. Finally, it should be noted that the overexpression of UCHL1 itself may cause the aggregation of other proteins as well as its own aggregation (Ardley *et al.*, 2004; Proctor *et al.*, 2010). Therefore the amount of overexpressed UCHL1 needs to be carefully gauged in clinical use.

rAAV-mediated UCHL1 gene therapy for AD. In chapter 4 we delivered UCHL1 into the hippocampal region of the mice by intracranial injection of rAAV. AAV-mediated gene therapy technique is being developed in recent years. Its safety and tolerability have been tested in a few Phase I clinical trials, including the AAV-GAD treatment for PD (Kaplitt *et al.*, 2007) and AAV2-NGF (Cere-110) treatment for AD (Sangamo BioSciences). There were no adverse events related to the gene therapy. Furthermore, long-term expression by AAV delivery was verified. Therefore, rAAV-mediated UCHL1 gene therapy will be a promising strategy for overexpressing UCHL1 in AD brains.

References

- Acquati, F., Accarino, M., Nucci, C., Fumagalli, P., Jovine, L., Ottolenghi, S. & Taramelli, R.
 (2000) The gene encoding DRAP (BACE2), a glycosylated transmembrane protein of the aspartic protease family, maps to the down critical region. *FEBS letters*, **468**, 59-64.
- ADI (2010) World Alzheimer Report 2010: The Global Economic Impact of Dementia. Alzheimer's Disease International, London.
- ADI (2013) World Alzheimer Report 2013: An Analysis of Long-term Care for Dementia. Alzheimer's Disease International, London.
- Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G.M., Cooper, N.R., Eikelenboom, P., Emmerling, M., Fiebich, B.L., Finch, C.E., Frautschy, S., Griffin, W.S., Hampel, H., Hull, M., Landreth, G., Lue, L., Mrak, R., Mackenzie, I.R., McGeer, P.L., O'Banion, M.K., Pachter, J., Pasinetti, G., Plata-Salaman, C., Rogers, J., Rydel, R., Shen, Y., Streit, W., Strohmeyer, R., Tooyoma, I., Van Muiswinkel, F.L., Veerhuis, R., Walker, D., Webster, S., Wegrzyniak, B., Wenk, G. & Wyss-Coray, T. (2000) Inflammation and Alzheimer's disease. *Neurobiology of aging*, 21, 383-421.
- Amerik, A., Swaminathan, S., Krantz, B.A., Wilkinson, K.D. & Hochstrasser, M. (1997) In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome. *The EMBO journal*, 16, 4826-4838.
- Amerik, A.Y. & Hochstrasser, M. (2004) Mechanism and function of deubiquitinating enzymes. *Biochim Biophys Acta*, 1695, 189-207.
- Andersson, F.I., Werrell, E.F., McMorran, L., Crone, W.J., Das, C., Hsu, S.T. & Jackson, S.E. (2011) The effect of Parkinson's-disease-associated mutations on the deubiquitinating enzyme UCH-L1. *Journal of molecular biology*, **407**, 261-272.
- Ando, K., Iijima, K.I., Elliott, J.I., Kirino, Y. & Suzuki, T. (2001) Phosphorylation-dependent regulation of the interaction of amyloid precursor protein with Fe65 affects the production of beta-amyloid. *The Journal of biological chemistry*, **276**, 40353-40361.
- Arancio, O., Zhang, H.P., Chen, X., Lin, C., Trinchese, F., Puzzo, D., Liu, S., Hegde, A., Yan, S.F., Stern, A., Luddy, J.S., Lue, L.F., Walker, D.G., Roher, A., Buttini, M., Mucke, L., Li, W., Schmidt, A.M., Kindy, M., Hyslop, P.A., Stern, D.M. & Du Yan, S.S. (2004) RAGE potentiates Abeta-induced perturbation of neuronal function in transgenic mice. *The EMBO journal*, 23, 4096-4105.
- Ardley, H.C., Scott, G.B., Rose, S.A., Tan, N.G. & Robinson, P.A. (2004) UCH-L1 aggresome formation in response to proteasome impairment indicates a role in inclusion formation in Parkinson's disease. *Journal of neurochemistry*, **90**, 379-391.
- Arnaiz, E. & Almkvist, O. (2003) Neuropsychological features of mild cognitive impairment and preclinical Alzheimer's disease. *Acta neurologica Scandinavica. Supplementum*, **179**, 34-41.

- Arriagada, P.V., Growdon, J.H., Hedley-Whyte, E.T. & Hyman, B.T. (1992) Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology*, 42, 631-639.
- Ashkenazi, A. & Dixit, V.M. (1998) Death receptors: signaling and modulation. *Science*, **281**, 1305-1308.
- Barrachina, M., Moreno, J., Juves, S., Moreno, D., Olive, M. & Ferrer, I. (2007) Target genes of neuron-restrictive silencer factor are abnormally up-regulated in human myotilinopathy. *The American journal of pathology*, **171**, 1312-1323.
- Bateman, R.J., Xiong, C., Benzinger, T.L., Fagan, A.M., Goate, A., Fox, N.C., Marcus, D.S., Cairns, N.J., Xie, X., Blazey, T.M., Holtzman, D.M., Santacruz, A., Buckles, V., Oliver, A., Moulder, K., Aisen, P.S., Ghetti, B., Klunk, W.E., McDade, E., Martins, R.N., Masters, C.L., Mayeux, R., Ringman, J.M., Rossor, M.N., Schofield, P.R., Sperling, R.A., Salloway, S., Morris, J.C. & Dominantly Inherited Alzheimer, N. (2012) Clinical and biomarker changes in dominantly inherited Alzheimer's disease. *The New England journal of medicine*, 367, 795-804.
- Beher, D., Hesse, L., Masters, C.L. & Multhaup, G. (1996) Regulation of amyloid protein precursor (APP) binding to collagen and mapping of the binding sites on APP and collagen type I. *The Journal of biological chemistry*, **271**, 1613-1620.
- Bell, K.F., Zheng, L., Fahrenholz, F. & Cuello, A.C. (2008) ADAM-10 over-expression increases cortical synaptogenesis. *Neurobiology of aging*, 29, 554-565.
- Bermejo-Pareja, F., Benito-Leon, J., Vega, S., Medrano, M.J., Roman, G.C. & Neurological Disorders in Central Spain Study, G. (2008) Incidence and subtypes of dementia in three elderly populations of central Spain. *Journal of the neurological sciences*, 264, 63-72.
- Bheda, A., Yue, W., Gullapalli, A., Whitehurst, C., Liu, R., Pagano, J.S. & Shackelford, J. (2009) Positive reciprocal regulation of ubiquitin C-terminal hydrolase L1 and beta-catenin/TCF signaling. *PloS one*, 4, e5955.
- Bilguvar, K., Tyagi, N.K., Ozkara, C., Tuysuz, B., Bakircioglu, M., Choi, M., Delil, S., Caglayan, A.O., Baranoski, J.F., Erturk, O., Yalcinkaya, C., Karacorlu, M., Dincer, A., Johnson, M.H., Mane, S., Chandra, S.S., Louvi, A., Boggon, T.J., Lifton, R.P., Horwich, A.L. & Gunel, M. (2013) Recessive loss of function of the neuronal ubiquitin hydrolase UCHL1 leads to early-onset progressive neurodegeneration. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 3489-3494.
- Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K.A., Gerhart, M., Davis, R., Fitzner, J.N., Johnson, R.S., Paxton, R.J., March, C.J. & Cerretti, D.P. (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature*, 385, 729-733.

- Boone, D.L., Turer, E.E., Lee, E.G., Ahmad, R.C., Wheeler, M.T., Tsui, C., Hurley, P., Chien, M., Chai, S., Hitotsumatsu, O., McNally, E., Pickart, C. & Ma, A. (2004) The ubiquitinmodifying enzyme A20 is required for termination of Toll-like receptor responses. *Nature immunology*, 5, 1052-1060.
- Borg, J.P., Ooi, J., Levy, E. & Margolis, B. (1996) The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein. *Molecular and cellular biology*, 16, 6229-6241.
- Borg, J.P., Yang, Y., De Taddeo-Borg, M., Margolis, B. & Turner, R.S. (1998) The X11alpha protein slows cellular amyloid precursor protein processing and reduces Abeta40 and Abeta42 secretion. *The Journal of biological chemistry*, 273, 14761-14766.
- Bourne, K.Z., Ferrari, D.C., Lange-Dohna, C., Rossner, S., Wood, T.G. & Perez-Polo, J.R. (2007) Differential regulation of BACE1 promoter activity by nuclear factor-kappaB in neurons and glia upon exposure to beta-amyloid peptides. *Journal of neuroscience research*, **85**, 1194-1204.
- Braak, H. & Braak, E. (1991) Neuropathological stageing of Alzheimer-related changes. Acta neuropathologica, 82, 239-259.
- Brinkmann, K., Zigrino, P., Witt, A., Schell, M., Ackermann, L., Broxtermann, P., Schull, S., Andree, M., Coutelle, O., Yazdanpanah, B., Seeger, J.M., Klubertz, D., Drebber, U., Hacker, U.T., Kronke, M., Mauch, C., Hoppe, T. & Kashkar, H. (2013) Ubiquitin Cterminal hydrolase-L1 potentiates cancer chemosensitivity by stabilizing NOXA. *Cell reports*, **3**, 881-891.
- Brookmeyer, R., Gray, S. & Kawas, C. (1998) Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset. *American journal of public health*, **88**, 1337-1342.
- Bu, G. (2009) Apolipoprotein E and its receptors in Alzheimer's disease: pathways, pathogenesis and therapy. *Nature reviews. Neuroscience*, **10**, 333-344.
- Butterworth, M.B., Edinger, R.S., Ovaa, H., Burg, D., Johnson, J.P. & Frizzell, R.A. (2007) The deubiquitinating enzyme UCH-L3 regulates the apical membrane recycling of the epithelial sodium channel. *The Journal of biological chemistry*, **282**, 37885-37893.
- Buxbaum, J.D., Liu, K.N., Luo, Y., Slack, J.L., Stocking, K.L., Peschon, J.J., Johnson, R.S., Castner, B.J., Cerretti, D.P. & Black, R.A. (1998) Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid protein precursor. *The Journal of biological chemistry*, **273**, 27765-27767.
- Caballero, O.L., Resto, V., Patturajan, M., Meerzaman, D., Guo, M.Z., Engles, J., Yochem, R., Ratovitski, E., Sidransky, D. & Jen, J. (2002) Interaction and colocalization of PGP9.5 with JAB1 and p27(Kip1). *Oncogene*, **21**, 3003-3010.

- Cai, H., Wang, Y., McCarthy, D., Wen, H., Borchelt, D.R., Price, D.L. & Wong, P.C. (2001) BACE1 is the major beta-secretase for generation of Abeta peptides by neurons. *Nature neuroscience*, 4, 233-234.
- Campion, D., Dumanchin, C., Hannequin, D., Dubois, B., Belliard, S., Puel, M., Thomas-Anterion, C., Michon, A., Martin, C., Charbonnier, F., Raux, G., Camuzat, A., Penet, C., Mesnage, V., Martinez, M., Clerget-Darpoux, F., Brice, A. & Frebourg, T. (1999) Early-onset autosomal dominant Alzheimer disease: prevalence, genetic heterogeneity, and mutation spectrum. *Am J Hum Genet*, 65, 664-670.
- Cao, X. & Sudhof, T.C. (2001) A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science*, **293**, 115-120.
- Caporaso, G.L., Gandy, S.E., Buxbaum, J.D. & Greengard, P. (1992) Chloroquine inhibits intracellular degradation but not secretion of Alzheimer beta/A4 amyloid precursor protein. *Proceedings of the National Academy of Sciences of the United States of America*, 89, 2252-2256.
- Catterall, W.A. (2000) From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron*, **26**, 13-25.
- Chen, C., Bharucha, V., Chen, Y., Westenbroek, R.E., Brown, A., Malhotra, J.D., Jones, D., Avery, C., Gillespie, P.J., 3rd, Kazen-Gillespie, K.A., Kazarinova-Noyes, K., Shrager, P., Saunders, T.L., Macdonald, R.L., Ransom, B.R., Scheuer, T., Catterall, W.A. & Isom, L.L. (2002) Reduced sodium channel density, altered voltage dependence of inactivation, and increased susceptibility to seizures in mice lacking sodium channel beta 2-subunits. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 17072-17077.
- Chen, C.H., Zhou, W., Liu, S., Deng, Y., Cai, F., Tone, M., Tone, Y., Tong, Y. & Song, W. (2011) Increased NF-kappaB signalling up-regulates BACE1 expression and its therapeutic potential in Alzheimer's disease. *Int J Neuropsychopharmacol*, 1-14.
- Cheng, I.H., Scearce-Levie, K., Legleiter, J., Palop, J.J., Gerstein, H., Bien-Ly, N., Puolivali, J., Lesne, S., Ashe, K.H., Muchowski, P.J. & Mucke, L. (2007) Accelerating amyloid-beta fibrillization reduces oligomer levels and functional deficits in Alzheimer disease mouse models. *The Journal of biological chemistry*, 282, 23818-23828.
- Choi, J., Levey, A.I., Weintraub, S.T., Rees, H.D., Gearing, M., Chin, L.S. & Li, L. (2004) Oxidative modifications and down-regulation of ubiquitin carboxyl-terminal hydrolase L1 associated with idiopathic Parkinson's and Alzheimer's diseases. *The Journal of biological chemistry*, 279, 13256-13264.
- Christensen, M.A., Zhou, W., Qing, H., Lehman, A., Philipsen, S. & Song, W. (2004) Transcriptional regulation of BACE1, the beta-amyloid precursor protein beta-secretase, by Sp1. *Molecular and cellular biology*, 24, 865-874.

- Ciechanover, A., Elias, S., Heller, H., Ferber, S. & Hershko, A. (1980a) Characterization of the heat-stable polypeptide of the ATP-dependent proteolytic system from reticulocytes. *The Journal of biological chemistry*, **255**, 7525-7528.
- Ciechanover, A., Heller, H., Elias, S., Haas, A.L. & Hershko, A. (1980b) ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. *Proceedings of the National Academy of Sciences of the United States of America*, **77**, 1365-1368.
- Clague, M.J. & Urbe, S. (2006) Endocytosis: the DUB version. Trends in cell biology, 16, 551-559.
- Clarris, H.J., Cappai, R., Heffernan, D., Beyreuther, K., Masters, C.L. & Small, D.H. (1997) Identification of heparin-binding domains in the amyloid precursor protein of Alzheimer's disease by deletion mutagenesis and peptide mapping. *Journal of neurochemistry*, 68, 1164-1172.
- Corder, E.H., Saunders, A.M., Risch, N.J., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Jr., Rimmler, J.B., Locke, P.A., Conneally, P.M., Schmader, K.E. & et al. (1994) Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. *Nature genetics*, 7, 180-184.
- Corder, E.H., Saunders, A.M., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Small, G.W., Roses, A.D., Haines, J.L. & Pericak-Vance, M.A. (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science*, 261, 921-923.
- Costes, S., Huang, C.J., Gurlo, T., Daval, M., Matveyenko, A.V., Rizza, R.A., Butler, A.E. & Butler, P.C. (2011) beta-cell dysfunctional ERAD/ubiquitin/proteasome system in type 2 diabetes mediated by islet amyloid polypeptide-induced UCH-L1 deficiency. *Diabetes*, **60**, 227-238.
- Cummins, J.M. & Vogelstein, B. (2004) HAUSP is required for p53 destabilization. *Cell cycle*, **3**, 689-692.
- Dahlmann, B. (2005) Proteasomes. Essays in biochemistry, 41, 31-48.
- Das, C., Hoang, Q.Q., Kreinbring, C.A., Luchansky, S.J., Meray, R.K., Ray, S.S., Lansbury, P.T., Ringe, D. & Petsko, G.A. (2006) Structural basis for conformational plasticity of the Parkinson's disease-associated ubiquitin hydrolase UCH-L1. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 4675-4680.
- Day, I.N., Hinks, L.J. & Thompson, R.J. (1990) The structure of the human gene encoding protein gene product 9.5 (PGP9.5), a neuron-specific ubiquitin C-terminal hydrolase. *The Biochemical journal*, 268, 521-524.
- Day, I.N. & Thompson, R.J. (1987) Molecular cloning of cDNA coding for human PGP 9.5 protein. A novel cytoplasmic marker for neurones and neuroendocrine cells. *FEBS letters*, 210, 157-160.

- De Jonghe, C., Cras, P., Vanderstichele, H., Cruts, M., Vanderhoeven, I., Smouts, I., Vanmechelen, E., Martin, J.J., Hendriks, L. & Van Broeckhoven, C. (1999) Evidence that Abeta42 plasma levels in presenilin-1 mutation carriers do not allow for prediction of their clinical phenotype. *Neurobiology of disease*, 6, 280-287.
- De Pietri Tonelli, D., Mihailovich, M., Di Cesare, A., Codazzi, F., Grohovaz, F. & Zacchetti, D. (2004) Translational regulation of BACE-1 expression in neuronal and non-neuronal cells. *Nucleic acids research*, **32**, 1808-1817.
- De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J.S., Schroeter, E.H., Schrijvers, V., Wolfe, M.S., Ray, W.J., Goate, A. & Kopan, R. (1999) A presenilin-1dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature*, **398**, 518-522.
- De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Annaert, W., Von Figura, K. & Van Leuven, F. (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature*, **391**, 387-390.
- De Vrij, F.M., Sluijs, J.A., Gregori, L., Fischer, D.F., Hermens, W.T., Goldgaber, D., Verhaagen, J., Van Leeuwen, F.W. & Hol, E.M. (2001) Mutant ubiquitin expressed in Alzheimer's disease causes neuronal death. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, **15**, 2680-2688.
- Deng, Y., Wang, Z., Wang, R., Zhang, X., Zhang, S., Wu, Y., Staufenbiel, M., Cai, F. & Song, W. (2013) Amyloid-beta protein (Abeta) Glu11 is the major beta-secretase site of beta-site amyloid-beta precursor protein-cleaving enzyme 1(BACE1), and shifting the cleavage site to Abeta Asp1 contributes to Alzheimer pathogenesis. *The European journal of neuroscience*, **37**, 1962-1969.
- Di Carlo, A., Baldereschi, M., Amaducci, L., Lepore, V., Bracco, L., Maggi, S., Bonaiuto, S., Perissinotto, E., Scarlato, G., Farchi, G., Inzitari, D. & Group, I.W. (2002) Incidence of dementia, Alzheimer's disease, and vascular dementia in Italy. The ILSA Study. *Journal of the American Geriatrics Society*, **50**, 41-48.
- Ding, W.X., Ni, H.M., Gao, W., Yoshimori, T., Stolz, D.B., Ron, D. & Yin, X.M. (2007) Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability. *The American journal of pathology*, **171**, 513-524.
- Doran, J.F., Jackson, P., Kynoch, P.A. & Thompson, R.J. (1983) Isolation of PGP 9.5, a new human neurone-specific protein detected by high-resolution two-dimensional electrophoresis. *Journal of neurochemistry*, 40, 1542-1547.
- Edbauer, D., Winkler, E., Regula, J.T., Pesold, B., Steiner, H. & Haass, C. (2003) Reconstitution of gamma-secretase activity. *Nature cell biology*, **5**, 486-488.
- El Ayadi, A., Stieren, E.S., Barral, J.M. & Boehning, D. (2012) Ubiquilin-1 regulates amyloid precursor protein maturation and degradation by stimulating K63-linked

polyubiquitination of lysine 688. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 13416-13421.

- Esch, F.S., Keim, P.S., Beattie, E.C., Blacher, R.W., Culwell, A.R., Oltersdorf, T., McClure, D. & Ward, P.J. (1990) Cleavage of amyloid beta peptide during constitutive processing of its precursor. *Science*, 248, 1122-1124.
- Farzan, M., Schnitzler, C.E., Vasilieva, N., Leung, D. & Choe, H. (2000) BACE2, a beta secretase homolog, cleaves at the beta site and within the amyloid-beta region of the amyloid-beta precursor protein. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 9712-9717.
- Fleisher, A.S., Chen, K., Quiroz, Y.T., Jakimovich, L.J., Gomez, M.G., Langois, C.M., Langbaum, J.B., Ayutyanont, N., Roontiva, A., Thiyyagura, P., Lee, W., Mo, H., Lopez, L., Moreno, S., Acosta-Baena, N., Giraldo, M., Garcia, G., Reiman, R.A., Huentelman, M.J., Kosik, K.S., Tariot, P.N., Lopera, F. & Reiman, E.M. (2012) Florbetapir PET analysis of amyloid-beta deposition in the presenilin 1 E280A autosomal dominant Alzheimer's disease kindred: a cross-sectional study. *Lancet neurology*, **11**, 1057-1065.
- Forstl, H. & Kurz, A. (1999) Clinical features of Alzheimer's disease. European archives of psychiatry and clinical neuroscience, 249, 288-290.
- Francis, R., McGrath, G., Zhang, J., Ruddy, D.A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M.C., Parks, A.L., Xu, W., Li, J., Gurney, M., Myers, R.L., Himes, C.S., Hiebsch, R., Ruble, C., Nye, J.S. & Curtis, D. (2002) aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation. *Developmental cell*, **3**, 85-97.
- Fraser, P.E., Levesque, G., Yu, G., Mills, L.R., Thirlwell, J., Frantseva, M., Gandy, S.E., Seeger, M., Carlen, P.L. & St George-Hyslop, P. (1998) Presenilin 1 is actively degraded by the 26S proteasome. *Neurobiology of aging*, **19**, S19-21.
- Galan, J.M. & Haguenauer-Tsapis, R. (1997) Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein. *The EMBO journal*, **16**, 5847-5854.
- Galcheva-Gargova, Z., Theroux, S.J. & Davis, R.J. (1995) The epidermal growth factor receptor is covalently linked to ubiquitin. *Oncogene*, **11**, 2649-2655.
- Gapuzan, M.E., Schmah, O., Pollock, A.D., Hoffmann, A. & Gilmore, T.D. (2005) Immortalized fibroblasts from NF-kappaB RelA knockout mice show phenotypic heterogeneity and maintain increased sensitivity to tumor necrosis factor alpha after transformation by v-Ras. *Oncogene*, 24, 6574-6583.
- Glenner, G.G. & Wong, C.W. (1984a) Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. *Biochemical and biophysical research communications*, **122**, 1131-1135.

- Glenner, G.G. & Wong, C.W. (1984b) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochemical and biophysical research communications*, **120**, 885-890.
- Goate, A., Chartier-Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L. & et al. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature*, **349**, 704-706.
- Goldgaber, D., Lerman, M.I., McBride, O.W., Saffiotti, U. & Gajdusek, D.C. (1987) Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease. *Science*, 235, 877-880.
- Goldstein, G. (1974) Isolation of bovine thymin: a polypeptide hormone of the thymus. *Nature*, **247**, 11-14.
- Goldstein, G., Scheid, M., Hammerling, U., Schlesinger, D.H., Niall, H.D. & Boyse, E.A. (1975) Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proceedings of the National Academy of Sciences* of the United States of America, **72**, 11-15.
- Gomez-Isla, T., Price, J.L., McKeel, D.W., Jr., Morris, J.C., Growdon, J.H. & Hyman, B.T. (1996) Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **16**, 4491-4500.
- Gong, B., Cao, Z., Zheng, P., Vitolo, O.V., Liu, S., Staniszewski, A., Moolman, D., Zhang, H., Shelanski, M. & Arancio, O. (2006) Ubiquitin hydrolase Uch-L1 rescues beta-amyloidinduced decreases in synaptic function and contextual memory. *Cell*, **126**, 775-788.
- Gong, B., Chen, F., Pan, Y., Arrieta-Cruz, I., Yoshida, Y., Haroutunian, V. & Pasinetti, G.M. (2010) SCFFbx2-E3-ligase-mediated degradation of BACE1 attenuates Alzheimer's disease amyloidosis and improves synaptic function. *Aging cell*, 9, 1018-1031.
- Goto, A., Wang, Y.L., Kabuta, T., Setsuie, R., Osaka, H., Sawa, A., Ishiura, S. & Wada, K. (2009) Proteomic and histochemical analysis of proteins involved in the dying-back-type of axonal degeneration in the gracile axonal dystrophy (gad) mouse. *Neurochem Int*, 54, 330-338.
- Goutte, C., Tsunozaki, M., Hale, V.A. & Priess, J.R. (2002) APH-1 is a multipass membrane protein essential for the Notch signaling pathway in Caenorhabditis elegans embryos. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 775-779.
- Gralle, M., Oliveira, C.L., Guerreiro, L.H., McKinstry, W.J., Galatis, D., Masters, C.L., Cappai, R., Parker, M.W., Ramos, C.H., Torriani, I. & Ferreira, S.T. (2006) Solution conformation and heparin-induced dimerization of the full-length extracellular domain of the human amyloid precursor protein. *Journal of molecular biology*, 357, 493-508.

- Graner, E., Tang, D., Rossi, S., Baron, A., Migita, T., Weinstein, L.J., Lechpammer, M., Huesken, D., Zimmermann, J., Signoretti, S. & Loda, M. (2004) The isopeptidase USP2a regulates the stability of fatty acid synthase in prostate cancer. *Cancer cell*, 5, 253-261.
- Granic, I., Dolga, A.M., Nijholt, I.M., van Dijk, G. & Eisel, U.L. (2009) Inflammation and NFkappaB in Alzheimer's disease and diabetes. *Journal of Alzheimer's disease : JAD*, **16**, 809-821.
- Gray, C., Hukkanen, M., Konttinen, Y.T., Terenghi, G., Arnett, T.R., Jones, S.J., Burnstock, G. & Polak, J.M. (1992) Rapid neural growth: calcitonin gene-related peptide and substance Pcontaining nerves attain exceptional growth rates in regenerating deer antler. *Neuroscience*, **50**, 953-963.
- Grisk, O., Grone, H.J., Rose, H.J. & Rettig, R. (2001) Sympathetic reinnervation of rat kidney grafts. *Transplantation*, 72, 1153-1155.
- Group, A.s.D.C. (1995) The structure of the presenilin 1 (S182) gene and identification of six novel mutations in early onset AD families. Alzheimer's Disease Collaborative Group. *Nature genetics*, **11**, 219-222.
- Guaiquil, V., Swendeman, S., Yoshida, T., Chavala, S., Campochiaro, P.A. & Blobel, C.P. (2009) ADAM9 is involved in pathological retinal neovascularization. *Molecular and cellular biology*, 29, 2694-2703.
- Haapasalo, A. & Kovacs, D.M. (2011) The many substrates of presenilin/gamma-secretase. *Journal of Alzheimer's disease : JAD*, **25**, 3-28.
- Haass, C., Koo, E.H., Mellon, A., Hung, A.Y. & Selkoe, D.J. (1992) Targeting of cell-surface beta-amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. *Nature*, **357**, 500-503.
- Haass, C. & Selkoe, D.J. (1993) Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide. *Cell*, 75, 1039-1042.
- Hadari, T., Warms, J.V., Rose, I.A. & Hershko, A. (1992) A ubiquitin C-terminal isopeptidase that acts on polyubiquitin chains. Role in protein degradation. *The Journal of biological chemistry*, 267, 719-727.
- Hardy, J. & Selkoe, D.J. (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*, **297**, 353-356.
- Hardy, J.A. & Higgins, G.A. (1992) Alzheimer's disease: the amyloid cascade hypothesis. *Science*, **256**, 184-185.
- Hartmann, D., de Strooper, B., Serneels, L., Craessaerts, K., Herreman, A., Annaert, W., Umans, L., Lubke, T., Lena Illert, A., von Figura, K. & Saftig, P. (2002) The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alphasecretase activity in fibroblasts. *Human molecular genetics*, 11, 2615-2624.

- He, G., Qing, H., Cai, F., Kwok, C., Xu, H., Yu, G., Bernstein, A. & Song, W. (2006) Ubiquitinproteasome pathway mediates degradation of APH-1. *Journal of neurochemistry*, 99, 1403-1412.
- He, G., Qing, H., Tong, Y., Cai, F., Ishiura, S. & Song, W. (2007) Degradation of nicastrin involves both proteasome and lysosome. *Journal of neurochemistry*, **101**, 982-992.
- He, X., Chang, W.P., Koelsch, G. & Tang, J. (2002) Memapsin 2 (beta-secretase) cytosolic domain binds to the VHS domains of GGA1 and GGA2: implications on the endocytosis mechanism of memapsin 2. *FEBS letters*, **524**, 183-187.
- He, X., Li, F., Chang, W.P. & Tang, J. (2005) GGA proteins mediate the recycling pathway of memapsin 2 (BACE). *The Journal of biological chemistry*, **280**, 11696-11703.
- Health, N.I.o.A.N.I.o., Health, U.S.D.o. & Services, H. (2002) *Alzheimer's Disease: Unraveling the Mystery*. Alzheimer's Disease Education and Referral (ADEAR) Center.
- Healy, D.G., Abou-Sleiman, P.M., Casas, J.P., Ahmadi, K.R., Lynch, T., Gandhi, S., Muqit, M.M., Foltynie, T., Barker, R., Bhatia, K.P., Quinn, N.P., Lees, A.J., Gibson, J.M., Holton, J.L., Revesz, T., Goldstein, D.B. & Wood, N.W. (2006) UCHL-1 is not a Parkinson's disease susceptibility gene. *Annals of neurology*, **59**, 627-633.
- Hendriks, L., van Duijn, C.M., Cras, P., Cruts, M., Van Hul, W., van Harskamp, F., Warren, A., McInnis, M.G., Antonarakis, S.E., Martin, J.J. & et al. (1992) Presenile dementia and cerebral haemorrhage linked to a mutation at codon 692 of the beta-amyloid precursor protein gene. *Nature genetics*, 1, 218-221.
- Hershko, A., Ciechanover, A., Heller, H., Haas, A.L. & Rose, I.A. (1980) Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis. *Proceedings of the National Academy of Sciences of the United States of America*, 77, 1783-1786.
- Herzig, M.C., Winkler, D.T., Burgermeister, P., Pfeifer, M., Kohler, E., Schmidt, S.D., Danner, S., Abramowski, D., Sturchler-Pierrat, C., Burki, K., van Duinen, S.G., Maat-Schieman, M.L., Staufenbiel, M., Mathews, P.M. & Jucker, M. (2004) Abeta is targeted to the vasculature in a mouse model of hereditary cerebral hemorrhage with amyloidosis. *Nature neuroscience*, 7, 954-960.
- Hibi, K., Liu, Q., Beaudry, G.A., Madden, S.L., Westra, W.H., Wehage, S.L., Yang, S.C., Heitmiller, R.F., Bertelsen, A.H., Sidransky, D. & Jen, J. (1998) Serial analysis of gene expression in non-small cell lung cancer. *Cancer research*, 58, 5690-5694.
- Hibi, K., Westra, W.H., Borges, M., Goodman, S., Sidransky, D. & Jen, J. (1999) PGP9.5 as a candidate tumor marker for non-small-cell lung cancer. *The American journal of pathology*, **155**, 711-715.
- Hicke, L. (1999) Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. *Trends in cell biology*, **9**, 107-112.

- Holsinger, R.M., McLean, C.A., Beyreuther, K., Masters, C.L. & Evin, G. (2002) Increased expression of the amyloid precursor beta-secretase in Alzheimer's disease. *Annals of neurology*, 51, 783-786.
- Hu, X., Hicks, C.W., He, W., Wong, P., Macklin, W.B., Trapp, B.D. & Yan, R. (2006) Bace1 modulates myelination in the central and peripheral nervous system. *Nature neuroscience*, 9, 1520-1525.
- Huse, J.T., Pijak, D.S., Leslie, G.J., Lee, V.M. & Doms, R.W. (2000) Maturation and endosomal targeting of beta-site amyloid precursor protein-cleaving enzyme. The Alzheimer's disease beta-secretase. *The Journal of biological chemistry*, 275, 33729-33737.
- Hussain, I., Powell, D., Howlett, D.R., Tew, D.G., Meek, T.D., Chapman, C., Gloger, I.S., Murphy, K.E., Southan, C.D., Ryan, D.M., Smith, T.S., Simmons, D.L., Walsh, F.S., Dingwall, C. & Christie, G. (1999) Identification of a novel aspartic protease (Asp 2) as beta-secretase. *Molecular and cellular neurosciences*, 14, 419-427.
- Hussain, S., Foreman, O., Perkins, S.L., Witzig, T.E., Miles, R.R., van Deursen, J. & Galardy, P.J. (2010) The de-ubiquitinase UCH-L1 is an oncogene that drives the development of lymphoma in vivo by deregulating PHLPP1 and Akt signaling. *Leukemia*, 24, 1641-1655.
- Hutton, M., Lendon, C.L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., Hackett, J., Adamson, J., Lincoln, S., Dickson, D., Davies, P., Petersen, R.C., Stevens, M., de Graaff, E., Wauters, E., van Baren, J., Hillebrand, M., Joosse, M., Kwon, J.M., Nowotny, P., Che, L.K., Norton, J., Morris, J.C., Reed, L.A., Trojanowski, J., Basun, H., Lannfelt, L., Neystat, M., Fahn, S., Dark, F., Tannenberg, T., Dodd, P.R., Hayward, N., Kwok, J.B., Schofield, P.R., Andreadis, A., Snowden, J., Craufurd, D., Neary, D., Owen, F., Oostra, B.A., Hardy, J., Goate, A., van Swieten, J., Mann, D., Lynch, T. & Heutink, P. (1998) Association of missense and 5'splice-site mutations in tau with the inherited dementia FTDP-17. *Nature*, **393**, 702-705.
- Ichihara, N., Wu, J., Chui, D.H., Yamazaki, K., Wakabayashi, T. & Kikuchi, T. (1995) Axonal degeneration promotes abnormal accumulation of amyloid beta-protein in ascending gracile tract of gracile axonal dystrophy (GAD) mouse. *Brain research*, 695, 173-178.
- Ivins, K.J., Thornton, P.L., Rohn, T.T. & Cotman, C.W. (1999) Neuronal apoptosis induced by beta-amyloid is mediated by caspase-8. *Neurobiology of disease*, **6**, 440-449.
- Iwata, A., Riley, B.E., Johnston, J.A. & Kopito, R.R. (2005) HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin. *The Journal of biological chemistry*, 280, 40282-40292.
- Jackson, P. & Thompson, R.J. (1981) The demonstration of new human brain-specific proteins by high-resolution two-dimensional polyacrylamide gel electrophoresis. *Journal of the neurological sciences*, 49, 429-438.
- Jackson, P., Thomson, V.M. & Thompson, R.J. (1985) A comparison of the evolutionary distribution of the two neuroendocrine markers, neurone-specific enolase and protein gene product 9.5. *Journal of neurochemistry*, 45, 185-190.

Jiang, X. & Wang, X. (2004) Cytochrome C-mediated apoptosis. Annu Rev Biochem, 73, 87-106.

- Joo, H.Y., Zhai, L., Yang, C., Nie, S., Erdjument-Bromage, H., Tempst, P., Chang, C. & Wang, H. (2007) Regulation of cell cycle progression and gene expression by H2A deubiquitination. *Nature*, 449, 1068-1072.
- Kabuta, T., Furuta, A., Aoki, S., Furuta, K. & Wada, K. (2008a) Aberrant interaction between Parkinson disease-associated mutant UCH-L1 and the lysosomal receptor for chaperonemediated autophagy. *The Journal of biological chemistry*, 283, 23731-23738.
- Kabuta, T., Setsuie, R., Mitsui, T., Kinugawa, A., Sakurai, M., Aoki, S., Uchida, K. & Wada, K. (2008b) Aberrant molecular properties shared by familial Parkinson's disease-associated mutant UCH-L1 and carbonyl-modified UCH-L1. *Human molecular genetics*, 17, 1482-1496.
- Kaltschmidt, B. & Kaltschmidt, C. (2009) NF-kappaB in the nervous system. *Cold Spring Harbor perspectives in biology*, **1**, a001271.
- Kamal, A., Almenar-Queralt, A., LeBlanc, J.F., Roberts, E.A. & Goldstein, L.S. (2001) Kinesinmediated axonal transport of a membrane compartment containing beta-secretase and presenilin-1 requires APP. *Nature*, 414, 643-648.
- Kamal, A., Stokin, G.B., Yang, Z., Xia, C.H. & Goldstein, L.S. (2000) Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. *Neuron*, 28, 449-459.
- Kaneko, M., Koike, H., Saito, R., Kitamura, Y., Okuma, Y. & Nomura, Y. (2010) Loss of HRD1mediated protein degradation causes amyloid precursor protein accumulation and amyloid-beta generation. *The Journal of neuroscience : the official journal of the Society* for Neuroscience, **30**, 3924-3932.
- Kang, E.L., Cameron, A.N., Piazza, F., Walker, K.R. & Tesco, G. (2010) Ubiquitin regulates GGA3-mediated degradation of BACE1. *The Journal of biological chemistry*, 285, 24108-24119.
- Kang, J., Lemaire, H.G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.H., Multhaup, G., Beyreuther, K. & Muller-Hill, B. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature*, **325**, 733-736.
- Kaplitt, M.G., Feigin, A., Tang, C., Fitzsimons, H.L., Mattis, P., Lawlor, P.A., Bland, R.J., Young, D., Strybing, K., Eidelberg, D. & During, M.J. (2007) Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. *Lancet*, 369, 2097-2105.
- Kay, J.E. & Korner, A. (1966) Effect of cycloheximide on protein and ribonucleic acid synthesis in cultured human lymphocytes. *The Biochemical journal*, **100**, 815-822.
- Keith B. J. Franklin, G.P. (2001) The Mouse Brain in Stereotaxic Coordinates. Academic Press.

- Keller, J.N., Hanni, K.B. & Markesbery, W.R. (2000) Impaired proteasome function in Alzheimer's disease. *Journal of neurochemistry*, **75**, 436-439.
- Kerridge, D. (1958) The effect of actidione and other antifungal agents on nucleic acid and protein synthesis in Saccharomyces carlsbergensis. *J Gen Microbiol*, **19**, 497-506.
- Kibbey, M.C., Jucker, M., Weeks, B.S., Neve, R.L., Van Nostrand, W.E. & Kleinman, H.K. (1993) beta-Amyloid precursor protein binds to the neurite-promoting IKVAV site of laminin. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 10150-10153.
- Kikuchi, T., Mukoyama, M., Yamazaki, K. & Moriya, H. (1990) Axonal degeneration of ascending sensory neurons in gracile axonal dystrophy mutant mouse. *Acta neuropathologica*, **80**, 145-151.
- Kim, D.Y., Carey, B.W., Wang, H., Ingano, L.A., Binshtok, A.M., Wertz, M.H., Pettingell, W.H., He, P., Lee, V.M., Woolf, C.J. & Kovacs, D.M. (2007) BACE1 regulates voltage-gated sodium channels and neuronal activity. *Nature cell biology*, 9, 755-764.
- Kim, D.Y., Ingano, L.A. & Kovacs, D.M. (2002) Nectin-1alpha, an immunoglobulin-like receptor involved in the formation of synapses, is a substrate for presenilin/gamma-secretase-like cleavage. *The Journal of biological chemistry*, 277, 49976-49981.
- Kim, H.J., Kim, Y.M., Lim, S., Nam, Y.K., Jeong, J., Kim, H.J. & Lee, K.J. (2009) Ubiquitin Cterminal hydrolase-L1 is a key regulator of tumor cell invasion and metastasis. *Oncogene*, 28, 117-127.
- Kimberly, W.T., LaVoie, M.J., Ostaszewski, B.L., Ye, W., Wolfe, M.S. & Selkoe, D.J. (2003) Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 6382-6387.
- Kirkin, V., Lamark, T., Sou, Y.S., Bjorkoy, G., Nunn, J.L., Bruun, J.A., Shvets, E., McEwan, D.G., Clausen, T.H., Wild, P., Bilusic, I., Theurillat, J.P., Overvatn, A., Ishii, T., Elazar, Z., Komatsu, M., Dikic, I. & Johansen, T. (2009) A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. *Molecular cell*, 33, 505-516.
- Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S. & Ito, H. (1988) Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. *Nature*, **331**, 530-532.
- Koh, Y.H., von Arnim, C.A., Hyman, B.T., Tanzi, R.E. & Tesco, G. (2005) BACE is degraded via the lysosomal pathway. *The Journal of biological chemistry*, **280**, 32499-32504.
- Koike, H., Tomioka, S., Sorimachi, H., Saido, T.C., Maruyama, K., Okuyama, A., Fujisawa-Sehara, A., Ohno, S., Suzuki, K. & Ishiura, S. (1999) Membrane-anchored metalloprotease MDC9 has an alpha-secretase activity responsible for processing the amyloid precursor protein. *The Biochemical journal*, **343 Pt 2**, 371-375.

- Komander, D., Clague, M.J. & Urbe, S. (2009) Breaking the chains: structure and function of the deubiquitinases. *Nature reviews. Molecular cell biology*, 10, 550-563.
- Koo, E.H., Squazzo, S.L., Selkoe, D.J. & Koo, C.H. (1996) Trafficking of cell-surface amyloid beta-protein precursor. I. Secretion, endocytosis and recycling as detected by labeled monoclonal antibody. *Journal of cell science*, **109** (Pt 5), 991-998.
- Koulich, E., Li, X. & DeMartino, G.N. (2008) Relative structural and functional roles of multiple deubiquitylating proteins associated with mammalian 26S proteasome. *Molecular biology* of the cell, 19, 1072-1082.
- Kraft, C., Peter, M. & Hofmann, K. (2010) Selective autophagy: ubiquitin-mediated recognition and beyond. *Nature cell biology*, **12**, 836-841.
- Kuperstein, I., Broersen, K., Benilova, I., Rozenski, J., Jonckheere, W., Debulpaep, M.,
 Vandersteen, A., Segers-Nolten, I., Van Der Werf, K., Subramaniam, V., Braeken, D.,
 Callewaert, G., Bartic, C., D'Hooge, R., Martins, I.C., Rousseau, F., Schymkowitz, J. &
 De Strooper, B. (2010) Neurotoxicity of Alzheimer's disease Abeta peptides is induced by
 small changes in the Abeta42 to Abeta40 ratio. *The EMBO journal*, 29, 3408-3420.
- Kwon, J., Mochida, K., Wang, Y.L., Sekiguchi, S., Sankai, T., Aoki, S., Ogura, A., Yoshikawa, Y.
 & Wada, K. (2005) Ubiquitin C-terminal hydrolase L-1 is essential for the early apoptotic wave of germinal cells and for sperm quality control during spermatogenesis. *Biology of reproduction*, **73**, 29-35.
- Kyratzi, E., Pavlaki, M. & Stefanis, L. (2008) The S18Y polymorphic variant of UCH-L1 confers an antioxidant function to neuronal cells. *Human molecular genetics*, **17**, 2160-2171.
- Laird, F.M., Cai, H., Savonenko, A.V., Farah, M.H., He, K., Melnikova, T., Wen, H., Chiang, H.C., Xu, G., Koliatsos, V.E., Borchelt, D.R., Price, D.L., Lee, H.K. & Wong, P.C. (2005) BACE1, a major determinant of selective vulnerability of the brain to amyloid-beta amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 25, 11693-11709.
- Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky, I., Trommer, B., Viola, K.L., Wals, P., Zhang, C., Finch, C.E., Krafft, G.A. & Klein, W.L. (1998) Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 6448-6453.
- Lammich, S., Kojro, E., Postina, R., Gilbert, S., Pfeiffer, R., Jasionowski, M., Haass, C. & Fahrenholz, F. (1999) Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 3922-3927.

- Lammich, S., Schobel, S., Zimmer, A.K., Lichtenthaler, S.F. & Haass, C. (2004) Expression of the Alzheimer protease BACE1 is suppressed via its 5'-untranslated region. *EMBO reports*, **5**, 620-625.
- Larsen, C.N., Krantz, B.A. & Wilkinson, K.D. (1998) Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases. *Biochemistry*, 37, 3358-3368.
- Larsen, C.N., Price, J.S. & Wilkinson, K.D. (1996) Substrate binding and catalysis by ubiquitin Cterminal hydrolases: identification of two active site residues. *Biochemistry*, 35, 6735-6744.
- Lee, B.H., Lee, M.J., Park, S., Oh, D.C., Elsasser, S., Chen, P.C., Gartner, C., Dimova, N., Hanna, J., Gygi, S.P., Wilson, S.M., King, R.W. & Finley, D. (2010) Enhancement of proteasome activity by a small-molecule inhibitor of USP14. *Nature*, 467, 179-184.
- Lee, J.H., Lau, K.F., Perkinton, M.S., Standen, C.L., Rogelj, B., Falinska, A., McLoughlin, D.M. & Miller, C.C. (2004) The neuronal adaptor protein X11beta reduces amyloid beta-protein levels and amyloid plaque formation in the brains of transgenic mice. *The Journal of biological chemistry*, 279, 49099-49104.
- Lee, J.H., Lau, K.F., Perkinton, M.S., Standen, C.L., Shemilt, S.J., Mercken, L., Cooper, J.D., McLoughlin, D.M. & Miller, C.C. (2003) The neuronal adaptor protein X11alpha reduces Abeta levels in the brains of Alzheimer's APPswe Tg2576 transgenic mice. *The Journal* of biological chemistry, 278, 47025-47029.
- Lee, M.J., Lee, B.H., Hanna, J., King, R.W. & Finley, D. (2011) Trimming of ubiquitin chains by proteasome-associated deubiquitinating enzymes. *Molecular & cellular proteomics : MCP*, **10**, R110 003871.
- Lee, Y.M., Lee, J.Y., Kim, M.J., Bae, H.I., Park, J.Y., Kim, S.G. & Kim, D.S. (2006) Hypomethylation of the protein gene product 9.5 promoter region in gallbladder cancer and its relationship with clinicopathological features. *Cancer science*, 97, 1205-1210.
- Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M.J., Jonnalagada, S., Chernova, T., Dehejia, A., Lavedan, C., Gasser, T., Steinbach, P.J., Wilkinson, K.D. & Polymeropoulos, M.H. (1998) The ubiquitin pathway in Parkinson's disease. *Nature*, **395**, 451-452.
- Lesne, S., Koh, M.T., Kotilinek, L., Kayed, R., Glabe, C.G., Yang, A., Gallagher, M. & Ashe, K.H. (2006) A specific amyloid-beta protein assembly in the brain impairs memory. *Nature*, 440, 352-357.
- Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D.M., Oshima, J., Pettingell, W.H., Yu, C.E., Jondro, P.D., Schmidt, S.D., Wang, K. & et al. (1995) Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science*, **269**, 973-977.
- Li, Q. & Sudhof, T.C. (2004) Cleavage of amyloid-beta precursor protein and amyloid-beta precursor-like protein by BACE 1. *The Journal of biological chemistry*, **279**, 10542-10550.

- Li, Y., Zhou, W., Tong, Y., He, G. & Song, W. (2006) Control of APP processing and Abeta generation level by BACE1 enzymatic activity and transcription. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, **20**, 285-292.
- Li, Y.M., Lai, M.T., Xu, M., Huang, Q., DiMuzio-Mower, J., Sardana, M.K., Shi, X.P., Yin, K.C., Shafer, J.A. & Gardell, S.J. (2000) Presenilin 1 is linked with gamma-secretase activity in the detergent solubilized state. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 6138-6143.
- Lim, G.P., Yang, F., Chu, T., Chen, P., Beech, W., Teter, B., Tran, T., Ubeda, O., Ashe, K.H., Frautschy, S.A. & Cole, G.M. (2000) Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **20**, 5709-5714.
- Lincoln, S., Vaughan, J., Wood, N., Baker, M., Adamson, J., Gwinn-Hardy, K., Lynch, T., Hardy, J. & Farrer, M. (1999) Low frequency of pathogenic mutations in the ubiquitin carboxyterminal hydrolase gene in familial Parkinson's disease. *Neuroreport*, **10**, 427-429.
- Liu, S., Bromley-Brits, K., Xia, K., Mittelholtz, J., Wang, R. & Song, W. (2008) TMP21 degradation is mediated by the ubiquitin-proteasome pathway. *The European journal of neuroscience*, 28, 1980-1988.
- Liu, X., Zeng, B., Ma, J. & Wan, C. (2009) Comparative proteomic analysis of osteosarcoma cell and human primary cultured osteoblastic cell. *Cancer investigation*, **27**, 345-352.
- Liu, Y., Fallon, L., Lashuel, H.A., Liu, Z. & Lansbury, P.T., Jr. (2002) The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. *Cell*, **111**, 209-218.
- Loo, D.T., Copani, A., Pike, C.J., Whittemore, E.R., Walencewicz, A.J. & Cotman, C.W. (1993) Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 7951-7955.
- Lopez Salon, M., Morelli, L., Castano, E.M., Soto, E.F. & Pasquini, J.M. (2000) Defective ubiquitination of cerebral proteins in Alzheimer's disease. *Journal of neuroscience research*, 62, 302-310.
- Lowe, J., McDermott, H., Landon, M., Mayer, R.J. & Wilkinson, K.D. (1990) Ubiquitin carboxylterminal hydrolase (PGP 9.5) is selectively present in ubiquitinated inclusion bodies characteristic of human neurodegenerative diseases. *The Journal of pathology*, **161**, 153-160.
- Luo, Y., Bolon, B., Kahn, S., Bennett, B.D., Babu-Khan, S., Denis, P., Fan, W., Kha, H., Zhang, J., Gong, Y., Martin, L., Louis, J.C., Yan, Q., Richards, W.G., Citron, M. & Vassar, R. (2001) Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation. *Nature neuroscience*, 4, 231-232.

- Ly, P.T., Cai, F. & Song, W. (2011) Detection of neuritic plaques in Alzheimer's disease mouse model. *Journal of visualized experiments : JoVE*.
- Ly, P.T., Wu, Y., Zou, H., Wang, R., Zhou, W., Kinoshita, A., Zhang, M., Yang, Y., Cai, F., Woodgett, J. & Song, W. (2013) Inhibition of GSK3beta-mediated BACE1 expression reduces Alzheimer-associated phenotypes. *The Journal of clinical investigation*, **123**, 224-235.
- Mackenzie, I.R. & Munoz, D.G. (1998) Nonsteroidal anti-inflammatory drug use and Alzheimertype pathology in aging. *Neurology*, **50**, 986-990.
- Maillard, I., Adler, S.H. & Pear, W.S. (2003) Notch and the immune system. *Immunity*, **19**, 781-791.
- Maillard, I. & Pear, W.S. (2003) Notch and cancer: best to avoid the ups and downs. *Cancer cell*, **3**, 203-205.
- Mann, D.A., Trowern, A.R., Lavender, F.L., Whittaker, P.A. & Thompson, R.J. (1996) Identification of evolutionary conserved regulatory sequences in the 5' untranscribed region of the neural-specific ubiquitin C-terminal hydrolase (PGP9.5) gene. *Journal of neurochemistry*, 66, 35-46.
- Maraganore, D.M., Farrer, M.J., Hardy, J.A., Lincoln, S.J., McDonnell, S.K. & Rocca, W.A. (1999) Case-control study of the ubiquitin carboxy-terminal hydrolase L1 gene in Parkinson's disease. *Neurology*, **53**, 1858-1860.
- Maraganore, D.M., Lesnick, T.G., Elbaz, A., Chartier-Harlin, M.C., Gasser, T., Kruger, R., Hattori, N., Mellick, G.D., Quattrone, A., Satoh, J., Toda, T., Wang, J., Ioannidis, J.P., de Andrade, M., Rocca, W.A. & Consortium, U.G.G. (2004) UCHL1 is a Parkinson's disease susceptibility gene. *Annals of neurology*, 55, 512-521.
- Marambaud, P., Shioi, J., Serban, G., Georgakopoulos, A., Sarner, S., Nagy, V., Baki, L., Wen, P., Efthimiopoulos, S., Shao, Z., Wisniewski, T. & Robakis, N.K. (2002) A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. *The EMBO journal*, **21**, 1948-1956.
- Martins, I.C., Kuperstein, I., Wilkinson, H., Maes, E., Vanbrabant, M., Jonckheere, W., Van Gelder, P., Hartmann, D., D'Hooge, R., De Strooper, B., Schymkowitz, J. & Rousseau, F. (2008) Lipids revert inert Abeta amyloid fibrils to neurotoxic protofibrils that affect learning in mice. *The EMBO journal*, 27, 224-233.
- Masters, C.L., Simms, G., Weinman, N.A., Multhaup, G., McDonald, B.L. & Beyreuther, K. (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proceedings of the National Academy of Sciences of the United States of America*, **82**, 4245-4249.

- May, P., Reddy, Y.K. & Herz, J. (2002) Proteolytic processing of low density lipoprotein receptorrelated protein mediates regulated release of its intracellular domain. *The Journal of biological chemistry*, 277, 18736-18743.
- Mc Donald, J.M., Savva, G.M., Brayne, C., Welzel, A.T., Forster, G., Shankar, G.M., Selkoe, D.J., Ince, P.G., Walsh, D.M., Medical Research Council Cognitive, F. & Ageing, S. (2010) The presence of sodium dodecyl sulphate-stable Abeta dimers is strongly associated with Alzheimer-type dementia. *Brain : a journal of neurology*, **133**, 1328-1341.
- McGeer, P.L., McGeer, E., Rogers, J. & Sibley, J. (1990) Anti-inflammatory drugs and Alzheimer disease. *Lancet*, 335, 1037.
- McLean, C.A., Cherny, R.A., Fraser, F.W., Fuller, S.J., Smith, M.J., Beyreuther, K., Bush, A.I. & Masters, C.L. (1999) Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Annals of neurology*, 46, 860-866.
- Meray, R.K. & Lansbury, P.T., Jr. (2007) Reversible monoubiquitination regulates the Parkinson disease-associated ubiquitin hydrolase UCH-L1. *The Journal of biological chemistry*, 282, 10567-10575.
- Meziane, H., Dodart, J.C., Mathis, C., Little, S., Clemens, J., Paul, S.M. & Ungerer, A. (1998) Memory-enhancing effects of secreted forms of the beta-amyloid precursor protein in normal and amnestic mice. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 12683-12688.
- Milani, M., Rzymski, T., Mellor, H.R., Pike, L., Bottini, A., Generali, D. & Harris, A.L. (2009) The role of ATF4 stabilization and autophagy in resistance of breast cancer cells treated with Bortezomib. *Cancer research*, **69**, 4415-4423.
- Miura, H., Oda, K., Endo, C., Yamazaki, K., Shibasaki, H. & Kikuchi, T. (1993) Progressive degeneration of motor nerve terminals in GAD mutant mouse with hereditary sensory axonopathy. *Neuropathology and applied neurobiology*, **19**, 41-51.
- Mizukami, H., Shirahata, A., Goto, T., Sakata, M., Saito, M., Ishibashi, K., Kigawa, G., Nemoto, H., Sanada, Y. & Hibi, K. (2008) PGP9.5 methylation as a marker for metastatic colorectal cancer. *Anticancer research*, 28, 2697-2700.
- Mori, S., Heldin, C.H. & Claesson-Welsh, L. (1992) Ligand-induced polyubiquitination of the platelet-derived growth factor beta-receptor. *The Journal of biological chemistry*, 267, 6429-6434.
- Mukoyama, M., Yamazaki, K., Kikuchi, T. & Tomita, T. (1989) Neuropathology of gracile axonal dystrophy (GAD) mouse. An animal model of central distal axonopathy in primary sensory neurons. *Acta neuropathologica*, **79**, 294-299.
- Mullan, M., Crawford, F., Axelman, K., Houlden, H., Lilius, L., Winblad, B. & Lannfelt, L. (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the Nterminus of beta-amyloid. *Nature genetics*, 1, 345-347.

- Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B.A. & Yuan, J. (2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature*, **403**, 98-103.
- Nemeth, L. & Puri, P. (2000) The innervation of human bowel mucosa and its alterations in Hirschsprung's disease using a whole-mount preparation technique. *Pediatric surgery international*, **16**, 277-281.
- Ni, C.Y., Murphy, M.P., Golde, T.E. & Carpenter, G. (2001) gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science*, **294**, 2179-2181.
- Nicassio, F., Corrado, N., Vissers, J.H., Areces, L.B., Bergink, S., Marteijn, J.A., Geverts, B., Houtsmuller, A.B., Vermeulen, W., Di Fiore, P.P. & Citterio, E. (2007) Human USP3 is a chromatin modifier required for S phase progression and genome stability. *Current biology* : CB, 17, 1972-1977.
- Nishikawa, K., Li, H., Kawamura, R., Osaka, H., Wang, Y.L., Hara, Y., Hirokawa, T., Manago, Y., Amano, T., Noda, M., Aoki, S. & Wada, K. (2003) Alterations of structure and hydrolase activity of parkinsonism-associated human ubiquitin carboxyl-terminal hydrolase L1 variants. *Biochemical and biophysical research communications*, **304**, 176-183.
- Ohno, M., Sametsky, E.A., Younkin, L.H., Oakley, H., Younkin, S.G., Citron, M., Vassar, R. & Disterhoft, J.F. (2004) BACE1 deficiency rescues memory deficits and cholinergic dysfunction in a mouse model of Alzheimer's disease. *Neuron*, 41, 27-33.
- Osaka, H., Wang, Y.L., Takada, K., Takizawa, S., Setsuie, R., Li, H., Sato, Y., Nishikawa, K., Sun, Y.J., Sakurai, M., Harada, T., Hara, Y., Kimura, I., Chiba, S., Namikawa, K., Kiyama, H., Noda, M., Aoki, S. & Wada, K. (2003) Ubiquitin carboxy-terminal hydrolase L1 binds to and stabilizes monoubiquitin in neuron. *Human molecular genetics*, **12**, 1945-1958.
- Pandey, U.B., Nie, Z., Batlevi, Y., McCray, B.A., Ritson, G.P., Nedelsky, N.B., Schwartz, S.L., DiProspero, N.A., Knight, M.A., Schuldiner, O., Padmanabhan, R., Hild, M., Berry, D.L., Garza, D., Hubbert, C.C., Yao, T.P., Baehrecke, E.H. & Taylor, J.P. (2007) HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. *Nature*, 447, 859-863.
- Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A., Bjorkoy, G. & Johansen, T. (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *The Journal of biological chemistry*, 282, 24131-24145.
- Pardossi-Piquard, R., Petit, A., Kawarai, T., Sunyach, C., Alves da Costa, C., Vincent, B., Ring, S., D'Adamio, L., Shen, J., Muller, U., St George Hyslop, P. & Checler, F. (2005) Presenilindependent transcriptional control of the Abeta-degrading enzyme neprilysin by intracellular domains of betaAPP and APLP. *Neuron*, 46, 541-554.
- Pastorino, L., Ikin, A.F., Lamprianou, S., Vacaresse, N., Revelli, J.P., Platt, K., Paganetti, P., Mathews, P.M., Harroch, S. & Buxbaum, J.D. (2004) BACE (beta-secretase) modulates the processing of APLP2 in vivo. *Molecular and cellular neurosciences*, 25, 642-649.

- Pastorino, L., Ikin, A.F., Nairn, A.C., Pursnani, A. & Buxbaum, J.D. (2002) The carboxylterminus of BACE contains a sorting signal that regulates BACE trafficking but not the formation of total A(beta). *Molecular and cellular neurosciences*, **19**, 175-185.
- Perez, R.G., Soriano, S., Hayes, J.D., Ostaszewski, B., Xia, W., Selkoe, D.J., Chen, X., Stokin, G.B. & Koo, E.H. (1999) Mutagenesis identifies new signals for beta-amyloid precursor protein endocytosis, turnover, and the generation of secreted fragments, including Abeta42. *The Journal of biological chemistry*, 274, 18851-18856.
- Pickart, C.M. (2000) Ubiquitin in chains. Trends in biochemical sciences, 25, 544-548.
- Pintelon, I., Brouns, I., De Proost, I., Van Meir, F., Timmermans, J.P. & Adriaensen, D. (2007) Sensory receptors in the visceral pleura: neurochemical coding and live staining in whole mounts. *American journal of respiratory cell and molecular biology*, **36**, 541-551.
- Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I. & Fuller, F. (1988) A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. *Nature*, **331**, 525-527.
- Popov, N., Wanzel, M., Madiredjo, M., Zhang, D., Beijersbergen, R., Bernards, R., Moll, R., Elledge, S.J. & Eilers, M. (2007) The ubiquitin-specific protease USP28 is required for MYC stability. *Nature cell biology*, 9, 765-774.
- Proctor, C.J., Tangeman, P.J. & Ardley, H.C. (2010) Modelling the role of UCH-L1 on protein aggregation in age-related neurodegeneration. *PloS one*, **5**, e13175.
- Qing, H., He, G., Ly, P.T., Fox, C.J., Staufenbiel, M., Cai, F., Zhang, Z., Wei, S., Sun, X., Chen, C.H., Zhou, W., Wang, K. & Song, W. (2008) Valproic acid inhibits Abeta production, neuritic plaque formation, and behavioral deficits in Alzheimer's disease mouse models. *The Journal of experimental medicine*, **205**, 2781-2789.
- Qing, H., Zhou, W., Christensen, M.A., Sun, X., Tong, Y. & Song, W. (2004) Degradation of BACE by the ubiquitin-proteasome pathway. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 18, 1571-1573.
- Ragland, M., Hutter, C., Zabetian, C. & Edwards, K. (2009) Association between the ubiquitin carboxyl-terminal esterase L1 gene (UCHL1) S18Y variant and Parkinson's Disease: a HuGE review and meta-analysis. *American journal of epidemiology*, **170**, 1344-1357.
- Raina, P., Santaguida, P., Ismaila, A., Patterson, C., Cowan, D., Levine, M., Booker, L. & Oremus, M. (2008) Effectiveness of cholinesterase inhibitors and memantine for treating dementia: evidence review for a clinical practice guideline. *Annals of internal medicine*, **148**, 379-397.
- Ring, S., Weyer, S.W., Kilian, S.B., Waldron, E., Pietrzik, C.U., Filippov, M.A., Herms, J., Buchholz, C., Eckman, C.B., Korte, M., Wolfer, D.P. & Muller, U.C. (2007) The secreted beta-amyloid precursor protein ectodomain APPs alpha is sufficient to rescue the anatomical, behavioral, and electrophysiological abnormalities of APP-deficient mice.

The Journal of neuroscience : the official journal of the Society for Neuroscience, **27**, 7817-7826.

- Robakis, N.K., Ramakrishna, N., Wolfe, G. & Wisniewski, H.M. (1987) Molecular cloning and characterization of a cDNA encoding the cerebrovascular and the neuritic plaque amyloid peptides. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 4190-4194.
- Roberds, S.L., Anderson, J., Basi, G., Bienkowski, M.J., Branstetter, D.G., Chen, K.S., Freedman, S.B., Frigon, N.L., Games, D., Hu, K., Johnson-Wood, K., Kappenman, K.E., Kawabe, T.T., Kola, I., Kuehn, R., Lee, M., Liu, W., Motter, R., Nichols, N.F., Power, M., Robertson, D.W., Schenk, D., Schoor, M., Shopp, G.M., Shuck, M.E., Sinha, S., Svensson, K.A., Tatsuno, G., Tintrup, H., Wijsman, J., Wright, S. & McConlogue, L. (2001) BACE knockout mice are healthy despite lacking the primary beta-secretase activity in brain: implications for Alzheimer's disease therapeutics. *Human molecular genetics*, 10, 1317-1324.
- Roch, J.M., Masliah, E., Roch-Levecq, A.C., Sundsmo, M.P., Otero, D.A., Veinbergs, I. & Saitoh, T. (1994) Increase of synaptic density and memory retention by a peptide representing the trophic domain of the amyloid beta/A4 protein precursor. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 7450-7454.
- Rogaev, E.I., Sherrington, R., Rogaeva, E.A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., Tsuda, T. & et al. (1995) Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature*, **376**, 775-778.
- Rogers, J. (2008) The inflammatory response in Alzheimer's disease. J Periodontol, 79, 1535-1543.
- Rohn, T.T., Head, E., Nesse, W.H., Cotman, C.W. & Cribbs, D.H. (2001) Activation of caspase-8 in the Alzheimer's disease brain. *Neurobiology of disease*, **8**, 1006-1016.
- Rohn, T.T., Rissman, R.A., Davis, M.C., Kim, Y.E., Cotman, C.W. & Head, E. (2002) Caspase-9 activation and caspase cleavage of tau in the Alzheimer's disease brain. *Neurobiology of disease*, 11, 341-354.
- Rolen, U., Kobzeva, V., Gasparjan, N., Ovaa, H., Winberg, G., Kisseljov, F. & Masucci, M.G. (2006) Activity profiling of deubiquitinating enzymes in cervical carcinoma biopsies and cell lines. *Molecular carcinogenesis*, 45, 260-269.
- Sabo, S.L., Lanier, L.M., Ikin, A.F., Khorkova, O., Sahasrabudhe, S., Greengard, P. & Buxbaum, J.D. (1999) Regulation of beta-amyloid secretion by FE65, an amyloid protein precursorbinding protein. *The Journal of biological chemistry*, 274, 7952-7957.
- Saigoh, K., Wang, Y.L., Suh, J.G., Yamanishi, T., Sakai, Y., Kiyosawa, H., Harada, T., Ichihara, N., Wakana, S., Kikuchi, T. & Wada, K. (1999) Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in gad mice. *Nature genetics*, 23, 47-51.

- Sakurai, M., Sekiguchi, M., Zushida, K., Yamada, K., Nagamine, S., Kabuta, T. & Wada, K. (2008) Reduction in memory in passive avoidance learning, exploratory behaviour and synaptic plasticity in mice with a spontaneous deletion in the ubiquitin C-terminal hydrolase L1 gene. *The European journal of neuroscience*, 27, 691-701.
- Sandoval, I.V. & Bakke, O. (1994) Targeting of membrane proteins to endosomes and lysosomes. *Trends in cell biology*, **4**, 292-297.
- Sastre, M., Turner, R.S. & Levy, E. (1998) X11 interaction with beta-amyloid precursor protein modulates its cellular stabilization and reduces amyloid beta-protein secretion. *The Journal of biological chemistry*, 273, 22351-22357.
- Saunders, A.J., Kim, T.-W. & Tanzi, R.E. (1999) BACE Maps to Chromosome 11 and a BACE Homolog, BACE2, Reside in the Obligate Down Syndrome Region of Chromosome 21. *Science*, **286**, 1255.
- Schlesinger, D.H., Goldstein, G. & Niall, H.D. (1975) The complete amino acid sequence of ubiquitin, an adenylate cyclase stimulating polypeptide probably universal in living cells. *Biochemistry*, 14, 2214-2218.
- Schoenfeld, A.R., Davidowitz, E.J. & Burk, R.D. (2000) Elongin BC complex prevents degradation of von Hippel-Lindau tumor suppressor gene products. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 8507-8512.
- Searfoss, G.H., Jordan, W.H., Calligaro, D.O., Galbreath, E.J., Schirtzinger, L.M., Berridge, B.R., Gao, H., Higgins, M.A., May, P.C. & Ryan, T.P. (2003) Adipsin, a biomarker of gastrointestinal toxicity mediated by a functional gamma-secretase inhibitor. *The Journal* of biological chemistry, **278**, 46107-46116.
- Setsuie, R., Wang, Y.L., Mochizuki, H., Osaka, H., Hayakawa, H., Ichihara, N., Li, H., Furuta, A., Sano, Y., Sun, Y.J., Kwon, J., Kabuta, T., Yoshimi, K., Aoki, S., Mizuno, Y., Noda, M. & Wada, K. (2007) Dopaminergic neuronal loss in transgenic mice expressing the Parkinson's disease-associated UCH-L1 I93M mutant. *Neurochem Int*, **50**, 119-129.
- Shankar, G.M., Li, S., Mehta, T.H., Garcia-Munoz, A., Shepardson, N.E., Smith, I., Brett, F.M., Farrell, M.A., Rowan, M.J., Lemere, C.A., Regan, C.M., Walsh, D.M., Sabatini, B.L. & Selkoe, D.J. (2008) Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nature medicine*, 14, 837-842.
- Shen, H., Sikorska, M., Leblanc, J., Walker, P.R. & Liu, Q.Y. (2006) Oxidative stress regulated expression of ubiquitin Carboxyl-terminal Hydrolase-L1: role in cell survival. *Apoptosis : an international journal on programmed cell death*, **11**, 1049-1059.
- Shen, J., Bronson, R.T., Chen, D.F., Xia, W., Selkoe, D.J. & Tonegawa, S. (1997) Skeletal and CNS defects in Presenilin-1-deficient mice. *Cell*, **89**, 629-639.
- Sherrington, R., Rogaev, E.I., Liang, Y., Rogaeva, E.A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J.F., Bruni, A.C., Montesi, M.P., Sorbi, S., Rainero, I., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P.,

Polinsky, R.J., Wasco, W., Da Silva, H.A., Haines, J.L., Perkicak-Vance, M.A., Tanzi, R.E., Roses, A.D., Fraser, P.E., Rommens, J.M. & St George-Hyslop, P.H. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*, **375**, 754-760.

- Shiba, T., Kametaka, S., Kawasaki, M., Shibata, M., Waguri, S., Uchiyama, Y. & Wakatsuki, S. (2004) Insights into the phosphoregulation of beta-secretase sorting signal by the VHS domain of GGA1. *Traffic*, 5, 437-448.
- Sinha, S., Anderson, J.P., Barbour, R., Basi, G.S., Caccavello, R., Davis, D., Doan, M., Dovey, H.F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberburg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Suomensaari, S.M., Wang, S., Walker, D., Zhao, J., McConlogue, L. & John, V. (1999) Purification and cloning of amyloid precursor protein beta-secretase from human brain. *Nature*, **402**, 537-540.
- Sisodia, S.S., Koo, E.H., Beyreuther, K., Unterbeck, A. & Price, D.L. (1990) Evidence that betaamyloid protein in Alzheimer's disease is not derived by normal processing. *Science*, **248**, 492-495.
- Small, S.A. & Gandy, S. (2006) Sorting through the cell biology of Alzheimer's disease: intracellular pathways to pathogenesis. *Neuron*, 52, 15-31.
- Smith, D.L., Pozueta, J., Gong, B., Arancio, O. & Shelanski, M. (2009) Reversal of long-term dendritic spine alterations in Alzheimer disease models. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 16877-16882.
- Song, W., Nadeau, P., Yuan, M., Yang, X., Shen, J. & Yankner, B.A. (1999) Proteolytic release and nuclear translocation of Notch-1 are induced by presenilin-1 and impaired by pathogenic presenilin-1 mutations. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 6959-6963.
- Spasic, D., Tolia, A., Dillen, K., Baert, V., De Strooper, B., Vrijens, S. & Annaert, W. (2006) Presenilin-1 maintains a nine-transmembrane topology throughout the secretory pathway. *The Journal of biological chemistry*, 281, 26569-26577.
- Spillantini, M.G., Bird, T.D. & Ghetti, B. (1998) Frontotemporal dementia and Parkinsonism linked to chromosome 17: a new group of tauopathies. *Brain pathology*, 8, 387-402.
- Springael, J.Y., Galan, J.M., Haguenauer-Tsapis, R. & Andre, B. (1999) NH4+-induced downregulation of the Saccharomyces cerevisiae Gap1p permease involves its ubiquitination with lysine-63-linked chains. *Journal of cell science*, **112** (**Pt 9**), 1375-1383.
- Stadelmann, C., Deckwerth, T.L., Srinivasan, A., Bancher, C., Bruck, W., Jellinger, K. & Lassmann, H. (1999) Activation of caspase-3 in single neurons and autophagic granules of granulovacuolar degeneration in Alzheimer's disease. Evidence for apoptotic cell death. *The American journal of pathology*, **155**, 1459-1466.

- Stanger, B.Z., Datar, R., Murtaugh, L.C. & Melton, D.A. (2005) Direct regulation of intestinal fate by Notch. Proceedings of the National Academy of Sciences of the United States of America, 102, 12443-12448.
- Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L. & Rotin, D. (1997) Regulation of stability and function of the epithelial Na+ channel (ENaC) by ubiquitination. *The EMBO journal*, **16**, 6325-6336.
- Strous, G.J., van Kerkhof, P., Govers, R., Ciechanover, A. & Schwartz, A.L. (1996) The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor. *The EMBO journal*, 15, 3806-3812.
- Strozyk, D., Blennow, K., White, L.R. & Launer, L.J. (2003) CSF Abeta 42 levels correlate with amyloid-neuropathology in a population-based autopsy study. *Neurology*, **60**, 652-656.
- Sturchler-Pierrat, C., Abramowski, D., Duke, M., Wiederhold, K.H., Mistl, C., Rothacher, S., Ledermann, B., Burki, K., Frey, P., Paganetti, P.A., Waridel, C., Calhoun, M.E., Jucker, M., Probst, A., Staufenbiel, M. & Sommer, B. (1997) Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 13287-13292.
- Sturchler-Pierrat, C. & Staufenbiel, M. (2000) Pathogenic mechanisms of Alzheimer's disease analyzed in the APP23 transgenic mouse model. *Ann N Y Acad Sci*, **920**, 134-139.
- Su, J.H., Anderson, A.J., Cummings, B.J. & Cotman, C.W. (1994) Immunohistochemical evidence for apoptosis in Alzheimer's disease. *Neuroreport*, 5, 2529-2533.
- Sun, X., He, G., Qing, H., Zhou, W., Dobie, F., Cai, F., Staufenbiel, M., Huang, L.E. & Song, W. (2006a) Hypoxia facilitates Alzheimer's disease pathogenesis by up-regulating BACE1 gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 18727-18732.
- Sun, X., He, G. & Song, W. (2006b) BACE2, as a novel APP theta-secretase, is not responsible for the pathogenesis of Alzheimer's disease in Down syndrome. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 20, 1369-1376.
- Sung, S., Yang, H., Uryu, K., Lee, E.B., Zhao, L., Shineman, D., Trojanowski, J.Q., Lee, V.M. & Pratico, D. (2004) Modulation of nuclear factor-kappa B activity by indomethacin influences A beta levels but not A beta precursor protein metabolism in a model of Alzheimer's disease. *The American journal of pathology*, **165**, 2197-2206.
- Takami, Y., Nakagami, H., Morishita, R., Katsuya, T., Cui, T.X., Ichikawa, T., Saito, Y., Hayashi, H., Kikuchi, Y., Nishikawa, T., Baba, Y., Yasuda, O., Rakugi, H., Ogihara, T. & Kaneda, Y. (2007) Ubiquitin carboxyl-terminal hydrolase L1, a novel deubiquitinating enzyme in the vasculature, attenuates NF-kappaB activation. *Arteriosclerosis, thrombosis, and* vascular biology, 27, 2184-2190.

- Takase, T., Hibi, K., Yamazaki, T., Nakayama, H., Taguchi, M., Kasai, Y., Ito, K., Akiyama, S., Nagasaka, T. & Nakao, A. (2003) PGP9.5 overexpression in esophageal squamous cell carcinoma. *Hepato-gastroenterology*, **50**, 1278-1280.
- Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G.
 & Iwatsubo, T. (2003) The role of presenilin cofactors in the gamma-secretase complex. *Nature*, 422, 438-441.
- Tamagno, E., Bardini, P., Obbili, A., Vitali, A., Borghi, R., Zaccheo, D., Pronzato, M.A., Danni, O., Smith, M.A., Perry, G. & Tabaton, M. (2002) Oxidative stress increases expression and activity of BACE in NT2 neurons. *Neurobiology of disease*, 10, 279-288.
- Tan, J. & Evin, G. (2012) Beta-site APP-cleaving enzyme 1 trafficking and Alzheimer's disease pathogenesis. *Journal of neurochemistry*, **120**, 869-880.
- Tan, Y.Y., Zhou, H.Y., Wang, Z.Q. & Chen, S.D. (2008) Endoplasmic reticulum stress contributes to the cell death induced by UCH-L1 inhibitor. *Molecular and cellular biochemistry*, **318**, 109-115.
- Tanzi, R.E., Gusella, J.F., Watkins, P.C., Bruns, G.A., St George-Hyslop, P., Van Keuren, M.L., Patterson, D., Pagan, S., Kurnit, D.M. & Neve, R.L. (1987) Amyloid beta protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus. *Science*, 235, 880-884.
- Tanzi, R.E., McClatchey, A.I., Lamperti, E.D., Villa-Komaroff, L., Gusella, J.F. & Neve, R.L. (1988) Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease. *Nature*, 331, 528-530.
- Taru, H., Kirino, Y. & Suzuki, T. (2002) Differential roles of JIP scaffold proteins in the modulation of amyloid precursor protein metabolism. *The Journal of biological chemistry*, 277, 27567-27574.
- Taylor, C.J., Ireland, D.R., Ballagh, I., Bourne, K., Marechal, N.M., Turner, P.R., Bilkey, D.K., Tate, W.P. & Abraham, W.C. (2008) Endogenous secreted amyloid precursor proteinalpha regulates hippocampal NMDA receptor function, long-term potentiation and spatial memory. *Neurobiology of disease*, **31**, 250-260.
- Tesco, G., Koh, Y.H., Kang, E.L., Cameron, A.N., Das, S., Sena-Esteves, M., Hiltunen, M., Yang, S.H., Zhong, Z., Shen, Y., Simpkins, J.W. & Tanzi, R.E. (2007) Depletion of GGA3 stabilizes BACE and enhances beta-secretase activity. *Neuron*, 54, 721-737.
- Tezel, E., Hibi, K., Nagasaka, T. & Nakao, A. (2000) PGP9.5 as a prognostic factor in pancreatic cancer. Clinical cancer research : an official journal of the American Association for Cancer Research, 6, 4764-4767.
- Thies, W. & Bleiler, L. (2011) 2011 Alzheimer's disease facts and figures. *Alzheimers Dement*, 7, 208-244.

- Thinakaran, G., Borchelt, D.R., Lee, M.K., Slunt, H.H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A.I., Gandy, S.E., Jenkins, N.A., Copeland, N.G., Price, D.L. & Sisodia, S.S. (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. *Neuron*, **17**, 181-190.
- Thinakaran, G., Harris, C.L., Ratovitski, T., Davenport, F., Slunt, H.H., Price, D.L., Borchelt, D.R. & Sisodia, S.S. (1997) Evidence that levels of presenilins (PS1 and PS2) are coordinately regulated by competition for limiting cellular factors. *The Journal of biological chemistry*, 272, 28415-28422.
- Thurston, T.L., Ryzhakov, G., Bloor, S., von Muhlinen, N. & Randow, F. (2009) The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. *Nature immunology*, **10**, 1215-1221.
- Tong, Y., Zhou, W., Fung, V., Christensen, M.A., Qing, H., Sun, X. & Song, W. (2005) Oxidative stress potentiates BACE1 gene expression and Abeta generation. *Journal of neural transmission*, **112**, 455-469.
- Touitou, R., Richardson, J., Bose, S., Nakanishi, M., Rivett, J. & Allday, M.J. (2001) A degradation signal located in the C-terminus of p21WAF1/CIP1 is a binding site for the C8 alpha-subunit of the 20S proteasome. *The EMBO journal*, **20**, 2367-2375.
- Trifa, F., Karray-Chouayekh, S., Jmaa, Z.B., Jmal, E., Khabir, A., Sellami-Boudawara, T., Frikha, M., Daoud, J. & Mokdad-Gargouri, R. (2013) Frequent CpG methylation of ubiquitin carboxyl-terminal hydrolase 1 (UCHL1) in sporadic and hereditary Tunisian breast cancer patients: clinical significance. *Medical oncology*, **30**, 418.
- Trowern, A.R. & Mann, D.A. (1999) A bi-functional activator/repressor element required for transcriptional activity of the human UCH-L1 gene assembles a neuron-specific protein: single-strand DNA complex. *Neuroscience letters*, **272**, 25-28.
- Troy, C.M., Rabacchi, S.A., Friedman, W.J., Frappier, T.F., Brown, K. & Shelanski, M.L. (2000) Caspase-2 mediates neuronal cell death induced by beta-amyloid. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **20**, 1386-1392.
- Turner, G.C. & Varshavsky, A. (2000) Detecting and measuring cotranslational protein degradation in vivo. *Science*, **289**, 2117-2120.
- Ummanni, R., Jost, E., Braig, M., Lohmann, F., Mundt, F., Barett, C., Schlomm, T., Sauter, G., Senff, T., Bokemeyer, C., Sultmann, H., Meyer-Schwesinger, C., Brummendorf, T.H. & Balabanov, S. (2011) Ubiquitin carboxyl-terminal hydrolase 1 (UCHL1) is a potential tumour suppressor in prostate cancer and is frequently silenced by promoter methylation. *Molecular cancer*, **10**, 129.
- Van Hoesen, G.W., Hyman, B.T. & Damasio, A.R. (1991) Entorhinal cortex pathology in Alzheimer's disease. *Hippocampus*, **1**, 1-8.
- van Leeuwen, F.W., de Kleijn, D.P., van den Hurk, H.H., Neubauer, A., Sonnemans, M.A., Sluijs, J.A., Koycu, S., Ramdjielal, R.D., Salehi, A., Martens, G.J., Grosveld, F.G., Peter, J.,
Burbach, H. & Hol, E.M. (1998) Frameshift mutants of beta amyloid precursor protein and ubiquitin-B in Alzheimer's and Down patients. *Science*, **279**, 242-247.

- Vanderstichele, H., Van Kerschaver, E., Hesse, C., Davidsson, P., Buyse, M.A., Andreasen, N., Minthon, L., Wallin, A., Blennow, K. & Vanmechelen, E. (2000) Standardization of measurement of beta-amyloid(1-42) in cerebrospinal fluid and plasma. *Amyloid : the international journal of experimental and clinical investigation : the official journal of the International Society of Amyloidosis*, 7, 245-258.
- Vassar, R., Bennett, B.D., Babu-Khan, S., Kahn, S., Mendiaz, E.A., Denis, P., Teplow, D.B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M.A., Biere, A.L., Curran, E., Burgess, T., Louis, J.C., Collins, F., Treanor, J., Rogers, G. & Citron, M. (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science*, 286, 735-741.
- Villemagne, V.L., Pike, K.E., Chetelat, G., Ellis, K.A., Mulligan, R.S., Bourgeat, P., Ackermann, U., Jones, G., Szoeke, C., Salvado, O., Martins, R., O'Keefe, G., Mathis, C.A., Klunk, W.E., Ames, D., Masters, C.L. & Rowe, C.C. (2011) Longitudinal assessment of Abeta and cognition in aging and Alzheimer disease. *Annals of neurology*, **69**, 181-192.
- von Arnim, C.A., Kinoshita, A., Peltan, I.D., Tangredi, M.M., Herl, L., Lee, B.M., Spoelgen, R., Hshieh, T.T., Ranganathan, S., Battey, F.D., Liu, C.X., Bacskai, B.J., Sever, S., Irizarry, M.C., Strickland, D.K. & Hyman, B.T. (2005) The low density lipoprotein receptorrelated protein (LRP) is a novel beta-secretase (BACE1) substrate. *The Journal of biological chemistry*, 280, 17777-17785.
- Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J. & Selkoe, D.J. (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature*, **416**, 535-539.
- Walters, B.J., Campbell, S.L., Chen, P.C., Taylor, A.P., Schroeder, D.G., Dobrunz, L.E., Artavanis-Tsakonas, K., Ploegh, H.L., Wilson, J.A., Cox, G.A. & Wilson, S.M. (2008) Differential effects of Usp14 and Uch-L1 on the ubiquitin proteasome system and synaptic activity. *Molecular and cellular neurosciences*, **39**, 539-548.
- Watanabe, T., Hikichi, Y., Willuweit, A., Shintani, Y. & Horiguchi, T. (2012) FBL2 regulates amyloid precursor protein (APP) metabolism by promoting ubiquitination-dependent APP degradation and inhibition of APP endocytosis. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **32**, 3352-3365.
- Weissman, A.M. (2001) Themes and variations on ubiquitylation. *Nature reviews. Molecular cell biology*, 2, 169-178.
- Wen, Y., Onyewuchi, O., Yang, S., Liu, R. & Simpkins, J.W. (2004) Increased beta-secretase activity and expression in rats following transient cerebral ischemia. *Brain research*, 1009, 1-8.
- Wenk, G.L. (2003) Neuropathologic changes in Alzheimer's disease. *The Journal of clinical psychiatry*, 64 Suppl 9, 7-10.

- Wertz, I.E., O'Rourke, K.M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D.L., Ma, A., Koonin, E.V. & Dixit, V.M. (2004) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. *Nature*, 430, 694-699.
- Wilkinson, K.D., Tashayev, V.L., O'Connor, L.B., Larsen, C.N., Kasperek, E. & Pickart, C.M. (1995) Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T. *Biochemistry*, 34, 14535-14546.
- Wilkinson, K.D., Urban, M.K. & Haas, A.L. (1980) Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. *The Journal of biological chemistry*, **255**, 7529-7532.
- Willem, M., Garratt, A.N., Novak, B., Citron, M., Kaufmann, S., Rittger, A., DeStrooper, B., Saftig, P., Birchmeier, C. & Haass, C. (2006) Control of peripheral nerve myelination by the beta-secretase BACE1. *Science*, **314**, 664-666.
- Wilson, P.O., Barber, P.C., Hamid, Q.A., Power, B.F., Dhillon, A.P., Rode, J., Day, I.N., Thompson, R.J. & Polak, J.M. (1988) The immunolocalization of protein gene product 9.5 using rabbit polyclonal and mouse monoclonal antibodies. *Br J Exp Pathol*, 69, 91-104.
- Wogulis, M., Wright, S., Cunningham, D., Chilcote, T., Powell, K. & Rydel, R.E. (2005) Nucleation-dependent polymerization is an essential component of amyloid-mediated neuronal cell death. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 25, 1071-1080.
- Wong, P.C., Zheng, H., Chen, H., Becher, M.W., Sirinathsinghji, D.J., Trumbauer, M.E., Chen, H.Y., Price, D.L., Van der Ploeg, L.H. & Sisodia, S.S. (1997) Presenilin 1 is required for Notch1 and DII1 expression in the paraxial mesoderm. *Nature*, **387**, 288-292.
- Wu, H.Y., Hudry, E., Hashimoto, T., Kuchibhotla, K., Rozkalne, A., Fan, Z., Spires-Jones, T., Xie, H., Arbel-Ornath, M., Grosskreutz, C.L., Bacskai, B.J. & Hyman, B.T. (2010) Amyloid beta induces the morphological neurodegenerative triad of spine loss, dendritic simplification, and neuritic dystrophies through calcineurin activation. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, **30**, 2636-2649.
- Wyss-Coray, T. (2006) Inflammation in Alzheimer disease: driving force, bystander or beneficial response? *Nature medicine*, **12**, 1005-1015.
- Xiang, T., Li, L., Yin, X., Yuan, C., Tan, C., Su, X., Xiong, L., Putti, T.C., Oberst, M., Kelly, K., Ren, G. & Tao, Q. (2012) The ubiquitin peptidase UCHL1 induces G0/G1 cell cycle arrest and apoptosis through stabilizing p53 and is frequently silenced in breast cancer. *PloS one*, **7**, e29783.
- Xiang, Z., Ho, L., Yemul, S., Zhao, Z., Qing, W., Pompl, P., Kelley, K., Dang, A., Qing, W., Teplow, D. & Pasinetti, G.M. (2002) Cyclooxygenase-2 promotes amyloid plaque deposition in a mouse model of Alzheimer's disease neuropathology. *Gene expression*, 10, 271-278.

- Yamazaki, K., Wakasugi, N., Tomita, T., Kikuchi, T., Mukoyama, M. & Ando, K. (1988) Gracile axonal dystrophy (GAD), a new neurological mutant in the mouse. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine*, 187, 209-215.
- Yamazaki, T., Hibi, K., Takase, T., Tezel, E., Nakayama, H., Kasai, Y., Ito, K., Akiyama, S., Nagasaka, T. & Nakao, A. (2002) PGP9.5 as a marker for invasive colorectal cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 8, 192-195.
- Yan, R., Bienkowski, M.J., Shuck, M.E., Miao, H., Tory, M.C., Pauley, A.M., Brashier, J.R., Stratman, N.C., Mathews, W.R., Buhl, A.E., Carter, D.B., Tomasselli, A.G., Parodi, L.A., Heinrikson, R.L. & Gurney, M.E. (1999) Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. *Nature*, 402, 533-537.
- Yang, L.B., Lindholm, K., Yan, R., Citron, M., Xia, W., Yang, X.L., Beach, T., Sue, L., Wong, P., Price, D., Li, R. & Shen, Y. (2003) Elevated beta-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. *Nature medicine*, 9, 3-4.
- Yao, T. & Cohen, R.E. (2002) A cryptic protease couples deubiquitination and degradation by the proteasome. *Nature*, 419, 403-407.
- Yin, Y.I., Bassit, B., Zhu, L., Yang, X., Wang, C. & Li, Y.M. (2007) {gamma}-Secretase Substrate Concentration Modulates the Abeta42/Abeta40 Ratio: IMPLICATIONS FOR ALZHEIMER DISEASE. *The Journal of biological chemistry*, 282, 23639-23644.
- Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y.Q., Rogaeva, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D.S., Holmes, E., Milman, P., Liang, Y., Zhang, D.M., Xu, D.H., Sato, C., Rogaev, E., Smith, M., Janus, C., Zhang, Y., Aebersold, R., Farrer, L.S., Sorbi, S., Bruni, A., Fraser, P. & St George-Hyslop, P. (2000) Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. *Nature*, **407**, 48-54.
- Zhang, X., Zhou, K., Wang, R., Cui, J., Lipton, S.A., Liao, F.F., Xu, H. & Zhang, Y.W. (2007) Hypoxia-inducible factor 1alpha (HIF-1alpha)-mediated hypoxia increases BACE1 expression and beta-amyloid generation. *The Journal of biological chemistry*, 282, 10873-10880.
- Zhang, X.Y., Varthi, M., Sykes, S.M., Phillips, C., Warzecha, C., Zhu, W., Wyce, A., Thorne, A.W., Berger, S.L. & McMahon, S.B. (2008) The putative cancer stem cell marker USP22 is a subunit of the human SAGA complex required for activated transcription and cell-cycle progression. *Molecular cell*, **29**, 102-111.
- Zhang, Z., Nadeau, P., Song, W., Donoviel, D., Yuan, M., Bernstein, A. & Yankner, B.A. (2000) Presenilins are required for gamma-secretase cleavage of beta-APP and transmembrane cleavage of Notch-1. *Nature cell biology*, 2, 463-465.

- Zheng, H., Jiang, M., Trumbauer, M.E., Sirinathsinghji, D.J., Hopkins, R., Smith, D.W., Heavens, R.P., Dawson, G.R., Boyce, S., Conner, M.W., Stevens, K.A., Slunt, H.H., Sisoda, S.S., Chen, H.Y. & Van der Ploeg, L.H. (1995) beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. *Cell*, 81, 525-531.
- Zhong, J., Zhao, M., Ma, Y., Luo, Q., Liu, J., Wang, J., Yuan, X., Sang, J. & Huang, C. (2012) UCHL1 acts as a colorectal cancer oncogene via activation of the beta-catenin/TCF pathway through its deubiquitinating activity. *International journal of molecular medicine*, **30**, 430-436.
- Zhou, W. & Song, W. (2006) Leaky scanning and reinitiation regulate BACE1 gene expression. *Molecular and cellular biology*, **26**, 3353-3364.
- Zhu, K., Dunner, K., Jr. & McConkey, D.J. (2010) Proteasome inhibitors activate autophagy as a cytoprotective response in human prostate cancer cells. *Oncogene*, **29**, 451-462.
- Zhu, P., Zhou, W., Wang, J., Puc, J., Ohgi, K.A., Erdjument-Bromage, H., Tempst, P., Glass, C.K.
 & Rosenfeld, M.G. (2007) A histone H2A deubiquitinase complex coordinating histone acetylation and H1 dissociation in transcriptional regulation. *Molecular cell*, 27, 609-621.