

**Studies on the use of  
glycosaminoglycans for the  
treatment of Alzheimer's disease**

**Hao Cui  
MPharmSc**

Submitted in fulfilment of the requirements for the  
degree of Doctor of Philosophy

University of Tasmania

10/2012

## DECLARATION OF ORIGINALITY

This thesis contains no material that has been accepted for the award of any other degree or diploma in any other tertiary institution.

To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person except where due reference is made in the text of the thesis

08.10.2012

## AUTHORITY OF ACCESS

This thesis may be available for loan and limited copying in accordance  
with the Copyright Act 1968

08.10.2012

# Table of Contents

<b>Abstract</b>	<b>i</b>
<b>Acknowledgements</b>	<b>v</b>
<b>List of Abbreviations</b>	<b>vii</b>
<b>Publication record</b>	<b>xii</b>
<b>Chapter 1. Literature review and introduction</b>	<b>1</b>
<b>1.1 Alzheimer's disease</b>	<b>2</b>
<b>1.2 Pathology of AD</b>	<b>2</b>
1.2.1 Neurofibrillary tangles (NFTs)	3
1.2.2 Amyloid plaque	4
1.2.3 Cerebral amyloid angiopathy (CAA)	5
1.2.4 Synaptic loss	6
<b>1.3 A<math>\beta</math> peptide</b>	<b>7</b>
1.3.1 A $\beta$ aggregation	9
1.3.2 Soluble A $\beta$ oligomer production and toxicity	10
1.3.3 A $\beta$ -induced calcium dysregulation	13
1.3.4 Physiological function of A $\beta$	15
<b>1.4 Biology of amyloid precursor protein (APP)</b>	<b>16</b>
1.4.1 Structure of APP	17

1.4.2 Functions of APP	20
1.4.2.1 Cell adhesion	20
1.4.2.2 Role as a cell surface receptor	21
1.4.2.3 Role in neurite outgrowth, synaptogenesis and neuroprotection	22
1.4.2.4 Interaction of APP with adapter proteins	24
1.4.2.5 Non-neuronal function of APP	27
1.4.3 Proteolytic processing of APP	28
1.4.3.1 APP trafficking and processing	28
1.4.3.2 APP processing secretases	29
1.4.3.2.1 $\alpha$ -secretase cleavage	29
1.4.3.2.2 $\beta$ -secretase cleavage	30
1.4.3.2.3 $\gamma$ -secretase cleavage	32
<b>1.5 Genetics and risk factors for AD</b>	<b>33</b>
1.5.1 Early onset AD (familial AD)	34
1.5.1.1 APP mutations	34
1.5.1.2 Presenilin mutations	36
1.5.2 Late onset AD (sporadic AD)	37
1.5.2.1 ApoE	38
1.5.2.2 Clusterin (apoJ)	43
1.5.2.3 Bridging integrator 1 (BIN1)	45
1.5.2.4 ATP-binding cassette transporter A7 (ABCA7)	46
<b>1.6 Mouse models of AD</b>	<b>47</b>
1.6.1 Transgenic mouse models of AD	48
1.6.2 APP knockout mice	52
<b>1.7 Current and prospective AD therapies</b>	<b>53</b>

1.7.1 Current therapeutic approaches of AD	54
1.7.1.1 Acetylcholinesterase (AChE) inhibitors	54
1.7.1.2 Memantine	55
1.7.2 Prospective therapeutic strategies in AD	56
1.7.2.1 A $\beta$ aggregation inhibitors	56
1.7.2.2 A $\beta$ immunotherapy	59
1.7.2.3 Suppression of A $\beta$ production	60
1.7.2.3.1 $\beta$ -secretase inhibitors	61
1.7.2.3.2 $\gamma$ -secretase inhibitors	61
1.7.2.3.3 Statins	63
1.7.2.4 Non-A $\beta$ -based approaches	63
<b>1.8 Proteoglycans</b>	<b>64</b>
1.8.1 Structure of proteoglycans and glycosaminoglycans	65
1.8.2 Synthesis and modification of proteoglycans	67
1.8.3 Expression of proteoglycans in the brain	68
1.8.3.1 Chondroitin sulfate proteoglycans (CSPGs)	68
1.8.3.2 Heparan sulfate proteoglycans (HSPGs)	72
1.8.4 Function of proteoglycans in the brain	73
1.8.4.1 Function of proteoglycans in the CNS development	74
1.8.4.1.1 Chondroitin sulfate proteoglycans	74
1.8.4.1.2 Heparan sulfate proteoglycans	76
1.8.4.2 Function of proteoglycans in plasticity	77
1.8.4.3 Function of proteoglycans in synaptogenesis	78
1.8.4.4 Function of proteoglycans in brain injury	79
1.8.5 Proteoglycans in neurodegeneration	80

1.8.6	Proteoglycans and GAGs in AD	81
1.8.6.1	PGs in AD pathology	81
1.8.6.2	Roles of PGs in AD	83
1.8.6.2.1	Interaction with APP	83
1.8.6.2.2	Interaction with A $\beta$	83
1.8.6.2.3	PGs or GAGs on A $\beta$ aggregation	84
1.8.6.2.4	Interaction with tau protein	84
1.8.6.3	Potential therapeutic implications	85
<b>1.9</b>	<b>Hypothesis and aims</b>	<b>88</b>
<b>Chapter 2. Effect of heparin and enoxaparin on APP processing and A<math>\beta</math> production in primary cortical neurons from Tg2576 mice</b>		<b>89</b>
<b>2.1</b>	<b>Introduction</b>	<b>90</b>
<b>2.2</b>	<b>Materials and methods</b>	<b>92</b>
2.2.1	Materials	92
2.2.2	Cell culture	93
2.2.3	SDS-PAGE and western blotting	93
2.2.4	Quantitative real-time PCR	96
<b>2.3</b>	<b>Results</b>	<b>97</b>
2.3.1	Effect of heparin and enoxaparin on A $\beta$	97
2.3.2	Characterisation of APP C-terminal fragments (CTFs)	97
2.3.3	Effects of heparin and enoxaparin on levels of APP, sAPP $\alpha$ and CTFs	104
2.3.4	Effects of heparin on the level of BACE1, ADAM10 and ADAM17	107
2.3.5	Effects of heparin fragments on A $\beta$ secretion	108
<b>2.4</b>	<b>Discussion</b>	<b>111</b>

## **Chapter 3. Size and sulfation are critical for the effect of heparin on APP processing and A $\beta$ production**

**117**

### **3.1 Introduction**

**118**

### **3.2 Materials and methods**

**119**

3.2.1 Materials 119

3.2.2 Primary cortical cell culture 121

3.2.3 SDS-polyacrylamide gel electrophoresis and western blotting 121

### **3.3 Results**

**122**

3.3.1 Effects of heparin fragments on APP processing 122

3.3.2 Effects of different class of GAGs on APP processing and A $\beta$  production 125

3.3.3 Effects of selectively desulfated and decarboxylated heparin on APP processing  
128

### **3.4 Discussion**

**133**

## **Chapter 4. Effects of enoxaparin on APP processing and A $\beta$ production in Tg2576 mice**

**137**

### **4.1 Introduction**

**138**

### **4.2 Materials and methods**

**139**

4.2.1 Materials 139

4.2.2 Animal and ENO treatment 139

4.2.3 Immunohistochemistry 140

4.2.4 SDS-PAGE and western blotting 141

### **4.3 Results**

**142**

4.3.1 Effects of ENO on the level of A $\beta$  142



4.3.2 Effects of ENO on levels of APP, APP C-terminal fragments and APP cleavage enzymes	142
4.3.3 Effect of ENO on the number of amyloid plaques and on total amyloid load	148
<b>4.4 Discussion</b>	<b>150</b>
<b>Chapter 5. Effects of endogenous heparan sulfate on APP processing and A<math>\beta</math> production</b>	<b>157</b>
<b>5.1 Introduction</b>	<b>158</b>
<b>5.2 Materials and methods</b>	<b>160</b>
5.2.1 Materials	160
5.2.2 Primary cortical cell culture	160
5.2.3 Western blotting of cell lysates and media	160
5.2.4 Immunocytochemistry	161
5.2.5 Heparin affinity chromatography	162
<b>5.3 Results</b>	<b>162</b>
5.3.1 Effects of endogenous HS on APP processing and A $\beta$ production	162
5.3.2 Heparin chromatography of ADAM10	167
<b>5.4 Discussion</b>	<b>169</b>
<b>Chapter 6. Discussion</b>	<b>173</b>
<b>6.1 A possible mechanism of the effect of GAG derivatives on APP processing</b>	<b>182</b>
<b>References</b>	<b>185</b>

# Abstract

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disorder that is commonly found in the elderly population. AD is characterized pathologically by the deposition of amyloid plaques and neurofibrillary tangles in the brain. The major component of amyloid plaque is the  $\beta$ -amyloid protein ( $A\beta$ ), a 40-42 amino-acid residue polypeptide that is generated from the  $\beta$ -amyloid precursor protein (APP) by the  $\beta$ -site APP cleaving enzyme-1 (BACE1) and  $\gamma$ -secretase. APP can also be cleaved by  $\alpha$ -secretase within the  $A\beta$  sequence to form sAPP $\alpha$  and C83, which thus precludes formation of  $A\beta$ .

Advances in AD research over the past three decades have not yet led to effective treatments to prevent or cure AD. Therefore, an effective drug for the treatment of AD is required. As oligomeric forms of  $A\beta$  are thought to be the major toxic species which cause AD, therapeutic approaches are now targeting the production, clearance or neurotoxicity of  $A\beta$ .

It has been reported that glycosaminoglycans (GAGs) such as heparin can influence  $A\beta$  production by disrupting APP proteolytic processing. Studies have reported that heparan sulfate and heparin can directly inhibit BACE1 activity in vitro and thereby decrease  $A\beta$  production in cell culture. Studies have also shown that heparin binds close to the prodomain of the BACE1 zymogen (proBACE1) and that this binding

stimulates proBACE1 activity. However, heparin can also inhibit mature BACE1 activity by binding close to the active site domain of the mature protein. In contrast, other groups have reported that heparin stimulates  $\beta$ -secretase cleavage of APP in a cultured cell line.

As there are conflicting reports on the effect of GAGs on APP processing and A $\beta$  production, the effects of heparin or enoxaparin on APP processing was first examined in primary cortical cells obtained from transgenic mice expressing human APP<sub>695</sub> with the Swedish familial AD mutant (Tg2576 mouse). The results showed that heparin or enoxaparin (ENO) treatment can lower A $\beta$  secretion from cortical cells by decreasing BACE1 and thereby inhibiting  $\beta$ -secretase processing of APP. Additionally, treatment with heparin or enoxaparin decreased the  $\alpha$ -secretase ADAM10 and inhibited  $\alpha$ -secretase processing of APP.

The development of GAG analogues which can be used for the treatment of AD will require the identification of highly potent and specific compounds that have the ability to cross the blood-brain barrier (BBB). Therefore, an aim of the studies in this thesis was to examine the structure specificity (molecular size and sulfation degree) of GAGs with the aim of identifying more potent and specific GAG-based compounds to inhibit APP processing and A $\beta$  production. The effects of various GAGs and sulfated polysaccharides on APP processing were tested in primary cortical cells derived from Tg2576 mice. The results showed that the effect of GAGs on APP processing was both size- and sulfation-dependent. Mucosal heparins (MHs) with small sizes (5 kDa and 3kDa) were less potent in reducing A $\beta$  than high molecular weight MHs (6 kDa and 12.5 kDa). 6-O-Sulfation was important for the effect on APP processing as

heparin lacking 6-O sulfate were less potent than native heparin. However, deletion of carboxyl groups on MH had no significant effect on APP processing. These data suggest that it might be possible to alter the structure of GAGs to achieve more potent and specific inhibitors of APP processing that can cross the blood-brain barrier.

It has been reported that peripheral administration of ENO can reduce the level of A $\beta$  and the amyloid plaque load in the brain of APP transgenic mice. However, the exact mechanism of these effects has been unclear. Therefore, an aim of this study was to examine whether the reduced amyloid plaque load reported to occur in the brains of the APP transgenic mice treated with ENO was due to decreased APP processing to A $\beta$  caused by ENO treatment. ENO was peripherally injected to Tg2576 mice, and the APP processing products and amyloid load in the brains of the mice were examined. The study found that ENO treatment decreased the A $\beta$ 40/A $\beta$ 42 ratio in cortex and increased the amyloid plaque load in both cortex and hippocampus, while overall APP processing was not significantly influenced by ENO. The exact mechanism of these effects remains unknown. These results suggest that the strategy of using ENO for the treatment of AD may need further assessment.

As GAGs such as HS are widely expressed in the brain in the form of proteoglycans, it is possible that the endogenous HS may also affect APP processing. Therefore, an aim of the study was to examine the role of endogenous HS on APP processing and A $\beta$  production. To examine this, primary cortical cells derived from Tg2576 mice were incubated with a drug or enzyme designed to degrade HS chains from endogenous proteoglycans. The results showed that deletion of endogenous HS can

reduce the level of BACE1 and ADAM10 and thus inhibit APP processing through  $\beta$ - and  $\alpha$ -secretase cleavage pathways similar to exogenous treatment of heparin. These findings suggest that regulation of endogenous HS to inhibit APP processing to A $\beta$  could be a novel approach for the treatment of AD.

Based on these results, modification of structures of GAGs or sulfate polysaccharides may achieve highly potent and specific BBB-permeable compounds which can inhibit APP processing to A $\beta$ . Moreover, regulation of endogenous HS can also affect APP processing and A $\beta$  production. Therefore, the studies reported in this thesis support the view that GAG-based compounds can regulate the A $\beta$  production and strategies based on administration of GAGs or the alteration of endogenous GAG metabolism may have value for the treatment of AD.

# Acknowledgements

I would like to express my gratitude to many people around me during the last three years.

Firstly, I would like to thank Professor David Small, Dr. Glenn Jacobson and Dr. Christian Narkowicz, for letting me be your student. Thank you to David for your patience, guidance, support and encouragement, without which none of this would be possible. Thank you to Glenn and Christian for offering me support and advice, you have helped me grow over the last 5 years.

I also would like to extend my special gratitude to Dr. Amos Hung. Thank you for your excellent and patient teaching when I started my degree. Thanks to Jenny Smith and also Claire Hadrill for keeping the lab running efficiently.

Thank you to Anna King for your assistance with tissue sectioning and immunohistochemistry. Thank you to Dr. Robert Gasperini for your help with the FPLC and DLS experiments. Thank you to Dr. Adele Vincent for your kind help when I started the degree. Thank you to Dr. Dave Klaver and Dr. Lisa Foa for their valuable advice.

Thanks to my team members Camilla Mitchell, Edgar Dawkins, Lila Landowski, Thanh Hoang, Yanling Hu and everyone else in the Menzies group, I had a great time working with you. Special thanks to Edgar for being my neighbour in the lab and

showing me how to do immunocytochemistry every day, and I also thank him for helping me to proofread this thesis.

Thank you to Professor Toshiharu Suzuki for providing us with the antibodies, and to Professor Craig Freeman for kindly supplying GAGs. Thank you to all staff at UTAS animal services for providing and caring for the animals used in this thesis.

Personally, I thank my parents from the bottom of my heart, although they are thousands of miles away. Their love beyond the distance, their support and care are always with me. Specially, I would also like to thank my partner, Wei Xiong, who was provided great support all these years.

# Abbreviations

ABCA1	ATP-binding cassette transporter A1
ABCA7	ATP-binding cassette transporter A7
AChE	Acetylcholinesterase
AChR	Acetylcholinesterase receptor
ADAM	A disintegrin and metalloproteinase
AICD	APP intracellular domain
APF	Annular protofibril
APH-1	Anterior pharynx-defective 1
APLP1	APP-like protein 1
APLP2	APP-like protein 2
apoE4	Apolipoprotein E4
APP	Amyloid precursor protein
BACE1	$\beta$ -site APP cleaving enzyme-1
BBB	Blood-brain barrier
BIN1	Bridging integrator 1
CAA	Cerebral amyloid angiopathy
CALHM1	Calcium homeostasis modulator 1
CAPPD	Central APP domain
CHD	Carbohydrate domain
ChS	Chondroitin sulfate
CNS	Central nervous system
CRP	Complement regulatory protein



CSF	Cerebrospinal fluid
CSPG	Chondroitin sulfate proteoglycan
CTFs	C-terminal fragments
CuBD	Copper-binding domain
Dab1	Disabled-1
DAPT	N-[N-3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine <i>t</i> -butyl ester
DIV	Day in vitro
DNase I	Deoxyribonuclease I
DRG	Dorsal root ganglion
DS	Dermatan sulfate
ECM	Extracellular matrix
ENO	Enoxaparin
ER	Endoplasmic reticulum
ES	Embryonic stem
EXT	Exostosin
FAD	Familial Alzheimer's disease
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GalNAc	N-acetylgalactosamine
GlcA	Glucuronic acid
GFLD	Growth-factor-like domain
GPI	Glycosylphosphatidylinositol
GWAS	Genome-wide association study

HA	Hyaluronic acid
HBD	Heparin-binding domain
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
HRP	Horseradish peroxidase
HS	Heparan sulfate
HS2ST	HS 2-O-sulfotransferase
HS6ST	HS 6-O-sulfotransferase
HSPG	Heparan sulfate proteoglycan
IdoA	Iduronic acid
JIP	JNK-interacting protein
KPI	Kunitz protease inhibitor
KS	Keratan sulfate
LDLR	Low-density lipoprotein receptor
LH	Lung heparin
LMW	Low molecular weight
LOAD	Late onset AD
LTD	Long-term depression
LTP	Long-term potentiation
MAP	Microtubule-associated protein
MCI	Mild cognitive impairment
MCSP	Melanoma-associated chondroitin sulfate proteoglycan
MH	Mucosal heparin
MH CR	MH with the carboxyl group removed
MH de 2S	MH without 2-O sulfate
MH de 6S	MH without 6-O sulfate

MH de NS	MH without N-sulfate
MH de S	MH without any sulfates
Mints	Munc-18-interacting proteins
NDST	N-deacetylase/N-sulfotransferase
NFT	Neurofibrillary tangle
NMDA	N-methyl-D-aspartate
NSAID	Non-steroidal anti-inflammatory drug
PBS	Phosphate-buffered saline
PSENEN	Presenilin enhancer 2
PGs	Proteoglycans
PHF	Paired helical filament
PNN	Perineuronal net
PPS	Pentosan polysulfate
PAT1	Protein interacting with APP tail 1
PS1	Presenilin 1
PS2	Presenilin 2
PTB	Phosphotyrosine binding domain
PVDF	Polyvinylidene difluoride
RAP	Receptor associated protein
RC	Random coil
RPTP $\beta$	Receptor-type protein-tyrosine phosphatase $\beta$
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SEA	Sperm protein-enterokinase-agrin
SERCA	Sarco ER Ca <sup>2+</sup> ATPase
Shc	Src homology collagen-like

TACE	Tumor necrosis factor- $\alpha$ converting enzyme
TBI	Traumatic brain injury
TM	Transmembrane
TTR	Transthyretin
xyloside	4-Methylumbelliferyl- $\beta$ -D-xyloside
ZnBD	Zinc-binding domain

# Publication record

## *Peer-reviewed Publications:*

**Cui, H.**, Hung, A. C., Freeman, C., Narkowicz, C., Jacobson, G. A. and Small, D. H. (2012). Size and sulfation are critical for the effect of heparin on APP processing and A $\beta$  production. *Journal of neurochemistry*, accepted.

**Cui, H.**, Hung, A. C., Klaver, D. W., Suzuki, T., Freeman, C., Narkowicz, C., Jacobson, G. A. and Small, D. H. (2011) Effects of heparin and enoxaparin on APP processing and A $\beta$  production in primary cortical neurons from Tg2576 mice. *PLoS One*, 6, e23007

Klaver, D. W., Wilce, M. C., **Cui, H.**, Hung, A. C., Gasperini, R., Foa, L. and Small, D. H. (2010) Is BACE1 a suitable therapeutic target for the treatment of Alzheimer's disease? Current strategies and future directions. *Biol Chem*, 391, 849-859.

## *Manuscripts in preparation:*

**Cui, H.**, Small, D. H. (2012). Proteoglycans in the central nervous system--role in development and aging. *IUBMB life*. Manuscript in preparation.

**Cui, H.**, King A. E., Gasperini, R. J., Hung, A. C., Dawkins E. H., Narkowicz, C., Jacobson, G. A. and Small, D. H. (2012). Effects of peripheral injection of enoxaparin on APP processing and A $\beta$  production in Tg2576 mice. Manuscript in preparation.

## *Conference publication:*

**Cui, H.**, Hung, A.C., Narkowicz, C., Jacobson, G. and Small, D.H., Effects of glycosaminoglycans on the level of  $\beta$ - and  $\alpha$ -secretase and APP processing in Tg2576 primary cortical cells, (2012), 32th annual meeting of Australia neuroscience society, Brisbane.

**Cui, H.**, Hung, A.C., Narkowicz, C., Jacobson, G. and Small, D.H., Effects of heparin and enoxaparin on APP processing and A $\beta$  production in Tg2576 primary cortical cells, (2011), 31th annual meeting of Australia neuroscience society, Auckland.

Hoang, Thanh, Gasperini, R., **Cui, H.**, Shepherd, C.E., Strickland, D.K., Foa, L. and Small, D.H., Interaction of the receptor-associated protein (RAP) with APP and A $\beta$ , (2011), 31th annual meeting of Australia neuroscience society, Auckland.

# **Chapter 1**

## **Literature review and introduction**

## **1.1 Alzheimer's disease**

Alzheimer's disease (AD) is the most common neurodegenerative disease in the elderly and is the major subtype of dementia. Currently, there are more than 20 million cases in the world and this is projected to increase to 42 million in 2020 [1]. About 2% of people aged between 65 and 69 years old suffer from AD [2]. Between 80 to 85 years of age, the prevalence dramatically increases to 25%-30% [2, 3]. Alzheimer's disease can be described symptomatically in three stages. In the early or mild stage, patients exhibit mild cognitive impairment (MCI) and have difficulty in recalling recent memories or storing information [4-6]. Most patients with MCI have mild behavioural and psychological symptoms including apathy, anxiety, delusion, hallucinations, aggression, depression and mood change [3, 7-9]. Other signs are problems with calculation, insight and judgment, but neurologic function in this stage is usually normal [10]. In the moderate stages of AD, the percentage of patients with behavioural and psychological symptoms increases [8]. Patients need assistance with their daily life [11-13], and the frequency of sleep disturbance is increased [14]. In the final stage of AD, patients only have fragments of memory and lose the ability to control bodily functions [12, 15]. Patients with severe AD need full-time care and assistance with their daily living [16].

## **1.2 Pathology of AD**

AD was first described by Alois Alzheimer in 1906, and neurofibrillary changes and deposition in the cortex were found after autopsy of a female patient [17]. AD is characterized pathologically by the formation of intracellular neurofibrillary tangles (NFTs) [18], extracellular deposition of amyloid plaques [19, 20] and the loss of

neurons and synapses in certain brain areas [21]. In addition, congophilic angiopathy (cerebral amyloid angiopathy) is a common pathology.

### *1.2.1 Neurofibrillary tangles (NFTs)*

Neurofibrillary tangles (NFTs), one of the cardinal neuropathologic hallmarks of AD, are made of paired helical filaments (PHF) [19, 22]. Studies show that the primary constituent of PHF is abnormally misfolded and hyperphosphorylated microtubule-associated protein tau [23-29]. Tau is encoded by a single gene located on the long arm of chromosome 17 [30]. It has six isoforms produced by alternative mRNA splicing, and all these isoforms has been found in NFTs [31-33]. Tau is highly enriched in nerve cells and mostly located in axons [34]. Tau is also found in dendrites and some subsets of glial cells [35-37]. Tau is a phosphoprotein and the degree of the phosphorylation is important for its biological activity [38]. In human brain, tau is the major microtubule-associated protein (MAP) [39-41] and is functionally involved in assembling of tubulin into the microtubules and maintaining its structure [39]. In the AD brain, all isoforms of tau are highly phosphorylated [42], and the level of abnormally phosphorylated tau is significantly higher in AD than in normal individuals [43, 44]. The level of normal tau in AD brain is similar to age-matched control brains [43, 45]. A possible explanation is that the abnormally hyperphosphorylated tau may be resistant to protease degradation and thus forms PHF which eventually forms NFT [42, 46]. In addition, studies indicate that the hyperphosphorylated form of tau loses the ability to promote assembly of tubulin into microtubules and that it may and even cause microtubule disruption [47, 48].



In the brain of AD patient, NFTs generally show a common and predictable pattern of distribution and progressive sequence, and can be distinguished into six stages. NFTs start from the transentorhinal region of the cortex (stage I) and then develop into entorhinal regions and CA1 regions of the hippocampus (stage II). Subsequently, NFTs accumulate in the limbic allocortex (stage III) and then the amygdala, thalamus and claustrum (stage IV). Finally, NFTs spread to all isocortical regions (stage V) and eventually affect the primary sensory, motor and visual areas (stage VI) [49, 50]. Several studies show that the extent and distribution of NFTs are correlated with the severity and duration of AD [51-53], suggesting that NFTs may have an important role in the progress of AD. In addition, it is important to note that NFTs are also present in other diseases [54]. The reason why different diseases have the same pathologic change is still unknown.

### *1.2.2 Amyloid plaque*

Another pathological lesion of AD is the amyloid plaque. Amyloid plaque is characterised by the accumulation and deposition of amyloid beta peptides ( $A\beta$ ) including  $A\beta_{40}$  and  $A\beta_{42}$  [55-57], which are derived from the normal proteolysis of  $\beta$ -amyloid precursor protein (APP) [58].

Amyloid plaques can be classified into two general types: dense core plaques and diffuse plaques. Studies show that the dense core plaques are usually associated with dystrophic neurites, synaptic loss, neuron loss and activation of astrocytes and microglia [59-63]. However, diffuse plaques, which are also commonly present in the

brains of elderly people without cognitive impairment, are not associated typically with dystrophic neuritis and synapse loss [59, 64, 65].

Unlike NFTs, amyloid plaques mainly accumulate in the isocortex, and the progression of spatiotemporal pattern is difficult to predict [50]. The progression of amyloid plaques can be roughly classified into three stages [50]. In stage A, amyloid deposits are firstly present in the isocortex and particularly in the basal portions of the frontal, temporal and occipital lobe, but the hippocampus is not affected. In stage B, all isocortex associated areas are affected and the hippocampus is only mildly involved. The primary sensory areas and primary motor regions are still free of amyloid deposits. Finally, in stage C, all isocortex regions are involved, but the hippocampus shows similar patterns of amyloid deposits as in stage B. Moreover, some regions outside the cerebral cortex, including the striatum, thalamus, hypothalamus, subthalamic nucleus and red nucleus, also show amyloid plaques [50].

Clinicopathological studies show that the presence of amyloid plaques is not associated with the severity and duration of AD [51, 66-68]. The deposition of A $\beta$  reaches a plateau early in the disease progress [53, 69], and recent studies demonstrate that significant plaque deposition occurs prior to clinical decline. These findings suggest that the presence of amyloid plaque alone may be not sufficient to induce cognitive decline [70].

### *1.2.3 Cerebral amyloid angiopathy (CAA)*

In AD, A $\beta$  also tends to form deposits in the walls of cerebral and leptomeningeal vessels (cerebral amyloid angiopathy). Cerebrovascular A $\beta$  deposits are mainly

present in leptomeningeal and cortical arteries, and less frequently in veins, capillaries and subcortical vessels [71]. The major component of vascular amyloid deposits is A $\beta$  [72, 73]. Other proteins, such as apoE, are also found in cerebrovascular amyloid deposits [74]. Moreover, studies indicate that AD individuals carrying apoE4 allele have more severe CAA than AD patients lacking the apoE4 allele [75, 76], suggesting an important role of apoE proteins in the development of CAA.

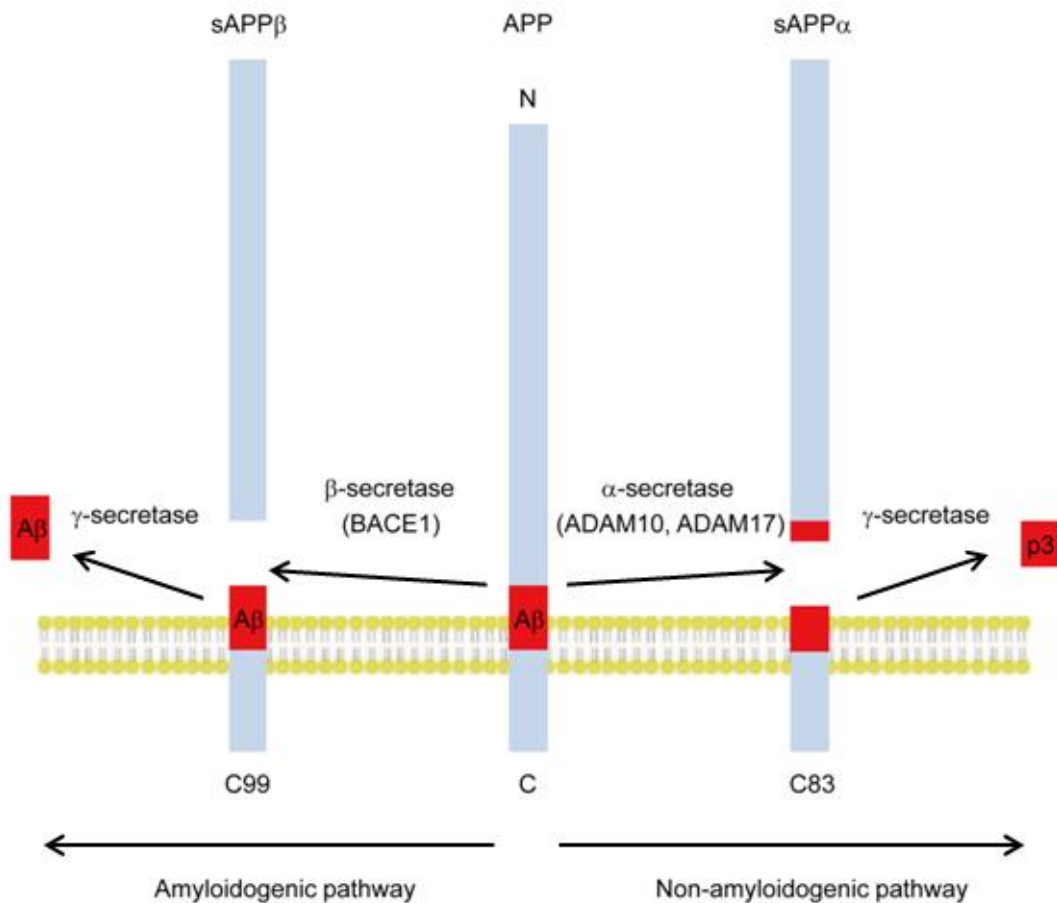
Morphologically, there are two types of CAA in AD: CAA with capillary involvement (CAA-type 1) and CAA lacking cortical capillary A $\beta$  deposition (CAA-type 2) [76]. Topographically, CAA starts in the leptomeningeal or neocortical vessel, and then expands into allocortical areas and the cerebellum, and finally subcortical nuclei such as the thalamus, basal ganglia, white matter and brain stem [77]. In addition, CAA is not specific to AD, other amyloidogenic proteins including transthyretin, cystatin C, gelsolin and prion protein can also accumulate in the cerebral blood vessels and form CAA in other diseases [78].

#### *1.2.4 Synaptic loss*

Synapse loss is believed to be the most reliable index of cognitive impairment in AD [79]. Studies consistently report that there is a substantial loss of synapses in the cortical and hippocampal region of AD patient during the disease progress [80-85]. Studies demonstrate that synaptic loss starts before tangle formation and neuronal loss [86]. Moreover, synaptic loss may also occur during normal aging, and it has been suggested that loss of synapses might be a consequence of normal aging that may contribute to overall synapse loss in AD [87].

### 1.3 A $\beta$ peptide

The deposition of amyloid plaques in the brain is the main pathologic hallmark of AD [19, 20]. The major component of plaques is the  $\beta$ -amyloid protein (A $\beta$ ) [56, 57] that is generated from amyloid precursor protein (APP) [58] by sequential cleavage of the  $\beta$ -site APP cleaving enzyme-1 (BACE1) [88-91] and  $\gamma$ -secretase complex [92] (Fig. 1.1). There are mainly two pathways involved in the proteolytic processing of APP. APP is predominantly cleaved by  $\alpha$ -secretase to form a soluble APP fragment (sAPP $\alpha$ ) and a membrane-bound fragment, C83. The cleavage site of  $\alpha$ -secretase is within the A $\beta$  sequence [93, 94], so  $\alpha$ -secretase cleavage precludes formation of A $\beta$  [95]. C83 is then cleaved by  $\gamma$ -secretase to generate p3 and the APP intracellular domain (AICD). Alternatively, cleavage of APP by BACE1 leads to the secretion of a large APP N-terminal fragment (sAPP $\beta$ ) and also yields a C-terminally truncated fragment (C99), which is subsequently cleaved by  $\gamma$ -secretase to produce A $\beta$ , containing from 37-42 amino-acid residues, and also AICD. The major A $\beta$  species are A $\beta$ 40 which contains 40-amino-acid residues and A $\beta$ 42 which contains an extra two amino-acid residues at its C-terminus. A $\beta$ 42 is the primary component of the amyloid plaques, and the two additional hydrophobic residues at the C-terminus makes A $\beta$ 42 aggregate more readily compared to A $\beta$ 40 [96, 97].



**Fig. 1.1.** Proteolytic processing of amyloid precursor protein (APP). APP can be processed through two distinct pathways: the amyloidogenic pathway and the non-amyloidogenic pathway. In the amyloidogenic pathway, APP is cleaved by  $\beta$ - and  $\gamma$ -secretase to produce A $\beta$ . However, in the non-amyloidogenic pathway, APP is cleaved by  $\alpha$ -secretases within the A $\beta$  sequence which precludes the formation of A $\beta$ .

### *1.3.1 A $\beta$ aggregation*

Theoretical and experimental studies show that A $\beta$  contains a central hydrophobic cluster from 17 to 21 of A $\beta$  and a completely hydrophobic C-terminal domain that shows high propensity to aggregate and form a  $\beta$ -sheet structure. The N-terminal sequence of A $\beta$  is hydrophilic and can exist as  $\alpha$ -helical or  $\beta$ -sheet conformation depending on the environmental conditions [98]. Structural studies have reported that A $\beta$  mainly adopts an  $\alpha$ -helical structure in organic solvents such as DMSO, while in aqueous buffers or in water it primarily demonstrates a  $\beta$ -sheet conformation [98]. The formation of amyloid fibril involves several steps and generates a range of A $\beta$  aggregates with different sizes and molecular weights. A $\beta$  oligomers, including dimers, trimers, or soluble higher molecular weight A $\beta$  oligomers, are soluble and non-fibrillar A $\beta$  aggregates with size of approximately 5 nm in diameter [99]. The precursor of full-length A $\beta$  fibrils are termed protofibrils, which are linear A $\beta$  aggregates approximately 4 to 11 nm in diameter and 20 to 200 nm in length [100, 101]. Another low molecular weight aggregated form of A $\beta$  may be annular in structure [102]. Compared with linear protofibrils, annular A $\beta$  is stable and cannot further convert to A $\beta$  fibrils in vitro [102]. The mature fibril has a size of approximately 14 nm in diameter and a length ranging from nanometres to microns [100].

The mechanism of A $\beta$  aggregation to form amyloid fibrils is still poorly understood. In vitro A $\beta$  aggregation studies have proposed some models to describe the A $\beta$  aggregation process. Amyloid fibrils could be formed following a nucleation-dependent polymerisation mechanism in which A $\beta$  monomers initially undergo a slow

nucleation phase (lag phase) to form A $\beta$  oligomers that can serve as the “seed”. Once the A $\beta$  “nucleus” is formed, it will undergo a rapid fibril elongation progress by adding A $\beta$  monomer to the “seed” (growth phase). Finally, the A $\beta$  aggregate will reach the steady state in which the A $\beta$  aggregates and monomers are at equilibrium [97]. A $\beta$  monomers may also aggregate to A $\beta$  fibrils via a template-dependent dock-lock mechanism [103]. Initially, A $\beta$  monomer may bind to pre-existing amyloid fibrils or other templates in a reversible manner (“dock” phase). Subsequently, this deposited A $\beta$  may undergo a conformation transition and become irreversibly associated with the amyloid fibril template in a time-dependent manner (“lock” phase). Finally, this irreversibly “locked” A $\beta$  may serve as a new binding site for “docking” the second A $\beta$  monomer [103, 104]. This model suggests that A $\beta$  fibril formation may occur without the formation of an A $\beta$  nucleus, and this theory is supported by the observation that a cell-released factor can promote A $\beta$  aggregation at physiological A $\beta$  concentrations, which is much less than critical concentration for forming A $\beta$  nucleus [105].

### *1.3.2 Soluble A $\beta$ oligomer production and toxicity*

Early studies suggested that the level of soluble A $\beta$  oligomers is strongly correlated with synaptic loss and the severity of AD [106, 107], while the level of insoluble A $\beta$  or amyloid plaque presented in the brain is poorly associated with the cognitive impairment of disease [106]. These findings indicate that the A $\beta$  oligomers may have important roles in the development and progress of AD, and thus should attract more attention in studies.

A $\beta$  dimer has been found in AD transgenic mouse models [108, 109]. In Tg2576 mice, the A $\beta$  dimers are highly located in lipid rafts and present before the amyloid plaques are detectable [108]. In human studies, A $\beta$  dimer can only be detected in the brains of patients with AD [108, 110], suggesting that A $\beta$  dimer is strongly associated with AD pathogenesis. Other studies show that A $\beta$  dimers extracted directly from the cerebral cortex of subjects with AD can potently inhibit long-term potentiation (LTP), elevate long-term depression (LTD) and decrease dendritic spine density in the normal rodent hippocampus [110]. However, insoluble A $\beta$  extractions from the cortex of AD patients have no effect on LTP unless they are disassembled to A $\beta$  dimers, suggesting that amyloid plaques may be relatively inactive but may act as a reservoir of A $\beta$  dimers [110]. More recently, studies show that A $\beta$  dimers can induce hyperphosphorylation of tau at AD-relevant epitopes in hippocampal neurons and also cause disruption of the microtubule cytoskeleton and neuritic degeneration. Knocking down endogenous tau fully prevents the A $\beta$  dimer-induced neuritic change, while overexpression of human tau can accelerate this process [111]. This finding firstly demonstrates the relationship between the A $\beta$  and NFTs, suggesting that NFTs may be a downstream consequence of A $\beta$ -induced toxicity.

A $\beta$  trimers have also been extracted from the brain tissue of AD transgenic mice (Tg2576) as early as the embryo stage, and trimers are stably expressed throughout the life of the mice [112]. Animal studies demonstrate that A $\beta$  trimers can more potently inhibit hippocampal LTP than A $\beta$  dimers and tetramers [113]. In addition, A $\beta$  trimers have a strong effect on inducing cofilin dephosphorylation and the formation of cofilin-actin rods within rat hippocampal neurons than other A $\beta$



oligomer species [114]. These findings suggest that A $\beta$  trimers could account for at least a portion of the A $\beta$  oligomer-induced neurotoxicity.

Dodecamers of A $\beta$  have a molecular weight of 56 kDa and have been termed A $\beta$ \*56 [112]. A $\beta$ \*56 was initially found to cause memory deficits in the Tg2576 mouse model. A cognitive decline of Tg2576 mice has been observed at 6 months of age when A $\beta$ \*56 is first detected, and administration of A $\beta$ \*56 purified from the brain of Tg2576 mice can impair memory in young rats. These results suggest that A $\beta$ \*56 may be an important neurotoxic form of A $\beta$  [112]. Interestingly, other studies show that longitudinal water-maze spatial training can improve learning performance and reduce the level of A $\beta$ \*56 in transgenic AD mice [115]. These studies further support the view that there may be a strong link between A $\beta$ \*56 and memory decline. However, a recent study reported that the level of A $\beta$ \*56 is stable and is not correlated with the memory deficits [116], suggesting that the relationship between A $\beta$ \*56 and memory impairment are correlative rather than causative.

Recently, annular protofibrils (APFs), which are structurally and functionally distinct amyloid oligomers, have been identified [102]. APFs have a pore-like structure with the molecular weight over 90 kDa. They have a structure similar to bacterial pore-forming toxins and thus have been proposed to alter permeability of the cell membrane and exhibit cellular toxicity [117]. However, in vitro studies indicate that APFs have less membrane-permeabilizing activity and toxicity compared with prefibrillar oligomers [102]. Studies demonstrate that APFs are also present in both AD transgenic mice and human brains [118, 119]. However, whether APFs have a neurotoxic effect in vivo is still unclear.

### *1.3.3 A $\beta$ -induced calcium dysregulation*

The neurotoxic effect of A $\beta$  was first reported approximately two decades ago, and subsequent studies indicate that the oligomeric species of A $\beta$ , rather than A $\beta$  fibrils, contribute to most A $\beta$ -induced neurotoxicity and are strongly correlated to the cognitive decline of AD. However, the precise cellular or molecular mechanism involved in A $\beta$ -induced neurotoxicity is still unclear. Recently, evidence shows that the dysregulation of calcium homeostasis may have a critical role in the pathogenesis of AD.

Initially, several studies indicated that the concentration of calcium in the brain increases with aging [120, 121]. However, the biochemical basis for this increase of Ca<sup>2+</sup> is unclear. Subsequently, studies have also linked the dysregulation of Ca<sup>2+</sup> homeostasis to AD. Mattson and colleagues firstly reported that A $\beta$  can disrupt neuronal calcium homeostasis and thereby render neurons more vulnerable to glutamate-induced excitotoxicity [122]. This effect was also observed in later studies [123, 124]. However, the exact mechanism underlying the A $\beta$ -induced dysregulation of Ca<sup>2+</sup> homeostasis is still unknown, but several hypotheses are proposed to explain these findings.

Several studies have suggested that A $\beta$  can trigger Ca<sup>2+</sup> entry by binding to the NMDA receptor [125], and that noncompetitive NMDA receptor antagonists can block the A $\beta$ -induced toxicity [126]. However, other groups demonstrate that A $\beta$  can promote Ca<sup>2+</sup> uptake through the stimulation of L-type voltage-sensitive Ca<sup>2+</sup> channels rather than NMDA channels in SH-SY5Y human neuroblastoma cells [127]. It has also been shown that A $\beta$  can open L-type Ca<sup>2+</sup> channels and induce the Ca<sup>2+</sup>

influx and eventually activate acetylcholinesterase, which is involved in the degradation of acetylcholine [128]. In addition, studies in rat hippocampal neurons show that A $\beta$  can also block voltage-gated fast-inactivating K $^{+}$  current and lead to prolonged cell depolarization which thereby increases Ca $^{2+}$  entry [129].

It has been demonstrated that A $\beta$  oligomers can increase membrane permeability to Ca $^{2+}$  [130], and other studies also suggest that A $\beta$  may form calcium channels in the bilayer membrane of the cell [131]. These findings raise the possibility that A $\beta$  may form pores in the membrane to allow Ca $^{2+}$  entry. This hypothesis has been supported by several studies [132-134]. However, recent in-vitro studies demonstrate that A $\beta$  oligomers could also increase membrane conductance by thinning the lipid membrane, and studies have failed to observe artificial ion channels or pores [135, 136]. To date, “A $\beta$  Ca $^{2+}$  channels” have not been observed in vivo, thus this artificial pore hypothesis still remains speculative.

Ca $^{2+}$  can influence AD progression by affecting APP processing and A $\beta$  production. Recent evidence shows that a polymorphism of the calcium homeostasis modulator 1 (CALHM1) gene may be associated with risk of AD [137]. This polymorphism of CALHM1 can elevate the A $\beta$  levels by altering Ca $^{2+}$  permeability and cytosolic Ca $^{2+}$  levels [137]. Recent studies also link familial AD mutations in presenilins to Ca $^{2+}$  homeostasis [138]. Studies show that presenilins can regulate Ca $^{2+}$  homeostasis via the inositol 1,4,5-trisphosphate (IP $_3$ ) receptor Ca $^{2+}$  release channel [139], while other studies demonstrate AD-related mutations of presenilin can reduce the activity of the sarco ER Ca $^{2+}$  ATPase (SERCA) pump, which is involved in the maintenance of the level of cytosolic Ca $^{2+}$  [140]. Importantly, regulation of IP $_3$  receptors or SERCA can

affect the A $\beta$  production [139, 140], suggesting that ER Ca<sup>2+</sup> homeostasis may have important roles in the A $\beta$  generation and familial AD.

#### *1.3.4 Physiological functions of A $\beta$*

A $\beta$  can be produced during normal cellular metabolism in vivo [92, 141], indicating that rather than only being a cause of neurotoxicity, A $\beta$  may have its own biological function at physiological concentrations in vivo. Several in-vitro studies report that A $\beta$  may have neuroprotective and neurotrophic effects at low concentrations [142-144]. A $\beta$  can also promote the proliferation and differentiation of primary neural progenitor cells derived from rat E18 cerebral cortex [145]. Several studies indicate that A $\beta$  may have a role in the regulation of synaptic activity [146]. Evidence shows that elevation of neuronal activity in hippocampal slices overexpressing APP can increase the production of A $\beta$ , and A $\beta$  can also selectively depress excitatory synaptic transmission onto neurons and thereby decrease the neuronal activity [146]. These observations thus suggest a role of A $\beta$  in a negative feedback to control the neuronal activity, and disruption of this feedback system could contribute to disease progression in AD. This hypothesis is supported by a recent study that reports that stimulation of NMDA receptors, which increase neuronal activity, can increase the expression of APP and promote A $\beta$  production [147]. These findings imply that A $\beta$  may have important physiological functions in the brain.

## **1.4 Biology of amyloid precursor protein (APP)**

In 1987, several groups used oligonucleotide probes corresponding to the amino acid sequence of A $\beta$  to isolate and sequence complementary DNA clones that encode the APP sequence, and mapped the APP gene to the long arm of human chromosome 21 [55, 58, 148, 149]. These findings suggest a genetic relationship between AD and Down syndrome, and explain why individuals with trisomy 21 also develop AD-like pathology. However, further studies have shown that familial Alzheimer's disease (FAD) doesn't result from the genetic defect causing increased gene dosage of APP [150, 151].

The human APP gene has at least 18 exons [152, 153]. Because of the alternative splicing of exons, APP mRNA encodes several isoforms ranging from 365 (APP365) to 770 (APP770) amino acid residues [154, 155]. APP365 and APP563 encode the secreted form of APP without the A $\beta$  domain [154, 156]. The three major isoforms of mammalian APP are APP695, APP751 and APP770. Compared with APP695, APP751 has a 168-nucleotide insert which introduce a 56 amino-acid Kunitz protease inhibitor (KPI) domain into the extracellular domain and also converts val289 into an isoleucine [155, 157, 158]. APP770 is identical to APP751 but has another 57 nucleotide insert which adds a 19 amino-acid OX-2 domain to the C-terminal of insert of APP751 [159]. These mammalian APP isoforms are expressed in both brain and peripheral tissues. In brain, APP695 normally expresses in neurons and is the primary source of APP in the brain, whereas APP751 and APP770 are mainly expressed in glial cells [160].

### *1.4.1 Structure of APP*

APP is a large type I transmembrane glycoprotein containing a cytoplasmic C-terminal domain and a large N-terminal ectodomain [161]. The structure of APP is organized into multiple distinct functional domains (Fig. 1.2). The extracellular domain of APP is divided into the E1 and E2 domains [162]. The E1 domain is stretched from the N-terminal signal peptide region and is followed by a cysteine-rich region. The cysteine-rich region can be further divided onto a number of subdomains, including a heparin-binding or growth-factor-like domain (HBD or GFLD) [163, 164], a small copper-binding domain (CuBD) [165] and a zinc-binding domain (ZnBD) [166]. Between the E1 and E2 domain is a highly acidic domain which is rich in glutamate and aspartate residues. In some APP mRNA split isoforms, the acidic domain is followed by the KPI domain [167]. The KPI domain may be formed adjacent to a 19-residue sequence which is homologous to the OX2 antigen (OX2 domain). The OX2 domain is only present in APP770 isoform [159].

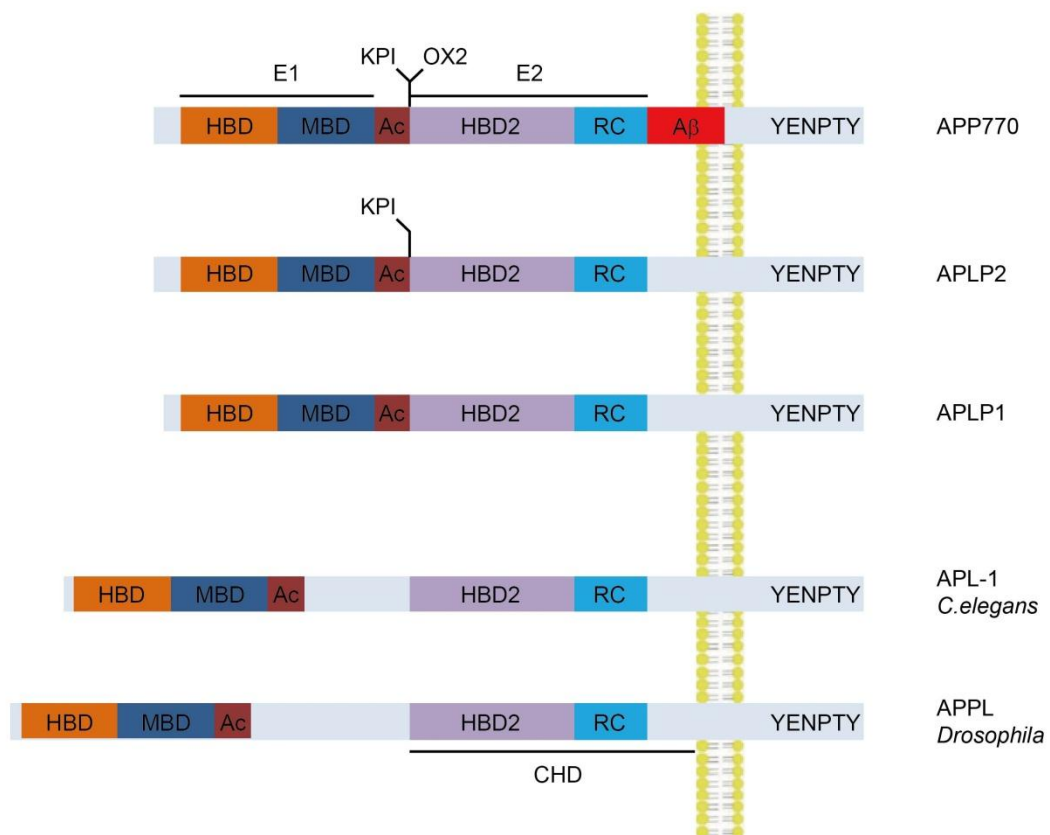
The remainder of the extracellular domain of APP is often termed the “carbohydrate domain” (CHD), which containing two potential N-glycosylation sites. The CHD can be divided into two subdomains. The first domain of CHD is the E2 domain or central APP domain (CAPPD). NMR and crystal structures of the E2 domain reveal that it is composed of six  $\alpha$ -helices which fold together as a coiled-coil substructure [168, 169]. The E2 domain contains a pentapeptide, RERMS, which may participate in APP dimerization and may relate to a role of APP in cell-cell adhesion. There is also a highly conserved heparin-binding site (HBD2) in the E2 domain [163], which is believed to mediate the binding of APP to the extracellular matrix (ECM) (e.g. collagen and laminin) [170, 171]. The E2 domain is followed by an unstructured

sequence called the random coil (RC) region which does not form a standard secondary structure of protein [172]. The RC contains the  $\beta$ - and  $\alpha$ -secretase cleavage sites.

The ectodomain of APP is followed by a transmembrane (TM) domain [58, 161], which contains the  $\gamma$ -secretase cleavage site. This site is located approximately in the middle of the TM domain [173]. The A $\beta$  sequence contains the last 29 C-terminal amino acids of the CHD and the first 11-13 amino acids of the TM domain.

The cytoplasmic domain of APP contains the APP intracellular domain (AICD) peptide sequence, which is derived from the  $\gamma$ -secretase cleavage. Most of the identified APP binding partners interact with the AICD [174]. The YENPTY motif present in the AICD is conserved in all APP homologues, and this sequence is also important for the clathrin-mediated endocytosis of APP [175].

APP homologues have been identified in several species, including *C. elegans* (APL-1) [162], *Drosophila* (APPL) [176], *Zebrafish* (APPa) [177], and *Xenopus laevis* (APP-A) [178]. Two APP homologues, APP-like protein 1 (APLP1) and APP-like protein 2 (APLP2) have been found in mammals [149, 179, 180]. These APP homologues have several conserved motifs in their sequence, including the E1, E2 domain and AICD. Importantly, the A $\beta$  sequence is not conserved and is only present in the APP sequence. *Drosophila* APPL and APLP1 are exclusively expressed in the nervous system [181, 182], while mammalian APP and APLP2 are not only highly abundant in the brain but also ubiquitously expressed outside of the brain [181, 183].



**Fig. 1.2.** Schematic illustration of domain structure of APP and its homologues. HBD, heparin-binding domain; MBD, metal-binding domain including copper-binding domain (CuBD) and zinc-binding domain (ZnBD); Ac, acidic domain; KPI, Kunitz protease inhibitor domain; OX2, immunoregulatory OX-2 antigen domain; HBD2, heparin-binding domain 2; RC, random coil; CHD, carbohydrate domain.



### *1.4.2 Functions of APP*

#### **1.4.2.1 Cell adhesion**

Studies have indicated that members of the APP family may participate in cell adhesion [184]. The E1 and E2 domain in the extracellular sequence of APP have been shown to interact with extracellular matrix proteins (e.g. collagen 1 and laminin) [170, 171] and heparin [163, 185, 186]. This property of APP suggests a role of APP in cell-matrix adhesion. Structural and functional studies also indicate that the E1 and E2 domain of APP are involved in cell-cell adhesion. X-ray crystallography analysis reveals that E2 domain of APP can form antiparallel dimers [169]. This implies that APP has a function in homophilic interaction and transcellular adhesion. Studies also show that heparin can bind to the E1 and E2 domain of APP and induce APP-APP dimerisation [187-189]. Further studies suggest that APP can form homodimers and heterodimers with APLP1 and APLP2 mainly via the conserved E1 region of APP [189].

Recently, studies demonstrate that the GxxxG motifs within APP transmembrane sequence provide another dimerisation site of APP [190, 191]. Mutagenesis of the glycine residues in GxxxG motif leads to a weaker dimerisation strength and causes a reduction in A $\beta$  production, but increases A $\beta$ 38 and shorter A $\beta$  species [190, 191]. As  $\beta$ -secretase cleavage of APP is not inhibited by mutation of the GxxxG motif. This suggests that  $\gamma$ -secretase cleavage of APP may be influenced by the APP dimerisation [190, 191].

The “RHDS” tetrapeptide sequence near the extralumenal region of APP, at the beginning of the A $\beta$  sequence, is also reported to promote cell adhesion in an

integrin-like manner [192]. Interestingly, APP colocalises with integrins on the surface of axons and at the sites of cell adhesion, suggesting that APP may either interact with integrins or has similar mechanism to promote cell adhesion [193, 194].

#### **1.4.2.2 Role as a cell surface receptor**

APP was initially predicted to function as a cell-surface receptor [58]. The similarity in structure and proteolytic processing between APP and notch, which is a cell-surface receptor involved in cell signaling, suggests that APP could also act as cell-surface receptor [195]. APP is reported to act as a G-protein coupled receptor by specially activating  $G_o$  in a ligand-dependent and ligand-specific manner [196]. Further studies show that APP can serve as a cell-surface receptor in cells and activate serine/threonine kinase (especially the MAPK pathway) [197]. Recently, F-spondin has been found to bind to the E2 domain of APP as well as APLP1 and APLP2, and this binding inhibits  $\beta$ -secretase cleavage of APP and thus could regulate APP processing [198]. The binding of F-spondin is also reported to promote neuronal survival by the activation of intracellular signaling pathways involving phosphorylation of Dab-1 and Akt and up-regulation of anti-apoptotic Bcl-2 family members [199]. There is evidence that reelin, an extracellular protein, can interact with the E1 domain of APP, leading to inhibition of  $A\beta$  production and promotion of neurite outgrowth [200, 201]. Nogo-66 receptor can interact with APP at a site close to the BACE1-cleavage site of APP. The binding of the Nogo-66 receptor to APP can reduce  $A\beta$  production and knockout of the Nogo-66 receptor can lead to  $A\beta$  accumulation in an APP transgenic mouse model [202]. Netrin-1, a multifunctional guidance and trophic factor, can bind to APP at the beginning of the  $A\beta$  sequence and

modulate APP signaling by triggering APP intracellular domain-dependent gene transcription. Interestingly, the binding of netrin-1 to APP also suppresses A $\beta$  production in brain slices from AD transgenic mice and A $\beta$  levels are elevated when netrin-1 expression is decreased [203]. In addition, another study suggests that sAPP $\alpha$  can serve as a physiologically relevant ligand of APP. The binding of neuroprotective sAPP $\alpha$  to APP can disrupt APP homodimerisation and is necessary for the protection of neuroblastoma cells against starvation-induced cell death [204].

#### **1.4.2.3 Role in neurite outgrowth, synaptogenesis and neuroprotection**

Several lines of evidence suggest a neurotrophic role for APP both in vitro and in vivo. Structurally, the N-terminal heparin-binding domain of APP is vaguely similar to cysteine-rich growth factors, suggesting that this region of APP could function as a growth factor in vivo [164]. Mice lacking APP, APLP1 and APLP2 have severe abnormalities in brain development, suggesting that APP family members have some roles in brain development [205].

After synthesis, APP can be rapidly transported to synaptic terminals [206-209], and APP mRNA and APP expression is progressively increased during the differentiation of brain cells when neurite outgrowth and synaptogenesis is maximal, suggesting that APP may be involved in synaptogenesis [210, 211]. In APP/APLP2 double knockout mice, synaptic vesicle density and active zone size are dramatically reduced, further supporting the idea that APP family proteins have important roles in regulating the formation and function of synapses [212, 213].

In addition, during traumatic brain injury (TBI), the expression of APP is elevated both in mammalian and *Drosophila* brain [214-216]. sAPP $\alpha$  treatment can reduce neuronal cell loss and axonal injury after TBI, and may antagonise dendritic degeneration and neuron death induced by proteasomal stress [217, 218].

The effect of APP on neurite outgrowth probably involves its cell adhesion properties. In particular, the binding of APP to HSPG in the extracellular matrix may stimulate the effects of APP on neurite outgrowth, and a peptide homologous to the heparin binding region of APP can block APP-induced neurite outgrowth [186]. When APP-transfected CHO cells are used as a substrate for the growth of rat hippocampal neurons, the increased surface APP on the CHO cells stimulates short-term neuronal adhesion and longer-term neurite outgrowth [219].

Several lines of evidence suggest that the  $\alpha$ -secretase cleaved form of APP, sAPP $\alpha$  also has important roles in neurite outgrowth. One of the earliest lines of evidence that soluble APP is important for cell growth came from the observation that an antisense APP construct can slow the growth of neuroblasts and that this growth retardation could be rescued by treatment with secreted sAPP $\alpha$  [220]. Subsequently, a pentapeptide motif “RERMS” in the E2 region of APP was reported to be responsible to promoting neurite outgrowth in vitro [221, 222]. Indeed, moderate overexpression of APP in mice, infusion of either an RERMS peptide or sAPP $\alpha$  into adult rat brain, or increasing sAPP $\alpha$  generation in vivo by overexpression of  $\alpha$ -secretase (ADAM10) can increase synaptic density and memory retention [223-226]. However, it has been suggested that both soluble and cell-associated APP can contribute to neurite outgrowth in a complex manner [194, 227, 228]. Studies show that the APP-deficient neurons have shorter axons at 1 day in vitro (DIV) but longer axons after 3 DIV

compared to wild-type neurons. This implies that cell-associated APP may primarily contribute to the onset of axon formation [228]. Young-Pearse *et al.*, [194] reported that deletion of APP, APLP1 or APLP2 or addition of sAPP $\alpha$  can promote neurite outgrowth in primary neuronal cells. However, sAPP $\alpha$  cannot stimulate neurite outgrowth in the absence of APP. These results suggest that sAPP $\alpha$  may inhibit the activity cell surface APP.

It should be noted that APP/APLP1/APLP2 triple knockout embryonic stem (ES) cell-derived neurons can form neurites and active excitatory synapses equally well compared to neurons derived from wild-type embryonic stem cells [229], a result which seems to be contradictory to this idea. Thus, it is still difficult to reconcile these findings into the neurotropic functions of APP.

#### **1.4.2.4 Interaction of APP with adaptor proteins**

The YENPTY motif of the intracellular domain of APP (AICD) has been shown to interact with many adaptor proteins possessing a phosphotyrosine binding domain (PTB) domain[230]. Considering that all APP family proteins contain a YENPTY sequence at the C-terminal tail, it is likely that all APP homologues may bind to similar adaptors and function in signal transduction and gene regulation.

FE65 was the first protein to be identified as an APP binding partner[231]. FE65 contains two PID/PTB domains and can bind to the YENPTY motif in a tyrosine phosphorylation independent manner [232]. The interaction of FE65 with APP may disrupt APP trafficking and processing [233, 234]. Studies show that overexpression of FE65 may increase translocation of APP to the plasma membrane and elevate the

secretion of both sAPP $\alpha$  and A $\beta$  [233]. However, in a study by Ando *et al.* (2001), expression of FE65 was found to suppress the maturation of APP and reduce A $\beta$  production. This study also suggested that the phosphorylation of APP at the thr668 position of APP695 may lead to a conformation change in AICD, which contains the YENPTY sequence and therefore diminishes the interaction of APP and FE65 [234]. APP may form a complex with FE65 and the histone acetyltransferase Tip60 and the interaction may have a function in gene expression [235]. The downstream targets of the APP/FE65/Tip65 complex may include KAI1 [236] [237], neprilysin [238], GSK3 $\beta$  [237, 239, 240], EGFR [241], LRP1 [242], APP and BACE1 [237] and P53 [243-245].

X11 family proteins, also called Munc-18-interacting proteins (Mints), are also known to bind to the YENPTY motif of APP [230]. The X11 family has three members including X11 $\alpha$ /Mint1, X11 $\beta$ /X11L/Mint2 and X11 $\gamma$ /X11L2/Mint3, and all of them are reported to bind to APP [230, 246]. Studies have shown that the binding of X11 proteins to APP can affect APP trafficking and reduce the production of A $\beta$  [247, 248]. In support of these findings, studies by Lee *et al.* (2003 and 2004) showed that overexpression of X11 can retard A $\beta$  production and amyloid deposition in the brain of APP transgenic mice [249, 250]. In mice lacking X11 and X11L, there is a significant increase in  $\beta$ -secretase cleavage of APP and accumulation of A $\beta$  in the brain [251, 252]. Importantly, knock-out of X11 and X11L shifts APP into lipid rafts, suggesting a possible mechanism whereby X11 could stabilise APP. This would inhibit its translocation into lipid rafts where it could be cleaved by BACE1, and thus reduce A $\beta$  production [252].

JNK-interacting proteins (JIPs), including JIP-1a, JIP-1b and JIP2, are reported to interact with the YENPTY motif of APP through a PTB domain [253, 254]. Further studies show that JIP-1b but not JIP-1a and JIP2 stabilises immature APP and inhibits APP processing, thus reducing the generation of A $\beta$ . This finding indicates that JIP-1b could directly bind to APP rather than to JNK and then activate the JNK signalling cascade [255]. It has been known that JNK can potentially phosphorylate APP at thr668, suggesting that JIP-1b may act as a scaffold protein and link JNK to APP, thus enhancing the phosphorylation of APP [256]. In addition, JIP-1 is reported to be involved in the normal axonal transportation of APP [257, 258].

Shc (Src homology collagen-like) A and Shc C, which contain a PTB domain, can bind to the YENPTY motif of APP in vitro and in vivo when APP is phosphorylated at tyr682. The phosphorylation at thr668 of APP also regulates the interaction of Shc A and APP, as mutation of tyr668 reduced this interaction [259]. It has been further demonstrated that knockdown of Shc C leads to a reduction of A $\beta$  and the level of BACE1, but silencing of Shc A has no effect on APP processing [260]. In human brain, C99 may form a complex with Shc along with growth factor receptor-bound protein 2 (Grb2). Interestingly both Shc and Grb2 are increased in the AD brain [261]. Subsequent studies have revealed that Grb2 can bind directly to tyr682 phosphorylated APP via its Src homology 2 region [262], and may play a role in the trafficking of APP and AICD [263]. In addition, studies show that Grb2 interacts with APP and PS1 and modulates the centrosomal mitotic activity of ERK1, 2 [264].

Disabled-1 (Dab1) protein, which is important in the development of nervous system, is reported to bind to all three APP family members [265, 266]. Expression of Dab1 can alter APP trafficking since the level of APP is increased on the cell surface. Dab1

can also affect APP cleavage and decrease the production of A $\beta$  [200]. Further studies have revealed that Fyn, a Src family-tyrosine kinase, can induce the phosphorylation of APP at tyr682 and facilitate interactions between APP and Dab1, and regulate the effect of Dab1 on APP trafficking and processing [267]. Recently, studies show that Fyn regulates the interaction of APP and Dab1 and modulates the targeting of APP and Dab1 to lipid rafts [268].

Numb, another PTB containing endocytic adaptor protein, has been shown to bind to the C-terminal YENPTY motif of APP, and has an effect on APP trafficking and processing in an isoform-dependent manner [269, 270]. There is evidence that the expression of Numb isoforms are elevated in both AD brain and APP transgenic mice compared with controls, suggesting a role for Numb in AD progression [271].

PAT1 (protein interacting with APP tail 1) has also been shown to interact with the C-terminus of APP and may be involved in the translocation of APP [272]. PAT1a, a homologue of PAT, is reported to bind to all three APP family proteins, and promote the processing of APP and APLPs [273]. In addition, PAT1 may bind to the Jcasp domain of APP and cause the accumulation of APP and APLP2 at the cell surface, thereby triggering a cell death signal [274].

#### **1.4.2.5 Non-neuronal function of APP**

APP and its homologues also have physiological functions outside the nervous system. Previously, the APP containing KPI domain has been reported to play a role in the regulation of blood coagulation [275, 276]. APP can be released by activated platelets and acts as a potent inhibitor of factor XIa [277, 278]. Studies in APP/APLP2 null



mice suggest functions of APP family proteins in insulin and glucose homeostasis [279]. Moreover, APP is highly expressed in adipose tissue and up-regulated in obesity, and its expression levels correlate with insulin resistance and adipocyte cytokine expression levels [280]. In addition, studies indicate that there is an up-regulation of APP in various cancers. Further studies reveal that APP may be associated with tumor growth as knockdown of APP results in inhibition of tumor cell growth [281].

### *1.4.3 Proteolytic processing of APP*

#### **1.4.3.1 APP trafficking and processing**

During the transition of APP from the endoplasmic reticulum (ER) to the plasma membrane through the constitutive secretory pathway, APP undergoes N-glycosylation and O-glycosylation [282]. The further addition of sulfate and phosphate in the late Golgi compartment and on the cell surface increases the molecular mass of APP. APP can be proteolytically processed at the cell surface by a set of  $\alpha$ -secretases and release sAPP $\alpha$  [94]. Activation of protein kinase C can increase sAPP $\alpha$  secretion, because protein kinase C is involved in the formation and release of secretory vesicles from the trans-Golgi network, activation of PKC increases the transport of APP to the cell surface [283]. After arriving at the cell surface, APP can be quickly internalised within minutes. This is achieved by the YENPTY motif at the intracellular region of APP. Mutation of the YENPTY motif can block the internalisation of APP and decrease A $\beta$  formation [284]. After internalisation of APP into the cytoplasm, APP is delivered to late endosome and a

fraction of the APP pool will be recycled back to cell surface. Internalised APP can also be delivered to lysosomes for degradation.

BACE1 is mainly localised in the late Golgi/TGN membrane and endosomes where APP can be cleaved during the endocytic/recycling step [285]. Studies show that the  $\gamma$ -secretase complex is present and has activity in multiple compartments including endoplasmic reticulum/intermediate compartment (ER/IC) [286], late Golgi/TGN [287, 288], endosomes [289] and plasma membrane [290, 291]. In non-neuronal and neuroblastoma cells, A $\beta$  is mainly generated in the TGN and endosomes during the trafficking of APP through the secretory and recycling pathways. In neurons, APP undergoes axonal transportation to nerve terminals by means of a fast anterograde component, and may be processed to A $\beta$  at those sites [207, 292]. Later studies show that the axonal transportation of APP may be mediated by direct binding of APP to the kinesin light chain subunit of kinesin-1 [293]. However, this conclusion is controversial and thus further investigation may be required [294].

### **1.4.3.2 APP processing secretases**

#### *1.4.3.2.1 $\alpha$ -Secretase cleavage*

The  $\alpha$ -secretase pathway is the primary route of APP processing and involves cleavage in the A $\beta$  sequence on the C- terminal side of the residue 16 of full A $\beta$  sequence [93, 94].  $\alpha$ -Secretase cleavage of APP releases a ~100 kDa soluble N-terminal APP fragment (sAPP $\alpha$ ) and generates an 83-residue membrane-bound fragment (C83) that has a molecular weight of ~9 kDa [95]. Thus, the  $\alpha$ -secretase cleavage of APP can preclude the generation of intact A $\beta$ . Evidence shows that  $\alpha$ -

secretase is a membrane-binding protein [94, 295], and can cleave APP at the cell surface [94, 296]. However, other studies indicate that  $\alpha$ -secretase cleavage of APP is also observed in intracellular compartments such as the Golgi apparatus [282, 297-299].

Tumor necrosis factor- $\alpha$  converting enzyme (TACE or ADAM17) is a type I integral membrane protein, which belongs to the disintegrin and metalloproteinase (ADAM) family [300, 301], and has been identified as a  $\alpha$ -secretase [302]. ADAM17-knockout mice show decreased production of sAPP $\alpha$ , and inhibition of TACE could affect APP secretion and A $\beta$  formation [302]. ADAM10 and metalloprotease disintegrin cysteine-rich protein 9 (MDC9)/ADAM9, another two members of ADAM family, also have been identified as  $\alpha$ -secretases [303, 304]. Overexpression of ADAM10 in cells or in mice results in a significant increase in  $\alpha$ -secretase cleavage of APP [303, 305]. Co-expression of ADAM9 and APP in COS cells leads to an increase of sAPP $\alpha$  production, but ADAM9 has no effect on secretion of sAPP $\alpha$  when the  $\alpha$ -secretase cleavage site in APP sequence is mutated [304]. However, deletion of both ADAM9 and ADAM17 in cells or in mice does not lead to significant reduction in sAPP $\alpha$ , indicating that ADAM9 and ADAM17 may be involved in regulated  $\alpha$ -secretase cleavage of APP rather than constitutive cleavage of APP [306, 307].

#### *1.4.3.2.2 $\beta$ -Secretase cleavage*

In the amyloidogenic pathway, APP is firstly cleaved by  $\beta$ -secretase at the N-terminus of A $\beta$  to release sAPP $\beta$  and to form a membrane-bound 99-residue C-terminal fragment of APP called C99. The A $\beta$  amino acid sequence begins at the asp1 residue

of A $\beta$  [308]. In HEK293 cells, however, there is a minority of A $\beta$  that starts at val3 and glu11 [92]. In contrast, glu11 species are mainly found in rat primary neuron culture [309]. Inhibitor studies show that val3 species of A $\beta$  are generated by a different protease whereas glu11 seem to be produced in parallel with asp1 A $\beta$  by the  $\beta$ -secretase [91, 309, 310].

In 1999 and 2000, a transmembrane aspartic protease was reported to have all known characteristics of  $\beta$ -secretase. This protease is now known as BACE1 (beta-site APP-cleaving enzyme). BACE1 is mainly expressed in the brain and plays the major role in  $\beta$ -secretase activity [88-90, 311, 312]. A homologue, BACE2, is widely expressed in peripheral tissue and can cleave APP within the A $\beta$  sequence [313]. However BACE2 is not the  $\beta$ -secretase because knockout of BACE2 does not abolish the production of A $\beta$  [314].

Full-length pre-pro-BACE1 has 501 amino residues and contains a signal peptide domain (residue 1-21) and a pro-domain (residue 22-45). A furin-like proprotein convertase is responsible for removing this prodomain and forming the mature BACE1 in the Golgi apparatus [315, 316]. BACE1 has two aspartic protease active site motifs which have optimal activity in acidic environments and mutation of either of these two residues inactivates the enzyme [312, 317]. Studies demonstrate that both the expression and enzymatic activity of BACE1 is elevated in AD patients [318, 319]. In BACE1 knockout mice, the deletion of BACE1 appears to cause no major phenotypic difference with the wide-type mice but significantly, BACE1 knockout block the production of A $\beta$  and sAPP $\beta$  [320, 321]. However, further precise studies show BACE1-null mice have abnormalities in cognitive, emotional, myelination and synaptic functions, and may have sensorimotor impairments, spatial memory deficits

and displayed seizures [322-326]. Knockout of BACE1 blocks the production of A $\beta$  and abolishes the formation of amyloid plaques in APP transgenic mice [321, 327]. This result indicates that BACE1 is the main  $\beta$ -secretase which initiates A $\beta$  generation, and drugs targeting BACE1 may have therapeutic potential for the treatment of AD.

#### *1.4.3.2.3 $\gamma$ -Secretase cleavage*

After  $\beta$ - and  $\alpha$ -secretase cleavage of APP, C99 and C83 can be cleaved by  $\gamma$ -secretase. The  $\gamma$ -secretase is a protein complex containing anterior pharynx-defective 1 (APH-1), nicastrin, presenilins (PS1 or PS2), presenilin enhancer 2 (PSENEN) [328]. The PSs are believed to be responsible for the main  $\gamma$ -secretase activity [329, 330].

PSs are multi-transmembrane proteins and synthesized as a ~50 kDa holoprotein. Subsequently, PS undergoes endoproteolytic cleavage and generates a ~30 kDa N-terminal fragment and a ~20 kDa C-terminal fragment. This cleavage forms the mature and active PS heterodimer [331-334]. Mutation of either transmembrane aspartates or deletion of PSs can inhibit A $\beta$  secretion and increase the C99 and C83 production [329, 335, 336]. Additionally, recent studies showed that mitochondria-associated membrane (MAM), which is involved in lipid metabolism and calcium homeostasis, is the predominant subcellular location for PS1 and PS2. The finding of localization of PS1 and PS2 in MAM may help reconcile the disparate hypotheses regarding the pathogenesis of Alzheimer disease, such as how mitochondrial oxidative damage is associated with abnormal APP processing [337].

Nicastrin is a type 1 membrane protein with a large ectodomain. Missense mutation of nicastrin causes a strong increase in A $\beta$  secretion, and deletion of certain domains or downregulation of nicastrin can significantly reduce A $\beta$  secretion [338, 339]. Nicastrin plays an important role in  $\gamma$ -secretase complex assembly and is required in the interaction between the  $\gamma$ -secretase complex and APP [340]. Nicastrin may not be a catalytic component in the  $\gamma$ -secretase complex. Instead, it may act as a receptor for the substrates of the  $\gamma$ -secretase complex [341].

APH-1 protein has two homologous forms (APH-1a and APH-1b) [342]. APH-1b is not involved in  $\gamma$ -secretase activity [343]. Deletion of APH-1 results in a decrease of levels of PS [343, 344], but overexpression of APH-1 can cause accumulation of PSs [345], suggesting that APH-1 has important function in the assembly, maturation and maintenance of  $\gamma$ -secretase complex [346, 347].

PSENEN has 101 amino acid residues containing two transmembrane domains [342]. PS, APH-1 and nicastrin can form a trimeric sub-complex in the cell, indicating that PSENEN may be the final protein binding to the  $\gamma$ -secretase complex [348]. In addition, PSENEN is required for the endoproteolysis and maturation of PSs [349]. Downregulation of PSENEN using RNA interference reduces the level of PS1 and leads to the accumulation of PS holoprotein [345, 350].

The  $\gamma$ -secretase complex is formed in sequential steps. APH-1 firstly forms a stable subcomplex with the immature form of nicastrin [348, 351]. Subsequently, the PS holoprotein is added into the complex to form a trimeric subcomplex. The addition of PSENEN leads to the maturation of nicastrin and PS, and finally activates  $\gamma$ -secretase complex [348]. In addition, recent evidence demonstrates that PS1 exhibits  $\gamma$ -secretase activity that is independent of other components of the complex [352].

## 1.5 Genetics and risk factors for AD

Mutations of APP and presenilins are causes of early-onset AD [353]. In late-onset AD, apolipoprotein E4 (apoE4) and other genotypes are known to increase the risk of AD [354]. In addition to these genetic factors, age is also a risk factor and the prevalence of AD doubles approximately every 5 years after the age of 60 in the normal population [355]. In addition, education, diet, physical activity, smoking, alcohol, and drugs are also reported to be associated with the development of AD [355, 356].

### 1.5.1 Early onset AD (*familial AD*)

#### 1.5.1.1 APP mutations

Mutations in the APP gene can cause early onset familial AD (FAD) [357], providing support for the view that APP and A $\beta$  are essential in the pathogenesis of AD. Several mutations have been found within the gene encoding APP [358]. A double mutation at codons 670 and 671 of APP770 transcript in Swedish kindred alters the lysine and methionine amino acid residue to asparagine and leucine, respectively close to the N-terminus of A $\beta$  [359]. Cells expressing APP with this Swedish mutation produce approximately 6-fold more A $\beta$  than cells expressing normal APP [360]. The substitution of leucine for methionine is sufficient to cause this increase in A $\beta$  and thus implying that this mutation increase sensitivity of beta cleavage site to the  $\beta$ -secretase [361, 362].

Mutation at position 692 of APP770 (near the  $\alpha$ -secretase cleavage site) converts an alanine to glycine. This mutation strongly decreases cleavage of APP by  $\alpha$ -secretase and leads to a significant increase in A $\beta$  production [358, 363]. Three mutations at codon 717 convert the valine to isoleucine [364, 365], phenylalanine [366], or glycine [367]. These mutations shift the cleavage of APP to the longer forms of A $\beta$  such as A $\beta$ 42 and A $\beta$ 43 which could form amyloid fibrils more readily than A $\beta$ 40 [97, 368].

In 1997, a mutation was reported at codon 717 in the APP gene in which an isoleucine residue is substituted by a valine. This mutation increases the generation of longer forms of A $\beta$  containing 42 or 43 amino acid residues (A $\beta$ 42/43) [357]. In addition, another mutation which converts the leucine to proline at the position of 723 of APP770 found in an Australian pedigree, also leads to marked increase in A $\beta$ 42/43 [369].

One early-onset AD mutation has been found in an Italian family which has a mutation at codon 715 of the APP770 gene. Interestingly, this mutation dramatically reduces A $\beta$ 40 release but has no effect on A $\beta$ 42 production in HEK293 cells. This implies that the ratio of A $\beta$ 42 to total A $\beta$  may be more critical for the development of AD than the total amount of A $\beta$  production [370].

In summary, all FAD-causing APP mutations cluster at or adjacent to the  $\alpha$ -,  $\beta$ - or  $\gamma$ -secretase cleavage site. All of these mutations are found to increase the production of A $\beta$ 42/43 or to increase the ratio of A $\beta$ 42/43 to total A $\beta$ . These findings indicate that long A $\beta$  products may contribute more to the pathology of AD than shorter A $\beta$  products (i.e. A $\beta$ 40).



### 1.5.1.2 Presenilin mutations

The APP mutations only account for a fraction of the cases of FAD, suggesting that there are other loci associated with FAD. Linkage studies showed a FAD locus on chromosome 14 [371]. Subsequently, presenilin 1 (PS1) was identified as the gene on chromosome 14 responsible for the disease [372]. Presenilin 2 (PS2), a homologue of PS1, located on chromosome 1, was identified as another gene causing FAD soon after [373-376]. Compared with PS1, mutations in PS2 are relatively rare [376]. So far, approximately 200 different PS1 mutations and over 20 PS2 mutations have been identified [377].

PS-associated mutations have been reported to increase the ratio of A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub>, which is a critical factor in the tendency of A $\beta$  aggregation [378-380], suggesting that PSs may influence the  $\gamma$ -secretase cleavage of APP. As no FAD-causing mutations have been identified in other  $\gamma$ -secretase substrates except APP, this suggests that the alteration of APP processing by mutations in PS is important for the development of AD.

In parallel with the identification of PSs as  $\gamma$ -secretases, AD-related PS mutations have been found to cause reduced  $\gamma$ -secretase activity with several different substrates [381-383], leading to a 'loss of function' hypothesis of the  $\gamma$ -secretase. This hypothesis is controversial, because the AD-associated mutations in PSs cause an increase in the ratio of A $\beta$ <sub>42</sub>/40 which would suggest a 'gain of function' of  $\gamma$ -secretase. However, recently, a model has been proposed to reconcile this paradox of 'loss' and 'gain' of  $\gamma$ -secretase function. Initially,  $\gamma$ -secretase cleaves APP in at least two positions. One is  $\gamma$ -site cleavage of APP and produces A $\beta$  peptides ranging from 39 to 43 amino acids. Another is  $\epsilon$ -site cleavage of APP which releases a long A $\beta$

(A $\beta$ 49) and a short AICD (AICD50). [173, 384]. Recent evidence indicates that the  $\epsilon$ -site cleavage of APP occurs before  $\gamma$ -site cleavage. The initial cleavage at the  $\epsilon$ -site generates A $\beta$ 48/49 which still contains the active site and is subsequently cleaved every three residues upstream until the  $\gamma$ -sites [385-388]. This hypothesis is supported by the identification of AICD sequence after  $\gamma$ -secretase cleavage, which shows that only two species of AICD, AICD50 and AICD51 (corresponding to A $\beta$ 49 and A $\beta$  48, respectively) are detected. These suggest that  $\epsilon$ -site cleavage is an early event in the  $\gamma$ -secretase cleavage of APP [388]. The model of successive  $\gamma$ -secretase cleavage of APP explains the ‘loss’ and ‘gain’ function of mutated PSs. FAD-mutations in PSs reduce the catalytic efficiency of the  $\gamma$ -secretase complex, and lead to proportionally more production of A $\beta$ 42 before it is further cleaved to shorter A $\beta$ s. Thus, a loss of function (reduced catalytic activity) could lead to a gain of function (increased A $\beta$ 42/40 ratio) of  $\gamma$ -secretase.

### *1.5.2 Late onset AD (sporadic AD)*

Late onset AD (LOAD) accounts for more than 95% of all AD cases and has an age of onset of at least 60-65 years old. The only gene that has been consistently found to be associated with LOAD is the apolipoprotein E (ApoE) gene [389-394]. However, other genes are also reported to be risk factors for the development of AD. The top four ranked genes which are associated with the risk of AD at the time of writing are reviewed and discussed below (for a complete list of the risk factors causing LOAD, see <http://www.alzgene.org/TopResults.asp> ).

### 1.5.2.1 ApoE

The human apoE gene is located on chromosome 19 and contains 4 exons which encode a 299 amino acid (34 kDa) glycoprotein [395]. ApoE is expressed in several organs, with the highest expression in liver, followed by the central nervous system (CNS). In the brain, apoE is mainly synthesised and released by astrocytes and microglia and it transports cholesterol and other lipids to neurons through low-density lipoprotein receptors (LDLRs) [396, 397]. Neurons also express apoE at very low levels under certain physiological and pathological conditions [398-402]. ApoE is polymorphic and has three major isoforms: apoE2, apoE3 and apoE4. These three ApoE isoforms differ from each other by one or two amino acids at position 112 and/or 158: apoE2 (cys112, cys158), apoE3 (cys112, arg158) and apoE4 (arg112, arg158). The most common apoE isoform is apoE3 followed by apoE4 and apoE2 [395]. These differences between apoE isoforms lead to different protein structure, lipid and receptor binding [403].

In general, apoE functions as a ligand for receptor-mediated endocytosis of lipoprotein particles. ApoE regulates transport, distribution and metabolism of lipids through apoE receptors [395, 404]. In the brain, apoE is the major apolipoprotein. ApoE-containing lipoprotein particles deliver lipids (e.g. cholesterol) and may be involved in supporting synaptogenesis and maintaining synaptic integrity [405]. In addition, apoE3, but not other apoE isoforms, may be involved in the stimulation of neurite outgrowth and synaptic plasticity [406-410].

In the early 1990s, apoE was identified as a major risk factor for AD [411, 412]. Subsequent studies showed that having one or two copies of the apoE4 allele increases the risk of AD by approximately 3- or 12-fold, respectively, compared to

individuals with no apoE4 alleles [413, 414]. It is important to note that while apoE4 allele increases the risk of AD, the apoE2 allele is associated with lower risk for developing AD [415-417]. However, apoE4 is not necessary and sufficient for development of AD [416-420], suggesting that other genes or risk factors may also contribute to increased risk of AD. In addition, although apoE isoforms are associated with increased risk and with onset age of AD, there is controversy over whether apoE alters the rate of cognitive decline of AD after disease onset [421, 422]. Some observations suggest that apoE4 may only have an influence at the early stage of the AD disease process [423, 424]. Further studies may be required to demonstrate a clear relationship between apoE and the rate of cognitive decline of AD.

In human studies, evidence indicates that apoE is associated with A $\beta$  deposition in the brain. Early on, apoE was co-localised with amyloid plaques in the brain [74, 425], and subsequent studies investigating the relationship between amyloid plaques and apoE suggested that there was a positive correlation between amyloid plaque burden and the number of apoE4 alleles [426, 427]. A recent study shows that fibrillar A $\beta$  burden in cognitively normal people is increased by the apoE4 gene dose [428], supporting the theory that apoE may accelerate amyloid plaque formation and that apoE carriers have more amyloid deposits although they are still cognitively normal. However, other studies also show conflicting results [429-431]. In addition, it is still unclear whether the protective role of apoE2 is mediated by reduced amyloid plaque formation [428] or whether some other mechanism, which is independent of the formation of AD pathology, is involved [432].

Whereas evidence from human studies suggest that apoE isoforms may have important roles in A $\beta$  deposition in the brain, the relationship between apoE isoforms

and A $\beta$  aggregation has also been examined in mouse models expressing human apoE isoforms. Early studies showed that APP transgenic mice lacking murine apoE have fewer amyloid deposits, cerebral amyloid angiopathy (CAA) and microhemorrhages also have a different anatomical distribution of amyloid plaques compared with the normal APP transgenic mice [433-436]. Furthermore, compared with APP transgenic mice lacking apoE or with murine apoE, the expression of human apoE delays amyloid plaque formation in an isoform-dependent manner (E4>E3>E2) [437, 438], suggesting that apoE isoforms may differently modulate the risk and pathology of AD. Further investigation of the mechanisms underlying the apoE-induced alteration in A $\beta$  pathology will be important for understanding the pathogenesis of AD.

From genetic and neuropathological studies in human and mouse models, it has been suggested that apoE and A $\beta$  may physically interact. Initial studies reported that lipid-free apoE can form SDS-stable complexes with synthetic A $\beta$ , and that apoE4 can bind to A $\beta$  more rapidly than apoE3 [439, 440]. Residues 244-272 of apoE and residues 12-28 of A $\beta$  are required for this interaction [440, 441]. However, further studies have revealed that lipid-associated apoE isolated from apoE transfected cell lines can also form a SDS-stable complex with A $\beta$ , and that this follows in the order apoE2 > apoE3 > apoE4 [442-445], suggesting that the lipidation status of apoE influences its ability to interact with A $\beta$ . Since the binding affinity between each of the apoE isoforms and A $\beta$  correlates inversely with the risk of developing AD, this suggests that apoE isoforms may be involved in the binding and clearance of A $\beta$  out of the CNS in vivo.

In addition to the studies of apoE and A $\beta$  binding, the role of apoE isoforms on A $\beta$  aggregation has also been examined in vitro. Studies demonstrated that apoE can

promote A $\beta$  aggregation in an isoform-dependent manner (E4 > E3 > E2) [446-448]. This is consistent with the increased amyloid plaque burden in both humans and mice carrying the apoE4 allele. However, other studies report that apoE can act as an amyloid nucleation inhibitor and they demonstrate that apoE inhibits A $\beta$  fibrillogenesis in vitro [449-452]. These conflicting findings may be due to differences in the preparation of the apoE (lipid free or lipid-bound) or A $\beta$  (A $\beta$ 40, A $\beta$ 42 or aggregation status of A $\beta$ ). Thus, further experiments should be carefully prepared and performed for a better understanding the effect of apoE isoforms on A $\beta$  aggregation. Moreover, it is interesting to note that several studies report that knockout of ATP-binding cassette transporter A1 (ABCA1), which is a cholesterol transporter involved in the lipidation of apoE, promotes amyloid plaque formation in APP transgenic mice without affecting APP processing to A $\beta$  [453-455]. However, overexpression of ABCA1 in APP transgenic mice inhibits A $\beta$  deposition in the brain [456]. Taken together, lipid status of apoE may be critical for amyloidogenesis of AD.

It is believed that impaired A $\beta$  clearance may be an important cause of LOAD [457]. There is evidence that apoE influences A $\beta$  clearance in the brain [458]. This could be achieved through two possible pathways. ApoE could form a complex with A $\beta$  and modulate the uptake and metabolism of A $\beta$  by cells through receptor-mediated endocytosis. In vitro studies show that apoE isoforms could enhance the internalisation of A $\beta$  [459-462]. In vivo studies also support this view and demonstrate that apoE promotes the proteolytic degradation of A $\beta$  by proteases (e.g. neprilysin) in an isoform- and lipidation-dependent manner [463]. ApoE may alternatively play important roles in A $\beta$  efflux across the blood brain barrier (BBB). Recent studies demonstrate that apoE can couple with A $\beta$  and slow A $\beta$  transport from

brain to blood compared to A $\beta$  alone [464, 465], and that apoE2-A $\beta$  and apoE3-A $\beta$  complex efflux is faster than that of the apoE4-A $\beta$  complex [458]. However, the opposite results have also been reported [466]. Further investigation may be required to demonstrate the precise role of apoE in A $\beta$  clearance in vivo. For instance, how and by what mechanism apoE isoforms modulate A $\beta$  clearance to different extents.

Many studies have studied the effect of apoE on APP processing and A $\beta$  production in culture systems. It has been reported that apoE can increase A $\beta$  production in an isoform-dependent manner when co-transfected with APP in cells [467, 468], while early studies showed that apoE has no effect on APP processing to A $\beta$  or that it may even reduce A $\beta$  production [469, 470]. These conflicting findings suggest that more work is needed to illustrate the role of apoE isoforms on APP processing and A $\beta$  production.

Although many studies focus on the effect of apoE isoforms on A $\beta$ -associated AD pathogenesis, other studies have provided evidence that apoE may be involved in A $\beta$ -independent AD pathogenesis. ApoE can be cleaved by a chymotrypsin-like serine protease and thereby generate a C-terminal-truncated form [471]. This C-terminal-truncated-form of apoE could directly induce neurotoxicity both in vitro and in vivo [471-473]. ApoE also has important roles in the phosphorylation and aggregation of tau in an isoform-dependent manner. Expression of either apoE4 or C-terminal truncated apoE4 leads to increased phosphorylation of tau and elevated tangle-like filaments in the brains of transgenic mice [471, 474]. Moreover, studies also suggest that apoE is isoform-dependently associated with the reduced glucose metabolism in AD brain, mitochondrial dysfunction in vitro and neuroinflammation in vitro and in vivo [472, 475-477].

In summary, apoE is associated with more than half of late-onset AD cases [419], and is believed to be a causative factor in AD pathogenesis. ApoE isoforms have multiple and diverse effects on AD pathology. Further investigation may be required for understanding the exact mechanism of how apoE isoforms participate in the progress of AD, and for developing therapeutic approaches for the treatment of AD based on apoE targets.

#### **1.5.2.2 Clusterin (apoJ)**

Clusterin or apoJ is another apolipoprotein that is also associated with LOAD [478]. The clusterin gene is located on chromosome 8 and encodes the 449-amino-acid clusterin precursor protein. Mature clusterin consists of approximately 80 kDa glycosylated heterodimers containing  $\alpha$ - and  $\beta$ -subunits linked by five disulfide bridges [479, 480]. Clusterin is expressed in almost all mammal tissues with the highest expression in brain than other tissues [481]. In brain, clusterin is mainly synthesised and secreted by astrocytes and neurons but not by microglia [482]. The primary function of clusterin is as a chaperone molecule, acting mainly in the extracellular space [481]. It also presents as a marker in responding to injuries and stress [483-485].

Initially, it was reported that the level of clusterin was elevated in the hippocampus of the AD patients [478]. Subsequent studies have confirmed this increase [486-488]. Moreover, clusterin is also found to co-localise with amyloid plaques but not with neurofibrillary tangles in the AD brain [488-490]. Recent studies demonstrate that the level of clusterin is also significantly increased in cerebrospinal fluid (CSF) of AD patients [491, 492]. In addition, plasma clusterin level is higher in AD patients



compared with non-AD individuals [493]. Subsequent studies have demonstrated that plasma clusterin levels are significantly associated with baseline prevalence and with severity of AD, but not with incidence of AD, suggesting that clusterin could be used as a potential biomarker of the presence, severity and risk of AD [494, 495].

Clusterin can interact with A $\beta$  both in vitro and in vivo [496, 497]. Later studies demonstrate that clusterin can also block the aggregation of A $\beta$  [498, 499]. Interestingly, the effect of clusterin on A $\beta$  aggregation is dependent on the ratio of clusterin and A $\beta$ . When a high molar excess (10-fold) of A $\beta$  is presented, clusterin can promote A $\beta$  aggregation [500, 501]. These findings indicate that clusterin could be an important factor in A $\beta$  fibrillogenesis. Knockout of clusterin in APP transgenic mice did not affect either the onset of A $\beta$  deposition or the level of brain A $\beta$  deposition compared to controls. However, knockout of clusterin significantly reduces fibrillar amyloid deposits and also decreases neuritic dystrophy associated with amyloid plaques [502]. This suggests that clusterin could influence amyloid plaque formation and neuritic toxicity in vivo. However, whether this occurs in human AD patients is unclear.

Similar to apoE, clusterin can also affect A $\beta$  clearance by promoting A $\beta$  uptake by cells. Studies show that aggregated A $\beta$  can increase the level of clusterin and then induce the endocytic uptake of A $\beta$  aggregates to astrocytes [503]. Alternatively, clusterin may form a complex with A $\beta$  and then increase A $\beta$  clearance from brain via megalin [464, 504].

In summary, clusterin has multiple potential roles in AD pathogenesis. Further studies are clearly required to demonstrate the exact role of clusterin in AD. Moreover,

clusterin is also an important marker in response to the pathology of AD. However, further investigation is needed to examine whether clusterin can be used as a reliable biomarker of AD.

### **1.5.2.3 Bridging integrator 1 (BIN1)**

The BIN1 gene is located on chromosome 2 and encodes several isoforms by alternate mRNA splicing in a cell type-specific manner [505-507]. Initially, BIN1 was reported to interact with the Myc oncoprotein and act as a putative tumor suppressor [508]. Subsequent studies showed that BIN1 is also associated with receptor-mediated endocytosis [509, 510], and that mutations in BIN1 disrupt its interaction with dynamin 2 which is involved in cell endocytosis [511]. However, BIN1-null mice do not have endocytosis defects, although they do have abnormal localization of the postsynaptic proteins, altered synaptic physiology and behavioural defects [512], indicating that BIN1 may act redundantly with other proteins in cell endocytosis.

In 2009, a genome-wide association study (GWAS) of AD revealed that BIN1 is associated with LOAD [513] and this finding was soon confirmed by another AD GWAS study in 2010 [514]. In 2011, several other GWAS studies reported the association of BIN1 with the risk of AD in different datasets [515-519]. Later studies have demonstrated that BIN1 is not associated with the levels of A $\beta$  and phosphorylated tau in CSF [520].

It is unclear how BIN1 is associated with AD pathogenesis. The YENTPY motif of APP is important for the clathrin-mediated APP endocytosis [175, 285], and deletion or mutation of this motif can reduce A $\beta$  secretion [284, 285]. As BIN1 has an

important role in cell endocytosis [509, 510], it is possible that BIN1 may have effects on A $\beta$  production and/or clearance. Moreover, while clathrin, dynamin and PICALM, which are involved in clathrin-mediated endocytosis, are up-regulated in APP transgenic mice, the level of BIN1 is unchanged. In addition, it is interesting to note that treatment of A $\beta$  causes impaired synaptic vesicle endocytosis and also leads to accumulation of BIN1 on cell surface [521]. Taken together, the role of BIN1 in AD progression is largely unexplored, further investigations are needed for understanding how BIN1 is associated with the risk of AD.

#### **1.5.2.4 ATP-binding cassette transporter A7 (ABCA7)**

ABCA7 belongs to the ATP-binding cassette (ABC) family of proteins and has the highest homology to ABCA1. The ABCA7 gene is located on chromosome 19 and encodes a 2146 amino-acid protein [522]. The ABCA7 gene is widely expressed in different species [522]. In humans, ABCA7 is highly expressed in the thymus, immune and hematopoietic tissues and brain [522, 523]. *In situ* hybridization studies show that ABCA7 is highly expressed in mouse hippocampal neurons [524]. ABCA7 is also expressed in human brain with the highest expression in microglia and neurons [525].

The role of ABCA7 in lipid transport is still not entirely clear. Some studies indicate that ABCA7 can promote the efflux of both cholesterol and phospholipids [523, 526, 527], while others reported that ABCA7 only promotes phospholipid efflux but not cholesterol to apolipoprotein A-I [528, 529]. Moreover, knockout of ABCA7 in mice does not cause defects in cholesterol and phospholipid efflux, suggesting that lipid transport may not be the primary function of ABCA7 [524]. Recently, studies suggest

a role of ABCA7 in the phagocytosis of apoptotic cell debris [530, 531]. As human microglia express the highest level of ABCA7 [525, 532], it is possible that ABCA7 also has important functions in phagocytosis of microglia in the brain.

In 2011, two GWAS of AD provide compelling evidence that ABCA7 is a susceptibility locus for AD [533, 534], and a later study also observed the association of ABCA7 with the risk of AD in an independent population [535]. It is unclear how ABCA7 is associated with the risk of AD. It has been hypothesised that ABCA7 may be associated with A $\beta$  production, clearance of A $\beta$  deposits, A $\beta$ -induced inflammation and A $\beta$ -induced neurotoxicity. However, further studies are required to explore the roles of ABCA7 in the development of AD.

## **1.6 Mouse models of AD**

Mouse models are widely used to study the pathogenesis of AD. Mice can selectively express proteins which are associated with AD (e.g. APP, tau, presenilin) and for this reason they can develop many features of AD [536]. APP transgenic mouse models have been valuable tools in AD research and have contributed to the development of potential therapeutic agents for the treatment of AD. APP transgenic mice also provide an important avenue for pathophysiology studies [537]. In addition, mouse models have also been used to investigate the function of APP family proteins and their proteolytic fragments. Many transgenic mouse models have been developed during the last two decades. However, none of them fully mimic the pathology of AD. APP transgenic mice can develop A $\beta$  deposits and memory loss but fail to replicate the neurofibrillary tangles and neuron loss seen in the human disease [538]. Here, the

major transgenic mouse models used in the AD research are summarised. For a more comprehensive list of AD mouse models, see <http://www.alzforum.org/res/com/tra/default.asp>.

### *1.6.1 Transgenic mouse models of AD*

In 1995, the PDAPP mouse line was generated [539]. PDAPP mice overexpress a human APP gene encoding the familial AD version of the protein containing a single mutation in which a valine is replaced by phenylalanine at residue 717 (Indiana mutation). The transgene contained a splicing cassette which allows for expression of three major isoforms of APP [539]. PDAPP mice have approximately a 5-fold increase in APP expression compared to wild-type mouse APP. The level of A $\beta$  is dramatically increased in the cerebral cortex and hippocampus of PDAPP mice. Amyloid deposit formation begins between 6 and 9 months of age in the hippocampus, corpus callosum and cerebral cortex and increases progressively with age [539]. PDAPP mice also develop other AD-like features, including dystrophic neuritic components, gliosis and loss of synaptic density [539]. However, no neurofibrillary tangles are observed [539, 540]. Behavioural tests demonstrate that PDAPP mice show cognitive deficits in learning and memory in an age- or plaque-dependent manner [541, 542]. For example, PDAPP mice present with deficits in visuospatial learning, sleep-wake states, thermoregulation and motor activity [543].

The Tg2576 mouse is one of the most widely used APP transgenic models and has been well studied [544]. Tg2576 mice overexpress the human APP695 isoform with the Swedish double mutation (K670N and M671L) and use the hamster prion protein promoter on a C57BL/6  $\times$  SJL background [545]. Tg2576 mice show a rapid increase

in A $\beta$  levels with age and start forming amyloid plaque from approximately 8 months of age [545, 546]. In addition to A $\beta$  deposits, Tg2576 mice also recapitulate A $\beta$ -induced microglial activation [547], oxidative stress [548, 549] and dystrophic neurites [550]. Tg2576 mice develop memory deficits and behavioural alterations, which are believed to be associated with the elevation of A $\beta$  levels [545]. However, Tg2576 mice have no significant loss of neurons [550] and do not show neurofibrillary tangle pathology.

As mutations in presenilin are associated with FAD, mouse models overexpressing mutated presenilin have also been developed. Although presenilin transgenic mice show an increase in A $\beta$  levels [551], no amyloid plaques are observed in these mice [379, 551]. Subsequently, double transgenic mice were developed that express both an APP and a presenilin transgene (APP/PS1 mice). Several different APP/PS1 mice have been developed by crossing APP transgenic mice with mice expressing mutated presenilin [552]. Mutations in presenilin lead to an increase in A $\beta$ <sub>42</sub> production and thus facilitate amyloid deposition. One of the most widely used APP/PS1 mouse lines has been created by crossing the Tg2576 mice with mice expressing presenilin with a M146L mutation [552]. This mouse line shows a 41% increase of A $\beta$ <sub>42</sub> before the amyloid deposits are present and the mice develop amyloid plaques at a very early age (approximately 2 to 3 months) [552, 553]. Generally, these APP/PS1 double transgenic mice show an early onset of AD-associated pathologies, including dystrophic synapses, hyperphosphorylated tau, gliosis and increased microglia activity [554]. Moreover, APP/PS1 mice also develop cognitive deficits at a very early age [555, 556].

The accumulation of hyperphosphorylated tau is an important feature of AD. Although the mutations in APP or presenilin result in amyloid plaque and neurofibrillary tangles in humans, and although APP transgenic mice have the ability to develop A $\beta$  deposits in the brain, it is unclear why the APP transgenic mice fail to produce neurofibrillary tangles. It is possible that the presence of A $\beta$  is not sufficient to induce tau-related pathologies in APP transgenic mice. Thus, it is important to understand the relationship between amyloid plaque and neurofibrillary tangles in AD progression. Initially, a number of single transgenic mice expressing wild-type human tau or mutant microtubule-associated protein tau (MAPT) were generated. These tau transgenic mice can produce neurofibrillary tangles and neuron loss in the brain, and also develop motor and behavioural deficits [557, 558]. It is interesting to note that in an inducible tau transgenic mouse model, suppression of mutant tau expression can rescue cognitive deficits and neuron loss even though neurofibrillary tangles continue to accumulate, suggesting that neurofibrillary tangles are not the primary determinant of the cause cognitive deficits and neuron loss [558]. Recently, transgenic mice expressing both APP and tau protein were generated by crossing Tg2576 mice with mice expressing mutant tau. Interestingly, these mice develop increased neurofibrillary tangles compared to tau transgenic mice, indicating that A $\beta$  may be involved in the development of neurofibrillary tangles [559, 560]. The association of A $\beta$  in tau pathology is also supported by a triple transgenic mouse model which expresses mutant APP, presenilin and tau. This mouse line develops both A $\beta$  and tau pathologies. However, the development of A $\beta$  deposits happens earlier than the tangle pathology [561]. This is consistent with the amyloid cascade hypothesis that tau pathology is a downstream effect of A $\beta$  production or deposits in AD. In summary, tau transgenic mice enable researchers to examine the role of tau pathology in AD or

other neurodegenerative disease. Moreover, the overexpression of tau in mice does not lead to A $\beta$  deposits, suggesting that the amyloid plaque is not a downstream symptom of tau pathology. However, it is still possible that A $\beta$  and tau may have a reciprocal relationship the development of AD.

Mice overexpressing various APP proteolytic products (A $\beta$ , C99, sAPP $\alpha$  and sAPP $\beta$ ) have also been generated for studying the toxic effects of these fragments or their functions in vivo. The overexpression of A $\beta$ 40 and A $\beta$ 42 in mouse in the absence of full length APP expression supports the view that A $\beta$ 42 is essential for amyloid plaque formation. While mice expressing high levels of A $\beta$ 40 alone fail to develop overt amyloid plaques, mice expressing A $\beta$ 42 can produce amyloid pathology [562]. The observation of neurotoxic effects of C99 in vitro has led to the generation of mice overexpressing C99 [563, 564]. C99 transgenic mice develop some neuropathological features vaguely resembling AD [565]. In addition, studies demonstrate that overexpression of C99 causes increased levels of A $\beta$  in the plasma even though there are no amyloid plaques developed in the brain [566]. Mice overexpressing sAPP $\alpha$  have also been created for the investigation of the trophic effects of sAPP $\alpha$  in vivo. The sAPP $\alpha$  knock-in mouse indicates that the expression of sAPP $\alpha$  can partially rescue deficits caused by the knockout of APP, suggesting that sAPP $\alpha$  may be sufficient to mimic the physiological functions of APP [567, 568]. Recently, studies show that a cleavage product of sAPP $\beta$  can bind to death receptor 6 and induce axon pruning and neuron death in vitro. A mouse line expressing sAPP $\beta$  has been generated for the investigation of the role of sAPP $\beta$  in vivo, in the absence APP and sAPP $\alpha$  [569]. The results indicate that sAPP $\beta$  is highly stable and is not normally cleaved in vivo. Moreover the expression of sAPP $\beta$  does not rescue the lethality and



neuromuscular synapse defects caused by the knockout of both APP and APLP2 [569], suggesting a distinct functional role of sAPP $\beta$  and sAPP $\alpha$  in vivo.

### *1.6.2 APP knockout mice*

In order to investigate the normal physiological function of APP family proteins, a number of APP or APLP1/2 knockout mice have been created. Initially, the APP single knockout mice were developed for exploring the functions of APP in vivo. APP knockout mice are viable and fertile but have reduced body and brain weight [570, 571]. They also suffer from increased frequency and severity of callosal agenesis and hippocampal commissure defects [571, 572]. These findings are consistent with the view that APP has an important role in neurite outgrowth or/and stem cell differentiation [186, 573]. APP knockout mice also demonstrate up-regulated levels of cholesterol, sphingolipid and copper in the brain [574, 575], and also show hyperinsulinaemia and decreased plasma glucose [279]. In addition, APP null mice have enhanced seizures [576], decreased locomotor activity and forelimb grip strength [570], suggesting a role for APP in neuronal and muscular function. However, no hippocampal neuron or synaptic bouton loss is observed in APP-null mice [577]. Behaviour studies show that APP knockout mice demonstrate learning and spatial memory defects which are possibly associated with impaired formation of long-term potentiation (LTP) [577-579]. The function of APLP1 and APLP2 in vivo has also been investigated by generating APLP1- or APLP2-null mice. Compared with APP knockout mice, no abnormalities are detected or observed in APLP1 or APLP2 knockout mice, and only APLP1-null mice have mild postnatal growth deficits [580,

581]. These findings suggest that there may be a functional redundancy within the APP family proteins.

In order to investigate the potential functional redundancies of APP family proteins, mice with combined knockout of APP family proteins have been created and examined. APP/APLP2 or APLP1/APLP2 double knockout mice are lethal shortly after birth [580, 581]. However, APP/APLP1 double knockout mice are viable and without compensatory elevation of APLP2 [581]. Moreover, APP/APLP1/APLP2 triple knockout mice are postnatally lethal. These results strongly suggest that there is a functional redundancy between APLP2 and the other two APP family members [581].

In summary, APP/APLP1/2 knockout mice provide a new avenue for the study of the function of APP. These loss-of-function studies indicate that APP and its homologues have essential functions in the development of both the peripheral and central nervous system. However, it is not clear whether the functions of APP homologues are mediated by the full-length proteins or by their proteolytic products. Thus, further investigation may be needed to examine the functions of proteolytic products of APP family members.

## **1.7 Current and prospective AD therapies**

Currently, there is no treatment that can slow or stop the progression of AD. During last two decades, the U.S. Food and Drug Administration (FDA) has approved five drugs for improving the symptoms of AD [582]. However, none of these drugs is able to alter the underlying causes of AD, and despite marginal symptomatic improvement,

these drugs are only effective in a subpopulation of patients. Therefore, development of therapeutic agents for AD treatment is a vital task for current research. Here, the current and prospective therapeutic approaches for AD are reviewed.

### *1.7.1 Current therapeutic approaches of AD*

#### **1.7.1.1 Acetylcholinesterase (AChE) inhibitors**

Initially, post-mortem studies showed that there is a loss of cholinergic neurons in the basal forebrain of AD patients [21, 583, 584]. Further studies of brain tissues from AD patients showed a reduction in choline acetyltransferase in the neocortex [585]. The finding of a cholinergic dysfunction led to the development of the “cholinergic hypothesis of geriatric memory dysfunction” [586]. The cholinergic hypothesis suggests that the loss of cholinergic neurons leads to dysfunction of cholinergic transmission in the brain and ultimately this dysfunction causes cognitive decline in AD individuals [586]. The cholinergic hypothesis provides a rationale for developing AChE inhibitors for AD treatment.

Acetylcholine is a neurotransmitter that can be rapidly degraded by AChE after its release into the synaptic cleft [587]. Inhibition of AChE causes an increase of acetylcholine level in the synaptic cleft and thus enhances cholinergic transmission and ameliorates cholinergic dysfunction in AD patients [588]. AChE inhibitors, including tacrine, donepezil, rivastigmine and galantamine, were the first compounds approved by FDA for treatment of AD [582]. All these four AChE inhibitors are approved for treatment of mild to moderate AD, as studies report that donepezil, rivastigmine and galantamine can effectively improve the cognitive symptoms at an

early stage of disease [589-593]. However, there are potentially some major side effects related to the gastrointestinal system [594]. Moreover, tacrine is now rarely used in clinical for the treatment of AD because of its unconfirmed efficacy and side effects [595-597]. Although AChE inhibitors may improve the cognitive symptoms in AD patients, the efficacy of these drugs decreases as AD progresses [594]. In addition, donepezil is reported to inhibit A $\beta$ -induced toxicity in vitro, suggesting that donepezil may not only increase cholinergic transmission but also is protective to neurons in the treatment of AD.

#### **1.7.1.2 Memantine**

Excessive activation of NMDA receptors can cause influx of Ca<sup>2+</sup> and lead to excitotoxicity and neuronal cell death [598]. In AD, *N*-methyl-*D*-aspartate (NMDA) receptor-mediated excitotoxicity may mediate neuron death in the brain [122, 599]. NMDA receptors are also known to play a role in synaptic plasticity which is altered in AD [600, 601]. Thus, it seems logical to use NMDA receptor antagonists for the treatment of AD.

Memantine, which was initially used in the treatment of Parkinson's disease, is the only NMDA receptor antagonist approved by FDA for the treatment of moderate to severe AD [602, 603]. Memantine is a non-competitive NMDA receptor antagonist and interacts with the NMDA receptors with moderate affinity at therapeutic concentrations [604]. Several studies indicate that memantine is well tolerated by patients and that it can improve cognitive function in patients with moderate to severe AD [603, 605]. In addition, memantine can be used in conjunction with the AChE inhibitor, donepezil, without adverse drug interaction, and this combination therapy

can significantly ameliorate the course of AD [603]. Interestingly, a recent study showed that AD triple transgenic mice treated with memantine have improved cognition, reduced A $\beta$  deposition and decreased hyperphosphorylated tau, indicating that memantine may have multi-targets and could modify disease progress in vivo [606].

### *1.7.2 Prospective therapeutic strategies in AD*

In recent years, a number of compounds acting on different mechanisms have been developed with the aim of modifying disease progression. Some of these compounds have reached the late stage of clinical trials in human and raise hope in the treatment of AD in the near future. These compounds can be simply divided into several different classes as reviewed below (Table 1.1).

#### **1.7.2.1 A $\beta$ aggregation inhibitors**

One of the hallmarks of AD is the presence of amyloid plaques in the brain, thus targeting A $\beta$  aggregation may have therapeutic potential for the treatment of AD. A number of endogenous molecules have been reported to inhibit A $\beta$  aggregation, including apoE [446-448], apoJ [498, 499], transthyretin (TTR) [607], the receptor associated protein (RAP) [608], proteoglycans (PGs) and glycosaminoglycans (GAGs) [609-612], phospholipids [613, 614], and metal ions [615, 616]. Targeting the interaction between these molecules and A $\beta$  to inhibit A $\beta$  aggregation may have therapeutic benefits for the treatment of AD. Synthetic compounds are also reported to influence aggregation of A $\beta$ . For instance, tramiprosate (Alzhemed<sup>®</sup>), a

glycosaminoglycan mimetic, can bind to soluble A $\beta$  and thereby inhibit its aggregation in vitro and in vivo [617]. In clinical trials, the initial phase II studies showed that administration of tramiprosate was well tolerated and could decrease CSF A $\beta$ 42 level, and also stabilise cognitive function [618]. However, the phase III clinical trials were discontinued in 2008 because of lack of efficacy.

It is important to note that it is still unclear which species of A $\beta$  aggregate contribute to A $\beta$ -induced toxicity. Recently, evidence suggests that the soluble oligomeric form of A $\beta$  is responsible for A $\beta$ -associated neurotoxicity [619-622]. Thus, A $\beta$  aggregation inhibitors could inhibit the formation of A $\beta$  fibril but may elevate the formation of oligomeric A $\beta$  species, which may be more toxic in vivo.

**Table 1.1 Selected Alzheimer's disease drug development programs**

<b>Drug name</b>	<b>Description</b>	<b>Company</b>	<b>Phase</b>
<b>Tramiprosate/ Alzhemed<sup>TM</sup></b>	Glycosaminoglycan mimetic, A $\beta$ antagonist, inhibits the aggregation of A $\beta$	Alzhemed (QU)	Discontinued
<b>AN1792/ AIP 001</b>	Synthetic aggregated A $\beta$ 42, active immunization	Elan Pharmaceuticals	Discontinued
<b>ACC-001</b>	Humanized monoclonal anti-A $\beta$ antibody, active immunization	Elan Wyeth	Phase II
<b>AAB-001/ bapineuzumab</b>	Humanized monoclonal anti-A $\beta$ antibody, passive immunization	Elan Wyeth	Phase III
<b>CTS-21166</b>	$\beta$ -Secretase inhibitor	CoMentis	Phase I complete
<b>r-Flurbiprofen/ tarenflurbil</b>	$\gamma$ -Secretase modulator	Myriad Pharmaceuticals Inc.	Discontinued
<b>LY450139/ semagacestat</b>	$\gamma$ -Secretase inhibitor	Eli Lilly	Discontinued

### 1.7.2.2 A $\beta$ immunotherapy

Since A $\beta$  plays a central role in AD pathogenesis, several types of A $\beta$  immunotherapy for the treatment of AD are under investigation. There are two different types of A $\beta$  immunotherapy: active immunotherapy and passive immunotherapy.

Active immunotherapy is achieved by immunising with synthetic intact A $\beta$  or synthetic A $\beta$  fragments conjugated to a carrier protein, to induce an immune response to A $\beta$ . In some pioneering studies, APP transgenic mice immunised with A $\beta$  have fewer amyloid plaques, less neuritic dystrophy, astrogliosis and improved cognitive function [623, 624]. The first clinical trials of A $\beta$  immunotherapy using human aggregated A $\beta$ 42 (AN1792) as the antigen found the treatment to be well tolerated and to elicit a positive antibody response in more than half of the patients [625]. However, this immunotherapy was terminated in phase II clinical trials due to the occurrence of meningoencephalitis in treated patients [625, 626]. This side effect of A $\beta$  immunotherapy was possibly due to a T-cell mediated autoimmune response [627]. Subsequently, another active immunotherapy treatment with fragments conjugated with a carrier protein has been developed. Some A $\beta$  fragments only have the B-cell epitopes and thus avoid A $\beta$ -induced T-cell response [628, 629]. ACC-001 is an A $\beta$ (1-6) fragment linked to a carrier protein. It has been found to be well tolerated and to increase A $\beta$  clearance in CNS in a phase I study. Phase II clinical trials are currently ongoing [629]. Recently, passive immunotherapy, which administrates the antibodies directly against A $\beta$ , has also been under investigation [630]. The first synthetic antibody against A $\beta$  in clinical trials is bapineuzumab (AAB-001). Phase III clinical trials have been initiated because of the finding of reduced A $\beta$  level and less brain volume loss in the phase II studies [631, 632]. In



addition, another humanised monoclonal antibody, solanezumab, is now under phase III clinical trials. The early studies indicate that it is well tolerated and has a dose-dependent effect on plasma and CSF A $\beta$ , although changes in cognitive function were not observed [633].

Several mechanisms are proposed for A $\beta$  immunotherapy. It is possible that A $\beta$  antibodies can bind to amyloid plaques and induce phagocytosis of amyloid by microglia. This view is supported by follow-up studies of AN1792-treated patients in which patients who develop anti-AN1792 titers have fewer amyloid plaques and more activated microglia [634, 635]. However, it is also possible that A $\beta$  antibodies may bind to A $\beta$  and prevent the formation of amyloid plaques [636]. In addition, studies in APP transgenic mice demonstrate that peripheral administration of an A $\beta$  antibody can rapidly increase the level of A $\beta$  in CSF, part of which may not due to the entry of the antibody into the CNS. These results suggest that treatment with A $\beta$  antibodies may alter the brain-plasma equilibrium of A $\beta$  and shift A $\beta$  from the CNS to the plasma [637]. In summary, A $\beta$  immunotherapy provides a promising approach for the treatment of AD, and also provides further support for the A $\beta$  hypothesis of AD. Further studies may need to optimise the A $\beta$  antigens and minimise unwanted immune response.

#### **1.7.2.3 Suppression of A $\beta$ production**

A $\beta$  is generated from APP by sequential  $\beta$ -secretase and  $\gamma$ -secretase cleavage. The direct involvement of these two enzymes in A $\beta$  generation makes them important targets for developing drugs for the treatment of AD.

#### *1.7.2.3.1 $\beta$ -Secretase inhibitors*

BACE1 initiates the first cleavage of APP in the generation of A $\beta$ . In animal studies, knockout of BACE1 produces a significant decrease in A $\beta$  generation and does not lead to severe phenotypic defects [321]. Moreover, knockout of BACE1 can rescue memory deficits in APP transgenic mice [322, 323]. These findings indicate that BACE1 could be a promising target for drug development. Although compounds based on the inhibition of BACE1 are under investigation [638], the development of agents inhibiting the BACE1 active site is problematic, as BACE1 has a large active site. This means designed protease inhibitors are too large to cross the blood-brain barrier (BBB) [639]. CTS-21166 is the first  $\beta$ -secretase inhibitor tested in clinical trials. The phase I studies report that CTS-21166 is well tolerated and can reduce the plasma A $\beta$  concentration [640, 641]. However, although CTS-21166 can cross the BBB, it is also possible that the reduced plasma A $\beta$  is caused by peripheral inhibition of BACE rather than inhibition of central BACE1.

The main concern in the development of BACE1 inhibitors is that BACE1 has multiple substrates *in vivo*. One of the BACE1 substrates is neuregulin-1 which is associated with the regulation of myelination in both CNS and PNS [326]. The deletion of BACE1 in mice leads to hypomyelination and impairs sciatic nerve remyelination [326, 642]. Thus, it is desirable to develop BACE1 inhibitors which may avoid other substrates and has specific inhibition of APP cleavage.

#### 1.7.2.3.2 $\gamma$ -Secretase inhibitors

After  $\beta$ -secretase cleavage, the C-terminal fragment of APP is subjected to  $\gamma$ -secretase cleavage to liberate A $\beta$ . Moreover,  $\gamma$ -secretase can also regulate the ratio of A $\beta$ 40 and A $\beta$ 42 production, which is an important factor in AD pathology. Thus, many compounds targeting  $\gamma$ -secretase and aiming to inhibit the release of A $\beta$  or modulate the generation of A $\beta$ 42 are being developed.

r-Flurbiprofen (tarenflurbil) is a non-steroidal anti-inflammatory drug (NSAID) derivative that can selectively modulate  $\gamma$ -secretase activity to lower the production of A $\beta$ 42 in favour of the shorter form of A $\beta$  that may be less toxic [643]. In phase II clinical trials, r-flurbiprofen was well tolerated and slowed the decline in daily living activities and global function in patients with mild AD, while it has no significant effect in patients with moderate AD [644]. Subsequent phase III clinical trials in patients with mild AD have been discontinued due to failure to prove efficacy [645], which may be due to the low brain penetration [646]. More recently, LY450139 (semagacestat), a  $\gamma$ -secretase inhibitor from Eli Lilly, is reported to be well tolerated and can decrease the plasma and CSF A $\beta$  concentration but has no effect on cognitive functions [647, 648]. However, LY450139 was terminated in phase III clinical trials due to the severe side effects including gastrointestinal symptoms, skin cancer or even worsening of cognitive functions [649, 650].

Similar to BACE1,  $\gamma$ -secretase is also involved in other proteolytic events. For example,  $\gamma$ -secretase can also cleave Notch, which is an important receptor involved in development and differentiation [651]. Disturbing the Notch signalling pathway could cause cancer [652] and intestinal goblet cell metaplasia [653] which may be an

explanation for the side effects caused by LY450139 treatment [649]. These non-selective functions of  $\gamma$ -secretase indicate that developing  $\gamma$ -secretase inhibitors which could specifically inhibit APP cleavage to A $\beta$  but have little or no effect on other  $\gamma$ -secretase substrates may be required.

#### *1.7.2.3.3 Statins*

Several lines of evidence indicated cholesterol metabolism is associated with APP processing to A $\beta$  [654]. Decreasing cholesterol could reduce A $\beta$  production in vitro and in vivo [655, 656]. In humans, treatment with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), a class of drugs used to inhibit cholesterol production, is reported to lower CSF and brain A $\beta$  levels and reduce the risk of developing dementia [657, 658]. In addition, diet-induced hypercholesterolemia in APP transgenic mice causes increased accumulation of A $\beta$  in the brain [659, 660]. Taken together, these findings suggest an important role for cholesterol in APP processing and A $\beta$  deposition in AD progression. However, in clinical trials, statins did not show positive effects in preventing AD or dementia compared to placebo [661, 662], suggesting that use of statins may be futile in the treatment of AD and further investigation may require to identify the exact relationship between cholesterol and AD pathogenesis.

#### **1.7.2.4 Non-A $\beta$ -based approaches**

In addition to the amyloid-modifying compounds, other therapeutic approaches including anti-inflammatory drugs, neuroprotective or neurotrophic agents, tau

phosphorylation inhibitors, antioxidants and mitochondrial neuroprotectants are under investigation or in the clinical trials, and these approaches may provide promising prospects in the treatment of AD [588, 663].

In summary, currently, there are limited therapeutic approaches for the treatment of AD in clinical and the existing therapies only improve the symptoms of AD and eventually lose efficacy as the disease progresses. Thus, the development of novel therapeutic strategies which could slow the disease progress or/and improve the cognitive function is now a primary focus of academic and industrial research. The prospective approaches summarised above may possibly lead to the development of drugs which are safe and effective for the treatment of AD.

## **1.8 Proteoglycans**

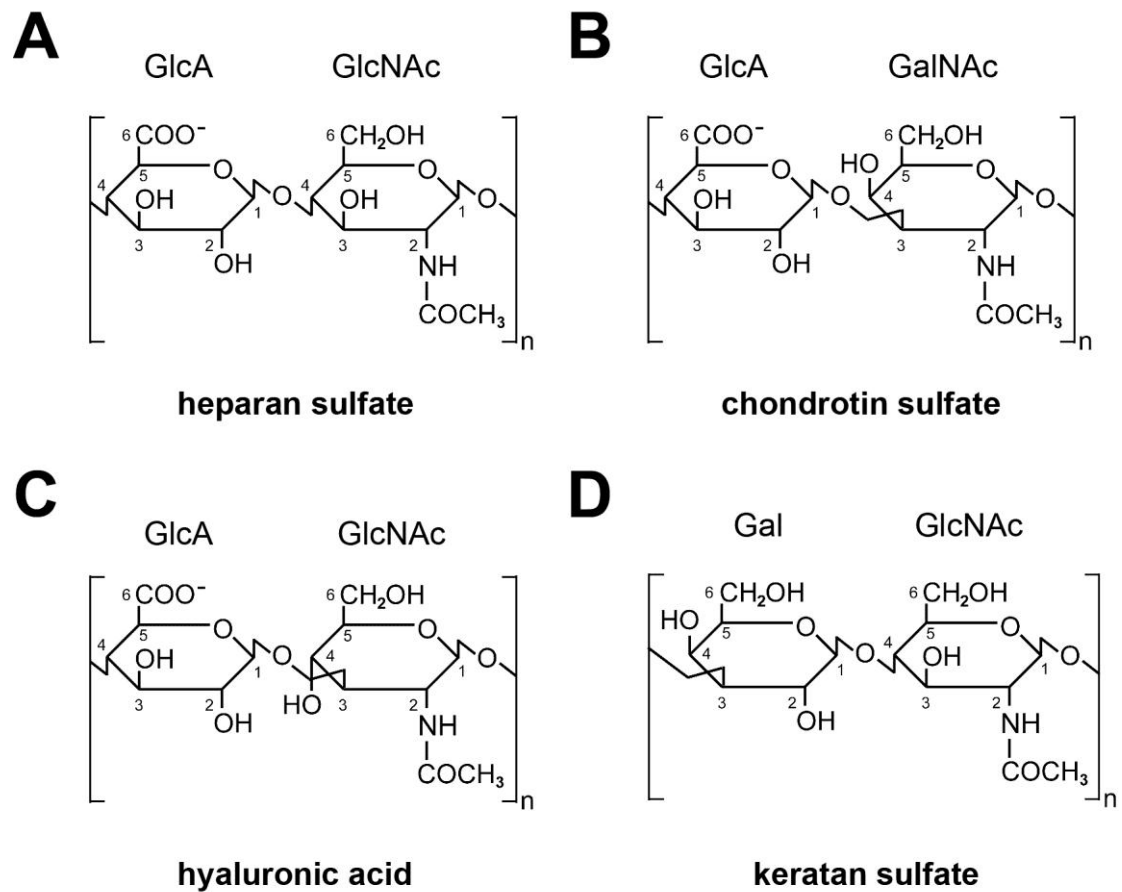
Proteoglycans (PGs) are high molecular weight complexes consisting of a core protein covalently attached to glycosaminoglycans (GAGs). PGs are mainly located on the cell surface or in the extracellular matrix, and participate in many important biological processes [664]. Here, the structure of GAGs and the major functions of heparan sulfate proteoglycans (HSPGs) and chondroitin sulfate proteoglycans (CSPGs) in the brain are discussed.

### *1.8.1 Structure of proteoglycans and glycosaminoglycans*

GAGs can be classified into several types according to their carbohydrate backbones which mainly include heparan sulfate (HS), chondroitin sulfates (ChSs), dermatan sulfate (DS), hyaluronic acid (HA) and keratan sulfate (KS).

Heparin is a linear polysaccharide with a repeating disaccharide unit of 1→4 linked uronic acid and glucosamine residues [665]. The uronic acid normally consists of approximately 90% iduronic acid and 10% glucuronic acid [666]. The 3- and 6-position of glucosamine can either be sulfated or remain unsubstituted, and the 2-position amino group of glucosamine residue can be sulfated, acetylated or unsubstituted. In addition, the 2- and 4-position of uronic acid can be substituted with a sulfate and a carboxyl group, respectively [666]. Indeed, the average sulfation degree of heparin is approximately 2.7 sulfate groups per disaccharide unit [667]. These sulfate groups and carboxyl groups make heparin highly negatively charged.

Heparan sulfate (HS) is a heparin analogue and has similar structures to heparin. The uronic acid residue of HS is mainly glucuronic acid but also contains iduronic acid (Fig. 1.3 A). The sulfation degree of HS is much less than heparin with an average of approximately 1 sulfate group per disaccharide unit [668]. In addition, heparin is predominantly synthesised in mast cells and basophils [669], while HS is expressed by almost all cell types [670].



**Fig. 1.3.** The repeating disaccharide units of GAGs. GlcA, glucuronic acid; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Gal, galactose.

Chondroitin sulfates (ChSs) and dermatan sulfate (DS) are composed of a repeating disaccharide unit in which N-acetylgalactosamine (GalNAc) links through 1→4 or 1→3 linkage to either glucuronic or iduronic acid [671] (Fig. 1.3 B). The ChS and DS can be sulfated to different degrees and at varying positions depending upon the tissue source. Sulfation at the 4- or 6- position of the GalNAc residue forms ChS A and ChS C, respectively. The sulfation at the 2-position of glucuronic acid and 6-position of GalNAc forms ChS D. ChS can also be sulfated at the 4- and 6-position of the GalNAc to form ChS E. DS has a sulfation group at the 2-position of the iduronic acid [671].

Hyaluronic acid (HA) comprises a repeating unit of GluNAc and glucuronic acid (GlcA) (Fig. 1.3 C). HA is not sulfated and does not attach to a core protein. In addition, HA is the only GAG which is synthesised on the cell surface [672].

Keratan sulfate (KS) is also a linear polymer containing a repeating disaccharide unit of N-acetylglucosamine (GluNAc) and galactose with the sulfation occurring on the 6-C of both sugar residues [673] (Fig. 1.3 D). KS can be further classified into three different types according to the tissue source or the different linkage between KS and the core protein [674, 675].

### *1.8.2 Synthesis and modification of proteoglycans*

The synthesis of GAGs starts with the attachment of a tetrasaccharide (xylose-galactose-galactose-uronic acid) to a core protein by several enzymes including xylosyl transferase, galactosyltransferase I, galactosyltransferase II and glucuronyltransferase I [676]. Next, in the case of ChSs and DS, the GAG chain is



elongated by attachment of GalNAc and GlcA repeating disaccharide units. Alternatively, attachment of GluNAc and GlcA repeating disaccharide units can generate HS or heparin. Several enzymes can catalyse proteoglycan GAG chain elongation. The polymerisation of HS is catalysed by the exostosin (EXT) family proteins including EXT1, EXT2, EXTL1, EXTL2 and EXTL3 [677, 678]. However, the elongation of ChS and DS require the chondroitin sulfate synthase complex [679].

After GAG chain polymerisation, HS undergoes several modifications including 5-position epimerisation of GlcA and several sulfations. Initially, HS is modified by replacement of acetyl group with a sulfate group by *N*-deacetylase/*N*-sulfotransferases (NDSTs) [680, 681]. This is a key step in the synthesis of HS and is also necessary for further structure modification. Then, GlcA residues of HS can be epimerised to iduronic acid (IdoA) by an epimerase [682]. After epimerisation, HS undergoes 2-position sulfation of uronic acid residues and 6- and 3-position sulfation of GluNAc by sulfotransferases. The sulfation of ChS is mainly on the 4- and 6 position of GalNAc by chondroitin sulfotransferases and generates different types of ChS. The conversion of GlcA residues to IdoA residues generates the DS that also can be sulfated at the 4-position of GalNAc residues.

### *1.8.3 Expression of proteoglycans in the brain*

#### **1.8.3.1 Chondroitin sulfate proteoglycans (CSPGs)**

CSPGs are the most abundant PGs in the mammalian CNS, and can be classified into several different types (Fig. 1.4B). Four members of the lectican family, including aggrecan [683], versican [684], neurocan [685] and brevican [686], have been

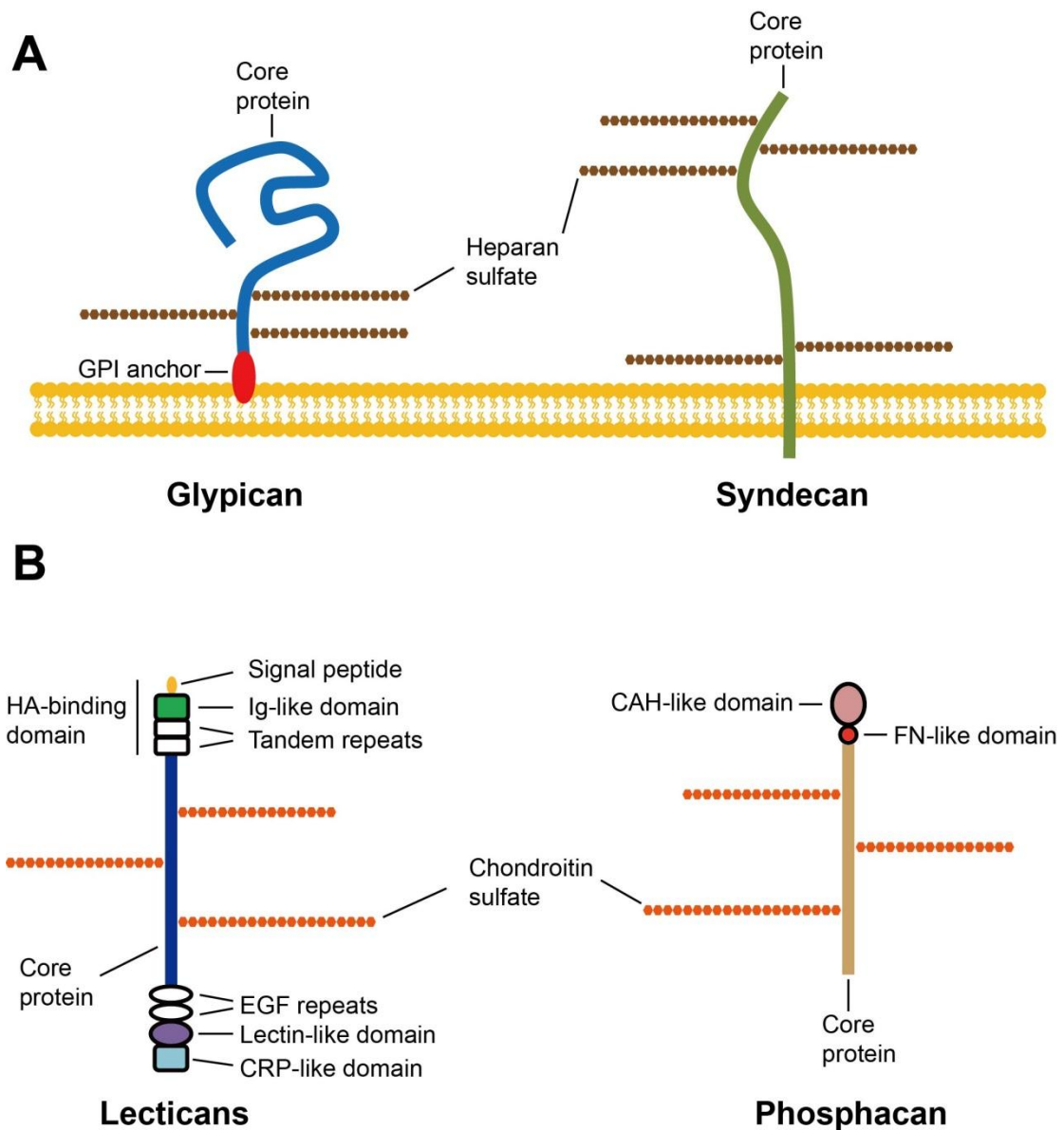
identified as CSPGs in the extracellular matrix of brain. Lecticans consist of a core protein ranging from 80 to 400 kDa with the presence of globular domains at the N-terminal and C-terminal ends and their sequences are highly homologous. The core proteins contain an N-terminal HA-binding domain which has important roles in the formation of extracellular matrix. In the C-terminal domain of the core protein, there are two EGF-like repeats, a C-type lectin domain and complement regulatory protein (CRP)-like domain. The central domain of the core proteins contains attachment sites for ChSs, and the numbers of potential ChSs attachment sites varies greatly among the lectican family members [687] (Fig. 1.4B).

Phosphacan and receptor-type protein-tyrosine phosphatase  $\beta$  (RPTP $\beta$ ) are typical CSPGs in the brain [688]. RPTP $\beta$  is a transmembrane proteoglycan and its core protein sequence has an N-terminal carbonic anhydrase-like domain, followed by a fibronectin type III domain, a chondroitin sulfate attachment region, a transmembrane segment and two intracellular tyrosine phosphatase domains [688]. Phosphacan is a secreted CSPG and is generated from the alternative splicing of RPTP $\beta$ . It has the same structure as the extracellular sequence of RPTP $\beta$  but lacks the two intracellular tyrosine phosphatase domains [689] (Fig. 1.4B). The alternative splicing of RPTP $\beta$  and phosphacan can generate a short form of both proteins that have less ChS attachment sites compared to full-length forms.

Decorin and biglycan are small leucine-rich proteoglycan family members and have a core protein containing leucine-rich repeats in the central region [690]. The leucine-rich repeats usually account for the majority of the core protein sequence and are flanked by cysteine clusters that may form disulfide bonds [690]. The N-terminal of decorin and biglycan contain one (decorin) or two (biglycan) ChS binding sites [688].

Melanoma-associated chondroitin sulfate proteoglycan (MCSP) or NG2 is a transmembrane CSPG and does not show significant sequence homology with other proteoglycans [691]. The expression of MCSP is developmentally regulated with the highest expression in immature, proliferating cells [691]. MCSP has a large ectodomain, followed by a transmembrane region and short cytoplasmic domain. The ectodomain can be further divided into three domains: the globular N-terminal cysteine-containing domain that is stabilized by several disulfide bonds, the central serine-glycine-containing domain that comprises of ChS attachment sites and another cysteine-containing domain [692].

In addition, several proteins such as neuroglycan C, thrombomodulin and APP can serve as “part-time” CSPGs. Neuroglycan C, which is predominantly expressed in the brain, is a transmembrane glycoprotein that contains an EGF-like extracellular domain with ChS attachment sites located at the N-terminal region [693]. Neuroglycan C can act as a proteoglycan with a single ChS chain attached during the development of CNS. However, in the mature CNS, it mainly exists in a non-proteoglycan form [694]. APP can also exist as the core protein of a CSPG called appican which is mainly expressed by glia cells in the brain [695]. Appican is generated by splicing out of exon 15 of APP mRNA, which forms the appropriate consensus sequence and allows a single ChS chain attachment [696]. The biological function of this ChS attached form of APP is unclear. However, evidence suggests that the attachment of ChS to APP may affect the processing of APP to A $\beta$  [697].



**Fig. 1.4.** The structure of representing CNS proteoglycans: heparan sulfate proteoglycans (A) and chondroitin sulfate proteoglycans (B). GPI, glycosylphosphatidylinositol. HA, hyaluronic acid. Ig, immunoglobulin. EGF, epidermal growth factor. CRP, complement regulatory protein. CAH, carbonic anhydrase. FN, fibronectin.

### **1.8.3.2 Heparan sulfate proteoglycans (HSPGs)**

HSPGs are present in all animal tissues and contain diverse core proteins with the HS chains attached. HSPGs can be simply classified into two types including cell surface HSPGs and extracellular matrix HSPGs [698]. The syndecan family includes four members (syndecan 1-4), which are the typical HSPGs. All syndecans are comprised of an N-terminal signal peptide, followed by a large ectodomain, a transmembrane domain, and a comparatively short cytoplasmic tail [699]. The HS attachment sites are located either on both the N-terminal and the C-terminal of the ectodomain (syndecan 1 and 3) or on the N-terminal of the ectodomain only (syndecan 2 and 4) [700] (Fig. 1.4A). The ectodomains of the syndecan family members only have a low amount of amino acid sequence homology, while the cytoplasmic domains are highly conserved [701]. The ectodomain of syndecans can undergo proteolytic cleavage by sheddases and generate a C-terminal fragment that can be further cleaved by the  $\gamma$ -secretase complex [702, 703].

The glypican family of HSPGs are linked to glycosylphosphatidylinositol (GPI) anchors which intercalate to the cell surface. The glypican family has six members (glypican 1-6), with molecular sizes ranging from 550 to 580 amino-acid residues [704]. Structurally, all glypicans contain an N-terminal signal peptide and a C-terminal hydrophobic domain that is required for the linkage of HSPG to the GPI anchor [705]. The HS attachment sites of glypicans are mainly located at the C-terminus of the core proteins, close to the GPI anchor or the cell surface [704] (Fig. 1.4A). In addition, most glypicans undergo proteolytic cleavage by a furin-like convertase which cleaves at the C-terminal end of the ectodomain, generating two subunits linked to each other by several disulfide bonds [706].

Perlecan (HSPG2) is a secreted and a basement membrane-associated HSPG [707]. Perlecan consists of a large core protein with a molecular weight at approximately 470 kDa. The structure of perlecan can be divided into five distinct domains. The N-terminal domain I contains the sperm protein-enterokinase-agrin (SEA) module and also HS attachment sites. The domain II contains four EGF-like domains. Domain III consists of laminin alpha-chains including three laminin IV-like modules followed by three sets of laminin EGF-like repeats. The large domain IV contains 21 immunoglobulin-like repeats. The domain V of perlecan consists of three laminin G-like domains followed by two sets of EGF-like domains. Additionally, the fifth domain of perlecan also contains HS attachment sites [708]. In addition to perlecan, other extracellular matrix-located HSPGs that have been identified include agrin [709], collagen XVIII [710] and the testican proteins [711].

#### *1.8.4 Function of proteoglycans in the brain*

ChSs and HS, which are the main proteoglycan components in the brain, are involved in many stages in mammalian CNS development [712]. Recent studies demonstrate that PGs may have important roles in the developing and mature brain by interacting with many growth factors, chemokines, morphogens, axon guidance molecules and extracellular matrix proteins [712]. In addition, PGs also have important roles in brain injury and neural regeneration [713].

### **1.8.4.1 Function of proteoglycans in the CNS development**

#### *1.8.4.1.1 Chondroitin sulfate proteoglycans*

During brain development, CSPGs interact with various proteins and are closely associated with cell adhesion, cell migration, neurite formation and elongation, synaptogenesis, axon guidance and barrier formation [714]. During CNS maturation, changes occur in both the level and composition of CSPG [715].

CSPGs have important roles in the formation of neural cell migration patterns [716]. In particular, several studies report that the migration patterns of neural crest cells are correlated with distribution patterns of CSPG. CSPG is predominantly located in the regions where neural crest cells do not migrate, suggesting an important role of CSPG is in restricting the regions conducive to migration [717]. Inhibition of CSPG synthesis alters the migration patterns of neural crest cells [717]. In vivo studies demonstrate that implantation of micro-membrane-bound aggrecans into the neural crest cell migration pathways can induce neural crest cells to spatiotemporally deviate from their normal migratory trajectory [718].

CSPGs are reported to act as barriers to axonal growth [719-722]. Studies indicate that extending axons avoid tissues with high CSPG expression and chemical removal of the ChS chains from CSPGs abolishes this phenomenon, suggesting that the GAG components of CSPG are associated with the axon guidance [719-722]. In vitro, CSPGs are inhibitory to both cerebellar granule neuron and dorsal root ganglion (DRG) axonal growth [723-725]. These findings further indicate that CSPG may participate in axonal guidance by restricting axonal extension of the developing neurons [726]. Further investigations suggest that specific sulfation patterns of CSPG affect axonal guidance in different ways. ChS sulfated at the 4-position (ChS A), but

not 6-position (ChS C), is a negative guidance cue for axons of developing neurons. Knockdown of 4-*O*-sulfotransferase, which is involved in the 4-position sulfation of ChS, significantly enhances axonal growth [727]. However, knockdown of 6-*O*-sulfotransferase, the enzyme responsible for the 6-position sulfation of GalNAc residues of ChS, has no effect on axonal outgrowth [727].

In vitro and in vivo studies also suggest a role of CSPGs in axon guidance during the development of the visual system. The evidence demonstrates that retinal axons can grow into the region which is normally repulsive to axons after treatment with chondroitinase [728]. Moreover, CSPGs are also reported to regulate the elongation of retinal ganglion cell (RGC) axons and the expression of CSPGs is undetectable after RGC axon elongation in the retina is complete [729]. Therefore, these findings suggest that CSPGs have an important role in the axon guidance and growth during brain development.

In situ hybridization histochemistry studies show that CSPG (particularly phosphacan) is mainly confined to regions of active cell proliferation, suggesting that CSPG may be involved in the cell proliferation in the developing CNS [730]. This idea is supported by the observation that ChS polysaccharides can promote the fibroblast growth factor-2-mediated proliferation of neural stem or progenitor cells [731]. In addition, CSPGs may be associated with axon myelination during CNS development. The expression of brevican coincides with the myelination of axon fibres [732], and a deficiency of brevican can cause a reorganization of the nodal matrices around the nodes of Ranvier of axons [733].



#### 1.8.4.1.2 Heparan sulfate proteoglycan

Early studies suggested an important role of HSPG in axon guidance and growth [734]. The addition of exogenous HS or removal of HS can perturb growth of the pioneer axon in cultured cockroach embryos by producing axon defasciculation and growth in incorrect directions [735]. HSPG can stimulate neurite outgrowth by binding and activating other proteins including APP [186, 736, 737], laminin [738] and FGF-2 [739]. Moreover, in the *Xenopus* optic systems, treatment with exogenous HS or enzymic digestion of HSPGs causes axons from the retina to bypass their primary target (optic tectum) [740, 741]. Further studies have demonstrated that there are two distinct roles of HS in retinal axon guidance [742]. HS is firstly involved in sorting of dorsal axons within the optic tract. Disturbing the synthesis of HS can cause the missorting of RGC axons in the optic tract [742]. HS is also associated with the path-finding of retinal axons to the tectum [742]. In addition, *C. elegans* syndecan 1 has an important role in neurons to ensure correct midline axon guidance [743]. The role of HSPG in axon guidance is also examined in transgenic mouse models. HS 2-*O*-sulfotransferase (HS2ST) and HS 6-*O*-sulfotransferase (HS6ST) are responsible for the 2-position sulfation of uronic acid residues and 6-position sulfation of GluNAc in HS, respectively. HS2ST or HS6ST-null mice can express HS with *N*-sulfation but completely lack 2-*O*-sulfation or 6-*O*-sulfation, and show specific axon guidance defects at the optic chiasm [744].

There is evidence that HSPG may also participate in the proliferation of neuronal precursor cells during early brain development. Studies demonstrate that syndecan-1 and glypican-4 are most highly expressed during the time of peak proliferation in the developing brain and that they are localised to ventricular regions of the brain where precursor cells are proliferating [745]. Further studies show that HS is required for the

FGF-stimulated brain precursor cell proliferation [745]. Knockout of HS2ST in mice also shows that HSPGs have a critical role in regulating cell proliferation during development of the cerebral cortex, since knockout of HS2ST results in alteration of HS structure and induces a significant decrease of neuronal cell proliferation [746]. In addition, studies show that HSPG (syndecan-1) also has an important function in regulating neuron migration in *C. elegans* [743].

#### **1.8.4.2 Function of proteoglycans in plasticity**

During postnatal development, various brain regions undergo a considerable level of plasticity that will decrease as the CNS matures until the end of the critical period [747]. Evidence shows that CSPGs have important role in regulating this alteration of plasticity. Perineuronal nets (PNNs) are highly condensed matrices surrounding the cell body and proximal dendrites of certain types of neurons and they contain a different composition of ChS chains than ECM [748]. PNNs are mainly assembled with CSPGs including the lectican family CSPGs, phosphacan, hyaluronan, tenascin-R and link proteins [749]. PNNs are thought to have important functions in restricting plasticity in the brain [750]. The organization of CSPGs into PNNs coincides with the termination of critical periods for plasticity. Sensory deprivation of rats or mice with dark rearing or whisker trimming results not only in a decreased number of PNNs but also in a delayed critical period [751, 752]. Degradation of CSPG in PNNs by chondroitinase ABC leads to a reactivation of experience-dependent plasticity in the adult rat visual cortex, suggesting an inhibitory role of CSPG in experience-dependent plasticity [751].

There is evidence that CSPGs are also involved in synaptic plasticity. Enzymic removal of CS chains reduces both long-term potentiation (LTP) and long-term depression (LTD) in hippocampal slice cultures [753]. Brevican- or neurocan-deficient mice showed significant deficits in the maintenance of hippocampal LTP [754, 755]. However, the mechanism underlying the role of CSPG on synaptic plasticity is currently unknown.

#### **1.8.4.3 Function of proteoglycans in synaptogenesis**

The synapse, which is a fundamental functional structure in the CNS, is formed between axons and dendrites, and is the place where neurotransmitters are released and bind to the postsynaptic receptors. Studies have demonstrated that HSPGs, and in particular agrin, are components of synapses [756, 757]. Subsequent studies indicate that agrin may have functions in stabilising the developing synapse by inducing and stabilising postsynaptic acetylcholinesterase receptor (AChR) clusters [758]. In cultured hippocampal neurons, the increased level of agrin precedes synaptogenesis. In contrast, inhibition of agrin synthesis by antisense oligonucleotide treatment or blocking agrin with a specific antibody leads to fewer functional synapses forming [759, 760]. Moreover, in neuron cultures from agrin knockout mice, the loss of agrin reduces synaptogenesis and selectively affects excitatory but not inhibitory synapses [761, 762]. In addition, recent evidence shows that agrin secreted by astrocytes could induce hippocampal neuron synapse formation in vitro [763].

Apart from agrin, other types of HSPGs also have function in synapse formation. Syndecan-2 has been reported to promote filopodia growth and dendritic spine

formation through the neurofibromin-PKA-Ena/VASP pathway [764]. These findings suggest that HSPGs play an important role in synaptogenesis in the CNS.

#### **1.8.4.4 Function of proteoglycans in brain injury**

A lesion to the CNS will induce the formation of glial scars that consist of activated astrocytes, microglia, oligodendrocyte precursors and up-regulated PGs [713]. Although the glial scar can initiate the healing process and may play a protective role after the CNS injury, it also has impediment effects on axon regeneration [765].

Direct evidence that the glial scar has detrimental effects on axon regeneration comes from the observation that axon extension is blocked by glial scars surrounding the lesion, while axons can grow normally along routes where the glial scar is absent [766]. Other studies suggest that CSPG may be associated with the inhibitory effects of glial scars on axon growth [767]. The level of CSPGs is up-regulated around the lesion area soon after the CNS injury [768, 769]. Inhibition of CSPG synthesis or removal of the CS chains with chondroitinase ABC increases axon elongation over the scar surface in vitro [770, 771]. In vivo, treatment with chondroitinase ABC promotes axon regeneration of dopaminergic neurons and enhances the regrowth of both sensory and motor axons after spinal cord injury [772-774].

Unlike CSPGs, the role of HSPG in response to CNS injury is mainly unknown. Several studies demonstrate that the level of HSPGs within or surrounding the lesion core is up-regulated following CNS injury [775-777]. Other studies indicate that there is an increase in 2-O-sulfated HS and syndecan-1 in the injured adult rat brain [778]. However, the role of the up-regulation of HSPGs following the CNS injury is still

unclear. Axons can sprout prolifically within lesion cores and up-regulated HSPGs are co-localised with neurotrophic fibroblast growth factor (FGF)-2 and FGF receptor 1 (FGFR1) [776, 777, 779], suggesting that HSPGs may have a supportive role in axon regeneration in the CNS lesion.

In summary, since the level of CSPGs and HSPGs are enhanced around injury regions and may have distinct roles on the axon regeneration after the CNS injury, understanding the mechanisms underlying these effects could be helpful in developing new therapeutic approaches for the treatment of brain or spinal cord injury.

### *1.8.5 Proteoglycans in neurodegeneration*

As proteoglycans are widely expressed in the brain and participate in diverse neurobiological events, PGs are thought to be involved in many neurodegenerative diseases during aging. PGs are closely involved in the development of AD. PGs initially have been reported to co-localise with the three typical AD pathology markers including amyloid plaques, neurofibrillary tangles and cerebral amyloid angiopathy [780, 781]. Subsequent studies have shown that PGs can bind to both A $\beta$  and tau and can promote their aggregation [782, 783]. PGs also have been implicated in the progression of other neurodegenerative diseases. There is evidence that PGs are involved in prion disease [784, 785], Parkinson's disease [786, 787], multiple sclerosis [788, 789], mucopolysaccharidoses [790] and other neuromuscular diseases [791]. Interestingly, all these diseases can develop plaque-like pathology, suggesting that there may be some common mechanisms involved. However, further investigation would be required to support this assertion.

### *1.8.6 Proteoglycans and GAGs in AD*

#### **1.8.6.1 PGs in AD pathology**

Several immunocytochemical studies have shown that HSPGs are localised to neuritic plaques [780, 792-794]. HSPGs are present in diffuse plaques in the hippocampus but not in the cerebellum in AD brains [795]. HSPGs subtypes including agrin, syndecan, and glypican and perlecan, are immunoreactive in senile plaques of AD brains [780, 782, 796]. Moreover, differently sulfated CSPGs are also found to be associated with amyloid plaques [797] and immunocytochemical studies demonstrate that decorin is also peripherally localised to amyloid deposits [798]. Recent studies demonstrate that HSPGs are preferentially accumulated around the A $\beta$ 40 dense cores of neuritic plaques, but remains largely absent from diffuse A $\beta$ 42 plaques [799].

PGs are not only found in amyloid plaques but also neurofibrillary tangles (NFTs). Several studies show that HSPGs, specially agrin, syndecan, and glypican, but not perlecan, are colocalised with NFTs [792, 793, 796]. Moreover, decorin and CSPGs with different sulfated side chains are also reported to be expressed in the filamentous structures within NFTs [797, 798].

Congophilic amyloid angiopathy (CAA), a pathological marker of AD, is also associated with PG in the brains of AD subjects. Several initial studies have reported that HSPGs are expressed in CAA, especially in capillaries [780, 792, 794]. Subsequently, studies revealed that several HSPGs including glypican-1, syndecan-2, collagen XVIII and agrin, but not syndecan-1, syndecan-3 and perlecan, are associated with CAA in the brains of AD patients [782, 800, 801].

More recent studies demonstrate that the total PG level in the brains of AD subjects is increased approximately 1.6-fold in the hippocampus and 4.3-fold in the gyrus frontalis superior compared to normal elderly subjects. HS showed a 9.3-fold increase in hippocampus and a 6.6-fold increase in the gyrus frontalis superior [802]. In mouse primary glial cultures, A $\beta$  treatment can increase the expression of glypican-1 and syndecan-3 [799]. Infusion of A $\beta$ 40 into rodent hippocampus leads to an accumulation of perlecan in microglia and macrophages within the A $\beta$  infusion site [803]. In addition, in Tg2576 mice, glypican-1 and syndecan-3 are found in glial cells associated with amyloid deposits proximal to the site of HS accumulation. This result is consistent with the previous finding in which expression of HSPGs was elevated in neurons and glia of the brains of AD patients in contrast to controls [793]. These findings suggest that HSPGs co-deposited with amyloid plaques may be mainly derived from glial cells and that the HSPGs are produced in response to the stimulation of A $\beta$ .

Several studies suggest that PGs may be associated with inhibition of A $\beta$  degradation [795]. In vitro studies demonstrate that both HSPGs and CSPGs could block the proteolytic degradation of fibrillar A $\beta$  but not non-fibrillar forms [804], implicating that PGs have an important role in the accumulation and persistence of amyloid plaques in AD. Moreover, other studies also indicate that microglia can uptake and degrade the plaque-like deposits on culture dishes. However, addition of CSPG inhibits this removal of A $\beta$  deposits by microglia [805]. These findings suggest that PGs co-localised with amyloid plaques may contribute to the resistance of amyloid plaques from degradation.

### **1.8.6.2 Roles of PGs in AD**

#### *1.8.6.2.1 Interaction with APP*

Early studies indicated that APP can bind to PGs and core proteins [171, 806-808]. Further investigations have demonstrated that heparin can bind to the N-terminal of APP (residues 96-110) [186]. Mutagenesis of three basic residues within this sequence decreases the heparin binding capacity, and a peptide homologous to this heparin-binding domain of APP can bind strongly to heparin [186]. Other studies have identified another heparin-binding site in the APP sequence (residues 316-447) [163, 185]. In addition, it has been shown that both HSPG and CSPG eluted from the culture medium of mouse brain cells can bind to APP, and this binding is required for APP-mediated neurite outgrowth [186, 737, 809].

#### *1.8.6.2.2 Interaction with A $\beta$*

Both HS chains and core proteins may bind to the N-terminal sequence of A $\beta$  [810, 811]. Further studies have shown the N-terminal residues 13-16 (His-His-Gln-Lys) are critical for the interaction between A $\beta$  and GAGs [812, 813]. In vitro studies also reveal that binding of A $\beta$  to GAGs is pH-dependent, with increasing interaction under pH 7.0 and weak binding at pH 8.0 [814]. In addition, studies by Watson *et al.*, [815] reported that heparin binds to fibrillar but not nonfibrillar A $\beta$ , suggesting that the heparin-A $\beta$  interaction depends on the conformation and aggregating state of A $\beta$  rather than the primary sequence alone.



#### *1.8.6.2.3 PGs or GAGs on A $\beta$ aggregation*

Since PGs and GAGs can bind to A $\beta$ , the effect of PGs or GAGs on A $\beta$  aggregation has also been investigated. It has been reported that sulfated GAGs could enhance the aggregation of A $\beta$  [812]. PGs, including perlecan and agrin, can accelerate A $\beta$  fibril formation and maintain the stability of the A $\beta$  fibrils, and their effects on A $\beta$  aggregation are mediated by GAG chains [609, 782]. Subsequent studies showed that the sulfate moieties of GAGs are critical for the enhancement of A $\beta$  aggregation. Removal of the *O*-sulfate from heparin leads to a partial loss of the effect on A $\beta$  aggregation. Complete deletion of sulfate groups results in a complete loss of enhancement of A $\beta$  fibril formation [668]. Moreover, other GAGs such as chondroitin-4-sulfate, dermatan sulfate, dextran sulfate and pentosan polysulfate, which are sulfated to different extents, can also promote A $\beta$  aggregation [668, 816]. In addition, it has been shown that chondroitin sulfate B (ChS B) can promote A $\beta$ <sub>42</sub> aggregation. ChS B-induced A $\beta$  fibrils have reportedly little toxicity on neuroblastoma cells in vitro [817].

#### *1.8.6.2.4 Interaction with tau protein*

GAGs are also reported to be associated with the formation of neurofibrillary tangles. Under physiological conditions in vitro, sulfated GAG heparin could promote assembly of non-phosphorylated recombinant tau to paired helical-like filaments [783, 818]. Several studies show that heparin can promote phosphorylation of tau by a number of protein kinases, prevent tau from binding to microtubules, and induce rapid microtubule disassembly in a sulfation-dependent manner [783, 819]. In addition, the

evidence shows that GAGs can induce a conformational change in tau, and affect PHF conformation and PHF-tau solubilisation [820, 821]. These findings suggest that GAGs may be an important factor in the development of the neurofibrillary lesions in AD.

### **1.8.6.3 Potential therapeutic implications**

Recently, several studies have demonstrated that a low molecular weight heparin, C3 or neuroparin may have therapeutic potential for the treatment of AD [822]. C3 is composed of 4-10 saccharides (approximately 2.1 kDa) and is derived from heparin by gamma irradiation [822]. C3 can penetrate the blood-brain barrier and has effects in the CNS [823]. Initial studies show that oral or subcutaneous administration of C3 can prevent A $\beta$ 25-35-induced appearance of tau-2-immunoreactivity in the hippocampus, suggesting that C3 may have potential to prevent abnormal tau protein formation in AD [824]. Further studies in rats indicate that C3 also effectively reduces cholinergic damage induced by a cholinotoxin, AF64A, in a dose- and time-dependent manner [825, 826]. In addition, injection of low molecular weight (LMW) heparin (enoxaparin or dalteparin), LMW anionic sulphonate or sulfate compounds can arrest inflammation-associated (AA) amyloid deposits in mice [827, 828]. Chronic subcutaneous administration of certoparin can prevent A $\beta$ 25-35-induced abnormal intracellular tau changes and reactive astrocytosis in rats [829].

In AD transgenic mice, peripheral administration of enoxaparin can reduce amyloid plaques and the level of A $\beta$  in the brain of APP23 mice [830]. Injection of enoxaparin also significantly decreases the number of activated astrocytes surrounding amyloid deposits and reduces the A $\beta$ -induced inflammatory response in the APP23 mouse

brain [830]. Recent studies demonstrate that administration of enoxaparin can improve cognition in APP<sup>swe</sup>/PS1<sup>dE9</sup> mice and influence A $\beta$  accumulation differently at different stages of amyloid plaque formation [831]. These in vivo studies suggest that low molecular weight heparins may offer a promising approach for the treatment of AD.

In 1997, Leveugle and colleagues first reported that heparin could dose-dependently promote the  $\beta$ -secretase cleavage of APP in a human neuroblastoma cell line [832]. In contrast, Scholefield *et al.*, [833, 834] reported that HS and heparin can bind to BACE1 and inhibit cleavage of APP. The inhibitory effect of heparin on BACE1 was reported to be dependent on size and the specific structure of the heparin. According to Scholefield *et al.*, [833] and in contrast to the results of Leveugle *et al.*, [832], treatment of heparin could reduce the production of sAPP $\beta$  and A $\beta$  but has no effect on sAPP $\alpha$ . These findings suggest several possibilities for the role of GAGs in the regulation of BACE1 cleavage of APP. GAGs like HS may bind to BACE1 and thereby prevent the access of APP to the active site of BACE1. Alternatively, as GAGs can interact with APP, it is possible that GAG-APP binding may sequester APP away from BACE1 and thereby prevent the A $\beta$  generation [835].

The conflict was resolved in subsequent in vitro studies which showed that low concentrations (1  $\mu$ g/mL) of heparin can stimulate recombinant human BACE1, while higher concentrations of heparin (10 or 100  $\mu$ g/mL) inhibit BACE1 activity [836]. Heparin could not activate the mature form (pro sequence cleaved) of BACE1, although it interacted strongly with the zymogen form of BACE1 (proBACE1) and bound to a peptide homologous to the N-terminal pro sequence of BACE1 [836]. These observations indicate that the pro domain is necessary for the activation effect

of heparin on BACE1. Further investigations show that effect of heparin on proBACE1 is dependent on the size, degree of sulfation and carboxylation of GAG [837].

In summary, GAGs may potentially attenuate A $\beta$ -induced inflammation in vitro and in vivo [827, 828, 838], prevent A $\beta$  aggregation [828], lower A $\beta$  generation and improve cognition in vivo [830, 831], and regulate the activity and level of BACE1 [833, 839]. Moreover, several lines of evidence also indicate that endogenous HS is involved in A $\beta$  uptake [840, 841]. LMW GAGs can cross the BBB [823, 842]. These observations suggest that GAGs may have multiple benefits for the treatment of AD, and it may be possible to design potent GAG derivatives that act specifically to prevent AD-related disorders.

## 1.9 Hypothesis and aims

Studies reviewed in this chapter suggest that GAGs and PGs are involved in the pathogenesis of AD. Recently, several studies reported that LMW heparins may cross the blood-brain barrier and can lower A $\beta$  generation, amyloid plaque load and improve cognition in APP transgenic mice. Importantly, GAGs such as heparin have also been reported to regulate BACE1 activity and affect APP processing in a structure-dependent pattern.

The central hypothesis of present study is that it may be possible to identify highly potent and specific GAG derivatives which may act specifically to inhibit A $\beta$  generation and can be used for the treatment of AD.

The specific aims of current work which will be covered by each chapter are:

1. To examine whether heparin and enoxaparin can affect APP processing and A $\beta$  production in primary cortical cells from Tg2576 mice by regulating BACE1 activity;
2. To determine the structural specificities (such as size, sulfation) of GAGs on APP processing and A $\beta$  production and also to identify highly potent and specific GAG derivatives for the treatment of AD;
3. To examine whether enoxaparin can affect APP processing and thus reduce A $\beta$  production in APP transgenic mice (Tg2576);
4. To examine the role of endogenous GAGs on APP processing and A $\beta$  generation which may help to understand the mechanism underlying the effects of GAGs on APP processing.

## **Chapter2**

### **Effects of heparin and enoxaparin on APP processing and A $\beta$ production in primary cortical neurons from Tg2576 mice**

## 2.1 Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease in the elderly and is a major cause of dementia [1]. AD is characterized by the deposition of amyloid plaques in the brain [19, 20], the major component of which is the  $\beta$ -amyloid protein ( $A\beta$ ), a 40-42 amino-acid residue polypeptide [56, 57] that is generated from the  $\beta$ -amyloid precursor protein (APP) [58] by the  $\beta$ -site APP cleaving enzyme-1 (BACE1) [88-91] and  $\gamma$ -secretase [92]. Cleavage of APP by BACE1 yields a C-terminally truncated fragment (C99) which is subsequently cleaved by  $\gamma$ -secretase to yield at least two  $A\beta$  species, the major product  $A\beta_{40}$ , which contains 40-amino-acid residues and  $A\beta_{42}$ , which contains an extra two amino-acid residues at its C-terminus. APP can also be cleaved by  $\alpha$ -secretase within the  $A\beta$  sequence [93, 94] to form sAPP $\alpha$  and C83, which thus precludes formation of  $A\beta$  [95]. Several members of the disintegrin and metalloprotease (ADAM) family have been proposed as  $\alpha$ -secretases although ADAM10 is likely to be the most important contributor to this activity [302-304, 843]. Oligomeric forms of  $A\beta$  are now thought to be the major toxic species [620, 844-846]. Therefore, therapeutically targeting the production, aggregation, clearance or neurotoxicity of  $A\beta$  is a central theme of current AD research [847].

A number of studies indicate that glycosaminoglycans (GAGs) may have value for the therapeutic treatment of AD. GAGs are linear polymers consisting of repeated disaccharide units. Heparin is a typical GAG and has a highly sulfated structure. A low molecular weight (LMW) derivative of heparin, enoxaparin, is generated by alkaline depolymerization of heparin benzyl ester. Both heparin and LMW heparins have been widely used as anticoagulant and antithrombotic drugs [848, 849]. LMW

heparins are now generally regarded as safer and more effective for the treatment of cardiovascular problems than unfractionated heparin [850].

LMW GAGs may also be suitable agents for the treatment of brain diseases as they can cross the blood-brain barrier (BBB) [823, 842]. GAGs can inhibit A $\beta$  toxicity [830, 840, 851, 852] and may have neuroprotective effects [825, 826]. In addition, peripheral administration of enoxaparin has been reported to reduce A $\beta$  load [830] and improve cognition in APP transgenic mice [831]. The mechanism of these effects is unclear. GAGs bind directly to APP [163, 185, 186, 813] and may influence its function [809]. In addition, GAGs can bind to aggregated A $\beta$  and accelerate amyloid fibril formation [611, 812].

Heparin may also influence A $\beta$  production by disrupting APP proteolytic processing. Scholefield et al. [833] first reported that heparan sulfate and heparin can directly inhibit BACE1 activity in vitro and thereby decrease A $\beta$  production in cell culture. Our own studies have shown that heparin binds close to the prodomain of the BACE1 zymogen (proBACE1) and that this binding stimulates proBACE1 activity [836, 837]. However, heparin can also inhibit mature BACE1 activity by binding close to the active site domain of the mature protein [837]. In contrast to the results of Scholefield et al. [44], Leveugle et al. [832] reported that heparin stimulates  $\beta$ -secretase cleavage of APP in a cultured cell line.

As there are conflicting reports on the effect of GAGs on APP processing and A $\beta$  production, the effects of heparin and enoxaparin on APP processing were examined in primary cortical cells obtained from transgenic mice expressing human APP<sub>695</sub> with



the Swedish familial AD mutation (Tg2576 mice) [545], because human APP and its fragments can be more easily detected with existing anti-human antibodies than rodent APP and A $\beta$ .

Studies reported in this chapter show that heparin and enoxaparin lower A $\beta$  secretion from cortical cells by decreasing BACE1 and thereby inhibiting  $\beta$ -secretase processing of APP. This effect is not specific for the amyloidogenic processing of APP, as heparin and enoxaparin also decrease the level of  $\alpha$ -secretase ADAM10 and inhibit  $\alpha$ -secretase processing of APP.

## **2.2 Materials and Methods**

### **2.2.1 Materials**

Porcine mucosal heparin, 5 kDa heparin, 3 kDa heparin, monoclonal anti- $\beta$ -actin antibody, rabbit anti-BACE1 (EE-17) antibody, polyclonal anti-APP C-terminal antibody (APP-CT) and N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester (DAPT) were purchased from Sigma-Aldrich Pty. Ltd. (Sydney, Australia). Rabbit anti-ADAM10 (ab1997) and rabbit anti-ADAM17 (ab2051) were purchased from Sapphire Bioscience Pty. Ltd. (Waterloo, Australia). Monoclonal anti-A $\beta$  antibody 6E10 was from Covance Pty. Ltd. (North Ryde, Australia). Neurobasal medium and B27 supplement were purchased from Invitrogen (Mulgrave, Australia). Mouse and rabbit HRP-conjugated secondary antibodies were purchased from DAKO (Campbellfield, Australia). Enoxaparin sodium (Clexane<sup>®</sup>) was from Sanofi-Aventis (Macquarie Park, Australia). Protein G Sepharose and complete mini protease inhibitor cocktail tablet were purchased from Roche Diagnostics (Castle Hill,

Australia). A polyclonal anti-phosphorylated APP antibody UT33 was prepared as previously described [853].

### **2.2.2 Cell culture**

Cortical cells were prepared from newborn (P<sub>0</sub>) Tg2576 mice. Cerebral cortices were dissected and incubated in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free Hank's balanced salts (HBSS) containing 0.25% (w/v) papain and 0.06% (w/v) deoxyribonuclease I (DNase I) for 30 min at 37 °C, followed by three washes with Neurobasal medium. Cells were then separated by gentle mechanical dissociation and 3×10<sup>5</sup> cells were plated onto poly-D-lysine-coated 12-well culture plates, and maintained in 1.2 mL complete Neurobasal medium containing 2% B27 supplement, 1 mM glutamine, and 1% penicillin/streptomycin (10,000 units of penicillin and 10,000 µg of streptomycin stock) in an atmosphere containing 5% CO<sub>2</sub> at 37 °C. After 3 days in vitro (DIV), half of the culture medium was replaced with fresh complete Neurobasal medium. All experiments were performed at 7 DIV cultures. Primary cortical cells were incubated with GAGs and inhibitors for 24 hours prior to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

### **2.2.3 SDS-PAGE and western blotting**

After treatment with GAGs or drugs, the medium was removed from cells for determination of A $\beta$  and sAPP $\alpha$ . The cells were incubated with cold RIPA buffer (150 mM NaCl, 50 mM Tris, 0.5% w/v Na-deoxycholate, 1% v/v Nonidet P-40, 0.1% SDS, pH 7.4) containing protease inhibitor cocktail on ice for 10 min and the cell lysates were then harvested for determination of C99, C83, BACE1, ADAM10, ADAM17 and APP.

The amount of A $\beta$ 40 or A $\beta$ 42 secreted into the cell medium was determined on 15% Tris-bicine-urea SDS-PAGE gels as described previously [854]. The cell medium was collected and cell debris was removed by centrifugation at 500 g for 5 min. The supernatant fractions were then transferred to 1.5 mL Eppendorf tubes. Monoclonal antibody 6E10 (1:1667 v/v) was added and the mixture was incubated overnight at 4 °C. Protein G agarose (20  $\mu$ L hydrated gel/mL medium) was then added and incubated for a further 3 hours at 4 °C. The beads were washed 3 times in 1 mL cold phosphate-buffered saline (PBS), and then 30  $\mu$ L of sample buffer was added to each pellet. After gentle mixing, the slurry was heated for 5 min at 95 °C. The sample was then centrifuged for 10 min at 15,000 rpm and 30  $\mu$ L supernatant fraction was loaded on 15% Tris-bicine-urea gels. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and stained with monoclonal antibody 6E10 (1:2,000 dilution) for A $\beta$ .

For the determination of sAPP $\alpha$ , BACE1, ADAM10, ADAM17 and APP, cell medium (the loading volumes were normalized from protein concentration of cell lysates) or 8  $\mu$ g protein cell lysate were applied to 8% Tris-glycine SDS-PAGE gels and subjected to western blotting using the anti-A $\beta$  monoclonal antibody 6E10 (1:2,000 dilution), anti-ADAM10 polyclonal antibody (1:1,000 dilution), anti-BACE1 polyclonal antibody (1:1,000 dilution), anti-ADAM17 polyclonal antibody (1:1,000 dilution) or an anti- $\beta$ -actin monoclonal antibody (1:16,000 dilution). The protein concentration was measured using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories Pty. Ltd., Gladesville, Australia) with bovine serum albumin as standard.

To determine the level of C99 and C83 in cells, cell lysates containing 12 µg of protein were applied to 16.5% Tris-tricine SDS-PAGE gels. After electrophoretic transfer of proteins onto a PVDF membrane, the membrane was cut into two pieces at the position of the 24 kDa molecular weight marker. The piece of PVDF membrane containing proteins from the upper region of the gel that migrated above an apparent molecular mass of 24 kDa was stained using a monoclonal antibody directed against  $\beta$ -actin (1:16,000 dilution). The piece containing proteins from the lower region of the gel which migrated below 24 kDa was stained for C99 and C83 using a polyclonal anti-APP C-terminal antibody (1:2,000 dilution), which was raised against residues 676-695 of the APP695 sequence.

For all western blotting, the bound primary antibody was detected using either a polyclonal goat anti-mouse or an anti-rabbit immunoglobulin conjugated to horseradish peroxidase (HRP) (1:6,000 dilution) and Immobilon<sup>TM</sup> Western chemiluminescent HRP substrate from Millipore Pty. Ltd. (North Ryde, Australia). Chemiluminescence was detected using a Chemi-Smart 5000 gel documentation system (Vilber Lourmat, Torcy, France). Images were taken and the density of staining was quantified using Image J software (RSB; NIH, <http://rsbweb.nih.gov/ij/index.html>). The ratio of immunoreactivity for each protein to the  $\beta$ -actin immunoreactivity was determined and then each ratio was used to calculate a percentage relative to mean values for control incubations lacking GAG. All experiments were performed at least six times and statistical tests were performed using SigmaPlot software (10.0v; Systat Software, Inc., San Jose, CA, USA). Statistical comparisons were made using one-way analysis of variance and Student's *t* tests. Values of  $p < 0.05$  were considered statistically significant.

#### 2.2.4 Quantitative real-time PCR

Total RNA was extracted from control or treatment cells using a SV total RNA isolation system from Promega (Sydney, Australia) following the manufacturer's instructions. The concentration and purity of the RNA was assessed spectrophotometrically at a wavelength of 260 and 280 nm using a NanoDrop<sup>TM</sup> 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA). RNA (250 ng) was reverse transcribed to form cDNA in a 20 µl reaction volume using a Fastlane cell cDNA kit from Qiagen Pty. Ltd. (Doncaster, Australia) following the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using a QuantiFast SYBR Green PCR kit (Qiagen Pty. Ltd., Doncaster, Australia). Each reaction contained 10 µl 2x QuantiFast SYBR Green PCR Master Mix, 1 µl forward and reverse primers (both from 10 µM stock), 2 µl template cDNA and 6 µl RNase-free water to a total volume of 20 µl. The reactions were carried out on a Rotor-Gene 6000 PCR cycler (Qiagen Pty. Ltd., Doncaster, Australia) according to the following protocol: pre-heat at 95 °C for 5 minutes to activate DNA polymerase, followed by 50 cycles of 10s at 95 °C and 30s at 60 °C. Primers with the following sequences were chosen: *BACE1*: Fw, 5'-CAGTGGGACCACCAACCTTC-3', Rev, 5'-GCTGCCTTGATGGACTTGAC-3'; *ADAM10*: Fw, 5'-TAAGGAATTATGCCATGTTTGCTGC-3', Rev, 5'-ACTGAACTGCTTGCTCCACTGCA-3'; *actin*: Fw, 5'-ATGCTCCCCGGGCTGTAT-3', Rev, 5'-CATAGGAGTCCTTCTGACCCATTC-3'; *GAPDH*: Fw, 5'-TGTGTCCGTCGTGGATCTGA-3', Rev, 5'-TTGCTGTTGAAGTCGCAGGAG-3'. All primers were obtained from GeneWorks Pty. Ltd. (Hindmarsh, Australia). Fluorescence data were acquired at the end of each cycle, and a melt curve was determined at the end of cycling. Comparative concentration of target genes was normalized to the comparative concentration of housekeeping gene actin and the

results were expressed as ratio of target gene and actin expression. Expression of additional housekeeping (e.g. GAPDH) genes was also analysed to verify the reliability of normalization relative to actin.

## **2.3 Results**

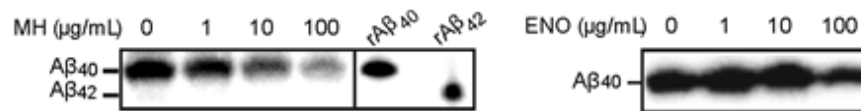
### **2.3.1 Effect of heparin and enoxaparin on A $\beta$**

Initially, the effect of heparin and enoxaparin on the secretion of A $\beta$  from cortical cells was examined. Primary cortical cells from Tg2576 mice were cultured and then treated with heparin or enoxaparin for 24 hours. The cell media were harvested and A $\beta$  was immunoprecipitated from the medium and then detected by western blotting. Although A $\beta$ 40 was easily detected in the cell culture medium, little A $\beta$ 42 was observed (Fig. 2.1 A). Therefore, in the subsequent experiments, only the level of A $\beta$ 40 was measured. A $\beta$ 40 and A $\beta$ 42 were not detectable in the cell lysate (data not shown). Incubations with 10  $\mu$ g/mL and 100  $\mu$ g/mL heparin significantly lowered levels of A $\beta$ 40 in the culture medium. Enoxaparin also reduced the level of A $\beta$ 40. However, enoxaparin was less effective than heparin and only had a significant effect on A $\beta$ 40 at a concentration of 100  $\mu$ g/mL (Fig. 2.1 B).

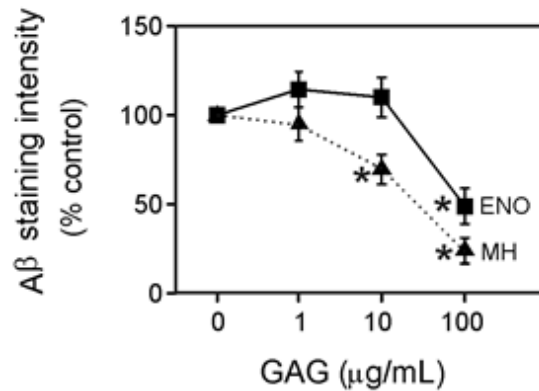
### **2.3.2 Characterization of APP C-terminal fragments (CTFs)**

To examine the effect of heparin and enoxaparin on APP processing, the major APP CTFs produced by the Tg2576 mouse cortical cells were characterized. The cell lysates were analysed by western blotting using a polyclonal anti-APP C-terminal antibody.

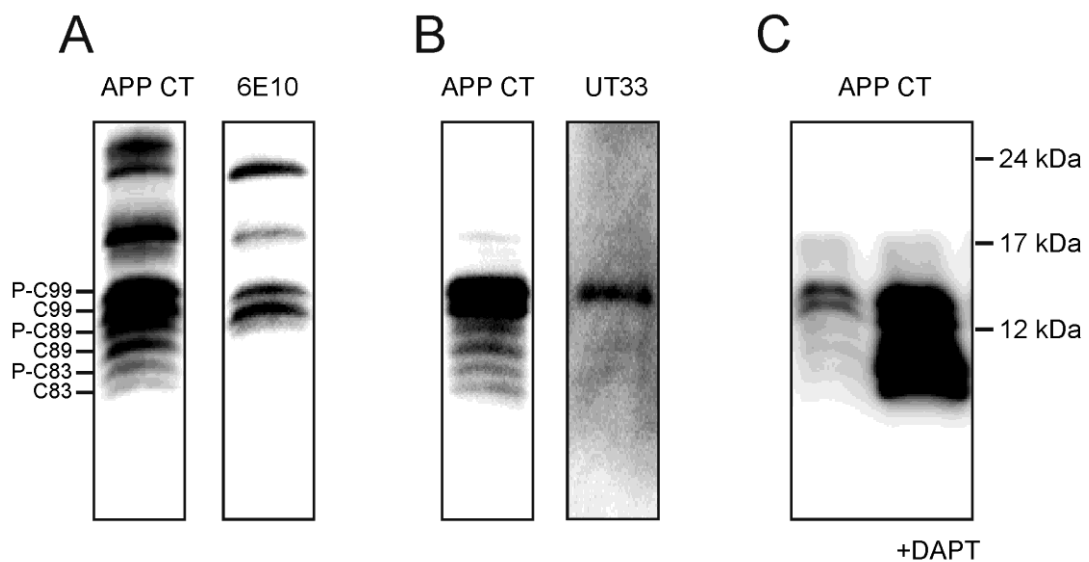
A



B



**Fig. 2.1.** Effect of heparin (MH) and enoxaparin (ENO) on Aβ secretion from Tg2576 mouse cortical cells. Cells were treated with 0, 1, 10, or 100 μg/mL heparin or enoxaparin for 24 hours. Aβ40 and Aβ42 in the culture medium were separated on Tris-bicine-urea SDS gels and visualized by western blotting with the anti-Aβ monoclonal antibody 6E10. (A) Typical western blots showing the effect of heparin and enoxaparin on Aβ40. The position of pure recombinant human Aβ40 (rAβ40) and Aβ42 (rAβ42) is also shown. (B) Quantification of Aβ40 immunoreactivity on the western blots. Asterisks show values that are significantly different from control incubations containing no GAG ( $p < 0.01$ ,  $n = 9$ ).

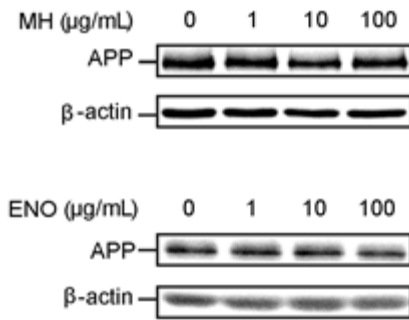


**Fig. 2.2.** Characterization of APP C-terminal fragments in Tg2576 mouse cortical cells. (A) Cell lysates from Tg2576 primary cortical cells at 7 DIV were analysed on 16.5% Tris-tricine SDS-PAGE and immunoblotted with polyclonal anti-APP C-terminal antibody (left). Membranes were stripped and reprobed with monoclonal antibody 6E10 which recognises the N-terminal region of A $\beta$  (right). (B) Cell lysates were immunoprecipitated using the anti-APP C-terminal antibody, and then separated on 16.5% Tris-tricine polyacrylamide gels. After blotting APP CTFs were visualized with the anti-APP C-terminal antibody (left). Blots were then stripped and reprobed with UT33 (right), an antibody which recognizes phosphorylated APP. (C) Cells were incubated in the absence (control) or presence of 0.5  $\mu$ M DAPT for 24 hours. Cell lysates were resolved on 16.5% Tris-tricine polyacrylamide gels. After blotting, the APP CTFs were visualized with the anti-APP C-terminal antibody. The positions of C99, C89 and C83 as well as their phosphorylation forms (P-C99, P-C89 and P-C83) are indicated.

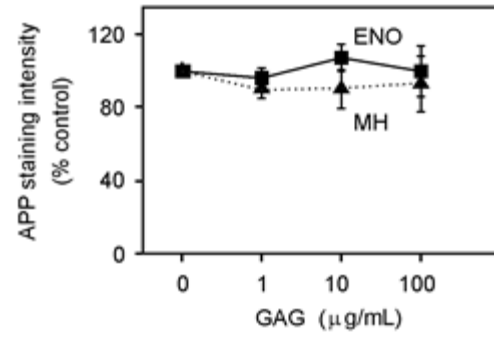


Analysis of the cell lysate fraction revealed six discrete protein bands migrating close to the 12-kDa molecular weight marker (Fig. 2.2 A, left panel). These bands corresponded to phospho-C99, C99, phospho-C89, C89 (a product of cleavage adjacent to residue 11 in A $\beta$  by BACE1), phospho-C83 and C83, based on their apparent molecular masses. To confirm their identities, the membrane was stripped and reprobed using monoclonal antibody 6E10 which recognizes C99 but not C89 and C83. As expected, it has been found that only the bands corresponding to phospho-C99 and C99 were stained (Fig. 2.2 A, right panel). Next, to confirm the identification of the phospho-C99 band, the cell lysate protein was immunoprecipitated with the anti-APP C-terminal antibody, and immunoblotted using the same polyclonal anti-APP C-terminal antibody (Fig. 2.2 B, left panel). The membrane was then stripped and restained using an anti-phospho-APP antibody UT33 which recognizes APP fragments phosphorylated at threonine 668 of the APP695 sequence [853]. Of the two 6E10-immunoreactive bands, only the upper band was immunoreactive (Fig. 2.2 B, right panel), confirming that it was phospho-C99. Levels of phosphorylated C89 and C83 were too faint to be easily visualised using UT33. The identities of the APP CTFs were also confirmed using the  $\gamma$ -secretase inhibitor DAPT. DAPT has been shown previously to cause the accumulation of both  $\beta$ - and  $\alpha$ -secretase-derived CTFs of APP (C99, C89 and C83) [855]. When primary cortical cells were treated with DAPT for 24 hours, there was a large increase in the levels of all protein bands around 12 kDa compared with the control (Fig. 2.2 C), confirming the identity of these bands as APP CTFs.

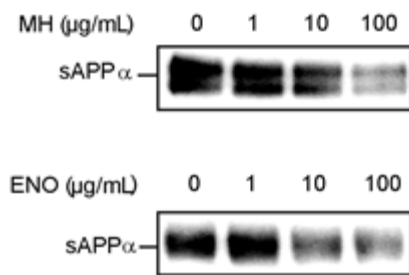
**A**



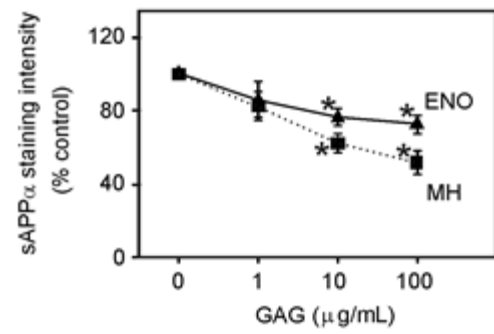
**B**



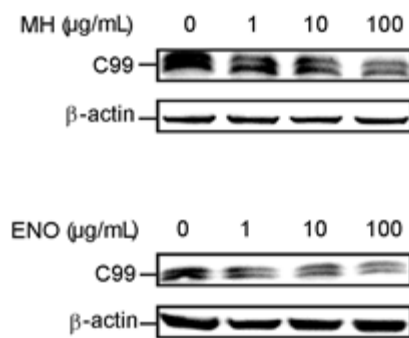
**C**



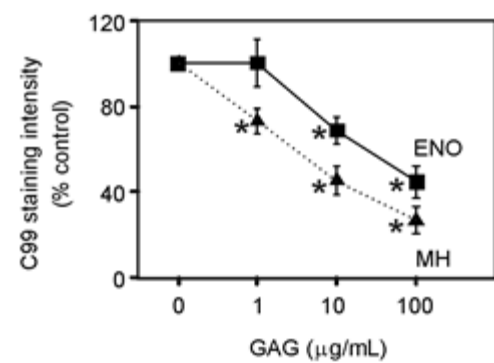
**D**



**E**

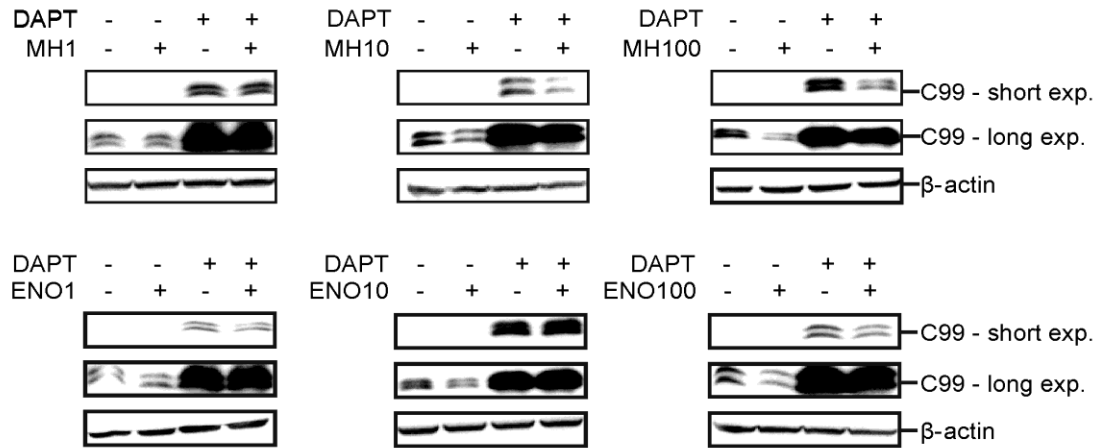


**F**

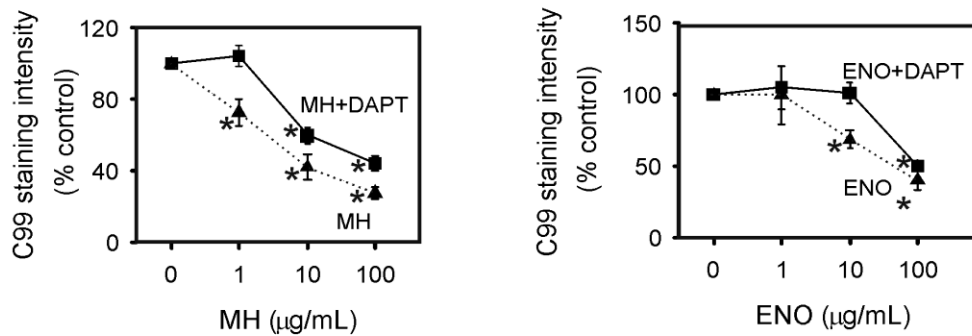


**Fig. 2.3.** Effects of heparin (MH) and enoxaparin (ENO) on APP (A, B), sAPP $\alpha$  (C, D) and C99 (E, F) in Tg2576 mouse cortical cell cultures. Cells were treated with 0, 1, 10, 100  $\mu$ g/mL heparin or enoxaparin for 24 hours. Figure shows typical western blots illustrating the effect of MH and ENO on APP (A), sAPP $\alpha$  (C) and C99 (E).  $\beta$ -Actin immunoreactivity is shown as a loading control. Figure also shows quantification of the level of APP (B), sAPP $\alpha$  (D) and C99 (F) immunoreactivity. Asterisks show values that are significantly different from controls ( $p < 0.05$ ,  $n = 12$ ).

A



B

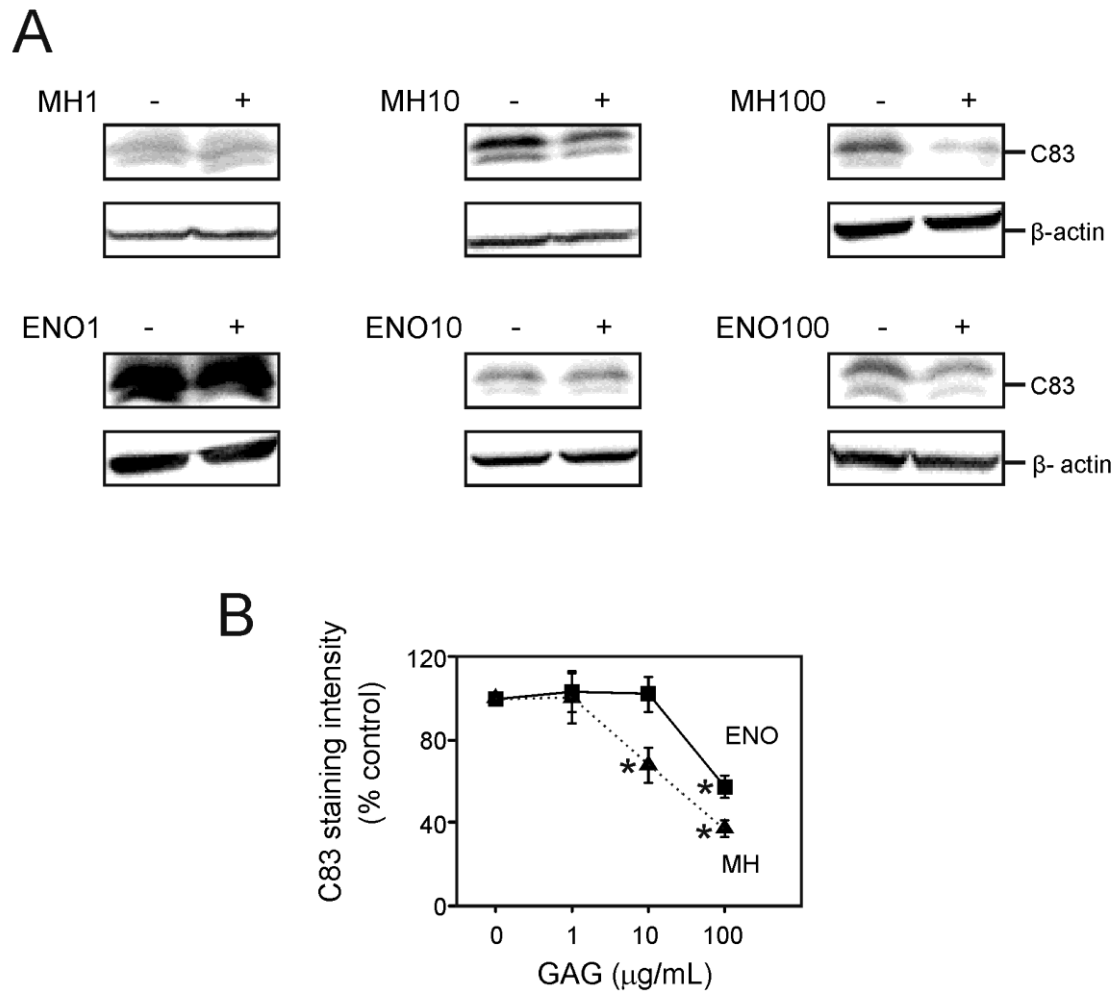


**Fig. 2.4.** Effects of DAPT, heparin and enoxaparin on C99 levels in Tg2576 mouse cortical cells. (A) Cells were incubated in the absence (control), or presence of 0.5  $\mu$ M DAPT and in the absence or presence of 1  $\mu$ g/mL heparin (MH1), 10  $\mu$ g/mL heparin (MH10) or 100  $\mu$ g/mL heparin (MH100), 1  $\mu$ g/mL enoxaparin (ENO1), 10  $\mu$ g/mL enoxaparin (ENO10) or 100  $\mu$ g/mL enoxaparin (ENO100) for 24 hours. The cell lysates were analysed for C99.  $\beta$ -Actin immunoreactivity is shown as a loading control. Chemiluminescence was detected with either short exposures (short exp.) or long exposures (long exp.) to visualise different levels of C99. (B) Quantitative analysis of C99 immunoreactivity. Asterisks show values that are significantly different from controls ( $p < 0.01$ ,  $n = 9$ ).

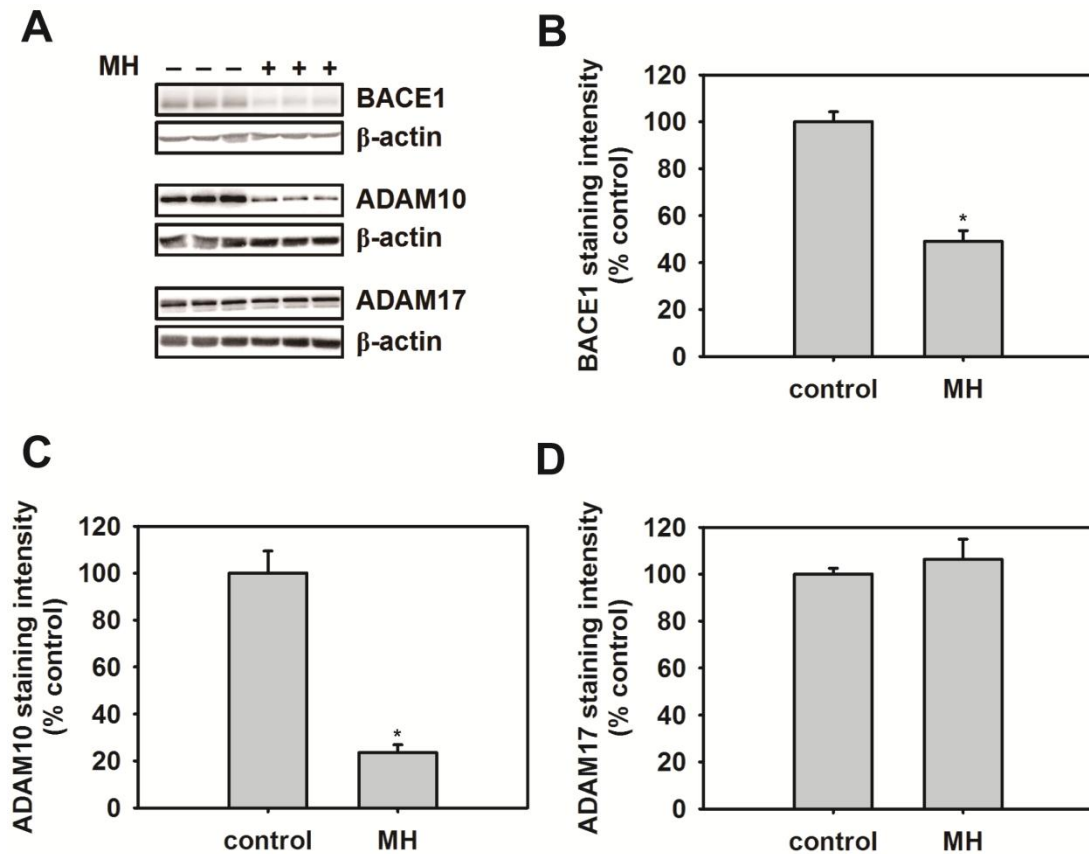
### **2.3.3 Effect of heparin and enoxaparin on levels of APP, sAPP $\alpha$ and CTFs**

Next, the effect of heparin and enoxaparin on the levels of APP and APP CTFs was examined. Heparin and enoxaparin had no significant effect on the level of APP in the cell lysate (Figs. 2.3 A and B). However, heparin decreased the amount of sAPP $\alpha$  secreted into the culture medium. Although 1  $\mu\text{g/mL}$  heparin did not significantly decrease the level of sAPP $\alpha$ , higher concentrations (10 and 100  $\mu\text{g/mL}$ ) of heparin significantly reduced sAPP $\alpha$  immunoreactivity by 40% and 50%, respectively (Figs. 2.3 C and D). Enoxaparin had a similar effect as heparin, but was less effective in its ability to reduce sAPP $\alpha$ . Enoxaparin (10 and 100  $\mu\text{g/mL}$ ) reduced the sAPP $\alpha$  immunoreactivity by approximately 25% and 30%, respectively (Figs. 2.3 C and D).

Subsequently, the effects of heparin and enoxaparin on C99 and C83 were determined. Cell lysates were analysed by western blotting using the anti-APP C-terminal antibody. Although C99 was clearly visualized using this method, the level of C83 was too low to measure accurately. Therefore, only C99 was quantified. C99 and phospho-C99 were measured together as they were often poorly separated. Heparin decreased C99 in a dose-dependent manner. At concentrations between 1 and 100  $\mu\text{g/mL}$ , heparin lowered C99 immunoreactivity by between 25% and 75% (Figs. 2.3 E and F). Enoxaparin also decreased the level of C99, but it was less potent in this regard. Enoxaparin, at concentrations of 10 and 100  $\mu\text{g/mL}$ , significantly reduced C99 immunoreactivity by 30% and 50% of the control value, respectively (Figs. 3E and F).



**Fig. 2.5.** Effects of heparin and enoxaparin in the presence of 0.5  $\mu$ M DAPT on C83 in Tg2576 mouse cortical cells. (A) Cells were incubated in the presence or absence of 1, 10 or 100  $\mu$ g/mL heparin (MH1, MH10 or MH100, respectively) or 1, 10 or 100  $\mu$ g/mL enoxaparin (ENO1, ENO10 or ENO100, respectively) for 24 hours. Cell lysates were then analysed by western blotting using polyclonal anti-APP C-terminal antibody to determine the level of C83. The level of  $\beta$ -actin immunoreactivity was used as a loading control. (B) Quantification of the level of C83 immunoreactivity. Asterisks show values that are significantly different from controls ( $p < 0.001$ ,  $n = 9$ ).



**Fig. 2.6.** Effect of heparin (MH) on the level of BACE1, ADAM10 and ADAM17 in Tg2576 mouse cortical cell cultures. Cells were treated with 100  $\mu\text{g}/\text{mL}$  MH for 24 hours. Figure shows typical western blots illustrating the effect of MH on BACE1, ADAM10 and ADAM17 (A).  $\beta$ -Actin immunoreactivity is shown as a loading control. Figure also shows quantification of the level of BACE1 (B), ADAM10 (C) and ADAM17 (D) immunoreactivity. Asterisks show values that are significantly different from controls ( $p < 0.05$ ,  $n = 8$ ).

As heparin and enoxaparin decreased levels of C99, studies in this chapter also examined whether this decrease might be attributed to an increase in  $\gamma$ -secretase activity, or to a decrease in C99 production. In the presence of the  $\gamma$ -secretase inhibitor DAPT (0.5  $\mu$ M), it was possible to accurately quantify C83 levels. Heparin (1  $\mu$ g/mL) had no significant effect on the level of C99. However, 10 and 100  $\mu$ g/mL heparin reduced C99 immunoreactivity by approximately 40% and 55%, respectively (Figs. 2.4 A and B) and heparin (10 and 100  $\mu$ g/mL) decreased C83 immunoreactivity by approximately 40% and 60% (Fig. 2.5 A and B). In the presence of DAPT, enoxaparin (1 and 10  $\mu$ g/mL) did not significantly decrease C99 or C83. However, 100  $\mu$ g/mL enoxaparin decreased both C99 and C83 to approximately 50% of control values (Fig. 2.4 and 2.5). Taken together, the results demonstrated that the reduced secretion of A $\beta$  was due to the decreased level of C99, and that treatment with heparin and enoxaparin also decreased  $\alpha$ -secretase processing.

#### **2.3.4 Effect of heparin on the level of BACE1, ADAM10 and ADAM17**

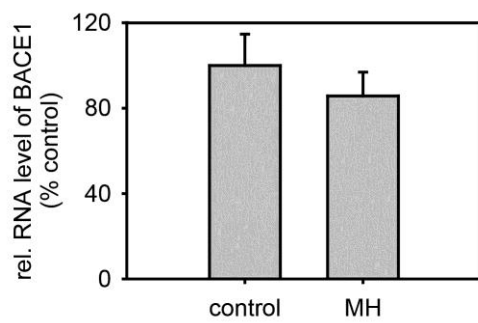
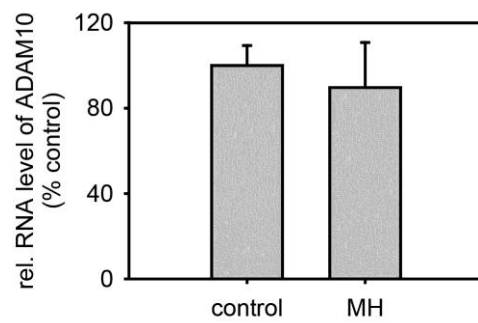
To examine whether the decrease in  $\alpha$ - and  $\beta$ -secretase processing of APP was due to a reduction in the level of  $\alpha$ - and  $\beta$ -secretase, primary cortical cells were treated with heparin (100  $\mu$ g/mL) for 24 hr and then the cells were lysed and the level of  $\beta$ -secretase (BACE1) and two putative  $\alpha$ -secretases (ADAM10 and ADAM17) were measured in the cell lysate by western blotting. Heparin (100  $\mu$ g/mL) significantly decreased the level of BACE1 and ADAM10 to approximately 50% and 25% of control values (Fig. 2.6 A-C). However, incubation with heparin did not lead to a significant change in ADAM17 levels compared with controls (Fig. 2.6 A and D).



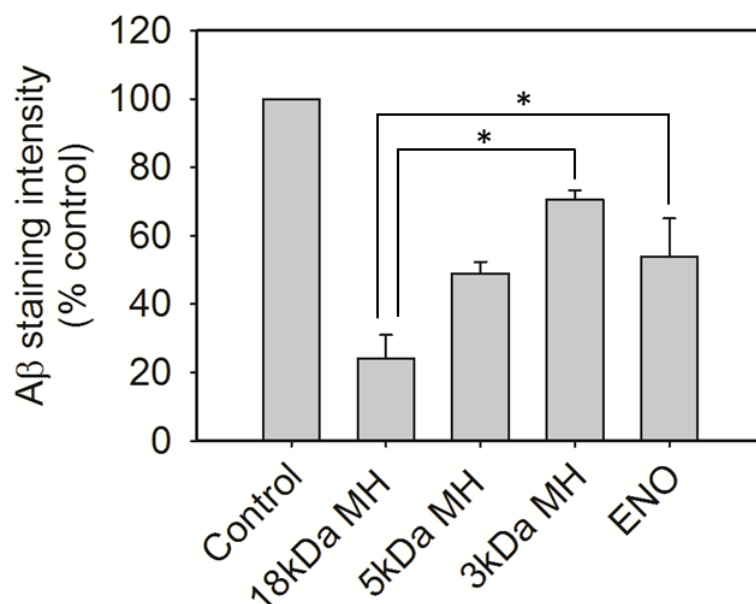
As the level of BACE1 and ADAM10 was reduced by treatment with heparin, it has been speculated that heparin might affect the expression of BACE1 and ADAM10 mRNA. The level of BACE1 and ADAM10 mRNA was measured by real-time PCR. However, it was unable to find any evidence that heparin treatment alters the expression of either BACE1 or ADAM10 (Fig. 2.7 A and B). This suggested that GAGs probably decrease BACE1 or ADAM10 at a post-translational level.

### **2.3.5 Effect of heparin fragments on A $\beta$ secretion**

As there were significant differences between heparin and enoxaparin in their effects on APP metabolism and A $\beta$  production, and because enoxaparin comprises a mixture of small-sized heparin fragments [849], the effect of heparin fragments of different molecular weight on secretion of A $\beta$  was examined. Primary cortical cells from Tg2576 mice were cultured and then treated with 18 kDa heparin, 5 kDa heparin, 3 kDa heparin or enoxaparin for 24 hours. The cell culture medium was harvested and A $\beta$  was immunoprecipitated from the medium and detected by western blotting. Incubations with native 18 kDa heparin, 5 kDa heparin, 3 kDa heparin or enoxaparin significantly lowered levels of A $\beta$ 40 in the culture medium. However, 5 kDa heparin, 3 kDa heparin and enoxaparin were less effective compared with native heparin (Fig. 2.8).

**A****B**

**Fig. 2.7.** Effects of heparin (MH) on mRNA level of BACE1 (A) and ADAM10 (B). The relative mRNA level of BACE1 and ADAM10 was determined by RT-PCR after 100  $\mu$ g/mL MH treatment.



**Fig. 2.8.** Effect of heparin fragments of different molecular weight on A $\beta$  secretion from Tg2576 mouse cortical cells. Cells were treated with 100  $\mu$ g/mL 18 kDa heparin, 5 kDa heparin, 3 kDa heparin or enoxaparin for 24 hours. A $\beta$ 40 and A $\beta$ 42 in the culture medium were visualized by western blotting using monoclonal antibody 6E10. Figure shows the quantification of A $\beta$ 40 immunoreactivity on the western blots. Asterisks show values that are significantly different from each other ( $p < 0.01$ ,  $n = 8$ ).

## 2.4 Discussion

In this chapter, the effect of heparin and enoxaparin on APP processing and A $\beta$  production was examined in primary cortical cells obtained from APP Tg2576 mice. The studies reported in this chapter showed that while heparin and enoxaparin had no effect on the level of APP, they decreased the level of C99, C83, sAPP $\alpha$  and secreted A $\beta$  in a dose-dependent manner. Heparin also decreased the level of  $\beta$ -secretase (BACE1) and a putative  $\alpha$ -secretase (ADAM10) but had no effect on the level of another putative  $\alpha$ -secretase (ADAM17).

As the level of APP was not decreased by heparin or enoxaparin, the decreased levels of C99, A $\beta$ , sAPP $\alpha$  and C83 were not due to a decrease in APP. Furthermore, as heparin and enoxaparin decreased the level of C83 and C99 in the presence of a  $\gamma$ -secretase inhibitor, and also treatment with heparin or enoxaparin decreased the secretion of A $\beta$  rather than increase A $\beta$  secretion. This indicated that the effect of GAGs in decreasing C99 and C83 could not be due to an increase in  $\gamma$ -secretase activity.

It was speculated that the decrease in C99, C83 and the decrease in A $\beta$  secretion might be due to a decrease in the level of  $\alpha$ - and  $\beta$ -secretases. Therefore, the level of both  $\alpha$ - and  $\beta$ -secretases was measured by western blotting. The results reported in this chapter showed that heparin significantly decreased the level of BACE1 and ADAM10, which has been shown to be the major contributor to total  $\alpha$ -secretase activity in many cells [843]. In contrast, the level of another putative  $\alpha$ -secretase, ADAM17 was not changed in the presence of heparin. Therefore, the results

demonstrate that the GAG-induced decrease in A $\beta$  secretion and the reduction in C99 were most likely due to a decrease in BACE1 levels within the cortical cells.

The mechanism by which heparin and enoxaparin decrease BACE1 and ADAM10 and alter APP processing is unclear. The decreased level of BACE1 and ADAM10 was probably not caused by a decrease in BACE1 or ADAM10 mRNA, because BACE1 and ADAM10 mRNA levels were not significantly changed after MH treatment (Fig. 2.7 A and B). This suggests that the effect was on some post-translational event such as BACE1 and ADAM10 turnover. However, further studies will be needed to delineate this mechanism.

The mechanism by which GAGs lower levels of BACE1 and ADAM10 is unclear. As BACE1 may bind to endogenous proteoglycans and because this interaction may be important for the trafficking [833] or processing of BACE1 [836], it is possible that heparin and enoxaparin may disrupt the normal trafficking or processing of the enzyme. However, it is unclear whether such a mechanism can also explain the effect of heparin on ADAM10 levels. Furthermore, it was interesting to note that the structurally related enzyme ADAM17 was not affected by heparin. To date, there have been no reports that ADAM10 can interact with GAGs or proteoglycans, although this possibility cannot be ruled out. GAGs could conceivably exert a less direct effect on BACE1 and ADAM10, possibly by acting on a specific secretory pathway. In this regard, it is interesting to note that there are differences in the roles of ADAM10 and ADAM17 in membrane protein shedding [856]. ADAM17 can be stimulated by protein kinase C and may be part of a regulated secretion pathway [857], whereas ADAM10 may be associated with a constitutive secretion pathway [858]. APP can be processed via both pathways [95, 303, 859]. Thus it is tempting to

speculate that GAGs may selectively alter the level of enzymes in the constitutive secretion pathway, rather than the regulated pathway, thus accounting for the selective decrease in ADAM10 but not in ADAM17.

The studies reported in this chapter showed that the smaller sized enoxaparin was less efficient in its ability to inhibit APP processing to A $\beta$  than native full-length heparin. To examine the reason for this difference, 18 kDa native heparin and enoxaparin with heparins of defined molecular weight were compared. The results showed that low molecular weight (3 kDa and 5 kDa) heparins were less effective in inhibiting the secretion of A $\beta$  than native heparin (Fig. 2.8). Enoxaparin possesses an average molecular weight of approximately 4.5 kDa and its efficacy for inhibiting A $\beta$  secretion was similar to that of 5 kDa heparin. Therefore, this result suggests that the differences between heparin and enoxaparin are probably due to differences in molecular weight.

In a previous preliminary study, it has been reported that heparin could decrease the level of A $\beta$  and sAPP $\alpha$  in cortical cells [860]. However, in that study, the results showed that heparin had no effect on C99 or C83. The failure to observe an effect on C99 and C83 previously was possibly due to the fact that the number of replicates in the earlier experiments was too low to observe significant differences in C99 and C83 levels. In a previous study, Scholefield et al. [833] reported that heparin and heparin analogues inhibit A $\beta$  and sAPP $\beta$  generation in APP-expressing cells, and they drew the conclusion that GAGs may bind to BACE1 and inhibit BACE1 cleavage activity. Our own studies have shown that at low concentrations (e.g. 1  $\mu$ g/mL) GAGs stimulate the activity of the zymogen pro-BACE1 whereas at higher concentrations

(e.g. 100  $\mu\text{g/mL}$ ) they inhibit the activity of the mature enzyme [836]. This biphasic effect of GAGs is most simply explained by the presence of a single heparin-binding site that lies adjacent to the both the pro sequence and active site regions [837]. Low concentrations of GAGs can alter the conformation of the prodomain, but higher concentrations are needed to block the active site [46].

In contrast to the studies reported in this chapter, Leveugle et al. [832], using SH-SY5Y neuroblastoma cells overexpressing APP with the Swedish mutation, reported that GAGs can increase APP secretion and processing through the  $\beta$ -secretase pathway. The difference in the effect of GAGs between the study of Leveugle et al. [832] and studies reported in this chapter is unclear, but it may possibly be explained by the fact that APP processing occurs differently in neuroblastoma cells overexpressing APP compared with primary cortical cells. This possibility emphasizes the need to use primary cells in culture, rather than cell lines for these types of studies.

Interestingly, studies reported in this chapter demonstrate that, in the absence of the  $\gamma$ -secretase inhibitor DAPT, C99 was more abundant than C83. This finding contrasts with reports from many studies using APP-transfected cells that C83 is more abundant than C99 [861, 862], in keeping with the concept that the  $\alpha$ -secretase pathway is a predominant route of APP processing than the  $\beta$ -secretase pathway. However, the observation that C99 was present in higher abundance than C83 is consistent with some previous studies in both APP23 and Tg2576 mice [546, 863, 864]. One possible explanation for the high level of C99 in the Tg2576 cells is that the transgene possesses the human Swedish NL double mutation at the beginning of the A $\beta$

encoding region. This mutation increases the susceptibility of APP for cleavage by BACE1 [361, 865]. Another reason why the level of C99 may have been higher than C83 is that the relative rate of cleavage of C99 and C83 by  $\gamma$ -secretase may be different. The level of C99 and C83 depends, in part, on the relative rates of  $\alpha$ - and  $\beta$ -secretase cleavage of APP [95]. However, it may also depend upon the capacity of the cells for  $\gamma$ -secretase cleavage. It has been reported that the  $\gamma$ -secretase degrades C83 much quicker than C99 [864]. Under conditions in which the  $\gamma$ -secretase is not saturated with its substrate, the relative rates of cleavage of C83 and C99 by  $\gamma$ -secretase may greatly influence the level of total C83 or C99. However, under conditions of very high APP overexpression, or under conditions where  $\gamma$ -secretase is almost completely inhibited, the  $\gamma$ -secretase would be saturated and the relative proportions of C99 and C83 would be dependent solely upon the relative rates of  $\beta$ - and  $\alpha$ -secretase cleavage, respectively. In support of this idea, we found that in the presence of the  $\gamma$ -secretase inhibitor DAPT, the proportion of C83 relative to C99 increased greatly.

In summary, studies in this chapter demonstrate that GAGs alter APP metabolism and decrease secretion of A $\beta$ . Low molecular weight heparins have also been shown to cross the blood-brain barrier [823, 842], to arrest amyloid-induced inflammation [827, 828], to decrease A $\beta$  aggregation [828] and to lower the A $\beta$  generation and improve cognition in AD transgenic mice [830, 831]. Therefore, the data in this chapter provide support for the view that heparin analogues may have value for the treatment of AD. However, it is worth considering the possibility that heparin analogues may have toxic side effects which lower their value as therapeutic agents. Studies reported



in this chapter showed that GAGs inhibited  $\alpha$ -secretase processing of APP, and this effect was associated with a decrease in the level of ADAM10. A number of studies have suggested that sAPP $\alpha$  may have important trophic functions [174]. Thus inhibition of sAPP $\alpha$  production could produce adverse effects in vivo. Such a possibility needs to be considered when examining the potential of heparin analogues for the treatment of AD.

## **Chapter 3**

**Size and sulfation are critical for the  
effect of heparin on APP processing and  
A $\beta$  production**

### 3.1 Introduction

The studies reported in Chapter 2 have shown that treatment with heparin can lower A $\beta$  secretion from primary cortical cells [839]. However, although heparin can bind directly to BACE1, decreased secretion of A $\beta$  in cell culture is due to a decrease in the level of BACE1, rather than to direct inhibition of the enzyme.

The development of GAG analogues which can be used for the treatment of AD will require the identification of high potency compounds that have the ability to cross the blood-brain barrier (BBB). Several reports indicate that low molecular weight GAGs can penetrate the BBB [823, 842] and this idea is further supported by the observation that peripheral administration of enoxaparin can lower brain amyloid load [830]. However, developing high-affinity compounds that can inhibit A $\beta$  production may be problematic. Studies reported in Chapter 2 showed that the most potent GAG heparin inhibits A $\beta$  production in cell culture at micromolar concentrations [839]. However, the development of GAG analogues which can be used for the treatment of AD may require high potency compounds acting in the nanomolar concentration range.

The pattern of sulfation of heparan sulfate (HS) may provide specificity for binding to certain proteins. For example, studies by Nurcombe et al. [866] have shown that the specificity of HS for binding to fibroblast growth factor receptors is controlled by the sulfation pattern. Similarly, the fine structure of HS may regulate syndecan-1 function [867], while a specific HS sulfation pattern regulates retinal axon targeting [741]. Studies by Patey et al. [834] show that specific sulfation patterns on heparin derivatives can result in high affinity compounds with great selectivity for inhibition of BACE1 activity and reduced activity against Factor Xa and other proteases. Indeed,

the different sulfation patterns of different HS species may reflect the need to bind specifically to different ligands. On this basis, then, it may be possible to alter the sulfate pattern of GAGs to achieve high affinity and specific effects on APP metabolism and A $\beta$  secretion.

In view of the relationship of GAG size to BBB permeability and of sulfation pattern to binding specificity, the aim of the present study in this chapter was to examine the role of molecular size and sulfation of GAGs on APP processing and A $\beta$  production in primary cell cultures. The effects of various GAGs and sulfated polysaccharides on APP processing were tested in cortical cells derived from transgenic mice expressing human APP<sub>695</sub> with the Swedish familial AD mutant (Tg2576 mouse). These mice were used for the study because human APP and its fragments can be more easily detected by existing anti-human antibodies than rodent APP and A $\beta$ . The results reported in this chapter showed that LMW heparin species can alter APP processing and that the effect of heparin on APP processing is dependent upon the degree of sulfation. Although no high potency GAG analogues were identified in this study, the results demonstrate that there is structural specificity to the effect of GAGs on APP, raising the possibility that high affinity BBB-permeable GAGs may eventually be identified.

## **3.2 Materials and Methods**

### **3.2.1 Materials**

Heparan sulfate (HO-10595) and 12.5 kDa heparin were purchased from Celsus Laboratories, Inc. (Cincinnati, OH, USA). Bovine lung heparin (LH) was from

Calbiochem (Melbourne, Australia). Porcine mucosal heparin, pentosan polysulfate (PPS), 6 kDa heparin (H2149), 3 kDa heparin (H3400), chondroitin sulfate A (ChS A), chondroitin sulfate B (ChS B), chondroitin sulfate C (ChS C), fucoidan, monoclonal anti- $\beta$ -actin antibody, rabbit anti-BACE1 (EE-17) antibody and polyclonal anti-APP C-terminal antibody (APP-CT) were purchased from Sigma-Aldrich Pty. Ltd. (Sydney, Australia). Rabbit anti-ADAM10 (ab1997) and rabbit anti-ADAM17 (ab2051) were purchased from Sapphire Bioscience Pty. Ltd. (Waterloo, Australia). Monoclonal anti-A $\beta$  antibody 6E10 was from Covance Pty. Ltd. (North Ryde, Australia). Neurobasal medium and B27 supplement were purchased from Invitrogen (Mulgrave, Australia). Mouse and rabbit HRP-conjugated secondary antibodies were purchased from DAKO (Campbellfield, Australia). Complete mini protease inhibitor cocktail tablet was from Roche Diagnostics (Castle Hill, Australia).

Mucosal heparin (5 kDa MH) was prepared by treatment of periodate-oxidized mucosal heparin with sodium hydroxide, followed by reduction with sodium borohydride and acid hydrolysis [868]. The resulting heparin fragments had an average degree of polymerization of 16, corresponding to an average molecular weight of 5 kDa. Mucosal heparin lacking the 2-O-sulfate group (MH de 2S), N-sulfate group (MH de NS), all sulfate groups (MH de S) or MH with the carboxyl group removed (MH CR) were prepared as described previously [837]. Mucosal heparin lacking 6-O sulfate (MH de 6S) was prepared by the treatment of the pyridinium salt of heparin with N,O-bis(trimethylsilyl)acetamide in pyridine for 2 h at 60 °C [869]. This procedure resulted in specific 6-O-desulfation of MH without depolymerisation or other chemical changes.

### **3.2.2 Primary cortical cell culture**

Cortical cells were prepared from brains of newborn (P<sub>0</sub>) Tg2576 mice. Cerebral cortices were dissected in Neurobasal medium and incubated with 0.25% (w/v) papain and 0.06% (w/v) deoxyribonuclease I (DNase I) mixture for 20 min at 37 °C, followed by three washes with Neurobasal medium. Cells were then separated by gentle mechanical dissociation and 3×10<sup>5</sup> cells/well were plated onto poly-D-lysine-coated 12-well culture plates, and maintained in 1.2 mL complete Neurobasal medium containing 2% B27 supplement, 1 mM glutamine, and 1% penicillin/streptomycin (10,000 units of penicillin and 10,000 µg of streptomycin stock) in an atmosphere containing 5% CO<sub>2</sub> at 37 °C. After 3 days in vitro (DIV), half of the culture medium was replaced with fresh complete Neurobasal medium. All experiments were performed at 7 DIV. Primary cortical cells were incubated with heparin derivatives or other compounds for 24 hours prior to analysis.

### **3.2.3 SDS-polyacrylamide gel electrophoresis and western blotting**

Culture medium was removed from cells for determination of A $\beta$  and sAPP $\alpha$ . The cells were incubated with cold RIPA buffer (150 mM NaCl, 50 mM Tris, 0.5% w/v Na-deoxycholate, 1% v/v Nonidet P-40, 0.1% SDS, pH 7.4) containing protease inhibitor cocktail on ice for 10 min and the cell lysates were then harvested for determination of C99, BACE1, ADAM10, ADAM17 and total APP. The protein concentration of cell lysate was measured using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories Pty. Ltd., Gladesville, Australia) with bovine serum albumin as standard.

The amount of A $\beta$ 40 or A $\beta$ 42 secreted into cell medium was determined using 15% Tris-bicine-urea SDS-PAGE as described previously [854]. The level of C99, sAPP $\alpha$ , BACE1, ADAM10, ADAM17 and APP was determined as described in Chapter 2. The density of protein staining was quantified using Image J software (Research Service Branch; National Institute of Health, <http://rsbweb.nih.gov/ij/index.html>). The ratio of staining intensity of each protein to the staining intensity of  $\beta$ -actin was determined and then each ratio was used to calculate a percentage relative to mean value for control incubations. The statistical tests were performed using SigmaPlot software (10.0v; Systat Software, Inc., San Jose, CA, USA). Statistical comparisons were made using one-way analysis of variance followed by Tukey test for *post hoc* comparisons. Values of  $p < 0.05$  were considered statistically significant.

### **3.3 Results**

#### **3.3.1 Effect of heparin fragments on APP processing**

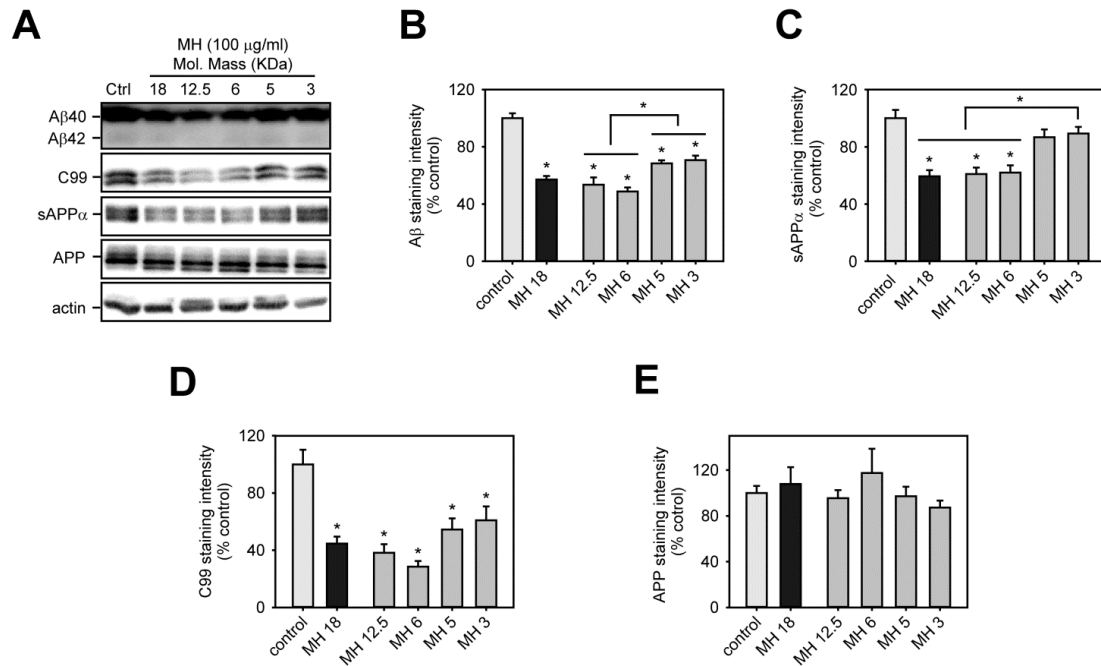
Initially, the relationship between the size of heparin fragments and the effect on APP processing was examined. Cortical cells derived from brains of newborn Tg2576 mice were treated with heparin fragments of different size (18 kDa, 12.5 kDa, 6 kDa, 5 kDa and 3 kDa) for 24 hours, and the level of total APP, sAPP $\alpha$ , C99 and A $\beta$  was measured. For the measurement of A $\beta$ , only the level of A $\beta$ 40 was quantified, because, as described in Chapter 2, very little A $\beta$ 42 was produced by the cells and the level could not be measured accurately (Fig. 3.1 A).

In agreement with the observations in Chapter 2, treatment of cells with 100  $\mu$ g/mL 18 kDa MH (MH 18) reduced the level of A $\beta$  secretion to approximately 60% of control values (Fig. 3.1 A and B). MH fragments ranging in molecular mass from 3

kDa to 12.5 kDa also decreased the level of A $\beta$  secretion when incubated at the same concentration. However, 3 kDa and 5 kDa MH were less potent in decreasing A $\beta$  secretion than 6 and 12.5 kDa MH (Fig. 3.1 A and B). Associated with the decrease in A $\beta$  secretion, there was also a decrease in the level of sAPP $\alpha$  secretion in the presence of 6, 12.5 and 18 kDa MH, although levels of sAPP $\alpha$  were not significantly reduced by 3 kDa and 5 kDa MH (Fig. 3.1 A and C). The effect of 6, 12.5 and 18 kDa MH on the level of sAPP $\alpha$  was greater than the effect of 3 kDa MH. All MH fragments decreased the level of C99 in the cells, but did not decrease the level of total APP (Fig. 3.1 A, D and E).

The results showed that MH with molecular masses between 3 kDa and 12.5 kDa can inhibit APP processing to A $\beta$ . As the studies reported in Chapter 2 demonstrated that the decrease in  $\alpha$ - and  $\beta$ -secretase processing of APP was due to a specific decrease in the level of BACE1 ( $\beta$ -secretase) and ADAM10 ( $\alpha$ -secretase), the effects of different sized MH fragments on the level of  $\beta$ -secretase (BACE1) and two major  $\alpha$ -secretases (ADAM10 and ADAM17) were examined (Fig. 3.2). The level of BACE1 was significantly reduced to approximately 30-50% of control values by treatment with 3, 5, 6, 12.5 and 18 kDa MH fragments (Fig. 3.2 A and B). The large MH fragments (6, 12.5 and 18 kDa) reduced the level of ADAM10 to approximately 10-20% of the control values (Fig. 3.2 A and C). MH (5 kDa) also decreased the level of ADAM10 but to a lesser extent than the large MH fragments, and 3 kDa MH did not have any significant effect on ADAM10 levels (Fig. 3.2 A and C). In contrast to ADAM10, the level of ADAM17 was not affected by any of the MH fragments tested (Fig. 3.2 A and D).



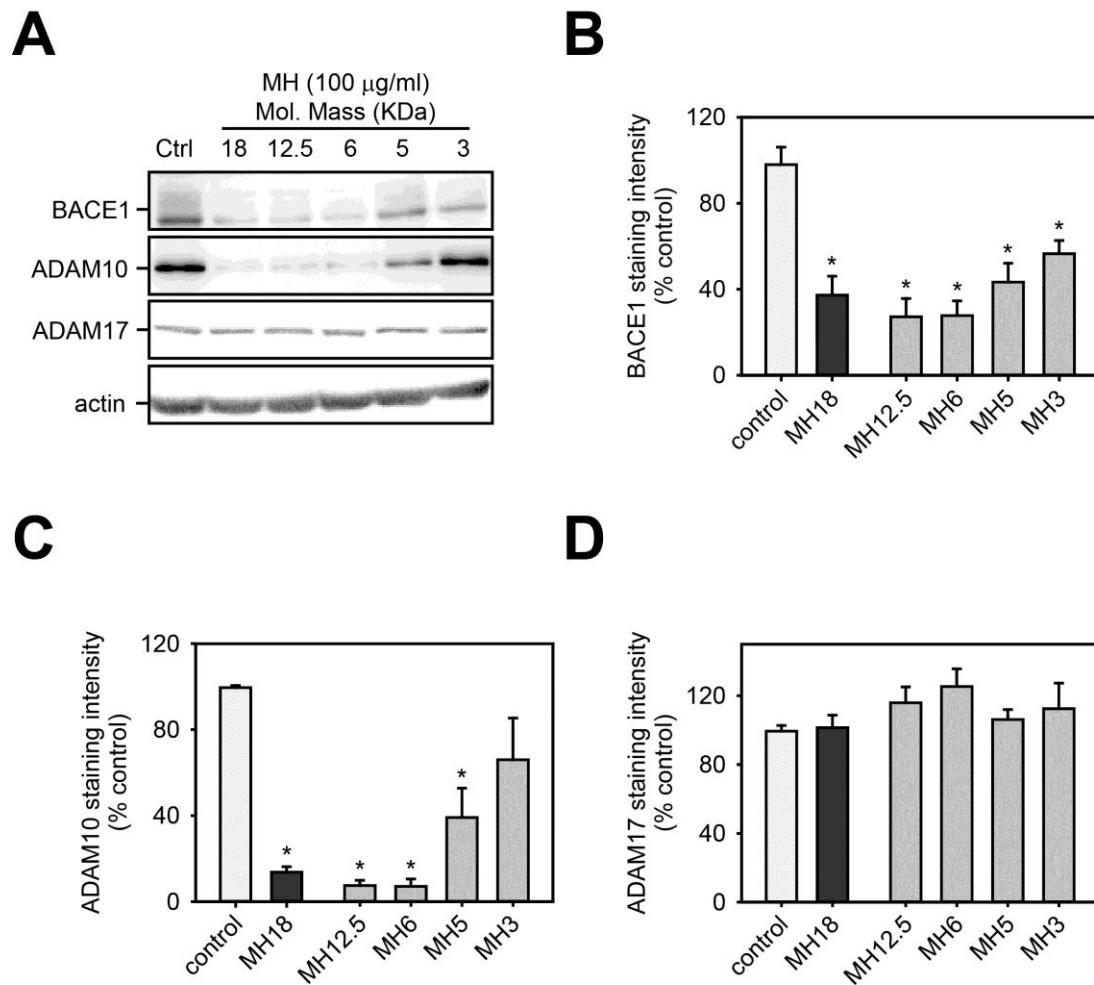


**Fig. 3.1.** Effects of mucosal heparin (MH) fragments on APP processing. Tg2576 mouse cortical cells were treated with different heparin fragments (100  $\mu\text{g/mL}$ ) for 24 hours. A $\beta$ 40, C99, sAPP $\alpha$  and total APP were measured using western blotting. (A) Typical western blots showing the effects of heparin fragments on the level of A $\beta$ 40, C99, sAPP $\alpha$  and total APP. Figure also shows quantification of A $\beta$ 40 immunoreactivity (B), sAPP $\alpha$  immunoreactivity (C), C99 immunoreactivity (D) and total APP immunoreactivity (E) on the western blots. Asterisks show values that are significantly different from control incubations ( $p < 0.05$ ,  $n = 8$ ).

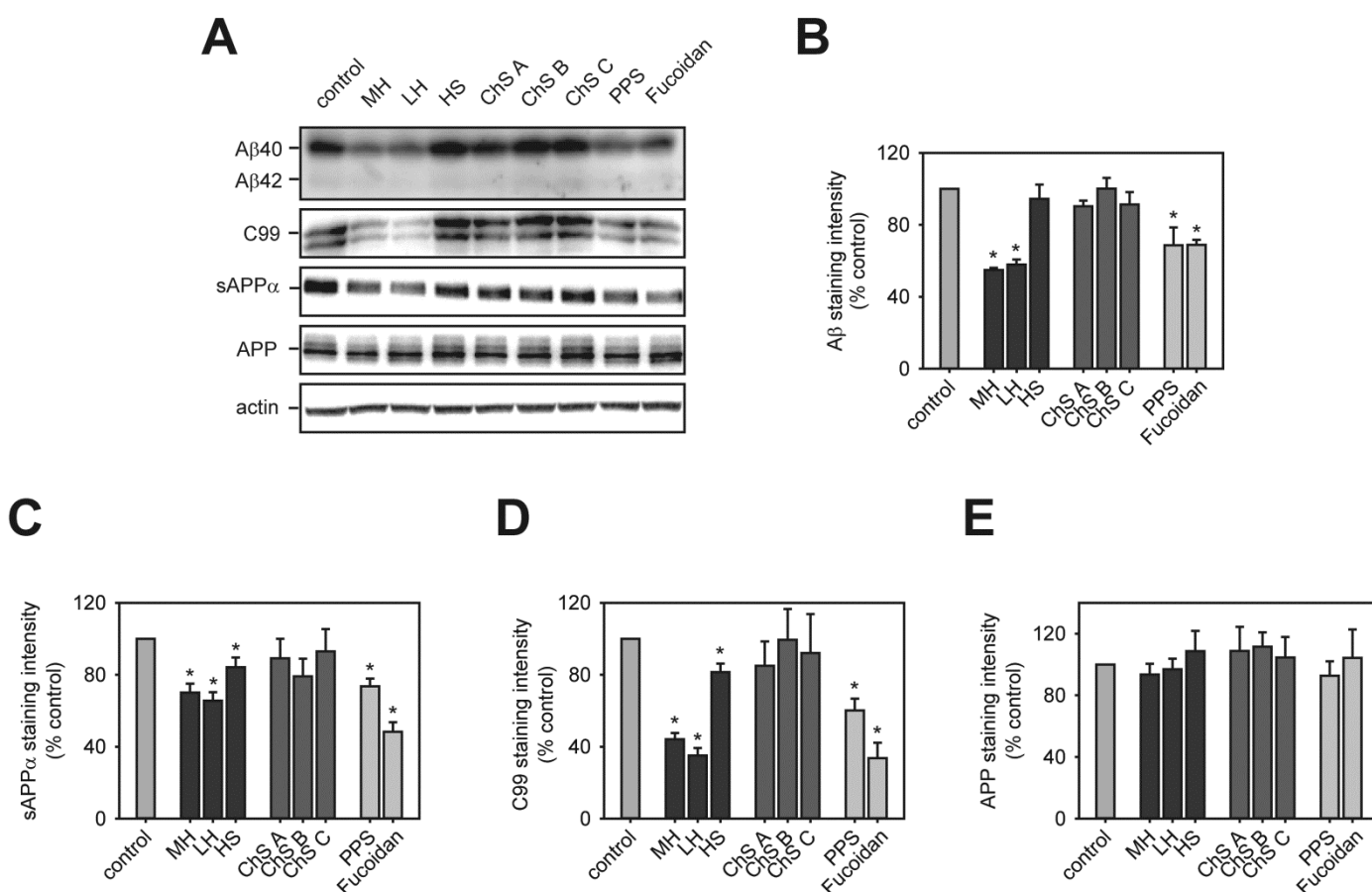
### 3.3.2 Effects of different classes of GAGs on APP processing and A $\beta$ production

To examine the specificity of the effect of MH on APP processing further, the ability of different classes of GAG and other polysulfated compounds to alter A $\beta$  production from APP were compared. Primary cortical cells were treated with mucosal heparin (MH), lung heparin (LH), heparan sulfate (HS), chondroitin sulfate A (ChS A), chondroitin sulfate B (ChS B), chondroitin sulfate C (ChS C) and sulfated polysaccharides (PPS and fucoidan). The relative amount of sAPP $\alpha$ , C99, A $\beta$ , BACE1, ADAM10 and ADAM17 was then measured by western blotting.

Initially, the effect of MH was compared with that of LH and HS. LH is a more highly sulfated form of heparin than MH, whereas HS from porcine mucosal tissue is less highly sulfated [666]. Incubation with LH led to a similar decrease in the level of A $\beta$  as was observed with MH (Fig. 3.3 A and B), whereas HS had no effect on A $\beta$  levels in the culture medium. The chondroitin sulfates (ChS A, ChS B and ChS C) did not reduce secretion of A $\beta$  when incubated at a concentration of 100  $\mu$ g/mL. Polysulfates, both PPS and fucoidan at a concentration of 100  $\mu$ g/mL, decreased A $\beta$  secretion to a similar extent as MH and LH (Fig. 3.3 A and B). Incubation with MH, LH, HS, PPS and fucoidan caused a significant decrease in sAPP $\alpha$  secretion. However, sAPP $\alpha$  secretion was not affected by treatment with ChS A, ChS B and ChS C (Fig. 3.3 A and C). Several GAGs (MH, LH and HS), PPS and fucoidan also significantly decreased the level of C99, although levels of C99 were not altered by ChS A, ChS B or ChS C treatment (Fig. 3.3 A and D). All of these different GAGs had no significant effect on the level of total APP (Fig. 3.3 A and E).



**Fig. 3.2.** Effects of mucosal heparin (MH) fragments on the level of BACE1, ADAM10 and ADAM17. Cells were treated with different heparin fragments (100  $\mu$ g/mL) for 24 hours and the level of BACE1, ADAM10 and ADAM17 were measured using western blotting. (A) Typical western blots showing the level of BACE1, ADAM10 and ADAM17 after MH fragments treatment. Figure shows quantification of BACE1 immunoreactivity (B), ADAM10 immunoreactivity (C) and ADAM17 immunoreactivity (D) on the western blots. Asterisks show values that are significantly different from control treatments. ( $p < 0.05$ ,  $n = 8$ ).



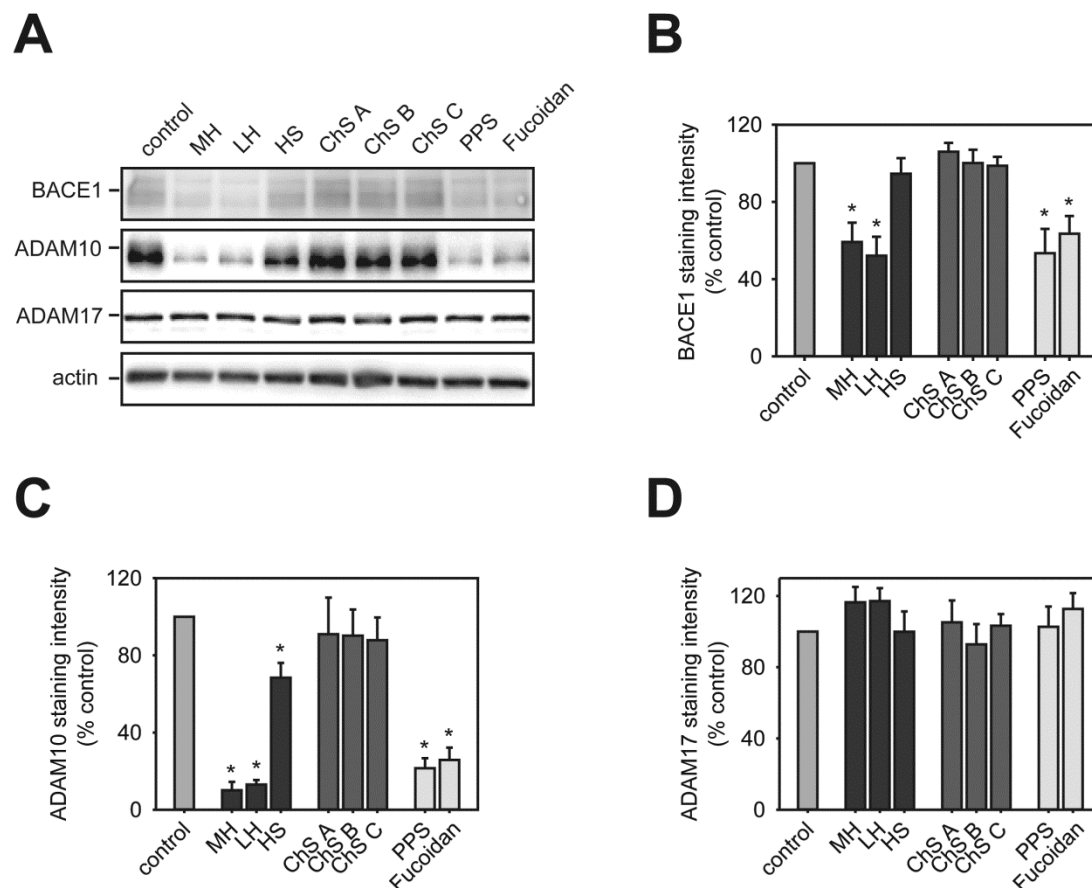
**Fig. 3.3.** Effects of different classes of glycosaminoglycans (GAGs) and sulfated polysaccharides on APP processing and Aβ production. Primary cortical cells from Tg2576 were treated with different GAGs or sulfated polysaccharides (100 μg/mL) for 24 hours. Levels of Aβ40, C99, sAPPα and total APP were measured using western blotting. (A) Western blots showing the effects of different GAGs and sulfated polysaccharides on the level of Aβ40, C99, sAPPα and total APP. Figure also shows quantification of Aβ40 immunoreactivity (B), sAPPα immunoreactivity (C), C99 immunoreactivity (D) and total APP immunoreactivity (E) on the western blots. Asterisks show values that are significantly different from control incubations (p<0.05, n=8).

Next, the effects of GAG analogues on BACE1, ADAM10 and ADAM17 were examined. MH, LH, PPS and fucoidan (100 µg/mL) decreased the level of BACE1 and ADAM10 to approximately 50% and 20% of the control values, respectively (Fig. 3.4 A, B and C). HS had a small but significant effect on ADAM10, decreasing the enzyme by approximately 20%, but HS had no effect on the level of BACE1. ChS A, ChS B and ChS C did not lower the levels of either BACE1 or ADAM10. None of the GAGs tested had any significant effect on the level of ADAM17 (Fig. 3.4 A and D).

### **3.3.3 Effects of selectively desulfated and decarboxylated heparin on APP processing**

The structure specificity studies indicated that the highly sulfated MH and LH are more potent in their ability to disrupt APP processing than HS, which is less sulfated. The studies also showed that ChS A, ChS B and ChS C, all of which are less sulfated than MH and LH [666], had no significant effect. Furthermore, the highly sulfated polysaccharides PPS and fucoidan had similar effects to the highly sulfated GAGs (MH and LH) on APP processing. These results suggested that the degree of sulfation could be important for the ability of GAGs to regulate APP processing.

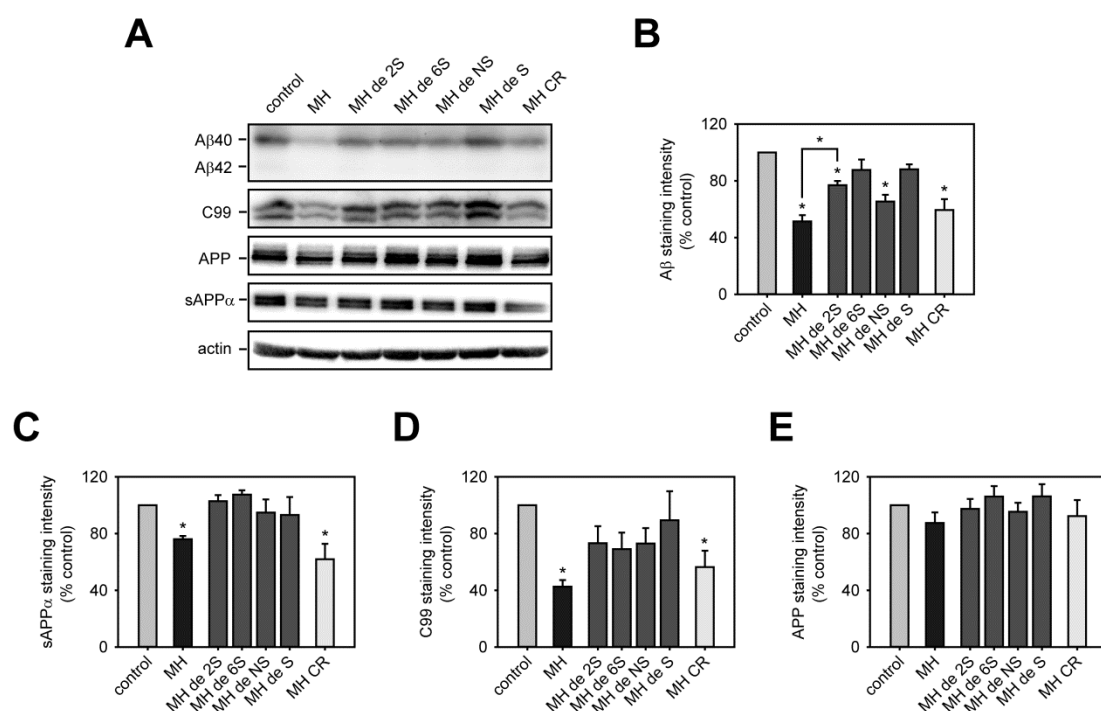
Therefore, to examine the role of specific negatively charged sulfate and carboxyl groups of heparin on APP processing, MH derivatives were generated without 2-O sulfate (MH de 2S), or 6-O sulfate (MH de 6S), or N-sulfate (MH de NS), or without any sulfates (MH de S) or with the carboxyl group removed (MH CR). Primary cortical cells were then treated with these MH derivatives and APP fragments and APP cleavage enzymes were examined by western blotting.



**Fig. 3.4.** Effects of different classes of glycosaminoglycans (GAGs) and sulfated polysaccharides on the level of BACE1, ADAM10 and ADAM17. Primary cortical cells were treated with different GAGs and sulfated polysaccharides (100  $\mu\text{g/mL}$ ) for 24 hours and the level of BACE1, ADAM10 and ADAM17 were measured by western blotting. (A) Typical western blots showing the level of BACE1, ADAM10 and ADAM17 after different types of GAGs and sulfated polysaccharides treatment. Figure also shows quantification of BACE1 immunoreactivity (B), ADAM10 immunoreactivity (C) and ADAM17 immunoreactivity (D) on the western blots. Asterisks show values that are significantly different from control treatments ( $p < 0.05$ ,  $n = 8$ ).

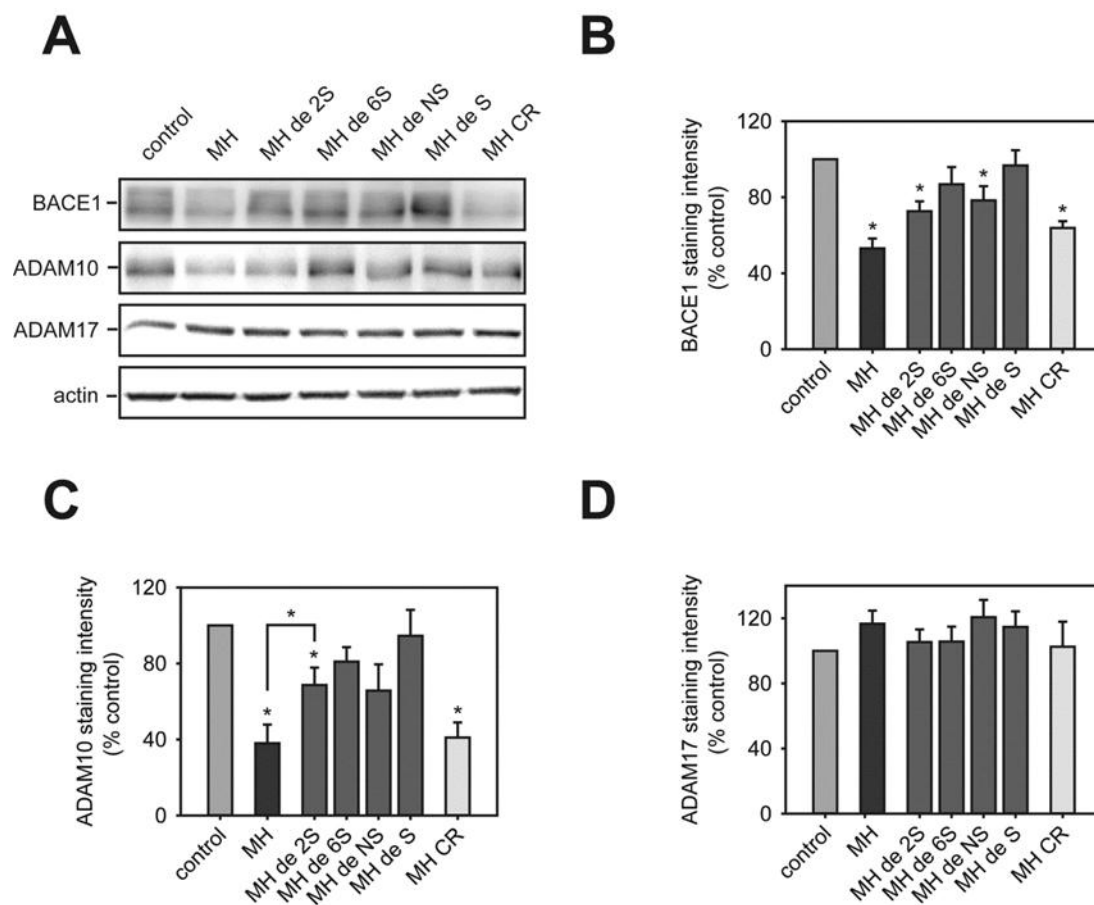
Compared with native MH, which reduced A $\beta$  secretion to approximately 50% of control values, both MH derivatives lacking one sulfate residue (MH de 2S and MH de NS) were less potent in lowering A $\beta$  (Fig. 3.5 A and B) compared to native MH. MH derivatives lacking 6-O sulfate (MH de 6S) or those lacking all sulfate groups were unable to decrease A $\beta$  secretion (Fig. 3.5 A and B). By contrast, MH lacking the carboxyl group (MH CR) had a similar effect to native MH on A $\beta$  secretion (Fig. 3.5 A and B). MH CR decreased sAPP $\alpha$  and C99 similarly to native MH, however, desulfated MH had no significant effect on either the level of sAPP $\alpha$  or C99 (Fig. 3.5 A, C and D). In addition, all of these MH derivatives had no effect on the level of total APP (Fig. 3.5 A and E).

The role of sulfate and carboxyl groups of MH on the level of BACE1, ADAM10 and ADAM17 were also examined. In contrast to MH, MH de 2S and MH de NS only weakly decreased BACE1 (Fig. 3.6 A and B). Neither MH de 6S nor MH de S lowered the level of BACE1. However, MH CR was similar to native MH in its ability to lower the level of BACE1 (Fig. 3.6 A and B). Native MH and MH CR possessed similar potency in decreasing ADAM10. MH de 2S significantly reduced ADAM10 but to a lesser extent than MH. By contrast, MH de 6S, MH de NS and MH de S did not affect the level of ADAM10 (Fig. 3.6 A and C). None of these MH derivatives had a significant effect on the level of ADAM17 (Fig. 3.6 A and D).



**Fig. 3.5.** Effects of selectively desulfated and decarboxylated MH on APP processing and Aβ production. Primary cortical cells from Tg2576 mice were treated with MH derivatives (100 μg/mL) lacking 2-O sulfate (MH de 2S), 6-O sulfate (MH de 6S), N-sulfate (MH de NS), all sulfates (MH de S) or carboxyl group (MH CR) for 24 hours. The levels of Aβ40, C99, sAPPα and total APP were measured using western blotting. (A) Western blots showing the effects of MH derivatives on the level of Aβ40, C99, sAPPα and total APP. Figure also shows quantification of Aβ40 immunoreactivity (B), sAPPα immunoreactivity (C), C99 immunoreactivity (D) and total APP immunoreactivity (E) on the western blots. Asterisks show values that are significantly different from control incubations ( $p < 0.05$ ,  $n = 8$ ).





**Fig. 3.6.** Effects of selectively desulfated and decarboxylated MH on the level of BACE1, ADAM10 and ADAM17. Tg2576 cortical cells were treated with 100  $\mu$ g/mL MH derivatives lacking 2-O sulfate (MH de 2S), 6-O sulfate (MH de 6S), N-sulfate (MH de NS), all sulfates (MH de S) or carboxyl group (MH CR) and the level of BACE1, ADAM10 and ADAM17 were measured using western blotting. (A) Western blots showing the level of BACE1, ADAM10 and ADAM17 after treatment of different MH derivatives. Figure also shows quantification of BACE1 immunoreactivity (B), ADAM10 immunoreactivity (C) and ADAM17 immunoreactivity (D) on the western blots. Asterisks show values that are significantly different from control treatments ( $p < 0.05$ ,  $n = 8$ ).

### 3.4 Discussion

The effects of different sulfated carbohydrates on APP processing and A $\beta$  production was examined in primary cortical cells derived from APP (SW) Tg2576 mice. Results reported in this chapter showed that both size and structure of GAGs are important for effects on APP processing. Indeed, the sulfation of MH is essential for an effect on APP processing. However, the carboxyl group on MH was not important since deletion of the carboxyl group did not block the effect of MH on APP processing.

LH, which contains more highly sulfated disaccharide units than MH [666], was similar to MH in its effects on APP processing. PPS and fucoidan, which are highly sulfated polysaccharides [870, 871] also reduced APP proteolytic processing. As PPS and fucoidan have a similar degree of sulfation to MH and LH, the results support the view that sulfation is important for the effect on APP processing. The results also suggest that the backbone structure of the carbohydrate may not be as important for the effect on APP processing, as PPS and fucoidan, which do not belong to the GAG family, were as potent as MH and LH. The fact that polysulfated compounds that are not GAGs also altered APP processing suggests that other sulfated polysaccharides could be candidates for the screening of therapeutic agents for AD. Compared with the highly sulfated GAGs and polysaccharides, HS and chondroitin sulfate A, B and C, which are less highly sulfated [666], were less potent in lowering BACE1 and ADAM10. Furthermore, they did not lower A $\beta$  levels. Taken together, these results indicate that sulfation is vital for the effect on the level of BACE1, ADAM10 and on APP processing.

To confirm the role of sulfation on APP processing further, individual sulfate groups were removed from MH and the effect of removal was determined. Results reported in this chapter indicated that the removal of any sulfate group decreased the potency of MH on APP processing. The 6-O sulfate was the most important of all the sulfate groups, as MH de 6S had no effect on the level of BACE1 and ADAM10. MH derivatives lacking all sulfate groups lacked the ability to disrupt APP processing. In contrast, removal of the carboxyl groups on MH did not attenuate the effects of MH on APP cleavage, suggesting that while sulfation of GAGs is important for inhibition of APP processing, the effect was not solely due to the negative charge on the carbohydrate.

The results reported in this chapter provide evidence that it may be possible to selectively modify the structure of GAGs and reduce the unwanted side effects without changing the potency of GAGs on APP processing. For instance, the carboxyl group is essential for the anticoagulant and vasodilatory activity of heparin [872, 873]. Chemical removal of the carboxyl group of heparin could reduce these unwanted effects but still potentially inhibit APP processing.

Results reported in this chapter also showed that the ability of GAGs to inhibit APP processing is dependent on chain length. A minimum GAG size of 6 kDa, which is equivalent to a length of 17 saccharide monomers, was necessary to achieve a similar effect on APP processing as that obtained with MH. While 6 kDa MH was similar to native 18 kDa MH in its effect on BACE1 and ADAM10, small heparin fragments (e.g. 3 kDa) only weakly reduced the level of BACE1 and ADAM10 and only weakly inhibited APP processing.

Heparin fragment size may influence its activity in vivo. It has been reported that a chain length of 17 saccharides (approximately 6 kDa) is required for efficient thrombin inhibition [874]. The chain length of heparin is also important for its binding to fibroblast growth factor (FGF)-2, as the tetrasaccharides or longer oligosaccharides of heparin are required to bind to FGF-2 and induce proliferation of chlorate-treated rat mammary fibroblasts [875]. These data suggest that it may be possible to design GAGs which can alter APP processing but which have fewer unwanted side effects.

It may also be possible to reduce the size of GAGs so that they can cross the BBB and still retain the ability to decrease A $\beta$  production. Previous studies have shown that full-length MH cannot cross the BBB, whereas 3 kDa or smaller heparins can cross [823, 842]. In the study of this chapter, the 3 kDa MH derivative decreased APP processing, albeit weakly, raising the possibility that small MH derivatives, without hemorrhagic side-effects, could pass through the BBB and inhibit APP processing.

Whether GAGs will have therapeutic value for the treatment of AD is still unclear. While it may be possible to design compounds which are more limited in their actions (i.e. affect APP processing and A $\beta$  production without unwanted side effects) and which cross the BBB, it is still unclear whether high potency compounds will be identified. In this regard, it was of particular interest to note that fucoidan was as potent as MH in lowering A $\beta$  levels. Fucoidans are a group of polysaccharides derived from algae and seaweed. Because of their considerable structural diversity, it seems logical to investigate further the effect of other fucoidans on A $\beta$  production.

Another consideration with regard to the suitability of GAGs as drugs for the treatment of AD is their action on sAPP $\alpha$ . GAGs also reduced the secretion of sAPP $\alpha$  in the studies reported in this chapter. However, some studies suggest that sAPP $\alpha$  may have neurotrophic actions [220, 224, 227, 876, 877]. To date, the side effects of decreasing sAPP $\alpha$  production are unknown. GAG derivatives which act specifically on the  $\beta$ -secretase cleavage pathway of APP may be needed.

In summary, this study shows that there is structural specificity to the effects of GAG on APP processing. LMW heparins can cross the BBB [823, 842] and potentially may attenuate A $\beta$ -induced inflammation [827, 828], decrease A $\beta$  aggregation [828], lower A $\beta$  generation and improve cognition [830, 831]. Ultimately, it may be possible to design more potent GAG derivatives which act specifically to inhibit  $\beta$ -secretase cleavage of APP that can be used for the treatment of AD.

## **Chapter 4**

### **Effects of enoxaparin on APP processing and A $\beta$ production in Tg2576 mice**

## 4.1 Introduction

The studies reported in Chapters 2 and 3 and those of other groups [780, 824, 828, 830, 833] raise the possibility that glycosaminoglycans (GAGs) or GAG analogues may have potential for the treatment of Alzheimer's disease. The studies reported in Chapter 2 have shown that treatment with heparin or enoxaparin (ENO) can lower A $\beta$  secretion from primary cortical cells. However, although heparin can bind directly to BACE1 and influence its activity [836, 837], decreased secretion of A $\beta$  in cell culture was found to be due to a decrease in the level of BACE1, rather than to direct inhibition of the enzyme.

Previously, peripheral administration of ENO has been reported to reduce the A $\beta$  level and amyloid plaque load in the brain of APP transgenic mice [830]. However, the exact mechanism of these effects is unclear. The studies in cell culture reported in Chapters 2 and 3 raise the possibility that administration of ENO to the APP transgenic mice may reduce APP processing by downregulating the level of BACE1 and thereby cause the decrease of A $\beta$  and amyloid plaque load. Therefore, the aim of this chapter was to examine whether the reduced amyloid plaque load in the brain of the APP transgenic mouse is due to the decreased APP processing to A $\beta$  caused by ENO treatment.

ENO was peripherally injected into APP transgenic mice (Tg2576) which can develop AD-like pathology such as amyloid plaque. After injection of ENO, the APP processing products and amyloid load in the brains of the Tg2576 mice were examined. The results indicate that ENO treatment decreases the A $\beta$ <sub>40</sub>/A $\beta$ <sub>42</sub> ratio in

cortex and increases the amyloid plaque load in both cortex and hippocampus, while the APP processing is not significantly influenced by ENO injection.

## **4.2 Material and Methods**

### **4.2.1 Materials**

Monoclonal anti- $\beta$ -actin antibody, rabbit anti-BACE1 (EE-17) antibody and polyclonal anti-APP C-terminal antibody (APP-CT) were purchased from Sigma-Aldrich Pty. Ltd. (Sydney, Australia). Rabbit anti-ADAM10 (ab1997) was purchased from Sapphire Bioscience Pty. Ltd. (Waterloo, Australia). Polyclonal rabbit anti- $\beta$ -amyloid (1-40) antibody was purchased from Invitrogen (Mulgrave, Australia). Mouse monoclonal SMI312 antibody and monoclonal anti-A $\beta$  antibody 6E10 were from Covance Pty. Ltd. (North Ryde, Australia). Mouse and rabbit HRP-conjugated secondary antibodies were purchased from DAKO (Campbellfield, Australia). Enoxaparin (ENO) sodium (Clexane<sup>®</sup>) was from Sanofi-Aventis (Macquarie Park, Australia).

### **4.2.2 Animals and ENO treatment**

Female Tg2576 mice were group housed in a controlled environment with a 12-hour light-dark cycle and a constant temperature of 21°C. Standard food pellets and water were given *ad libitum*.

Tg2576 mice (8 months of age) were injected intraperitoneally with 100  $\mu$ l of water or ENO (60  $\mu$ g), three times per week for 5 months. At the end of the treatment, all the mice were anaesthetised with 140 mg/kg sodium pentobarbitone and transcardially



perfused with PBS. Mice were decapitated and their brains were collected. The cortex and hippocampus from one hemisphere were dissected and kept at -80 °C for biochemical assays. The other hemisphere was fixed in 4% paraformaldehyde, the tissues were then cryoprotected and 40 µm coronal sections were cut on a cryostat.

#### **4.2.3 Immunohistochemistry**

To examine the number of amyloid plaques and amyloid load in the brains of Tg2576 mice, five tissue sections from each Tg2576 mouse were assessed. These five sections were spaced 800 µm apart. All sections chosen for examination of amyloid load were treated with formic acid (90%) for 20 minutes followed by 7 washes in PBS and double immunolabeled with polyclonal rabbit anti-β-amyloid (1-40) antibody (1:500) and mouse monoclonal SMI312 antibody against a pan-axonal neurofilament marker (1:2000) for 4 hours at room temperature and then overnight at 4°C. The sections were washed 3 times with PBS and then incubated in the dark with a goat anti-rabbit IgG Alexa Fluor 488 and goat anti-mouse IgG Alexa Fluor 594 secondary antibody (1:1000) for 2 hours at room temperature. The sections were then washed 3 times with PBS, and then dried and mounted with mounting medium (Dako). Images were acquired with an Olympus BX50 microscope with 20x objective (NA 0.6), cooled CCD camera (Photometrics) and commercial software (NIS Elements, Nikon).

The number of amyloid plaques and amyloid load were determined for the entire neocortex superior to the rhinal fissure, and the hippocampus (when present) in one hemisphere of each section of tissue. The size of amyloid plaque and the area of neocortex and hippocampus were quantified using Image J software (Research Service Branch; National Institute of Health, <http://rsbweb.nih.gov/ij/index.html>). The

low magnification images were obtained to determine the total area of neocortex and hippocampus in each tissue section, and high magnification (20x) images were used to quantify the total A $\beta$  plaque number and area for each tissue section. Plaques smaller than 5  $\mu$ m in core diameter were ignored. All image collection, plaque number counts and area quantification were performed blindly.

#### **4.2.4 SDS-PAGE and western blotting**

Frozen cortex and hippocampus were homogenized using a disposable polypropylene pestle in 1 ml sample buffer (10%  $\beta$ -mercaptoethanol, 2% SDS, 50 mM Tris and 10% glycerol, pH 6.8). Samples were sonicated for 4 min and centrifuged for 10 minutes at 13,000 rpm in 4  $^{\circ}$ C, the supernatant fraction was collected. The protein concentration of the supernatant fraction was measured using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories Pty. Ltd., Gladesville, Australia) with bovine serum albumin as standard.

The level of A $\beta$ 40, A $\beta$ 42, C99, C83, BACE1, ADAM10 and total APP was measured as described in Chapter 2. The density of protein staining was quantified using Image J software. The ratio of staining intensity of each protein to the staining intensity of  $\beta$ -actin was determined and then each ratio was used to calculate a percentage relative to mean value for control incubations. The statistical tests were performed using SigmaPlot software (10.0v; Systat Software, Inc., San Jose, CA, USA). Statistical comparisons were made using Student's *t* tests. Values of  $p < 0.05$  were considered statistically significant.

## **4.3 Results**

### **4.3.1 Effect of ENO on the level of A $\beta$**

The Tg2576 mice were treated with 60mg ENO or water three times per week for 5 months. After ENO treatment, the level of A $\beta$  in cortex and hippocampus was examined by western blotting. The results showed that in the ENO injection group, the mean A $\beta$ 40 level in cortex was 90% the control value, but the mean A $\beta$ 42 level was approximately 115% of the mean control value (Fig. 4.1 A, C and E). However, these differences were not statistically significant. In the hippocampus, the mean A $\beta$ 40 and A $\beta$ 42 levels were 50% greater than control levels, although the difference was not statistically significant (Fig. 4.1 B, D and F). As the A $\beta$ 40/A $\beta$ 42 ratio is an important factor in determining A $\beta$  aggregation in vitro [97], the ratio of A $\beta$ 40 to A $\beta$ 42 was also calculated. Compared with the vehicle group, the A $\beta$ 40/A $\beta$ 42 ratio was significantly decreased to approximately 70% of the mean control value in the cortex after ENO treatment, while the ratio in hippocampus was not influenced by the administration of ENO (Fig. 4.1 G and H).

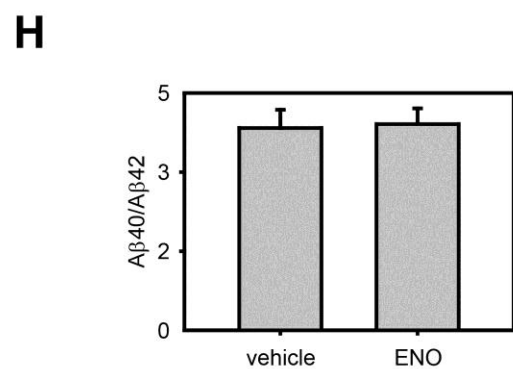
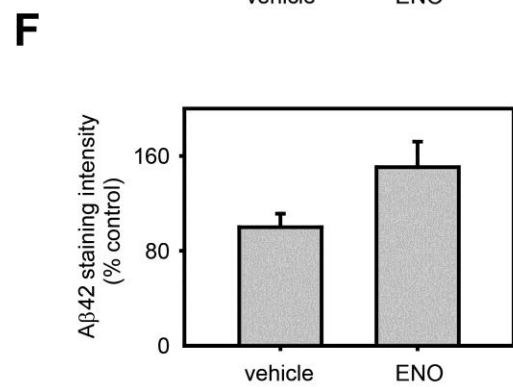
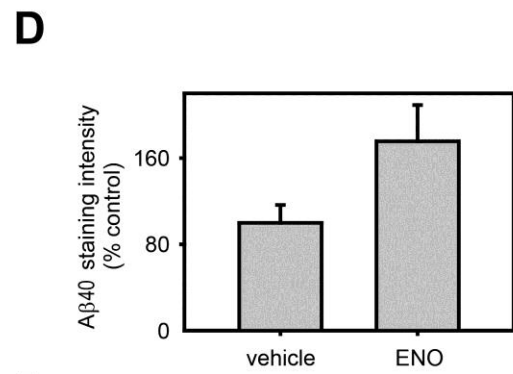
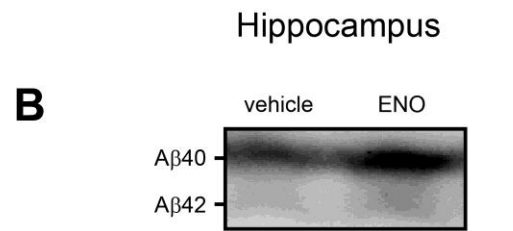
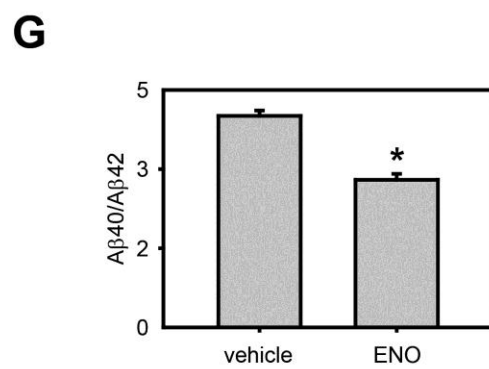
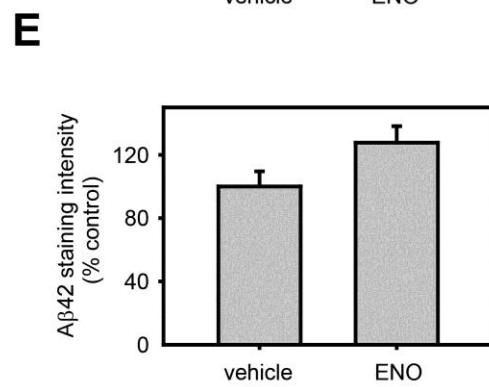
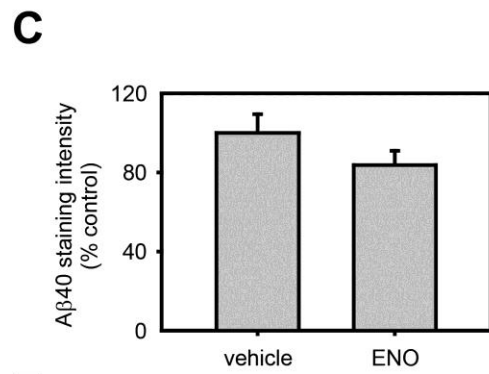
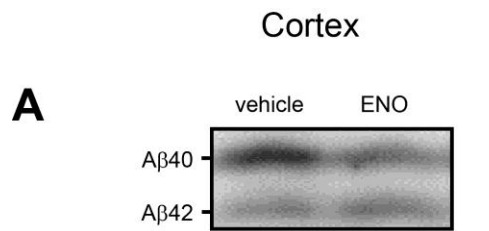
### **4.3.2 Effect of ENO on levels of APP, APP C-terminal fragments and APP cleavage enzymes**

Cell culture studies reported in Chapter 2 showed that ENO treatment could inhibit APP processing in vitro. In the studies reported in this chapter, the effect of ENO injection on APP processing was examined by measuring the APP proteolytic products C99 and C83. Similar to the observations reported in Chapter 2, there were two bands observed on the western blots corresponding to C99 or C83. The upper

band was previously shown be the phosphorylated form of C99 or C83. Both bands of C99 or C83 were quantified.

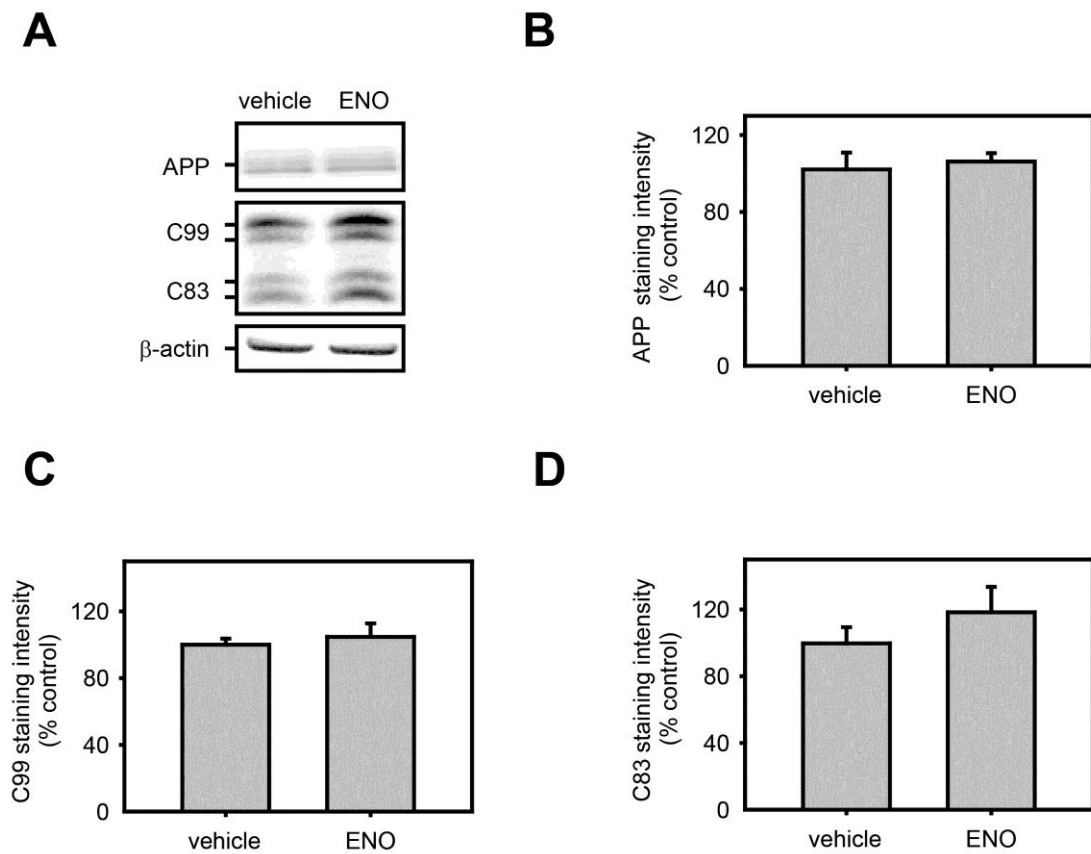
In cortex, after ENO injection, there was no significant alteration in the level of APP (Fig. 4.2 A and B). The level of C99 was also not influenced by ENO treatment, while the level of C83 was slightly greater compared to vehicle treatment, but was not statistically significant (Fig. 4.2 A, C and D). In hippocampus, the level of APP, C99 and C83 was also determined by western blotting. Administration of ENO to Tg2576 mice had no significant effect on the level of APP (Fig. 4.3 A and B). The level of C99 and C83 was approximately 15 % lower in the ENO-treated mice compared to vehicle-treated mice. However, this difference was also not statistically significant (Fig. 4.3 A, C and D).

The cell culture studies in Chapters 2 and 3 suggested that ENO treatment could decrease the level of both BACE1 and ADAM10 and therefore inhibit APP processing through both  $\beta$ - and  $\alpha$ -secretase cleavage pathways. In the study in this chapter, the effect of ENO injection on the level of BACE1 and ADAM10 in Tg2576 mice was also examined, and the results indicated that there was no significant alteration on the level of both BACE1 and ADAM10 in cortex and also hippocampus after injection of ENO (Fig. 4.4).



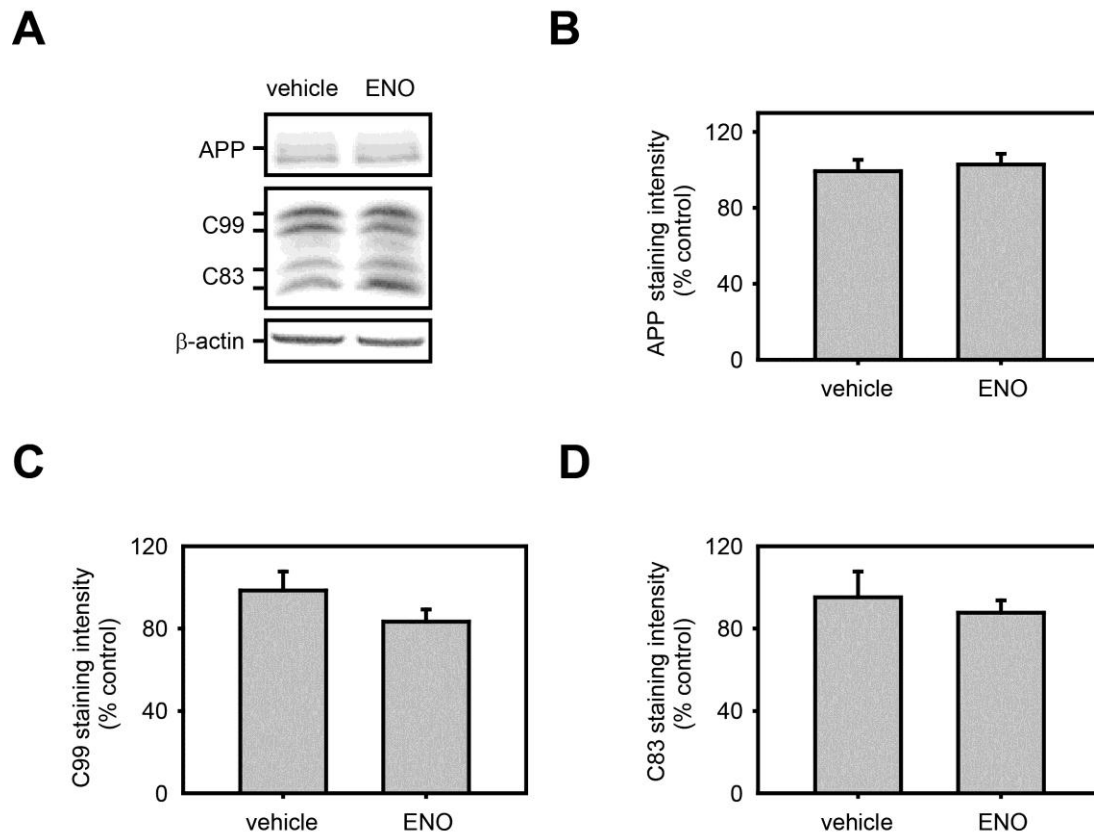
**Fig. 4.1.** Effect of peripheral injection enoxaparin (ENO) on A $\beta$  in cortex (A, C, E, G) and hippocampus (B, D, F, H) of Tg2576 mice. The level of A $\beta$ 40 and A $\beta$ 42 was measured using western blotting. Figure shows the representative western blots showing the effects of ENO on the level of A $\beta$ 40 and A $\beta$ 42 immunoreactivity in cortex (A) and hippocampus (B). Figure also shows level of A $\beta$ 40 (C) and A $\beta$ 42 (E) immunoreactivity in cortex and A $\beta$ 40 (D) and A $\beta$ 42 (F) immunoreactivity in hippocampus. The ratio of A $\beta$ 40 to A $\beta$ 42 in cortex (G) and hippocampus (H) was calculated. Asterisks show values that are significantly different from controls ( $p < 0.05$ , Student's  $t$  test,  $n = 10$ ).

## Cortex



**Fig. 4.2.** Effect of enoxaparin (ENO) on the level of APP and its proteolytic products in cortex. After ENO injection, the level of APP, C99 and C83 in cortex was measured using western blotting. Figure shows the representative western blots showing the effects of ENO on the level of APP, C99 and C83 in cortex (A). Figure also shows quantification of APP (B), C99 (C) and C83 (D) immunoreactivity in cortex. (Student's *t* test, *n*=10).

## Hippocampus



**Fig. 4.3.** Effect of enoxaparin (ENO) on the level of APP and its proteolytic products in hippocampus. After ENO injection, the level of APP, C99 and C83 in hippocampus was measured using western blotting. Figure shows the representative western blots showing the effects of ENO on the level of APP, C99 and C83 in hippocampus (A). Figure also shows quantification of APP (B), C99 (C) and C83 (D) immunoreactivity in hippocampus. (Student's *t* test, *n*=10).

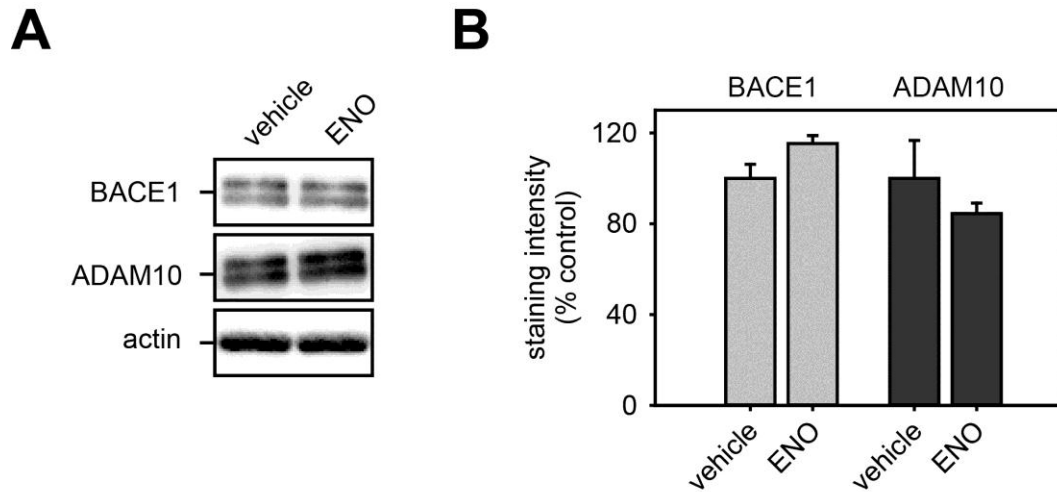


### **4.3.3 Effect of ENO on the number of amyloid plaques and on total amyloid load**

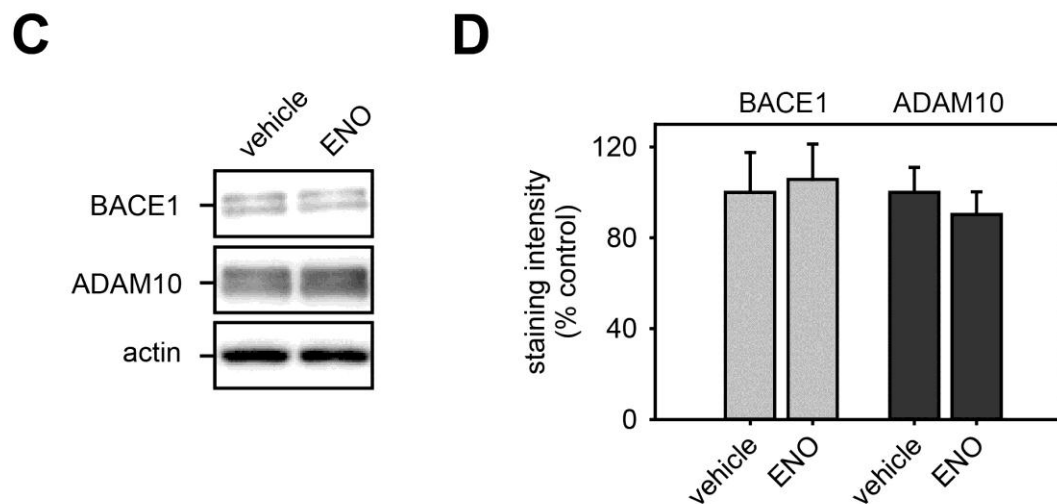
Immunohistochemical studies were conducted to analyse amyloid plaque pathology in both cortex and neocortex after ENO injection. Sections from the brains of Tg2576 mice were stained for amyloid plaques, and the number of plaques and also the area covered by amyloid plaques (amyloid load) were measured in both cortex and hippocampus.

The results indicated that administration of ENO caused a significant increase in the number of amyloid plaques in neocortex compared to the vehicle treatment (Fig. 4.5 A and B, Fig. 4.6 A). In hippocampus, the number of amyloid plaques was also greater after ENO injection (Fig. 4.5 C and D). However, the difference was not statistically significant (Fig. 4.6 C). The area occupied by amyloid plaques in neocortex and hippocampus was measured and normalised to the total area of neocortex and hippocampus, respectively (amyloid load). The result showed that amyloid load in cortex was significantly increased by approximately 60% of control value after the treatment with ENO (Fig. 4.6 B). In hippocampus, the amyloid load was also dramatically elevated by ENO treatment to approximately 4-fold of control value (Fig. 4.6 D).

## Cortex



## Hippo.



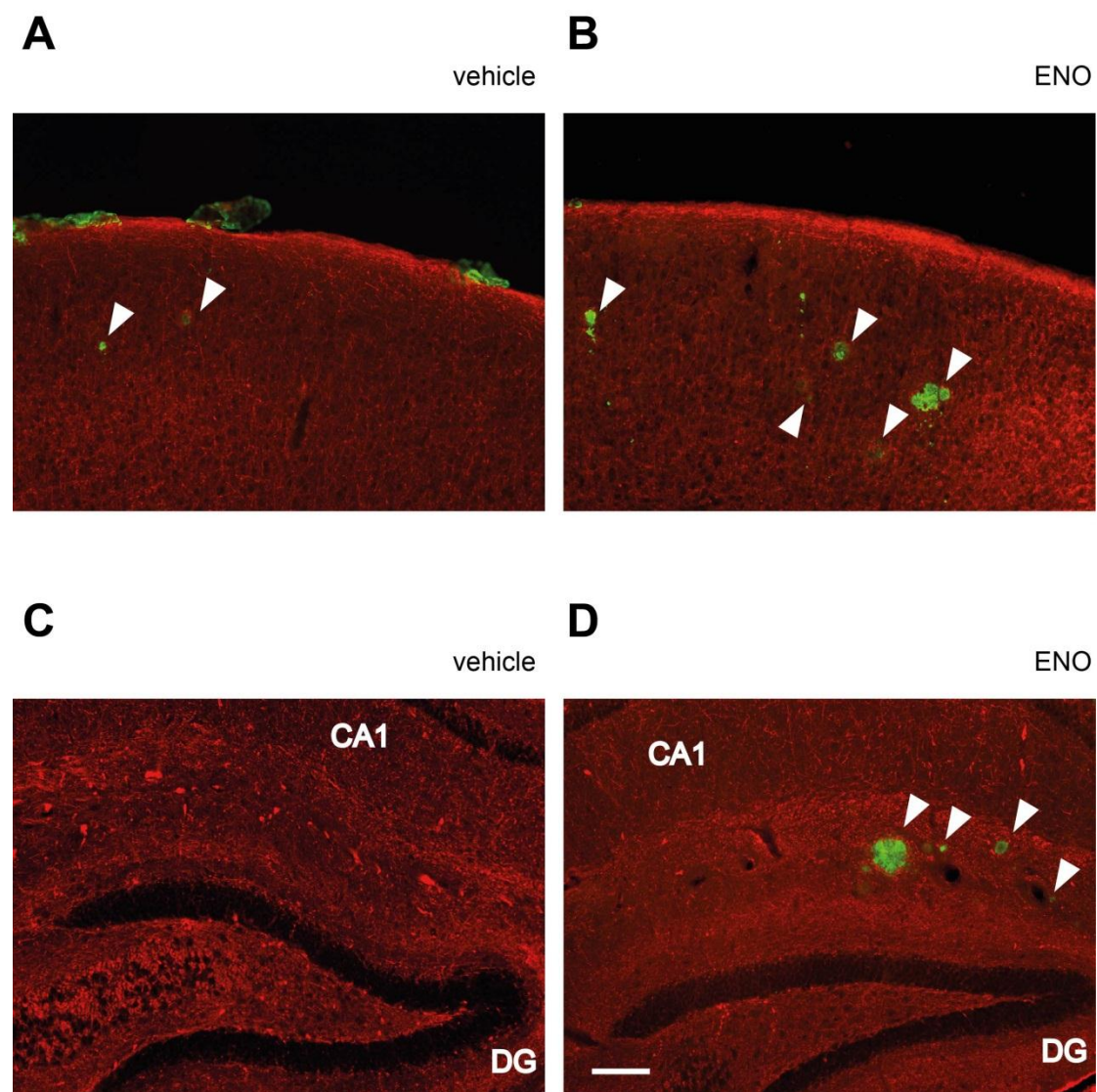
**Fig. 4.4.** Effect of enoxaparin (ENO) on the level of BACE1 and ADAM10 in cortex and hippocampus (hippo.). After ENO injection, the level of BACE1 and ADAM10 in cortex and hippocampus was measured using western blotting. Figure shows the representative western blots illustrating the effects of ENO on the level of BACE1 and ADAM10 in cortex (A) and hippocampus (C). Figure also shows quantification of BACE1 and ADAM10 immunoreactivity in cortex (B) and hippocampus (D). (Student's *t* test, *n*=10).

## 4.4 Discussion

In the present study, Tg2576 mice expressing human APP<sub>695</sub> protein with the Swedish familial AD mutant were used to examine the effects of ENO on APP processing in vivo. This mouse line can develop amyloid plaques in the cortex and hippocampus starting from approximately 8 months of age [545], and the amyloid plaques can be easily observed after 12 months of age. Moreover, APP and APP proteolytic products expressed by this mouse can also be easily examined by western blotting. Therefore, it is possible to assess the relationship between amyloid plaque load and altered APP processing, which was the aim of the present study.

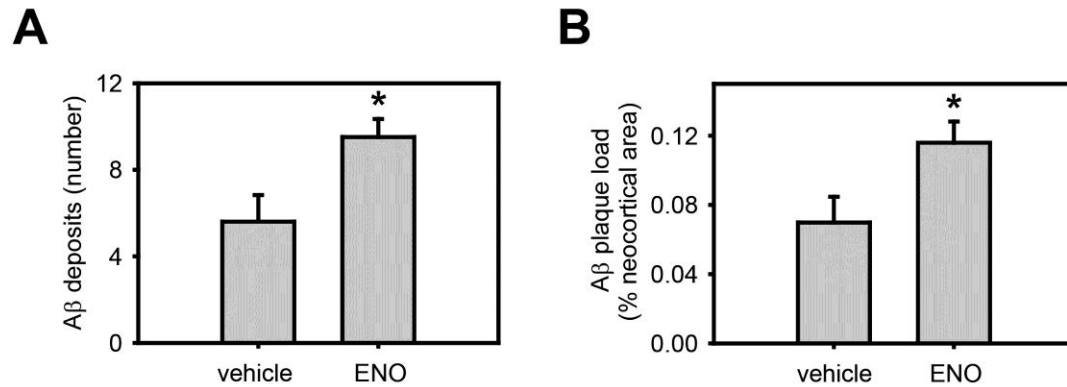
ENO was injected to Tg2576 mice over a 5 month period, and biochemical and immunohistochemical analysis were then performed. The results showed that treatment of ENO significantly decreased the ratio of A $\beta$ 40 to A $\beta$ 42 in cortex, and the number of amyloid plaques and amyloid load were also increased in the cortex of ENO-treated mice. In hippocampus, ENO injection had no significant effect on the ratio of A $\beta$ 40 to A $\beta$ 42 and the number of amyloid plaques, but it increased the amyloid load.

Previous studies by Bergamaschini et al. (2004) reported that ENO-treated APP23 mice (expressing human APP<sub>751</sub> with Swedish mutation) developed less amyloid plaque pathology and had a lower level of A $\beta$ 40 in cortex compared to saline-treated APP23 mice [830]. However, the results reported in this chapter showed that ENO injection increases both the number of amyloid plaques and the amyloid load in cortex of Tg2576 mice.

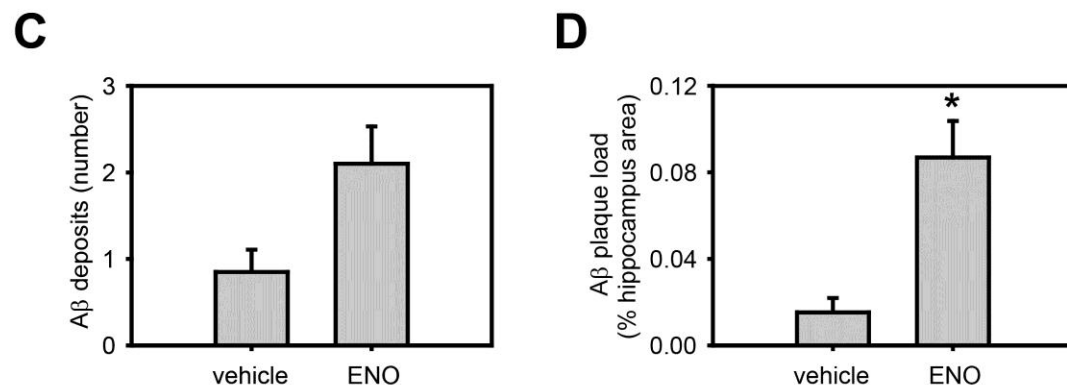


**Fig. 4.5.** Effect of enoxaparin (ENO) on amyloid plaque load in cortex and hippocampus. After administration of ENO, brain sections were prepared and stained with polyclonal rabbit anti- $\beta$ -amyloid (1-40) antibody (green) and with a monoclonal mouse antibody (SMI312) against axonal neurofilament protein (red). Figure shows the amyloid plaque load in cortex of control mice (A) and ENO-treated mice (B). Figure also shows the amyloid plaque load in hippocampus of control mice (C) and ENO-treated mice (D). The black arrows indicate the amyloid plaque. Figure shows CA1 region and dentate gyrus (DG). Scale bar = 50  $\mu$ m.

## Cortex



## Hippo.



**Fig. 4.6.** Effect of enoxaparin (ENO) injection on amyloid plaque number and amyloid plaque load in cortex and hippocampus. After injection of ENO, the brain sections were prepared and stained by polyclonal rabbit anti- $\beta$ -amyloid (1-40) antibody and monoclonal mouse antibody (SMI312) for axonal neurofilament protein. The number of amyloid plaques and amyloid plaque load were measured in both cortex and hippocampus. Figure shows the quantification of amyloid plaque number (A) and amyloid load (B) in cortex. Figure also shows the quantification of amyloid plaque number (C) and amyloid load (D) in hippocampus. Asterisks show values that are significantly different from controls ( $p < 0.05$ , Student's  $t$  test,  $n = 10$ ).

It is unclear how ENO treatment could cause a different effect on the amyloid plaque pathology compared to the results reported by Bergamaschini and colleagues [830], as the experimental procedures were similar. However, in Bergamaschini's study, they quantified and compared the total area of amyloid plaques between ENO-treated and saline-treated group without normalising the plaque area to the total cortex area. In addition, only a small number of mice were used in their study. The small number of mice may have led to an inaccurate quantification of plaque load and amyloid plaque number. In the present study, the total plaque area was normalised to the area of neocortex or hippocampus, which can accurately reflect the amyloid load. Moreover, more mice were used in the present study, which may have led to less error caused by individual variation. In addition, Tg2576 mice expressing human APP<sub>695</sub> with the Swedish mutation were used in the studies reported in this chapter, while another APP transgenic mouse APP23 expressing human APP<sub>751</sub> with the Swedish mutation was used in Bergamaschini's study [830]. This difference between the APP transgenes of these two different APP transgenic mice may possibly cause the different effects of ENO treatment. However, if this is the case, the exact mechanism is unclear.

The results reported in this chapter showed that treatment of ENO can increase the number of amyloid deposits and amyloid load in the cortex. It is likely that the increased amyloid load might be caused by the increased number of plaques rather than an increase in the average size of each amyloid plaque, because the average size of amyloid deposits was not significantly influenced by ENO treatment (Fig. 4.6 A and B). Evidence shows that A $\beta$  may aggregate via the nucleation-dependent polymerization mechanism, and that amyloid formation is nucleated or "seeded" by the long form of A $\beta$ , A $\beta$ <sub>42</sub> [96, 97]. Moreover, studies in mice have shown that expression of A $\beta$ <sub>40</sub> alone does not lead to overt amyloid pathology, whereas mice

expressing low levels of A $\beta$ 42 in the absence of A $\beta$ 40 can form insoluble A $\beta$ 42 and develop compact amyloid plaques in the brain [562]. Furthermore, mice generated by crossing the mice expressing A $\beta$ 40 with Tg2576 mice or mice expressing A $\beta$ 42 have lower A $\beta$  deposition compared to control Tg2576 mice or mice expressing A $\beta$ 42 only [878]. These studies suggest that the ratio of A $\beta$ 42 to A $\beta$ 40 is essential for amyloid plaque formation in the brain [562, 878], as increasing the ratio of A $\beta$ 42 to A $\beta$ 40 may cause more A $\beta$  deposits. Studies reported in this chapter showed that ENO injection significantly increased the number of amyloid deposits in cortex (Fig. 4.5 A and B). This could be caused by the increased ratio of A $\beta$ 42/A $\beta$ 40 (Fig. 4.1 G), which may lead to the formation of more A $\beta$  nuclei and increase amyloid formation in cortex. In hippocampus, administration of ENO did not alter the ratio of A $\beta$ 42/A $\beta$ 40 and thus had no significant effect on the number of amyloid plaques (Fig. 4.1 F and Fig. 4.6 C). However, ENO treatment still significantly increased the amyloid load in hippocampus. This result is inconsistent with the finding that the ratio of A $\beta$ 42/A $\beta$ 40 was not affected by ENO injection. These results suggest that the increased amyloid load may not be simply due to the increased ratio of A $\beta$ 42 to A $\beta$ 40. Other mechanisms may also exist.

Leveugle *et al.* [842] reported that small heparin derivatives which have a size smaller than 3 kDa may have the ability to penetrate the blood-brain barrier. ENO used in this study was a mixture that consisted of different sizes of heparins of which approximately 20% of the fragments possesses a molecular size smaller than 3 kDa. These small fragments of heparin should cross the blood-brain barrier, if the previous report [842] is correct. Moreover, many studies reported that heparin and LMWHs can accelerate the A $\beta$  aggregation in vitro [812, 831]. Therefore, it is possible that

ENO may cross the blood-brain barrier after peripheral injection, and increase aggregation of A $\beta$  in the brains of Tg2576 mice and thereby exacerbate the brain A $\beta$  plaque pathology.

Studies reported in this chapter indicate that APP processing is not affected by injection of ENO to Tg2576 mice. This is may be because only a small fraction of low molecular weight heparins in ENO may cross the BBB. Moreover, the studies reported in Chapter 2 showed that the effect of ENO on APP processing is dose-dependent. Thus, it is possible that the concentration of ENO in the brain after peripheral injection may not have been high enough to cause a significant reduction on BACE1 and to influence APP processing. Alternatively, the results reported in Chapter 3 indicate that small MHs (3 kDa) have only a very weak effect on APP processing. Therefore, it is possible that although a small fraction of ENO with a size below 3 kDa may cross the BBB, they may not have the ability to cause the reduction of BACE1 and result in a significant effect on APP processing.

Recently, studies indicate that A $\beta$  oligomers, rather than A $\beta$  fibrils, are mainly responsible for the A $\beta$ -induced neurotoxicity [620, 844-846]. It has also been reported that the level of soluble A $\beta$  oligomers is strongly correlated with the synaptic loss and the severity of AD [106, 107], while the level of insoluble A $\beta$  or amyloid plaque presented in the brain is poorly associated with the cognitive impairment of disease [106]. In this chapter, ENO treatment increased amyloid plaque load in the brain of APP transgenic mice, suggesting that injection of ENO may transfer the A $\beta$  oligomers to insoluble A $\beta$  aggregates and thus prevent mice from the more toxic oligomeric A $\beta$ . These results also suggest that further studies may need to measure the soluble and



insoluble A $\beta$  separately and examine the effect of ENO on the proportion of soluble and insoluble A $\beta$ .

In addition, studies in this chapter showed that administration of ENO could reduce the ratio of A $\beta$ 40/A $\beta$ 42. This effect is consistent with the study by Timmer and colleagues [831] which reported that the ENO treatment can significantly enhance the level of A $\beta$ 42 in 10 month old APP transgenic mice compared to untreated controls. However, the mechanism of this effect remains unknown. As heparin can bind to A $\beta$  [811, 812] and ENO treatment did not affect APP processing to A $\beta$ , it is possible that the binding of ENO to A $\beta$ 40 or A $\beta$ 42 in the brain may affect the clearance of A $\beta$ 40 and A $\beta$ 42 to different extents, and thereby alter the proportion of A $\beta$ 42 and A $\beta$ 40.

In conclusion, studies reported in this chapter showed that ENO treatment increased amyloid plaque load in both cortex and hippocampus, and also increased the ratio of A $\beta$ 42 to A $\beta$ 40 in cortex. However, these effects were not due to the effect of ENO treatment on APP processing. The exact mechanism of these effects caused by ENO injection remains unknown. Thus, the use of ENO for the treatment of AD may need further assessment.

## **Chapter 5**

### **Effects of endogenous heparan sulfate on APP processing and A $\beta$ production**

## 5.1 Introduction

The studies reported in Chapters 2 and 3 and those of other groups [780, 824, 828, 830, 833] suggest that GAG-based compounds may have potential for the treatment of AD. The studies reported in Chapter 2 have shown that heparin and enoxaparin treatment can lower A $\beta$  secretion from primary cortical cells. The studies in Chapter 3 demonstrate that the effects of heparin on APP processing are dependent on size, and sulfation degree of GAGs, but not on the carboxylation or the structure backbone of GAGs. This result suggests that potent and specific heparin derivatives may be identified and used for the treatment of AD.

The results reported in Chapters 2 and 3 demonstrate that decreased secretion of A $\beta$  after GAG treatment is primarily due to a decrease in the level of BACE1 which decreases the production of C99. In addition, studies reported in Chapters 2 and 3 also showed that treatment with heparin decreases the level of ADAM10 and inhibits  $\alpha$ -secretase cleavage of APP. As GAGs such as HS are widely expressed in the brain in the form of proteoglycans [879], it is possible that the endogenous HS may also affect APP processing by regulating the level of BACE1 and ADAM10.

Previous studies reported that both heparin and endogenous heparan sulfate (HS) can bind to BACE1 [833, 837]. According to the results reported in Chapter 2, it was hypothesized that heparin may bind to BACE1 and cause downregulation of BACE1, which thus inhibits APP processing and A $\beta$  production. Studies reported in Chapters 2 and 3 also showed that treatment of heparin decreased the level of ADAM10. This raises the possibility that ADAM10 may also bind to heparin, and that the binding of heparin to BACE1 and ADAM10 may cause a decrease in the level of BACE1 and

ADAM10, which thus inhibits APP processing through  $\beta$ - and  $\alpha$ -secretase cleavage pathways.

The aim of the study described in this chapter was to examine the role of endogenous HS on APP processing and A $\beta$  production. To examine this, primary cortical cells derived from Tg2576 mice were incubated with a drug or enzyme designed to degrade HS chains from endogenous proteoglycans. Moreover, the heparin-binding capacity of ADAM10 was also examined by applying ADAM10 to a heparin column and then determining the binding affinity of ADAM10 to heparin.

The results show that deletion of endogenous HS can reduce the level of BACE1 and ADAM10 and thus inhibit APP processing through  $\beta$ - and  $\alpha$ -secretase cleavage pathways similar to exogenous treatment of heparin. These findings suggest that endogenous HS may be involved in regulating APP processing by altering the level of BACE1 and ADAM10. The data also show that heparin does not bind directly to ADAM10. Therefore, the results indicate that the heparin-induced decrease in ADAM10 is not due to a direct binding of heparin to ADAM10, and may also suggest that a direct binding of BACE1 to heparin may also not be necessary for the heparin-induced decrease of BACE1. In summary, these findings suggest that regulation of endogenous HS to reduce APP processing to A $\beta$  could be a novel approach for the treatment of AD, and the precise mechanism underlying this need to be further investigated.

## **5.2 Materials and Methods**

### **5.2.1 Materials**

Monoclonal anti-heparan sulfate antibody (10E4 epitope) and heparitinase (EC 4.2.2.8) were purchased from Seikagaku Corporation (Tokyo, Japan). Monoclonal anti- $\beta$ -actin antibody, rabbit anti-BACE1 (EE-17) antibody and polyclonal anti-APP C-terminal antibody (APP-CT), 4-methylumbelliferyl- $\beta$ -D-xyloside (xyloside) were purchased from Sigma-Aldrich Pty. Ltd. (Sydney, Australia). Rabbit anti-ADAM10 (ab1997) and rabbit anti-ADAM17 (ab2051) were purchased from Sapphire Bioscience Pty. Ltd. (Waterloo, Australia). Monoclonal anti-A $\beta$  antibody 6E10 was from Covance Pty. Ltd. (North Ryde, Australia). Neurobasal medium and B27 supplement were purchased from Invitrogen (Mulgrave, Australia). Mouse and rabbit HRP-conjugated secondary antibodies were purchased from DAKO (Campbellfield, Australia). Goat anti-mouse IgG Alexa Fluor 596 antibody and Alexa Fluor 488 phalloidin were purchased from Invitrogen (Oregon, USA). Complete mini protease inhibitor cocktail tablet was from Roche Diagnostics (Castle Hill, Australia).

### **5.2.2 Primary cortical cell culture**

Primary cortical cells were prepared and maintained as described in Chapters 2 and 3.

### **5.2.3 Western blotting of cell lysates and media**

Culture medium was removed from cells for determination of A $\beta$  and sAPP $\alpha$  levels. The cells were incubated with cold RIPA buffer (150 mM NaCl, 50 mM Tris, 0.5% w/v Na-deoxycholate, 1% v/v Nonidet P-40, 0.1% SDS, pH 7.4) containing protease

inhibitor cocktail on ice for 10 min and the cell lysates were then harvested for determination of C99, BACE1, ADAM10, ADAM17 and APP.

The level of A $\beta$ 40, A $\beta$ 42, sAPP $\alpha$ , C99, BACE1, ADAM10, ADAM17 and APP was determined by western blotting as described in Chapters 2 and 3. The density of staining was quantified using Image J software (RSB; NIH, <http://rsbweb.nih.gov/ij/index.html>). The ratio of staining intensity of each protein to the staining intensity of  $\beta$ -actin was determined and then each ratio was used to calculate a percentage relative to mean value for control incubations lacking GAG. The statistical tests were performed using SigmaPlot software (10.0v; Systat Software, Inc., San Jose, CA, USA). Statistical comparisons were made using Student's *t* tests. Values of *p*<0.05 were considered statistically significant.

#### **5.2.4 Immunocytochemistry**

To validate the activity of heparitinase, endogenous HS was visualised in 7-DIV primary cortical cells in the presence or absence of heparitinase. The primary cortical cells from Tg2576 mice were grown on poly-D-lysine-coated coverslips. After treatment with vehicle or 8 mU/mL heparitinase for 24 hours, the cells were washed twice with ice-cold PBS and then fixed for 10 min with 4% paraformaldehyde. After washing 3 times with PBS, cells were then permeabilized with 0.3% (v/v) Triton-X100 in PBS for 10 min. The fixed cells were washed 3 times with PBS and blocked with 4% (v/v) goat serum in PBS for 30 min. The cells were then incubated overnight in cool room with monoclonal anti-HS (10E4) antibody (1:250) in 2% goat serum in PBS. Cells were then washed 3 times with PBS and then incubated with goat anti-mouse IgG Alexa Fluor 594 secondary antibody (1:1000) and Alexa Fluor 488 Phalloidin (1:50) in 2% goat serum in PBS at room temperature. Cells were

counterstained with DAPI for 5 min and washed 2 times with PBS. Cells were then rinsed with distilled water, and dried and mounted with mounting medium (Dako). Images were acquired with an Olympus BX50 microscope with 20x objective (NA 0.6), cooled CCD camera (Photometrics) and commercial software (NIS Elements, Nikon).

### **5.2.5 Heparin affinity chromatography**

For heparin affinity chromatography of ADAM10, 30 µg of recombinant human ADAM10 (270 µg/mL in 25 mM MES, 10mM CaCl<sub>2</sub>, 150mM NaCl and 2.5 mM ZnCl<sub>2</sub>, pH 5.2) was diluted into 5 mL with 10 mM sodium phosphate buffer, pH 7.0 (phosphate buffer), and then applied to a 1 mL HiTrap heparin HP column (GE Healthcare Bio-Sciences, Australia) pre-equilibrated with phosphate buffer for 5 min at a flow rate of 2 mL/min using a BioLogic DuoFlow chromatography system (Bio-Rad). Unbound material was eluted with 2 mL phosphate buffer at a rate of 2 mL/min, and then bound material was eluted with gradient elution buffer (2 M NaCl in phosphate buffer, pH 7.0) at a flow rate of 2 mL/min for 2.5 min. The absorbance of the effluent was monitored at 280 nm. Fractions (1 mL each) were collected from 5 min to 17 min of elution time. To determine ADAM10, a 50 µl sample from each fraction was analysed by western blotting with a rabbit anti-ADAM10 (ab1997) antibody.

## **5.3 Results**

### **5.3.1 Effects of endogenous HS on APP processing and A $\beta$ production**

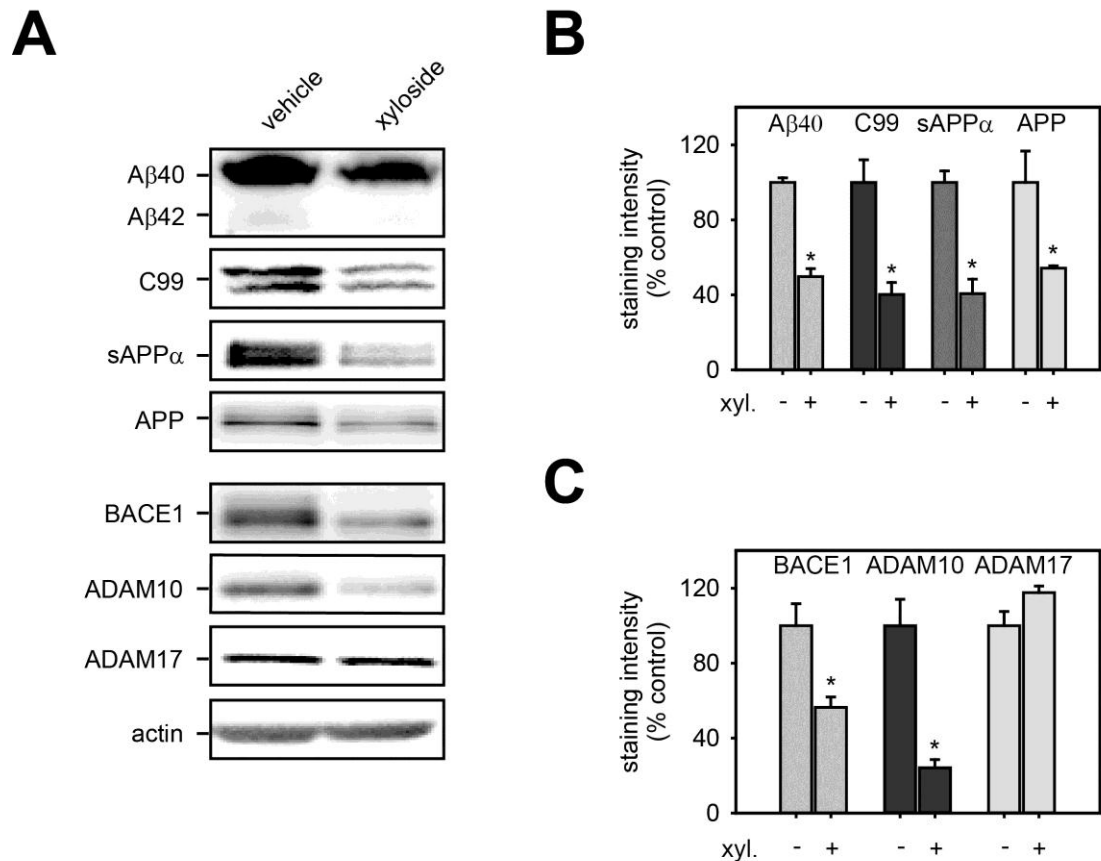
To examine the role of endogenous GAG on APP processing and A $\beta$  production, the primary cortical cells were firstly treated with 1 mM xyloside, an inhibitor of

proteoglycan biosynthesis [880]. The levels of APP, APP proteolytic processing products ( $A\beta$ , C99, sAPP $\alpha$ ) and APP cleavage enzymes (BACE1, ADAM10 and ADAM17) were determined using western blotting. Although  $A\beta$ 40 was easily detected in the cell culture medium, little  $A\beta$ 42 was observed (Fig. 5.1 A). Therefore, only the level of  $A\beta$ 40 was measured.

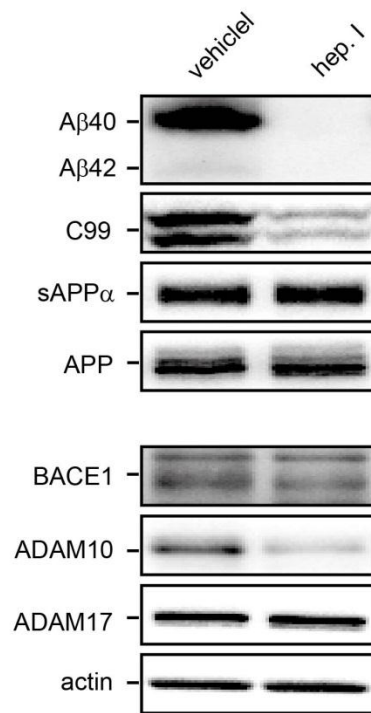
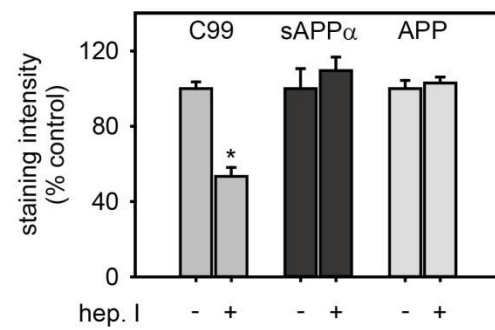
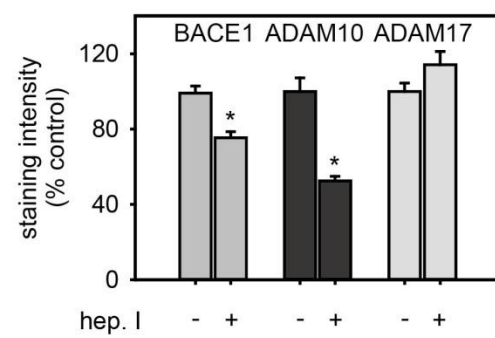
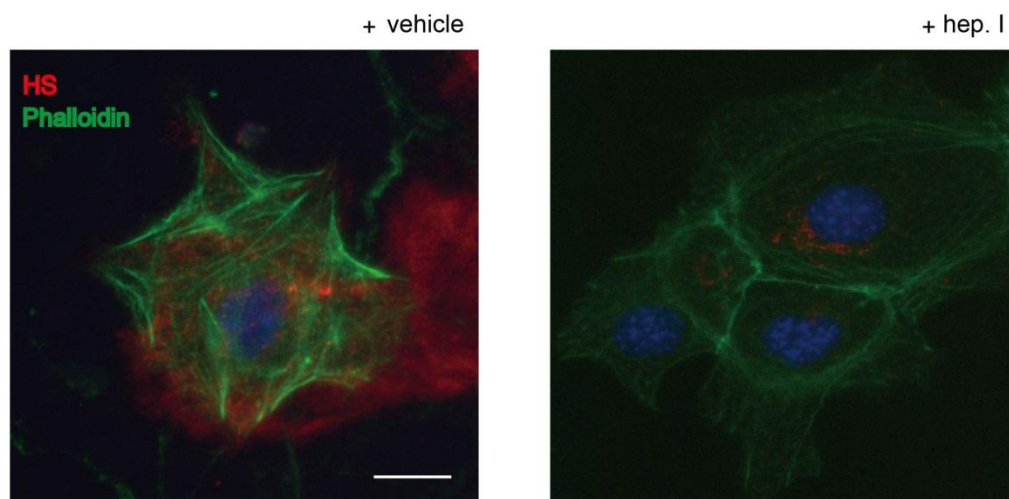
Treatment of cells with 1 mM xyloside decreased the level of  $A\beta$ , C99 and sAPP $\alpha$  to approximately 50%, 40% and 40% of controls, respectively (Fig. 5.1 A and B). Interestingly, treatment with xyloside also decreased the level of APP to approximately 55% of the mean control value (Fig. 5.1 A and B). After treatment with 1 mM xyloside, the level of BACE1, ADAM10 and ADAM17 were also measured, and the results showed that the level of BACE1 and ADAM10 were dramatically reduced to approximately 55% and 30% of control values after the treatment of xyloside, respectively (Fig. 5.1 A and C). However, incubation of xyloside had no significant effect on the level of ADAM17 (Fig. 5.1 A and C).

The effect of endogenous HS on APP processing was also examined by treatment of heparitinase which can specifically digest endogenous HS chain. Firstly, the activity of heparitinase was validated using immunocytochemistry, and the results showed that heparitinase treatment efficiently degraded the endogenous HS compared to the vehicle treatment (Fig. 5.2 D).





**Fig. 5.1.** Effect of xyloside (xyl.) on APP processing. Primary cortical cells from Tg2576 mice were treated with xyloside (1 mM) for 24 hours. The level of A $\beta$ 40 and A $\beta$ 42, C99, sAPP $\alpha$ , APP, BACE1, ADAM10 and ADAM17 was measured using western blotting. (A) Western blots showing the level of A $\beta$ 40, C99, sAPP $\alpha$ , total APP, BACE1, ADAM10 and ADAM17 in the presence or absence of xyloside. Figure also shows quantification of A $\beta$ 40, C99, sAPP $\alpha$ , and APP immunoreactivity (B), and BACE1, ADAM10 and ADAM17 immunoreactivity (C) on the western blots. Asterisks show values that are significantly different from control incubations (p < 0.05, Student's t test, n=6).

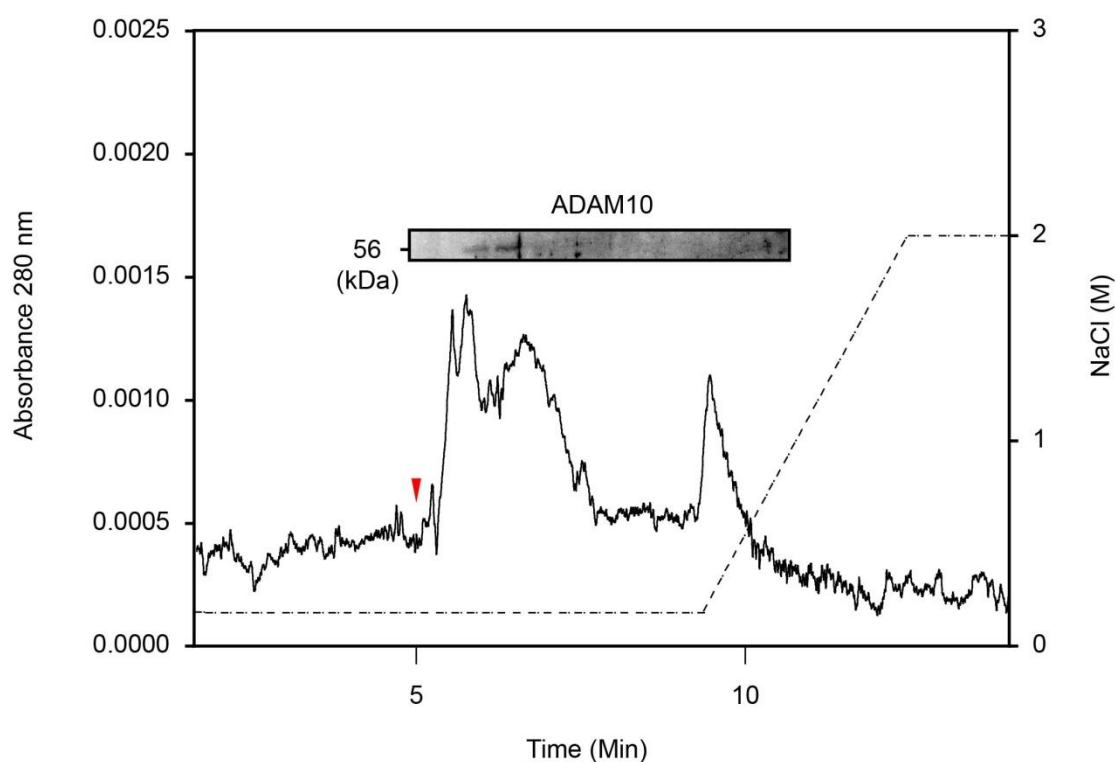
**A****B****C****D**

**Fig. 5.2.** Effect of heparitinase (hep. I) on APP processing. Primary cortical cells from Tg2576 mice were treated with heparitinase (8 mU/mL) for 24 hours. The level of A $\beta$ 40 and A $\beta$ 42, C99, sAPP $\alpha$ , APP, BACE1, ADAM10 and ADAM17 was determined by western blotting. (A) Typical western blots showing the effect of heparitinase treatment on the level of C99, sAPP $\alpha$ , total APP, BACE1, ADAM10 and ADAM17. Figure shows quantification of C99, sAPP $\alpha$ , and APP immunoreactivity (B), and BACE1, ADAM10 and ADAM17 immunoreactivity (C) on the western blots. The activity of heparitinase was validated in the cell culture by immunocytochemistic staining using monoclonal anti-heparan sulfate antibody (D). Asterisks show values that are significantly different from control treatments ( $p < 0.05$ , Student's t test,  $n = 6$ ). Scale bar = 20  $\mu$ m.

Next, the primary cortical cells were treated with 8 mU/mL heparitinase and the level of APP, A $\beta$ , C99, sAPP $\alpha$ , BACE1, ADAM10 and ADAM17 was determined by western blotting. Treatment of the cells with heparitinase resulted in a marked reduction of both A $\beta$ 40 and A $\beta$ 42 compared to controls, as the level of A $\beta$ 40 and A $\beta$ 42 was too low to be quantified accurately (Fig. 5.2 A). Heparitinase treatment also decreased the level of C99, but had no significant effect on the level of sAPP $\alpha$  and APP (Fig. 5.2 A and B). The deletion of endogenous HS by heparitinase also led to a reduction of BACE1 and ADAM10 to approximately 80% and 50% of control values, respectively. However, heparitinase treatment did not alter the level of ADAM17 (Fig. 5.2 A and C).

### **5.3.2 Heparin chromatography of ADAM10**

To examine the heparin-binding capacity of ADAM10, ADAM10 (30  $\mu$ g) was applied to a 1 mL HiTrap heparin column. The absorbance of the effluent was monitored at 280 nm. The fractions were collected and the ADAM10 in each fraction was determined by western blotting. The immunoreactivity of ADAM10 was observed in the fractions corresponding to the wash step containing unbound material, and there was no significant immunoreactivity of ADAM10 in any subsequent fractions during the elution of bound material with gradient elution buffer containing NaCl (Fig. 5.3). The results suggest that ADAM10 does not bind to heparin, at least under the conditions of the method used.



**Fig. 5.3.** Heparin affinity chromatography of ADAM10. ADAM10 (30  $\mu$ g) was applied to a 1 ml HiTrap heparin HP column. The column was then eluted with a linear gradient (0-2.0 M NaCl) over 2.5 min at a flow rate of 2.0 mL/min using a BioLogic DuoFlow chromatography system (Bio-Rad). The absorbance of the effluent was monitored at 280 nm. Figure shows the concentration of NaCl eluting from the column of the effluent as calculated from conductivity measurements (dash line). The red arrow head shows the time point when the ADAM was loaded. The UV absorbance (solid line) was subtracted to control (phosphate buffer only) values. Figure also shows the western blot of ADAM10 (inset).

## 5.4 Discussion

In the study presented in this chapter, the role of endogenous HS on APP processing was studied to examine whether endogenous HS can affect APP processing like the exogenously added heparin. Moreover, the heparin-binding capacity of ADAM10 was also examined to test the possibility that the heparin-induced decrease in ADAM10 might be due to the direct binding of heparin to ADAM10.

First, the role of endogenous HS on APP processing was studied in primary cortical cells from Tg2576 mice. The data indicated that disruption of proteoglycan biosynthesis or removal of endogenous HS decreases the level of BACE1 and ADAM10, and inhibits APP processing to A $\beta$ . Next, the possibility that ADAM10 may bind to heparin was examined. The results showed that there was no obvious binding of ADAM10 to heparin observed in the heparin affinity chromatography study.

The results demonstrated that treatment of xyloside or heparitinase could reduce the level of BACE1 and ADAM10 and thus inhibit APP processing similar to the exogenous treatment of heparin. These findings suggest that the endogenous HS may be involved in regulation of level of BACE1 and ADAM10. In Chapter 3, it was suggested that the identification of potent and specific GAG-based compounds which are useful for the treatment of AD is problematic, because GAGs have a relatively large molecular weight, which makes them unable to cross the blood-brain barrier and efficiently affect APP processing inside the brain. In this chapter, the results showed that digestion of endogenous HS could reduce the level of BACE1 and inhibit APP processing to A $\beta$ . These results suggest that regulation of the endogenous HS may

have potential for the treatment of AD, because drugs which target to reduce endogenous HS may decrease the level of BACE1 and thereby lower the production of A $\beta$ . However, the studies also showed that digestion of endogenous HS led a decrease of the level of ADAM10 which thus reduces the secretion of sAPP $\alpha$ . Previous studies suggest that sAPP $\alpha$  may have neurotrophic actions [220, 224, 227, 876, 877]. Therefore, regulation of endogenous HS may result in unwanted side effects caused by the down-regulated level of sAPP $\alpha$ .

The present study also examined the heparin-binding capacity of ADAM10. The results indicated that there was no obvious binding of ADAM10 to heparin under the methods used. This result suggests that the decrease of ADAM10 after heparin treatment may not require a direct binding of ADAM10 to heparin. The data also imply that the binding of heparin to BACE1 may be not necessary for the heparin-induced decrease of BACE1. Although it is possible that the decrease in BACE1 and ADAM10 occurs via different mechanisms, the fact that ADAM10 does not bind heparin suggested that at least for ADAM10, the effect could not be mediated by a direct interaction. Moreover, the studies showed that the degradation of endogenous HS can also reduce the level of BACE1 and ADAM10 similar to exogenous treatment of heparin, suggesting that endogenous GAGs may be involved. However, to determine the exact mechanism of downregulation may require further investigation.

The studies reported in this chapter showed that treatment with xyloside significantly decreased the level of APP. This data suggests that although the treatment of xyloside reduced the secretion of A $\beta$ , an effect that may be caused by the reduced level of BACE1, the decreased level of APP may also partially contribute to the observed

decrease of A $\beta$  and other APP proteolytic products such as C99 and sAPP $\alpha$ . It is unclear how the treatment of xyloside decreases the level of APP. It has been reported that proteoglycans can bind to APP [163, 186]. This raises the possibility that disrupting the endogenous HS by xyloside may affect its binding to APP, which may influence APP trafficking and turnover. However, the studies reported in this chapter showed that the level of APP was not affected by heparitinase treatment, suggesting that the reduced level of APP caused by xyloside may not be due to the digestion of endogenous HS. The studies show that xyloside treatment can affect the biosynthesis of both HS and chondroitin sulfate [880]. Previous studies have also reported that APP can serve as a core protein of proteoglycan called appican in which the chondroitin sulfate chain is attached to a serine residue of APP [696]. It is possible that xyloside treatment may inhibit the addition of the chondroitin sulfate chains to APP and thereby influence the turnover of APP.

The studies reported in Chapters 2 and 3 indicate that heparin treatment can decrease the level of ADAM10 and thus reduce the secretion of sAPP $\alpha$ . The studies reported in this chapter demonstrated that heparitinase treatment reduced the level of ADAM10 but had no significant effect on the secretion of sAPP $\alpha$ . It remains unclear how treatment of heparitinase causes a decrease in ADAM10 without affecting the level of sAPP $\alpha$ . Previous studies have reported that other ADAM family proteins, including ADAM17, ADAM9 and ADAM19, may also have  $\alpha$ -secretase activity [881-883]. Although the studies reported in this chapter showed that the level of ADAM17 was not significantly affected by the treatment of xyloside and heparitinase, it is possible that the cleavage of APP by other  $\alpha$ -secretases such as ADAM9 and ADAM19 may



be elevated and thus generate sAPP $\alpha$  in compensation. Therefore, further investigation is required to examine this hypothesis.

In summary, the studies reported in this chapter suggest that endogenous HS can reduce the level of BACE1 and ADAM10 and thereby inhibit APP processing, and these effects may not due to the binding of endogenous HS to BACE1 and ADAM10. These results suggest that degradation of endogenous HS can inhibit APP processing to A $\beta$ , implying that therapeutic approaches targeting to the synthesis of HS or digestion of endogenous HS may be potentially valuable for the treatment of AD.

## **Chapter 6**

### **Discussion**

Alzheimer's disease (AD) was described in 1906 by the German neuropathologist Alois Alzheimer [17]. Subsequently, AD was histologically characterised by the deposition of amyloid plaques and neurofibrillary tangles in the brain of patient [18, 19]. Although molecular research on AD began several decades ago, the precise mechanism of the development of AD still remains unclear.

There are still no effective treatments to prevent, reverse or cure AD [663]. Currently, several pharmaceutical agents, including acetylcholinesterase inhibitors (AChEIs) and an NMDA-receptor antagonist, are available for the treatment of AD [582]. However, these compounds only marginally improve the symptoms of AD, without retarding the underlying progression of disease, and thus will not be effective for the treatment of advanced cases of AD [847].

Over the last two decades, the amyloid hypothesis has received significant attention, and proposes that the chronic imbalance of production and clearance of A $\beta$  triggers the development of AD [884]. Based on the amyloid hypothesis, several therapeutic strategies, which are mainly purported to reduce A $\beta$  production or increase the A $\beta$  clearance, have been proposed, and many compounds targeting A $\beta$  have entered human clinical trials [847]. However, new drugs are still greatly needed for the treatment of AD.

In the late 1980s and the early 1990s, several groups reported that proteoglycans (PGs) or glycosaminoglycans (GAGs) are co-localised with amyloid plaques and also other pathological hallmarks of AD including neurofibrillary tangles and cerebral amyloid angiopathy [780, 781, 798]. PGs or GAGs can bind to A $\beta$  [810, 811], and accelerate A $\beta$  aggregation [609, 812, 885]. Furthermore, several studies demonstrate that low molecular weight heparin (LMWH) derivatives can inhibit the amyloid fibril

formation [617, 827, 828]. These studies suggested that PGs or GAGs may have important roles in the development of AD pathology, and also opened the possibility that GAG-based compounds may inhibit A $\beta$  aggregation and thus have benefits for the treatment of AD.

Several lines of evidence indicated that GAGs, such as heparin and heparan sulfate (HS), can bind to BACE1, which initiates APP processing to A $\beta$  [832, 833, 836, 837]. These studies suggested that PGs or GAGs may be associated with APP proteolytic processing, and raised the possibility that GAG-related compounds could be useful for the treatment of AD as they might regulate the production of A $\beta$ . Recently, it was reported that administration of a LMWH, enoxaparin (ENO), could not only reduce A $\beta$  levels and amyloid plaque load in the brain [830], but also improve cognition in APP transgenic mice [831]. These findings could be logically explained by the hypothesis that ENO inhibited the activity of BACE1 and thereby reduced the production of A $\beta$ , which thus lead to decreased amyloid plaque load and improved cognition in APP transgenic mice [833].

Based on these findings in this study, it was hypothesized that GAG-based compounds may have therapeutic value for the treatment of AD by regulating APP processing to A $\beta$ . The studies reported in this thesis aimed to assess the potential of GAG-based compounds for the treatment of AD by examining the effects of heparin derivatives such as heparin or ENO on APP processing in vitro and in vivo, and also by investigating the structural specificity of GAGs to identify highly potent and specific GAGs on APP processing.

The studies reported in Chapter 2 demonstrated that treatment of cells with heparin or ENO can reduce the secretion of A $\beta$  and other APP proteolytic processing products

including C99, C83 and sAPP $\alpha$ . These findings confirmed the results reported by Scholefield and colleagues [833], that treatment of GAGs like heparin or HS can reduce the level of C99 and A $\beta$  in cells overexpressing human APP. However, the decreased secretion of A $\beta$  was found to be due to a decrease in the level of BACE1, rather than a direct inhibition of the enzyme by heparin [833]. These studies suggest a novel mechanism whereby GAGs like heparin can regulate the level of BACE1 and thereby affect A $\beta$  production. The studies reported in Chapter 2 therefore further support the view that GAG-based compounds can be used for the treatment of AD.

$\gamma$ -Secretase is the enzyme that cleaves C99 and generates A $\beta$ , which thus has become a major target for reducing the production of A $\beta$  [886]. However, there is major concern regarding the therapeutic value of  $\gamma$ -secretase inhibition as multiple  $\gamma$ -secretase substrates exist other than APP [886], such as Notch 1 [651]. Therefore, drugs that can regulate  $\gamma$ -secretase activity may also cause severe side effects. This view is supported by the observation in clinical trials that treatment of a  $\gamma$ -secretase inhibitor, LY450139, causes side effects including gastrointestinal symptoms, skin cancer, and worsening of cognitive function [649, 650]. Studies in Chapter 2 indicate that the inhibition of APP processing after heparin treatment is not mediated by  $\gamma$ -secretase, as heparin still decreased the  $\beta$ -secretase cleavage of APP, even after the inhibition of  $\gamma$ -secretase. This suggests that GAGs may be suitable for the treatment of AD, because the effect of heparin derivatives on APP processing is not dependent on  $\gamma$ -secretase, and thus it may be possible to avoid the severe side effects associated with the disturbance of  $\gamma$ -secretase activity.

The studies reported in Chapter 2 show that heparin or ENO treatment also decreases the  $\alpha$ -secretase cleavage of APP by reducing the level of the major  $\alpha$ -secretase,

ADAM10, resulting in a decreased level of sAPP $\alpha$ . Previous studies have indicated that sAPP $\alpha$  may have neuroprotective and neurotrophic functions [220, 224, 227, 876, 877]. These results raise the possibility that decreasing levels of sAPP $\alpha$  may cause side effects when using GAG-based compounds for the treatment of AD, although the toxic effects of reducing the production of sAPP $\alpha$  are unclear. These results suggest that GAG derivatives which specifically inhibit the  $\beta$ -secretase cleavage of APP may be needed. In addition, treatment with heparin has no significant effect on the level of another  $\alpha$ -secretase, ADAM17, which has a similar structure, active site and substrate specificity as ADAM10 [887]. The mechanism underlying the different response of ADAM10 and ADAM17 after the treatment of heparin is unknown, and further studies are required to investigate how differences between ADAM10 and ADAM17 makes them respond differently to GAG treatment.

It is generally believed that there are mainly two cleavage pathways involved in APP processing, the amyloidogenic ( $\beta$ -secretase cleavage) and the non-amyloidogenic ( $\alpha$ -secretase cleavage) pathways. However, other APP cleavage pathways which are independent of  $\beta$ - and  $\alpha$ -secretase cleavage have also been reported [888-890]. Identification of new cleavage pathways or fragments of APP may help us to understand the general biological function of APP, and may also provide novel therapeutic targets for the treatment of AD. The studies in Chapter 2 identified a 9 kDa APP fragment termed APP-X which contains the N-terminal region of the A $\beta$  sequence but does not have an intact APP C-terminus. Further studies indicated that generation of APP-X is independent of  $\beta$ - and  $\gamma$ -secretase and that the level of APP-X is increased by heparin or ENO treatment. These results suggest that GAGs may promote APP processing through a novel proteolytic pathway without involving the

$\alpha$ - and  $\beta$ -secretases directly, and also imply that compounds such as GAG derivatives which activate this pathway may indirectly inhibit APP processing through the  $\beta$ -secrease pathway and thus have therapeutic values for the treatment of AD. However, several questions are raised in relation to the identification of APP-X. The first is how APP-X is generated or what enzymes are involved in the cleavage of APP to produce APP-X. Identification of the enzymes which produce APP-X may help in understanding the general metabolism of APP and also provide novel targets for designing drugs for the treatment of AD. Further studies are also needed to examine whether APP-X can be naturally produced in vivo, because this is important for assessing the biological significance of the identification of APP-X. In addition, it is also important to examine the mechanism underlying the heparin-induced increase of APP-X. This may provide more information for designing other drugs which can activate the pathway that generates APP-X, and thus prevent APP processing through  $\beta$ -secretase pathway and produce A $\beta$ .

Recently, several promising drugs designed for the treatment of AD have failed in the clinical trials because of a lack of efficacy or because of side effects [891]. The design of GAG-based compounds for the treatment of AD may require identifying highly potent and specific compounds. Strong ionic interaction is the common mechanism for binding of heparin to proteins and the highly acidic sulfate groups in the structure of heparin are the main contributor to the binding affinity [892]. Moreover, studies reported that the pattern of sulfation of HS may provide specificity for binding to certain proteins [741, 834, 866, 867]. Together with the results reported in Chapter 2, these studies raise the possibility of designing highly potent and specific GAG-based drugs to inhibit APP processing to A $\beta$ .

Studies reported in Chapter 3 demonstrate that the effect of GAGs on APP processing is both size- and sulfation-dependent. The smaller sizes (5 kDa and 3kDa) of mucosal heparin (MH) were less potent in reducing A $\beta$  than high molecular weight heparins (6 kDa and 12.5 kDa MH), and GAGs with a higher degree of sulfation were more potent in inhibiting APP processing compared to less sulfated GAGs. Although no high potency GAG analogues were identified in the studies reported in Chapter 3, these findings provide a logical basis for the rational design of therapeutic agent based on GAG. For example, over-sulfated GAGs, which in theory are more potent in their ability to inhibit APP processing, can be designed and synthesized for the treatment of AD. However, caution must be taken when increasing the sulfation of GAGs to achieve more potent effects on APP processing, as over-sulfated chondroitin sulfate has been associated with an acute onset of serious side effects and was eventually cause death [893].

Previous studies have shown that full-length MH cannot cross the blood-brain barrier (BBB), whereas 3 kDa or smaller heparins can cross [823, 842]. Studies reported in Chapter 3 showed that the 3 kDa MH derivative decreased APP processing, albeit weakly. These results suggest that small GAG derivatives may cross the BBB and still retain the ability to decrease A $\beta$  production, and also indicate that more potent small GAG derivatives may need to be identified as the heparin derivatives smaller than 3 kDa may only have a weak effect on APP processing.

Studies reported in Chapter 3 demonstrate that the effect of GAGs on APP processing is not mediated by the structure backbone of GAG, as highly sulfated polysaccharides such as pentosan polysulfate (PPS), which do not belong to the GAG family, are as potent as heparin. PPS is a semi-synthesized and highly sulfated polysaccharide with a



relatively low molecular weight of 4000-5000 Da. It is a drug approved by U.S. Food and Drug administration (FDA) for the treatment of interstitial cystitis with few side effects [894]. These indicate that compounds based on PPS (e.g. smaller than PPS) may have the ability to cross the BBB and more potently inhibit APP processing, and thus they may potentially be useful for treatment of AD.

Cell culture studies reported in Chapters 2 and 3 indicated that GAGs such as ENO can reduce A $\beta$  secretion by reducing the level of BACE1. Studies reported by Bergamaschini [830] demonstrate that administration of ENO can reduce the level of A $\beta$  and amyloid plaque load in the brain of the APP transgenic mice. Therefore, it is logical to hypothesize that the decreased A $\beta$  level in the brains of APP transgenic mice may result from the decreased BACE1 level resulting from the treatment with ENO. However, in vivo studies reported in Chapter 4 do not support this view, as APP processing was not affected by injection of ENO to Tg2576 mice. ENO is a mixture containing different sizes of heparins of which a small fraction of the fragments possessed a molecular size smaller than 3 kDa, which is thought to be the largest size for heparin to cross the BBB [842]. Moreover, studies reported in Chapter 2 showed that the effect of ENO on APP processing is dose-dependent. Thus, it is possible that the concentration of ENO in the brain after peripheral injection may not be high enough to cause a significant reduction in BACE1 and thereby have no effect on APP processing. Alternatively, the results reported in Chapter 3 indicate that small MHs (3 kDa) only have very weak effect on APP processing. Therefore, it is possible that although a small fraction of ENO with the size below 3 kDa may cross the BBB, they may not have the ability to cause the reduction of BACE1 and result in a significant effect on APP processing.

Studies reported in Chapter 4 show that treatment with ENO caused an increase in amyloid plaque load. Evidence suggests that A $\beta$  oligomers, rather than A $\beta$  fibrils, are mainly responsible for A $\beta$ -induced toxicity [620, 844-846]. Thus, the increase of plaque load in the brain may be a protective consequence caused by the treatment of ENO. However, the use of ENO for the treatment of AD may require further assessment. For example, after ENO treatment, the proportion of soluble and insoluble A $\beta$  in the brain may need to be measured to examine whether ENO can reduce the level of toxic A $\beta$  oligomers. In addition, although the plaque load was elevated by ENO treatment, animal behaviour studies may be needed to examine whether ENO treatment can improve the cognitive function of Tg2576 mice, because the amyloid plaque load is thought to be poorly associated with cognitive impairment [106].

The studies on the role of endogenous HS on APP processing (Chapter 5) suggest that endogenous HS may be involved in regulating APP processing. The results suggest that the effect of heparin on ADAM10 may not due to the direct binding of heparin to ADAM10. Therefore, the effects of endogenous HS cannot be due to a direct interaction. As the effect of heparin, GAG analogues, xyloside or heparitinase treatment on BACE1 was similar to that of ADAM10, the effect of GAG derivatives on BACE1 may also not due to a direct binding of GAG derivatives to BACE1. The results reported in Chapter 5 also suggest that drugs targeting to the synthesis or degradation of endogenous HS, which can regulate the A $\beta$  production, may be potentially useful for the treatment of AD.

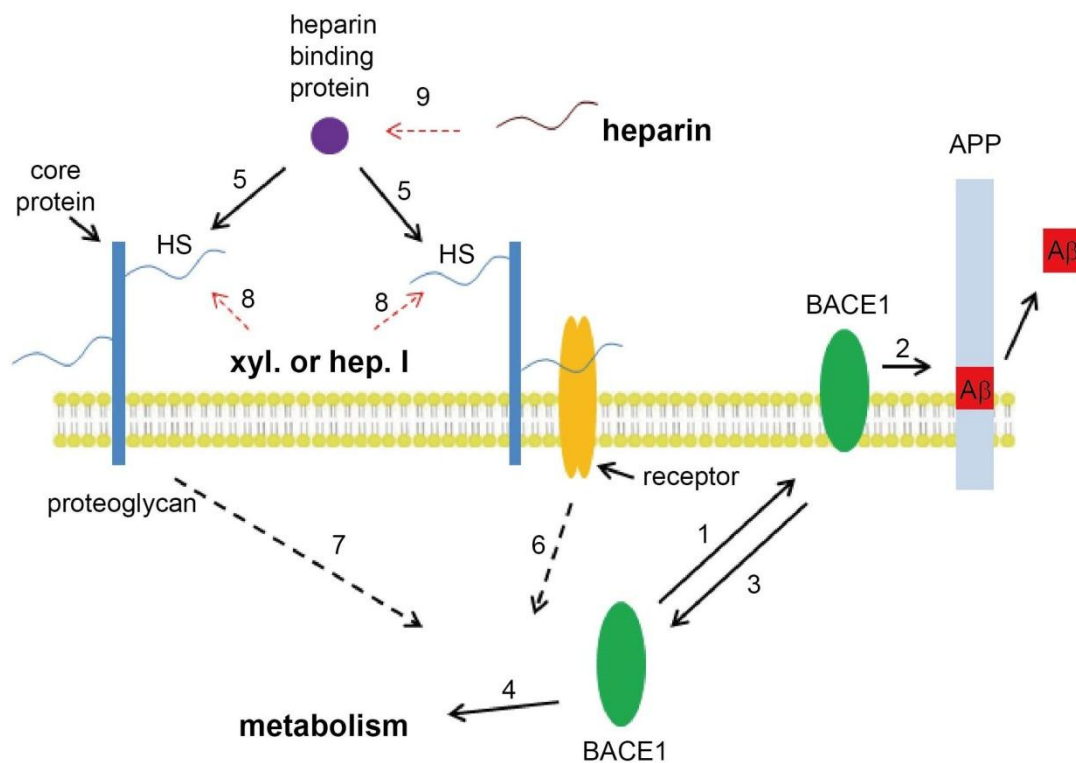
According to the studies reported in this thesis, treatment of GAGs or deletion of endogenous HS inhibits APP processing to A $\beta$  by reducing the level of BACE1.

However, caution is needed when targeting the BACE1 cleavage of APP. Studies indicate that BACE1 has multiple substrates including neuregulin family proteins which are involved in axon myelination [326, 642]. Mice deficient in BACE1 display hypomyelination [326, 642] and downregulation of BACE1 may disrupt neuregulin signalling and cause behavioural deficits similar to schizophrenia [895]. These suggest that decreasing the level of BACE1 by GAG-related therapeutics may potentially cause unwanted side effects, and the benefits and risks of down-regulation of BACE1 by GAG-related therapeutics for the treatment of AD need to be considered.

### **6.1 A possible mechanism of the effect of GAG derivatives on APP processing**

The exact mechanism underlying the effects of GAGs on APP processing reported in this thesis remains unclear. However, a possible mechanism is proposed here based on the results reported in this thesis (Fig. 6.1). The studies indicate that many signalling pathways involve not only ligands and receptors but also various types of co-receptors such as proteoglycans [698]. Endogenous HS may play a role in cellular signalling, such as fibroblast growth factor signalling, either as receptor or co-receptor [698]. Therefore, it is hypothesized that the level of BACE1 and ADAM10 may be mediated by endogenous HS through an unknown signalling pathway. Exogenously added heparin or deletion of endogenous HS may block the binding of endogenous HS to ligands or receptors and thus disrupt this endogenous HS mediated signal transduction. The disruption of endogenous HS-mediated signalling may cause the increase of BACE1 and ADAM10 turnover which thereby inhibit APP processing to A $\beta$ . However, other mechanisms may exist and further studies are needed. Ultimately,

based on the studies reported in this thesis and further studies in the future, it may be possible to design potent GAG derivatives which act specifically to inhibit  $\beta$ -secretase cleavage of APP that can be used for the treatment of AD.



**Fig. 6.1.** Simplified model of the proposed mechanism of the effects of GAGs or endogenous heparan sulfate (HS) on APP processing. Briefly, BACE1 is synthesized and modified inside the cell. The mature BACE1 is then transported to cell surface (1) and initiates APP proteolytic processing (2). The cell surface BACE1 can be internalised into the cytoplasm (3) for metabolism (4). Heparin-binding protein may serve as a ligand, which bind to the cell surface heparan sulfate (5) and trigger downstream cell signalling (6 or 7), and regulate BACE1 metabolism (4). Exogenously added GAGs such as heparin may bind to the heparin-binding protein (8) and thereby block their binding to endogenous heparan sulfate (HS) which may disturb the downstream cell signalling and cause an increase in BACE1 metabolism. Alternatively, inhibition of endogenous HS synthesis by xyloside (xyl.) or digestion of endogenous HS by heparitinase (hep. I) (9) can also block the binding of heparin-binding protein to proteoglycan and increase BACE1 turnover.

## References

1. Ferri, C.P., et al., *Global prevalence of dementia: a Delphi consensus study*. Lancet, 2005. **366**(9503): p. 2112-7.
2. Vandenberghe, R. and J. Tournoy, *Cognitive aging and Alzheimer's disease*. Postgrad Med J, 2005. **81**(956): p. 343-52.
3. Gauthier, S., et al., *Alzheimer's disease: current knowledge, management and research*. CMAJ, 1997. **157**(8): p. 1047-52.
4. Morris, J.C., et al., *Mild cognitive impairment represents early-stage Alzheimer disease*. Arch Neurol, 2001. **58**(3): p. 397-405.
5. McCormick, W.C., et al., *Symptom patterns and comorbidity in the early stages of Alzheimer's disease*. J Am Geriatr Soc, 1994. **42**(5): p. 517-21.
6. Bayley, P.J., et al., *Comparison of the serial position effect in very mild Alzheimer's disease, mild Alzheimer's disease, and amnesia associated with electroconvulsive therapy*. J Int Neuropsychol Soc, 2000. **6**(3): p. 290-8.
7. Weiner, M.F., et al., *Early behavioral symptoms and course of Alzheimer's disease*. Acta Psychiatr Scand, 2005. **111**(5): p. 367-71.
8. Mega, M.S., et al., *The spectrum of behavioral changes in Alzheimer's disease*. Neurology, 1996. **46**(1): p. 130-5.
9. Landes, A.M., S.D. Sperry, and M.E. Strauss, *Prevalence of apathy, dysphoria, and depression in relation to dementia severity in Alzheimer's disease*. J Neuropsychiatry Clin Neurosci, 2005. **17**(3): p. 342-9.
10. Tarawneh, R. and D.M. Holtzman, *The clinical problem of symptomatic Alzheimer disease and mild cognitive impairment*. Cold Spring Harb Perspect Med, 2012. **2**(5): p. a006148.
11. Emery, V.O., *Language impairment in dementia of the Alzheimer type: a hierarchical decline?* Int J Psychiatry Med, 2000. **30**(2): p. 145-64.

12. Scian, S.G. and B. Reisberg, *Functional assessment staging (FAST) in Alzheimer's disease: reliability, validity, and ordinality*. Int Psychogeriatr, 1992. **4 Suppl 1**: p. 55-69.
13. De Vreese, L.P., et al., *1995 IPA/Bayer Research Awards in Psychogeriatrics. Bihemispheric language disorders in early-stage dementia of the Alzheimer type: evidence from a novel metalinguistic task*. Int Psychogeriatr, 1996. **8**(1): p. 63-81.
14. Rebok, G.W., B.W. Rovner, and M.F. Folstein, *Sleep disturbance and Alzheimer's disease: relationship to behavioral problems*. Aging (Milano), 1991. **3**(2): p. 193-6.
15. Reisberg, B., et al., *The Global Deterioration Scale for assessment of primary degenerative dementia*. Am J Psychiatry, 1982. **139**(9): p. 1136-9.
16. Herrmann, N. and S. Gauthier, *Diagnosis and treatment of dementia: 6. Management of severe Alzheimer disease*. CMAJ, 2008. **179**(12): p. 1279-87.
17. Alzheimer, A., *Über eine eigenartige Erkrankung der Hirnrinde*. Allgemeine Zeitschrift für Psychiatrie und phychisch-Gerichtliche Medizin, (Berlin), 1907. **64**: p. 146-148.
18. Terry, R.D., *The Fine Structure of Neurofibrillary Tangles in Alzheimer's Disease*. J Neuropathol Exp Neurol, 1963. **22**: p. 629-42.
19. Kidd, M., *Alzheimer's Disease--an Electron Microscopical Study*. Brain, 1964. **87**: p. 307-20.
20. Terry, R.D., N.K. Gonatas, and M. Weiss, *Ultrastructural Studies in Alzheimer's Presenile Dementia*. Am J Pathol, 1964. **44**: p. 269-97.
21. Whitehouse, P.J., et al., *Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain*. Science, 1982. **215**(4537): p. 1237-9.



22. Kidd, M., *Paired helical filaments in electron microscopy of Alzheimer's disease*. Nature, 1963. **197**: p. 192-3.
23. Delacourte, A. and A. Defossez, *Alzheimer's disease: Tau proteins, the promoting factors of microtubule assembly, are major components of paired helical filaments*. J Neurol Sci, 1986. **76**(2-3): p. 173-86.
24. Nukina, N. and Y. Ihara, *One of the antigenic determinants of paired helical filaments is related to tau protein*. J Biochem, 1986. **99**(5): p. 1541-4.
25. Wood, J.G., et al., *Neurofibrillary tangles of Alzheimer disease share antigenic determinants with the axonal microtubule-associated protein tau (tau)*. Proc Natl Acad Sci U S A, 1986. **83**(11): p. 4040-3.
26. Kosik, K.S., C.L. Joachim, and D.J. Selkoe, *Microtubule-associated protein tau (tau) is a major antigenic component of paired helical filaments in Alzheimer disease*. Proc Natl Acad Sci U S A, 1986. **83**(11): p. 4044-8.
27. Grundke-Iqbal, I., et al., *Microtubule-associated protein tau. A component of Alzheimer paired helical filaments*. J Biol Chem, 1986. **261**(13): p. 6084-9.
28. Wischik, C.M., et al., *Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease*. Proc Natl Acad Sci U S A, 1988. **85**(12): p. 4506-10.
29. Goedert, M., et al., *Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated protein tau*. Proc Natl Acad Sci U S A, 1988. **85**(11): p. 4051-5.
30. Neve, R.L., et al., *Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2*. Brain Res, 1986. **387**(3): p. 271-80.

31. Goedert, M., et al., *Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease*. Neuron, 1989. **3**(4): p. 519-26.
32. Goedert, M., et al., *Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain*. EMBO J, 1989. **8**(2): p. 393-9.
33. Himmler, A., et al., *Tau consists of a set of proteins with repeated C-terminal microtubule-binding domains and variable N-terminal domains*. Mol Cell Biol, 1989. **9**(4): p. 1381-8.
34. Kowall, N.W. and K.S. Kosik, *Axonal disruption and aberrant localization of tau protein characterize the neuropil pathology of Alzheimer's disease*. Ann Neurol, 1987. **22**(5): p. 639-43.
35. Migheli, A., et al., *Light and electron microscope localization of the microtubule-associated tau protein in rat brain*. J Neurosci, 1988. **8**(6): p. 1846-51.
36. Papasozomenos, S.C. and L.I. Binder, *Phosphorylation determines two distinct species of Tau in the central nervous system*. Cell Motil Cytoskeleton, 1987. **8**(3): p. 210-26.
37. Riederer, B.M. and G.M. Innocenti, *Differential Distribution of Tau Proteins in Developing Cat Cerebral Cortex and Corpus Callosum*. Eur J Neurosci, 1991. **3**(11): p. 1134-1145.
38. Lindwall, G. and R.D. Cole, *Phosphorylation affects the ability of tau protein to promote microtubule assembly*. J Biol Chem, 1984. **259**(8): p. 5301-5.

39. Weingarten, M.D., et al., *A protein factor essential for microtubule assembly*. Proc Natl Acad Sci U S A, 1975. **72**(5): p. 1858-62.
40. Murphy, D.B. and G.G. Borisy, *Association of high-molecular-weight proteins with microtubules and their role in microtubule assembly in vitro*. Proc Natl Acad Sci U S A, 1975. **72**(7): p. 2696-700.
41. Sloboda, R.D., et al., *Cyclic AMP-dependent endogenous phosphorylation of a microtubule-associated protein*. Proc Natl Acad Sci U S A, 1975. **72**(1): p. 177-81.
42. Kopke, E., et al., *Microtubule-associated protein tau. Abnormal phosphorylation of a non-paired helical filament pool in Alzheimer disease*. J Biol Chem, 1993. **268**(32): p. 24374-84.
43. Khatoon, S., I. Grundke-Iqbal, and K. Iqbal, *Brain levels of microtubule-associated protein tau are elevated in Alzheimer's disease: a radioimmuno-slot-blot assay for nanograms of the protein*. J Neurochem, 1992. **59**(2): p. 750-3.
44. Goedert, M., et al., *Tau proteins of Alzheimer paired helical filaments: abnormal phosphorylation of all six brain isoforms*. Neuron, 1992. **8**(1): p. 159-68.
45. Mah, V.H., et al., *In situ hybridization of calcium/calmodulin dependent protein kinase II and tau mRNAs; species differences and relative preservation in Alzheimer's disease*. Brain Res Mol Brain Res, 1992. **12**(1-3): p. 85-94.
46. Lee, V.M., et al., *A68: a major subunit of paired helical filaments and derivatized forms of normal Tau*. Science, 1991. **251**(4994): p. 675-8.
47. Iqbal, K., et al., *Alzheimer paired helical filaments. Restoration of the biological activity by dephosphorylation*. FEBS Lett, 1994. **349**(1): p. 104-8.

48. Alonso Adel, C., et al., *Polymerization of hyperphosphorylated tau into filaments eliminates its inhibitory activity*. Proc Natl Acad Sci U S A, 2006. **103**(23): p. 8864-9.
49. Braak, H., et al., *Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry*. Acta Neuropathol, 2006. **112**(4): p. 389-404.
50. Braak, H. and E. Braak, *Neuropathological staging of Alzheimer-related changes*. Acta Neuropathol, 1991. **82**(4): p. 239-59.
51. Bierer, L.M., et al., *Neocortical neurofibrillary tangles correlate with dementia severity in Alzheimer's disease*. Arch Neurol, 1995. **52**(1): p. 81-8.
52. Arriagada, P.V., K. Marzloff, and B.T. Hyman, *Distribution of Alzheimer-type pathologic changes in nondemented elderly individuals matches the pattern in Alzheimer's disease*. Neurology, 1992. **42**(9): p. 1681-8.
53. Ingelsson, M., et al., *Early Abeta accumulation and progressive synaptic loss, gliosis, and tangle formation in AD brain*. Neurology, 2004. **62**(6): p. 925-31.
54. Wisniewski, K., et al., *Alzheimer neurofibrillary tangles in diseases other than senile and presenile dementia*. Ann Neurol, 1979. **5**(3): p. 288-94.
55. Robakis, N.K., et al., *Molecular cloning and characterization of a cDNA encoding the cerebrovascular and the neuritic plaque amyloid peptides*. Proc Natl Acad Sci U S A, 1987. **84**(12): p. 4190-4.
56. Glenner, G.G. and C.W. Wong, *Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein*. Biochem Biophys Res Commun, 1984. **120**(3): p. 885-90.
57. Masters, C.L., et al., *Amyloid plaque core protein in Alzheimer disease and Down syndrome*. Proc Natl Acad Sci U S A, 1985. **82**(12): p. 4245-9.

58. Kang, J., et al., *The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor*. Nature, 1987. **325**(6106): p. 733-6.
59. Masliah, E., et al., *Diffuse plaques do not accentuate synapse loss in Alzheimer's disease*. Am J Pathol, 1990. **137**(6): p. 1293-7.
60. Masliah, E., et al., *Synaptic and neuritic alterations during the progression of Alzheimer's disease*. Neurosci Lett, 1994. **174**(1): p. 67-72.
61. Pike, C.J., B.J. Cummings, and C.W. Cotman, *Early association of reactive astrocytes with senile plaques in Alzheimer's disease*. Exp Neurol, 1995. **132**(2): p. 172-9.
62. Urbanc, B., et al., *Neurotoxic effects of thioflavin S-positive amyloid deposits in transgenic mice and Alzheimer's disease*. Proc Natl Acad Sci U S A, 2002. **99**(22): p. 13990-5.
63. Vehmas, A.K., et al., *Immune reactive cells in senile plaques and cognitive decline in Alzheimer's disease*. Neurobiol Aging, 2003. **24**(2): p. 321-31.
64. Yamaguchi, H., et al., *Diffuse type of senile plaques in the cerebellum of Alzheimer-type dementia demonstrated by beta protein immunostain*. Acta Neuropathol, 1989. **77**(3): p. 314-9.
65. Morris, J.C., et al., *Cerebral amyloid deposition and diffuse plaques in "normal" aging: Evidence for presymptomatic and very mild Alzheimer's disease*. Neurology, 1996. **46**(3): p. 707-19.
66. Arriagada, P.V., et al., *Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease*. Neurology, 1992. **42**(3 Pt 1): p. 631-9.
67. Hyman, B.T., K. Marzloff, and P.V. Arriagada, *The lack of accumulation of senile plaques or amyloid burden in Alzheimer's disease suggests a dynamic*

- balance between amyloid deposition and resolution. J Neuropathol Exp Neurol*, 1993. **52**(6): p. 594-600.
68. Giannakopoulos, P., et al., *Tangle and neuron numbers, but not amyloid load, predict cognitive status in Alzheimer's disease. Neurology*, 2003. **60**(9): p. 1495-500.
  69. Serrano-Pozo, A., et al., *Reactive glia not only associates with plaques but also parallels tangles in Alzheimer's disease. Am J Pathol*, 2011. **179**(3): p. 1373-84.
  70. Jack, C.R., Jr., et al., *Serial PIB and MRI in normal, mild cognitive impairment and Alzheimer's disease: implications for sequence of pathological events in Alzheimer's disease. Brain*, 2009. **132**(Pt 5): p. 1355-65.
  71. Revesz, T., et al., *Cerebral amyloid angiopathies: a pathologic, biochemical, and genetic view. J Neuropathol Exp Neurol*, 2003. **62**(9): p. 885-98.
  72. Herzig, M.C., et al., *Abeta is targeted to the vasculature in a mouse model of hereditary cerebral hemorrhage with amyloidosis. Nat Neurosci*, 2004. **7**(9): p. 954-60.
  73. Tekirian, T.L., et al., *N-terminal heterogeneity of parenchymal and cerebrovascular Abeta deposits. J Neuropathol Exp Neurol*, 1998. **57**(1): p. 76-94.
  74. Namba, Y., et al., *Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in Creutzfeldt-Jakob disease. Brain Res*, 1991. **541**(1): p. 163-6.
  75. Chalmers, K., G.K. Wilcock, and S. Love, *APOE epsilon 4 influences the pathological phenotype of Alzheimer's disease by favouring cerebrovascular*

- over parenchymal accumulation of A beta protein. Neuropathol Appl Neurobiol*, 2003. **29**(3): p. 231-8.
76. Thal, D.R., et al., *Two types of sporadic cerebral amyloid angiopathy. J Neuropathol Exp Neurol*, 2002. **61**(3): p. 282-93.
  77. Thal, D.R., et al., *Vascular pathology in Alzheimer disease: correlation of cerebral amyloid angiopathy and arteriosclerosis/lipohyalinosis with cognitive decline. J Neuropathol Exp Neurol*, 2003. **62**(12): p. 1287-301.
  78. Revesz, T., et al., *Genetics and molecular pathogenesis of sporadic and hereditary cerebral amyloid angiopathies. Acta Neuropathol*, 2009. **118**(1): p. 115-30.
  79. DeKosky, S.T., S.W. Scheff, and S.D. Styren, *Structural correlates of cognition in dementia: quantification and assessment of synapse change. Neurodegeneration*, 1996. **5**(4): p. 417-21.
  80. Masliah, E., et al., *Immunohistochemical quantification of the synapse-related protein synaptophysin in Alzheimer disease. Neurosci Lett*, 1989. **103**(2): p. 234-9.
  81. Masliah, E., et al., *Immunoelectron microscopic study of synaptic pathology in Alzheimer's disease. Acta Neuropathol*, 1991. **81**(4): p. 428-33.
  82. Scheff, S.W., L. Sparks, and D.A. Price, *Quantitative assessment of synaptic density in the entorhinal cortex in Alzheimer's disease. Ann Neurol*, 1993. **34**(3): p. 356-61.
  83. Scheff, S.W. and D.A. Price, *Synaptic density in the inner molecular layer of the hippocampal dentate gyrus in Alzheimer disease. J Neuropathol Exp Neurol*, 1998. **57**(12): p. 1146-53.

84. Scheff, S.W. and D.A. Price, *Alzheimer's disease-related alterations in synaptic density: neocortex and hippocampus*. J Alzheimers Dis, 2006. **9**(3 Suppl): p. 101-15.
85. Scheff, S.W., et al., *Hippocampal synaptic loss in early Alzheimer's disease and mild cognitive impairment*. Neurobiol Aging, 2006. **27**(10): p. 1372-84.
86. Masliah, E., *Mechanisms of synaptic dysfunction in Alzheimer's disease*. Histol Histopathol, 1995. **10**(2): p. 509-19.
87. Masliah, E., et al., *Quantitative synaptic alterations in the human neocortex during normal aging*. Neurology, 1993. **43**(1): p. 192-7.
88. Vassar, R., et al., *Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE*. Science, 1999. **286**(5440): p. 735-41.
89. Yan, R., et al., *Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity*. Nature, 1999. **402**(6761): p. 533-7.
90. Sinha, S., et al., *Purification and cloning of amyloid precursor protein beta-secretase from human brain*. Nature, 1999. **402**(6761): p. 537-40.
91. Cai, H., et al., *BACE1 is the major beta-secretase for generation of A $\beta$  peptides by neurons*. Nat Neurosci, 2001. **4**(3): p. 233-4.
92. Haass, C., et al., *Amyloid beta-peptide is produced by cultured cells during normal metabolism*. Nature, 1992. **359**(6393): p. 322-5.
93. Sisodia, S.S., et al., *Evidence that beta-amyloid protein in Alzheimer's disease is not derived by normal processing*. Science, 1990. **248**(4954): p. 492-5.
94. Sisodia, S.S., *Beta-amyloid precursor protein cleavage by a membrane-bound protease*. Proc Natl Acad Sci U S A, 1992. **89**(13): p. 6075-9.



95. Esch, F.S., et al., *Cleavage of amyloid beta peptide during constitutive processing of its precursor*. Science, 1990. **248**(4959): p. 1122-4.
96. Jarrett, J.T., E.P. Berger, and P.T. Lansbury, Jr., *The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease*. Biochemistry, 1993. **32**(18): p. 4693-7.
97. Jarrett, J.T., E.P. Berger, and P.T. Lansbury, Jr., *The C-terminus of the beta protein is critical in amyloidogenesis*. Ann N Y Acad Sci, 1993. **695**: p. 144-8.
98. Serpell, L.C., *Alzheimer's amyloid fibrils: structure and assembly*. Biochim Biophys Acta, 2000. **1502**(1): p. 16-30.
99. Losic, D., et al., *High resolution scanning tunnelling microscopy of the beta-amyloid protein (Abeta1-40) of Alzheimer's disease suggests a novel mechanism of oligomer assembly*. J Struct Biol, 2006. **155**(1): p. 104-10.
100. Arimon, M., et al., *Fine structure study of Abeta1-42 fibrillogenesis with atomic force microscopy*. FASEB J, 2005. **19**(10): p. 1344-6.
101. Harper, J.D., et al., *Observation of metastable Abeta amyloid protofibrils by atomic force microscopy*. Chem Biol, 1997. **4**(2): p. 119-25.
102. Kaye, R., et al., *Annular protofibrils are a structurally and functionally distinct type of amyloid oligomer*. J Biol Chem, 2009. **284**(7): p. 4230-7.
103. Esler, W.P., et al., *Alzheimer's disease amyloid propagation by a template-dependent dock-lock mechanism*. Biochemistry, 2000. **39**(21): p. 6288-95.
104. Cannon, M.J., et al., *Kinetic analysis of beta-amyloid fibril elongation*. Anal Biochem, 2004. **328**(1): p. 67-75.

105. Podlisny, M.B., et al., *Oligomerization of endogenous and synthetic amyloid beta-protein at nanomolar levels in cell culture and stabilization of monomer by Congo red*. Biochemistry, 1998. **37**(11): p. 3602-11.
106. McLean, C.A., et al., *Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease*. Ann Neurol, 1999. **46**(6): p. 860-6.
107. Lue, L.F., et al., *Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease*. Am J Pathol, 1999. **155**(3): p. 853-62.
108. Kawarabayashi, T., et al., *Dimeric amyloid beta protein rapidly accumulates in lipid rafts followed by apolipoprotein E and phosphorylated tau accumulation in the Tg2576 mouse model of Alzheimer's disease*. J Neurosci, 2004. **24**(15): p. 3801-9.
109. Shankar, G.M., et al., *Biochemical and immunohistochemical analysis of an Alzheimer's disease mouse model reveals the presence of multiple cerebral Abeta assembly forms throughout life*. Neurobiol Dis, 2009. **36**(2): p. 293-302.
110. Shankar, G.M., et al., *Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory*. Nat Med, 2008. **14**(8): p. 837-42.
111. Jin, M., et al., *Soluble amyloid beta-protein dimers isolated from Alzheimer cortex directly induce Tau hyperphosphorylation and neuritic degeneration*. Proc Natl Acad Sci U S A, 2011. **108**(14): p. 5819-24.
112. Lesne, S., et al., *A specific amyloid-beta protein assembly in the brain impairs memory*. Nature, 2006. **440**(7082): p. 352-7.

113. Townsend, M., et al., *Effects of secreted oligomers of amyloid beta-protein on hippocampal synaptic plasticity: a potent role for trimers*. J Physiol, 2006. **572**(Pt 2): p. 477-92.
114. Davis, R.C., et al., *Amyloid beta dimers/trimers potently induce cofilin-actin rods that are inhibited by maintaining cofilin-phosphorylation*. Mol Neurodegener, 2011. **6**: p. 10.
115. Billings, L.M., et al., *Learning decreases A beta\*56 and tau pathology and ameliorates behavioral decline in 3xTg-AD mice*. J Neurosci, 2007. **27**(4): p. 751-61.
116. Lefterov, I., et al., *Memory deficits in APP23/Abca1+/- mice correlate with the level of Abeta oligomers*. ASN Neuro, 2009. **1**(2).
117. Lashuel, H.A. and P.T. Lansbury, Jr., *Are amyloid diseases caused by protein aggregates that mimic bacterial pore-forming toxins?* Q Rev Biophys, 2006. **39**(2): p. 167-201.
118. Kokubo, H., et al., *Amyloid Beta annular protofibrils in cell processes and synapses accumulate with aging and Alzheimer-associated genetic modification*. Int J Alzheimers Dis, 2009. **2009**.
119. Lasagna-Reeves, C.A., C.G. Glabe, and R. Kaye, *Amyloid-beta annular protofibrils evade fibrillar fate in Alzheimer disease brain*. J Biol Chem, 2011. **286**(25): p. 22122-30.
120. Khachaturian, Z.S., *Hypothesis on the regulation of cytosol calcium concentration and the aging brain*. Neurobiol Aging, 1987. **8**(4): p. 345-6.
121. Landfield, P.W., *'Increased calcium-current' hypothesis of brain aging*. Neurobiol Aging, 1987. **8**(4): p. 346-7.

122. Mattson, M.P., et al., *beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity*. J Neurosci, 1992. **12**(2): p. 376-89.
123. Mark, R.J., et al., *Amyloid beta-peptide impairs ion-motive ATPase activities: evidence for a role in loss of neuronal Ca<sup>2+</sup> homeostasis and cell death*. J Neurosci, 1995. **15**(9): p. 6239-49.
124. Price, S.A., B. Held, and H.A. Pearson, *Amyloid beta protein increases Ca<sup>2+</sup> currents in rat cerebellar granule neurones*. Neuroreport, 1998. **9**(3): p. 539-45.
125. De Felice, F.G., et al., *Abeta oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine*. J Biol Chem, 2007. **282**(15): p. 11590-601.
126. Domingues, A., et al., *Toxicity of beta-amyloid in HEK293 cells expressing NR1/NR2A or NR1/NR2B N-methyl-D-aspartate receptor subunits*. Neurochem Int, 2007. **50**(6): p. 872-80.
127. Ho, R., D. Ortiz, and T.B. Shea, *Amyloid-beta promotes calcium influx and neurodegeneration via stimulation of L voltage-sensitive calcium channels rather than NMDA channels in cultured neurons*. J Alzheimers Dis, 2001. **3**(5): p. 479-483.
128. Sberna, G., et al., *The amyloid beta-protein of Alzheimer's disease increases acetylcholinesterase expression by increasing intracellular calcium in embryonal carcinoma P19 cells*. J Neurochem, 1997. **69**(3): p. 1177-84.

129. Good, T.A., D.O. Smith, and R.M. Murphy, *Beta-amyloid peptide blocks the fast-inactivating K<sup>+</sup> current in rat hippocampal neurons*. *Biophys J*, 1996. **70**(1): p. 296-304.
130. Arispe, N., E. Rojas, and H.B. Pollard, *Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum*. *Proc Natl Acad Sci U S A*, 1993. **90**(2): p. 567-71.
131. Demuro, A., et al., *Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers*. *J Biol Chem*, 2005. **280**(17): p. 17294-300.
132. Lin, H., Y.J. Zhu, and R. Lal, *Amyloid beta protein (1-40) forms calcium-permeable, Zn<sup>2+</sup>-sensitive channel in reconstituted lipid vesicles*. *Biochemistry*, 1999. **38**(34): p. 11189-96.
133. Lin, H., R. Bhatia, and R. Lal, *Amyloid beta protein forms ion channels: implications for Alzheimer's disease pathophysiology*. *FASEB J*, 2001. **15**(13): p. 2433-44.
134. Lal, R., H. Lin, and A.P. Quist, *Amyloid beta ion channel: 3D structure and relevance to amyloid channel paradigm*. *Biochim Biophys Acta*, 2007. **1768**(8): p. 1966-75.
135. Kaye, R., et al., *Permeabilization of lipid bilayers is a common conformation-dependent activity of soluble amyloid oligomers in protein misfolding diseases*. *J Biol Chem*, 2004. **279**(45): p. 46363-6.
136. Sokolov, Y., et al., *Soluble amyloid oligomers increase bilayer conductance by altering dielectric structure*. *J Gen Physiol*, 2006. **128**(6): p. 637-47.

137. Dreses-Werringloer, U., et al., *A polymorphism in CALHM1 influences Ca<sup>2+</sup> homeostasis, Aβ levels, and Alzheimer's disease risk*. Cell, 2008. **133**(7): p. 1149-61.
138. Chan, S.L., et al., *Presenilin-1 mutations increase levels of ryanodine receptors and calcium release in PC12 cells and cortical neurons*. J Biol Chem, 2000. **275**(24): p. 18195-200.
139. Cheung, K.H., et al., *Mechanism of Ca<sup>2+</sup> disruption in Alzheimer's disease by presenilin regulation of InsP3 receptor channel gating*. Neuron, 2008. **58**(6): p. 871-83.
140. Green, K.N., et al., *SERCA pump activity is physiologically regulated by presenilin and regulates amyloid beta production*. J Cell Biol, 2008. **181**(7): p. 1107-16.
141. Seubert, P., et al., *Isolation and quantification of soluble Alzheimer's beta-peptide from biological fluids*. Nature, 1992. **359**(6393): p. 325-7.
142. Whitson, J.S., D.J. Selkoe, and C.W. Cotman, *Amyloid beta protein enhances the survival of hippocampal neurons in vitro*. Science, 1989. **243**(4897): p. 1488-90.
143. Yankner, B.A., L.K. Duffy, and D.A. Kirschner, *Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides*. Science, 1990. **250**(4978): p. 279-82.
144. Giuffrida, M.L., et al., *Beta-amyloid monomers are neuroprotective*. J Neurosci, 2009. **29**(34): p. 10582-7.
145. Chen, Y. and C. Dong, *Aβ<sub>40</sub> promotes neuronal cell fate in neural progenitor cells*. Cell Death Differ, 2009. **16**(3): p. 386-94.

146. Kamenetz, F., et al., *APP processing and synaptic function*. Neuron, 2003. **37**(6): p. 925-37.
147. Lesne, S., et al., *NMDA receptor activation inhibits alpha-secretase and promotes neuronal amyloid-beta production*. J Neurosci, 2005. **25**(41): p. 9367-77.
148. Tanzi, R.E., et al., *Amyloid beta protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus*. Science, 1987. **235**(4791): p. 880-4.
149. Goldgaber, D., et al., *Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease*. Science, 1987. **235**(4791): p. 877-80.
150. St George-Hyslop, P.H., et al., *The genetic defect causing familial Alzheimer's disease maps on chromosome 21*. Science, 1987. **235**(4791): p. 885-90.
151. Podlisny, M.B., G. Lee, and D.J. Selkoe, *Gene dosage of the amyloid beta precursor protein in Alzheimer's disease*. Science, 1987. **238**(4827): p. 669-71.
152. Yoshikai, S., et al., *Genomic organization of the human amyloid beta-protein precursor gene*. Gene, 1990. **87**(2): p. 257-63.
153. Lamb, B.T., et al., *Introduction and expression of the 400 kilobase amyloid precursor protein gene in transgenic mice [corrected]*. Nat Genet, 1993. **5**(1): p. 22-30.
154. Jacobsen, J.S., et al., *A novel species-specific RNA related to alternatively spliced amyloid precursor protein mRNAs*. Neurobiol Aging, 1991. **12**(5): p. 575-83.
155. Kitaguchi, N., et al., *Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity*. Nature, 1988. **331**(6156): p. 530-2.

156. de Sauvage, F. and J.N. Octave, *A novel mRNA of the A4 amyloid precursor gene coding for a possibly secreted protein*. Science, 1989. **245**(4918): p. 651-3.
157. Tanzi, R.E., et al., *Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease*. Nature, 1988. **331**(6156): p. 528-30.
158. Ponte, P., et al., *A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors*. Nature, 1988. **331**(6156): p. 525-7.
159. Donnelly, R.J., et al., *Multiple forms of beta-amyloid peptide precursor RNAs in a single cell type*. Neurobiol Aging, 1988. **9**(4): p. 333-8.
160. Palmert, M.R., et al., *Amyloid protein precursor messenger RNAs: differential expression in Alzheimer's disease*. Science, 1988. **241**(4869): p. 1080-4.
161. Dyrks, T., et al., *Identification, transmembrane orientation and biogenesis of the amyloid A4 precursor of Alzheimer's disease*. EMBO J, 1988. **7**(4): p. 949-57.
162. Daigle, I. and C. Li, *apl-1, a Caenorhabditis elegans gene encoding a protein related to the human beta-amyloid protein precursor*. Proc Natl Acad Sci U S A, 1993. **90**(24): p. 12045-9.
163. Mok, S.S., et al., *Expression and analysis of heparin-binding regions of the amyloid precursor protein of Alzheimer's disease*. FEBS Lett, 1997. **415**(3): p. 303-7.
164. Rossjohn, J., et al., *Crystal structure of the N-terminal, growth factor-like domain of Alzheimer amyloid precursor protein*. Nat Struct Biol, 1999. **6**(4): p. 327-31.



165. Hesse, L., et al., *The beta A4 amyloid precursor protein binding to copper*. FEBS Lett, 1994. **349**(1): p. 109-16.
166. Bush, A.I., et al., *A novel zinc(II) binding site modulates the function of the beta A4 amyloid protein precursor of Alzheimer's disease*. J Biol Chem, 1993. **268**(22): p. 16109-12.
167. Hynes, T.R., et al., *X-ray crystal structure of the protease inhibitor domain of Alzheimer's amyloid beta-protein precursor*. Biochemistry, 1990. **29**(43): p. 10018-22.
168. Dulubova, I., et al., *Three-dimensional structure of an independently folded extracellular domain of human amyloid-beta precursor protein*. Biochemistry, 2004. **43**(30): p. 9583-8.
169. Wang, Y. and Y. Ha, *The X-ray structure of an antiparallel dimer of the human amyloid precursor protein E2 domain*. Mol Cell, 2004. **15**(3): p. 343-53.
170. Beher, D., et al., *Regulation of amyloid protein precursor (APP) binding to collagen and mapping of the binding sites on APP and collagen type I*. J Biol Chem, 1996. **271**(3): p. 1613-20.
171. Narindrasorasak, S., et al., *Characterization of high affinity binding between laminin and Alzheimer's disease amyloid precursor proteins*. Lab Invest, 1992. **67**(5): p. 643-52.
172. Gralle, M., et al., *Solution studies and structural model of the extracellular domain of the human amyloid precursor protein*. Biophys J, 2002. **83**(6): p. 3513-24.

173. Weidemann, A., et al., *A novel epsilon-cleavage within the transmembrane domain of the Alzheimer amyloid precursor protein demonstrates homology with Notch processing*. Biochemistry, 2002. **41**(8): p. 2825-35.
174. Reinhard, C., S.S. Hebert, and B. De Strooper, *The amyloid-beta precursor protein: integrating structure with biological function*. EMBO J, 2005. **24**(23): p. 3996-4006.
175. Chen, W.J., J.L. Goldstein, and M.S. Brown, *NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor*. J Biol Chem, 1990. **265**(6): p. 3116-23.
176. Rosen, D.R., et al., *A Drosophila gene encoding a protein resembling the human beta-amyloid protein precursor*. Proc Natl Acad Sci U S A, 1989. **86**(7): p. 2478-82.
177. Musa, A., H. Lehrach, and V.A. Russo, *Distinct expression patterns of two zebrafish homologues of the human APP gene during embryonic development*. Dev Genes Evol, 2001. **211**(11): p. 563-7.
178. Okado, H. and H. Okamoto, *A Xenopus homologue of the human beta-amyloid precursor protein: developmental regulation of its gene expression*. Biochem Biophys Res Commun, 1992. **189**(3): p. 1561-8.
179. Wasco, W., et al., *Identification of a mouse brain cDNA that encodes a protein related to the Alzheimer disease-associated amyloid beta protein precursor*. Proc Natl Acad Sci U S A, 1992. **89**(22): p. 10758-62.
180. Wasco, W., et al., *Isolation and characterization of APLP2 encoding a homologue of the Alzheimer's associated amyloid beta protein precursor*. Nat Genet, 1993. **5**(1): p. 95-100.

181. Lorent, K., et al., *Expression in mouse embryos and in adult mouse brain of three members of the amyloid precursor protein family, of the alpha-2-macroglobulin receptor/low density lipoprotein receptor-related protein and of its ligands apolipoprotein E, lipoprotein lipase, alpha-2-macroglobulin and the 40,000 molecular weight receptor-associated protein*. Neuroscience, 1995. **65**(4): p. 1009-25.
182. Martin-Morris, L.E. and K. White, *The Drosophila transcript encoded by the beta-amyloid protein precursor-like gene is restricted to the nervous system*. Development, 1990. **110**(1): p. 185-95.
183. Thinakaran, G., et al., *Distribution of an APP homolog, APLP2, in the mouse olfactory system: a potential role for APLP2 in axogenesis*. J Neurosci, 1995. **15**(10): p. 6314-26.
184. Breen, K.C., M. Bruce, and B.H. Anderton, *Beta amyloid precursor protein mediates neuronal cell-cell and cell-surface adhesion*. J Neurosci Res, 1991. **28**(1): p. 90-100.
185. Clarris, H.J., et al., *Identification of heparin-binding domains in the amyloid precursor protein of Alzheimer's disease by deletion mutagenesis and peptide mapping*. J Neurochem, 1997. **68**(3): p. 1164-72.
186. Small, D.H., et al., *A heparin-binding domain in the amyloid protein precursor of Alzheimer's disease is involved in the regulation of neurite outgrowth*. J Neurosci, 1994. **14**(4): p. 2117-27.
187. Dahms, S.O., et al., *Structure and biochemical analysis of the heparin-induced E1 dimer of the amyloid precursor protein*. Proc Natl Acad Sci U S A, 2010. **107**(12): p. 5381-6.

188. Gralle, M., et al., *Solution conformation and heparin-induced dimerization of the full-length extracellular domain of the human amyloid precursor protein*. J Mol Biol, 2006. **357**(2): p. 493-508.
189. Soba, P., et al., *Homo- and heterodimerization of APP family members promotes intercellular adhesion*. EMBO J, 2005. **24**(20): p. 3624-34.
190. Munter, L.M., et al., *GxxxG motifs within the amyloid precursor protein transmembrane sequence are critical for the etiology of Abeta42*. EMBO J, 2007. **26**(6): p. 1702-12.
191. Kienlen-Campard, P., et al., *Amyloidogenic processing but not amyloid precursor protein (APP) intracellular C-terminal domain production requires a precisely oriented APP dimer assembled by transmembrane GXXXG motifs*. J Biol Chem, 2008. **283**(12): p. 7733-44.
192. Ghiso, J., et al., *A 109-amino-acid C-terminal fragment of Alzheimer's-disease amyloid precursor protein contains a sequence, -RHDS-, that promotes cell adhesion*. Biochem J, 1992. **288** ( Pt 3): p. 1053-9.
193. Yamazaki, T., E.H. Koo, and D.J. Selkoe, *Cell surface amyloid beta-protein precursor colocalizes with beta 1 integrins at substrate contact sites in neural cells*. J Neurosci, 1997. **17**(3): p. 1004-10.
194. Young-Pearse, T.L., et al., *Secreted APP regulates the function of full-length APP in neurite outgrowth through interaction with integrin beta1*. Neural Dev, 2008. **3**: p. 15.
195. Selkoe, D. and R. Kopan, *Notch and Presenilin: regulated intramembrane proteolysis links development and degeneration*. Annu Rev Neurosci, 2003. **26**: p. 565-97.

196. Okamoto, T., et al., *Ligand-dependent G protein coupling function of amyloid transmembrane precursor*. J Biol Chem, 1995. **270**(9): p. 4205-8.
197. Murayama, Y., et al., *Cell surface receptor function of amyloid precursor protein that activates Ser/Thr kinases*. Gerontology, 1996. **42 Suppl 1**: p. 2-11.
198. Ho, A. and T.C. Sudhof, *Binding of F-spondin to amyloid-beta precursor protein: a candidate amyloid-beta precursor protein ligand that modulates amyloid-beta precursor protein cleavage*. Proc Natl Acad Sci U S A, 2004. **101**(8): p. 2548-53.
199. Peterziel, H., et al., *F-spondin regulates neuronal survival through activation of disabled-1 in the chicken ciliary ganglion*. Mol Cell Neurosci, 2011. **46**(2): p. 483-97.
200. Hoe, H.S., et al., *DAB1 and Reelin effects on amyloid precursor protein and ApoE receptor 2 trafficking and processing*. J Biol Chem, 2006. **281**(46): p. 35176-85.
201. Hoe, H.S., et al., *Interaction of reelin with amyloid precursor protein promotes neurite outgrowth*. J Neurosci, 2009. **29**(23): p. 7459-73.
202. Park, J.H., et al., *Alzheimer precursor protein interaction with the Nogo-66 receptor reduces amyloid-beta plaque deposition*. J Neurosci, 2006. **26**(5): p. 1386-95.
203. Lourenco, F.C., et al., *Netrin-1 interacts with amyloid precursor protein and regulates amyloid-beta production*. Cell Death Differ, 2009. **16**(5): p. 655-63.
204. Gralle, M., M.G. Botelho, and F.S. Wouters, *Neuroprotective secreted amyloid precursor protein acts by disrupting amyloid precursor protein dimers*. J Biol Chem, 2009. **284**(22): p. 15016-25.

205. Herms, J., et al., *Cortical dysplasia resembling human type 2 lissencephaly in mice lacking all three APP family members*. EMBO J, 2004. **23**(20): p. 4106-15.
206. Sisodia, S.S., et al., *Identification and transport of full-length amyloid precursor proteins in rat peripheral nervous system*. J Neurosci, 1993. **13**(7): p. 3136-42.
207. Koo, E.H., et al., *Precursor of amyloid protein in Alzheimer disease undergoes fast anterograde axonal transport*. Proc Natl Acad Sci U S A, 1990. **87**(4): p. 1561-5.
208. Lyckman, A.W., et al., *Post-translational processing and turnover kinetics of presynaptically targeted amyloid precursor superfamily proteins in the central nervous system*. J Biol Chem, 1998. **273**(18): p. 11100-6.
209. Moya, K.L., et al., *The amyloid precursor protein is developmentally regulated and correlated with synaptogenesis*. Dev Biol, 1994. **161**(2): p. 597-603.
210. Hung, A.Y., et al., *Increased expression of beta-amyloid precursor protein during neuronal differentiation is not accompanied by secretory cleavage*. Proc Natl Acad Sci U S A, 1992. **89**(20): p. 9439-43.
211. Yoshikawa, K., T. Aizawa, and K. Maruyama, *Neural differentiation increases expression of Alzheimer amyloid protein precursor gene in murine embryonal carcinoma cells*. Biochem Biophys Res Commun, 1990. **171**(1): p. 204-9.
212. Wang, P., et al., *Defective neuromuscular synapses in mice lacking amyloid precursor protein (APP) and APP-Like protein 2*. J Neurosci, 2005. **25**(5): p. 1219-25.

213. Yang, G., et al., *Reduced synaptic vesicle density and active zone size in mice lacking amyloid precursor protein (APP) and APP-like protein 2*. Neurosci Lett, 2005. **384**(1-2): p. 66-71.
214. Murakami, N., et al., *Experimental brain injury induces expression of amyloid precursor protein, which may be related to neuronal loss in the hippocampus*. J Neurotrauma, 1998. **15**(11): p. 993-1003.
215. Van den Heuvel, C., et al., *Upregulation of amyloid precursor protein messenger RNA in response to traumatic brain injury: an ovine head impact model*. Exp Neurol, 1999. **159**(2): p. 441-50.
216. Leyssen, M., et al., *Amyloid precursor protein promotes post-developmental neurite arborization in the Drosophila brain*. EMBO J, 2005. **24**(16): p. 2944-55.
217. Thornton, E., et al., *Soluble amyloid precursor protein alpha reduces neuronal injury and improves functional outcome following diffuse traumatic brain injury in rats*. Brain Res, 2006. **1094**(1): p. 38-46.
218. Copanaki, E., et al., *sAPPalpha antagonizes dendritic degeneration and neuron death triggered by proteasomal stress*. Mol Cell Neurosci, 2010. **44**(4): p. 386-93.
219. Qiu, W.Q., et al., *Cell-surface beta-amyloid precursor protein stimulates neurite outgrowth of hippocampal neurons in an isoform-dependent manner*. J Neurosci, 1995. **15**(3 Pt 2): p. 2157-67.
220. Saitoh, T., et al., *Secreted form of amyloid beta protein precursor is involved in the growth regulation of fibroblasts*. Cell, 1989. **58**(4): p. 615-22.

221. Ninomiya, H., et al., *Amino acid sequence RERMS represents the active domain of amyloid beta/A4 protein precursor that promotes fibroblast growth.* J Cell Biol, 1993. **121**(4): p. 879-86.
222. Jin, L.W., et al., *Peptides containing the RERMS sequence of amyloid beta/A4 protein precursor bind cell surface and promote neurite extension.* J Neurosci, 1994. **14**(9): p. 5461-70.
223. Roch, J.M., et al., *Increase of synaptic density and memory retention by a peptide representing the trophic domain of the amyloid beta/A4 protein precursor.* Proc Natl Acad Sci U S A, 1994. **91**(16): p. 7450-4.
224. Meziane, H., et al., *Memory-enhancing effects of secreted forms of the beta-amyloid precursor protein in normal and amnesic mice.* Proc Natl Acad Sci U S A, 1998. **95**(21): p. 12683-8.
225. Bell, K.F., et al., *ADAM-10 over-expression increases cortical synaptogenesis.* Neurobiol Aging, 2008. **29**(4): p. 554-65.
226. Mucke, L., et al., *Synaptotrophic effects of human amyloid beta protein precursors in the cortex of transgenic mice.* Brain Res, 1994. **666**(2): p. 151-67.
227. Milward, E.A., et al., *The amyloid protein precursor of Alzheimer's disease is a mediator of the effects of nerve growth factor on neurite outgrowth.* Neuron, 1992. **9**(1): p. 129-37.
228. Perez, R.G., et al., *The beta-amyloid precursor protein of Alzheimer's disease enhances neuron viability and modulates neuronal polarity.* J Neurosci, 1997. **17**(24): p. 9407-14.
229. Bergmans, B.A., et al., *Neurons generated from APP/APLP1/APLP2 triple knockout embryonic stem cells behave normally in vitro and in vivo: lack of*



- evidence for a cell autonomous role of the amyloid precursor protein in neuronal differentiation. Stem Cells, 2010. 28(3): p. 399-406.*
230. Borg, J.P., et al., *The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein. Mol Cell Biol, 1996. 16(11): p. 6229-41.*
  231. Fiore, F., et al., *The regions of the Fe65 protein homologous to the phosphotyrosine interaction/phosphotyrosine binding domain of Shc bind the intracellular domain of the Alzheimer's amyloid precursor protein. J Biol Chem, 1995. 270(52): p. 30853-6.*
  232. Scheinfeld, M.H., et al., *Processing of beta-amyloid precursor-like protein-1 and -2 by gamma-secretase regulates transcription. J Biol Chem, 2002. 277(46): p. 44195-201.*
  233. Sabo, S.L., et al., *Regulation of beta-amyloid secretion by FE65, an amyloid protein precursor-binding protein. J Biol Chem, 1999. 274(12): p. 7952-7.*
  234. Ando, K., et al., *Phosphorylation-dependent regulation of the interaction of amyloid precursor protein with Fe65 affects the production of beta-amyloid. J Biol Chem, 2001. 276(43): p. 40353-61.*
  235. Cao, X. and T.C. Sudhof, *A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. Science, 2001. 293(5527): p. 115-20.*
  236. Baek, S.H., et al., *Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein. Cell, 2002. 110(1): p. 55-67.*

237. von Rotz, R.C., et al., *The APP intracellular domain forms nuclear multiprotein complexes and regulates the transcription of its own precursor*. J Cell Sci, 2004. **117**(Pt 19): p. 4435-48.
238. Pardossi-Piquard, R., et al., *Presenilin-dependent transcriptional control of the Abeta-degrading enzyme neprilysin by intracellular domains of betaAPP and APLP*. Neuron, 2005. **46**(4): p. 541-54.
239. Kim, H.S., et al., *C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3beta expression*. FASEB J, 2003. **17**(13): p. 1951-3.
240. Ryan, K.A. and S.W. Pimplikar, *Activation of GSK-3 and phosphorylation of CRMP2 in transgenic mice expressing APP intracellular domain*. J Cell Biol, 2005. **171**(2): p. 327-35.
241. Zhang, Y.W., et al., *Presenilin/gamma-secretase-dependent processing of beta-amyloid precursor protein regulates EGF receptor expression*. Proc Natl Acad Sci U S A, 2007. **104**(25): p. 10613-8.
242. Liu, Q., et al., *Amyloid precursor protein regulates brain apolipoprotein E and cholesterol metabolism through lipoprotein receptor LRP1*. Neuron, 2007. **56**(1): p. 66-78.
243. Alves da Costa, C., et al., *Presenilin-dependent gamma-secretase-mediated control of p53-associated cell death in Alzheimer's disease*. J Neurosci, 2006. **26**(23): p. 6377-85.
244. Checler, F., et al., *The gamma/epsilon-secretase-derived APP intracellular domain fragments regulate p53*. Curr Alzheimer Res, 2007. **4**(4): p. 423-6.

245. Ozaki, T., et al., *The intracellular domain of the amyloid precursor protein (AICD) enhances the p53-mediated apoptosis*. Biochem Biophys Res Commun, 2006. **351**(1): p. 57-63.
246. Tanahashi, H. and T. Tabira, *X11L2, a new member of the X11 protein family, interacts with Alzheimer's beta-amyloid precursor protein*. Biochem Biophys Res Commun, 1999. **255**(3): p. 663-7.
247. Borg, J.P., et al., *The X11alpha protein slows cellular amyloid precursor protein processing and reduces Abeta40 and Abeta42 secretion*. J Biol Chem, 1998. **273**(24): p. 14761-6.
248. Sastre, M., R.S. Turner, and E. Levy, *X11 interaction with beta-amyloid precursor protein modulates its cellular stabilization and reduces amyloid beta-protein secretion*. J Biol Chem, 1998. **273**(35): p. 22351-7.
249. Lee, J.H., et al., *The neuronal adaptor protein X11alpha reduces Abeta levels in the brains of Alzheimer's APPswe Tg2576 transgenic mice*. J Biol Chem, 2003. **278**(47): p. 47025-9.
250. Lee, J.H., et al., *The neuronal adaptor protein X11beta reduces amyloid beta-protein levels and amyloid plaque formation in the brains of transgenic mice*. J Biol Chem, 2004. **279**(47): p. 49099-104.
251. Sano, Y., et al., *Enhanced amyloidogenic metabolism of the amyloid beta-protein precursor in the X11L-deficient mouse brain*. J Biol Chem, 2006. **281**(49): p. 37853-60.
252. Saito, Y., et al., *X11 proteins regulate the translocation of amyloid beta-protein precursor (APP) into detergent-resistant membrane and suppress the amyloidogenic cleavage of APP by beta-site-cleaving enzyme in brain*. J Biol Chem, 2008. **283**(51): p. 35763-71.

253. Matsuda, S., et al., *c-Jun N-terminal kinase (JNK)-interacting protein-1b/islet-brain-1 scaffolds Alzheimer's amyloid precursor protein with JNK*. J Neurosci, 2001. **21**(17): p. 6597-607.
254. Scheinfeld, M.H., et al., *Jun NH2-terminal kinase (JNK) interacting protein 1 (JIP1) binds the cytoplasmic domain of the Alzheimer's beta-amyloid precursor protein (APP)*. J Biol Chem, 2002. **277**(5): p. 3767-75.
255. Taru, H., Y. Kirino, and T. Suzuki, *Differential roles of JIP scaffold proteins in the modulation of amyloid precursor protein metabolism*. J Biol Chem, 2002. **277**(30): p. 27567-74.
256. Inomata, H., et al., *A scaffold protein JIP-1b enhances amyloid precursor protein phosphorylation by JNK and its association with kinesin light chain 1*. J Biol Chem, 2003. **278**(25): p. 22946-55.
257. Horiuchi, D., et al., *Control of a kinesin-cargo linkage mechanism by JNK pathway kinases*. Curr Biol, 2007. **17**(15): p. 1313-7.
258. Muresan, Z. and V. Muresan, *Coordinated transport of phosphorylated amyloid-beta precursor protein and c-Jun NH2-terminal kinase-interacting protein-1*. J Cell Biol, 2005. **171**(4): p. 615-25.
259. Tarr, P.E., et al., *Tyrosine phosphorylation of the beta-amyloid precursor protein cytoplasmic tail promotes interaction with Shc*. J Biol Chem, 2002. **277**(19): p. 16798-804.
260. Xie, Z., et al., *RNA interference silencing of the adaptor molecules ShcC and Fe65 differentially affect amyloid precursor protein processing and Abeta generation*. J Biol Chem, 2007. **282**(7): p. 4318-25.
261. Russo, C., et al., *Signal transduction through tyrosine-phosphorylated C-terminal fragments of amyloid precursor protein via an enhanced interaction*

- with Shc/Grb2 adaptor proteins in reactive astrocytes of Alzheimer's disease brain.* J Biol Chem, 2002. **277**(38): p. 35282-8.
262. Zhou, D., et al., *Growth factor receptor-bound protein 2 interaction with the tyrosine-phosphorylated tail of amyloid beta precursor protein is mediated by its Src homology 2 domain.* J Biol Chem, 2004. **279**(24): p. 25374-80.
  263. Raychaudhuri, M. and D. Mukhopadhyay, *Grb2-mediated alteration in the trafficking of AbetaPP: insights from Grb2-AICD interaction.* J Alzheimers Dis, 2010. **20**(1): p. 275-92.
  264. Nizzari, M., et al., *Amyloid precursor protein and Presenilin1 interact with the adaptor GRB2 and modulate ERK 1,2 signaling.* J Biol Chem, 2007. **282**(18): p. 13833-44.
  265. Howell, B.W., et al., *The disabled 1 phosphotyrosine-binding domain binds to the internalization signals of transmembrane glycoproteins and to phospholipids.* Mol Cell Biol, 1999. **19**(7): p. 5179-88.
  266. Homayouni, R., et al., *Disabled-1 binds to the cytoplasmic domain of amyloid precursor-like protein 1.* J Neurosci, 1999. **19**(17): p. 7507-15.
  267. Hoe, H.S., et al., *Fyn modulation of Dab1 effects on amyloid precursor protein and ApoE receptor 2 processing.* J Biol Chem, 2008. **283**(10): p. 6288-99.
  268. Minami, S.S., H.S. Hoe, and G.W. Rebeck, *Fyn kinase regulates the association between amyloid precursor protein and Dab1 by promoting their localization to detergent-resistant membranes.* J Neurochem, 2011. **118**(5): p. 879-90.
  269. Roncarati, R., et al., *The gamma-secretase-generated intracellular domain of beta-amyloid precursor protein binds Numb and inhibits Notch signaling.* Proc Natl Acad Sci U S A, 2002. **99**(10): p. 7102-7.

270. Kyriazis, G.A., et al., *Numb endocytic adapter proteins regulate the transport and processing of the amyloid precursor protein in an isoform-dependent manner: implications for Alzheimer disease pathogenesis*. J Biol Chem, 2008. **283**(37): p. 25492-502.
271. Chigurupati, S., et al., *Evidence for altered Numb isoform levels in Alzheimer's disease patients and a triple transgenic mouse model*. J Alzheimers Dis, 2011. **24**(2): p. 349-61.
272. Zheng, P., et al., *PAT1, a microtubule-interacting protein, recognizes the basolateral sorting signal of amyloid precursor protein*. Proc Natl Acad Sci U S A, 1998. **95**(25): p. 14745-50.
273. Kuan, Y.H., et al., *PAT1a modulates intracellular transport and processing of amyloid precursor protein (APP), APLP1, and APLP2*. J Biol Chem, 2006. **281**(52): p. 40114-23.
274. Briand, S., et al., *PAT1 induces cell death signal and SET mislocalization into the cytoplasm by increasing APP/APLP2 at the cell surface*. Neurobiol Aging, 2011. **32**(6): p. 1099-113.
275. Smith, R.P., D.A. Higuchi, and G.J. Broze, Jr., *Platelet coagulation factor XIa-inhibitor, a form of Alzheimer amyloid precursor protein*. Science, 1990. **248**(4959): p. 1126-8.
276. Van Nostrand, W.E., et al., *Protease nexin-II (amyloid beta-protein precursor): a platelet alpha-granule protein*. Science, 1990. **248**(4956): p. 745-8.
277. Bush, A.I., et al., *The amyloid precursor protein of Alzheimer's disease is released by human platelets*. J Biol Chem, 1990. **265**(26): p. 15977-83.

278. Smith, R.P. and G.J. Broze, Jr., *Characterization of platelet-releasable forms of beta-amyloid precursor proteins: the effect of thrombin*. Blood, 1992. **80**(9): p. 2252-60.
279. Needham, B.E., et al., *Identification of the Alzheimer's disease amyloid precursor protein (APP) and its homologue APLP2 as essential modulators of glucose and insulin homeostasis and growth*. J Pathol, 2008. **215**(2): p. 155-63.
280. Lee, Y.H., et al., *Amyloid precursor protein expression is upregulated in adipocytes in obesity*. Obesity (Silver Spring), 2008. **16**(7): p. 1493-500.
281. Venkataramani, V., et al., *Histone deacetylase inhibitor valproic acid inhibits cancer cell proliferation via down-regulation of the alzheimer amyloid precursor protein*. J Biol Chem, 2010. **285**(14): p. 10678-89.
282. Tomita, S., Y. Kirino, and T. Suzuki, *Cleavage of Alzheimer's amyloid precursor protein (APP) by secretases occurs after O-glycosylation of APP in the protein secretory pathway. Identification of intracellular compartments in which APP cleavage occurs without using toxic agents that interfere with protein metabolism*. J Biol Chem, 1998. **273**(11): p. 6277-84.
283. Xu, H., P. Greengard, and S. Gandy, *Regulated formation of Golgi secretory vesicles containing Alzheimer beta-amyloid precursor protein*. J Biol Chem, 1995. **270**(40): p. 23243-5.
284. Perez, R.G., et al., *Mutagenesis identifies new signals for beta-amyloid precursor protein endocytosis, turnover, and the generation of secreted fragments, including Abeta42*. J Biol Chem, 1999. **274**(27): p. 18851-6.
285. Koo, E.H. and S.L. Squazzo, *Evidence that production and release of amyloid beta-protein involves the endocytic pathway*. J Biol Chem, 1994. **269**(26): p. 17386-9.

286. Cook, D.G., et al., *Alzheimer's A beta(1-42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells*. Nat Med, 1997. **3**(9): p. 1021-3.
287. Greenfield, J.P., et al., *Endoplasmic reticulum and trans-Golgi network generate distinct populations of Alzheimer beta-amyloid peptides*. Proc Natl Acad Sci U S A, 1999. **96**(2): p. 742-7.
288. Xu, H., et al., *Generation of Alzheimer beta-amyloid protein in the trans-Golgi network in the apparent absence of vesicle formation*. Proc Natl Acad Sci U S A, 1997. **94**(8): p. 3748-52.
289. Vetrivel, K.S., et al., *Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes*. J Biol Chem, 2004. **279**(43): p. 44945-54.
290. Kaether, C., et al., *Presenilin-1 affects trafficking and processing of betaAPP and is targeted in a complex with nicastrin to the plasma membrane*. J Cell Biol, 2002. **158**(3): p. 551-61.
291. Chyung, J.H., D.M. Raper, and D.J. Selkoe, *Gamma-secretase exists on the plasma membrane as an intact complex that accepts substrates and effects intramembrane cleavage*. J Biol Chem, 2005. **280**(6): p. 4383-92.
292. Buxbaum, J.D., et al., *Alzheimer amyloid protein precursor in the rat hippocampus: transport and processing through the perforant path*. J Neurosci, 1998. **18**(23): p. 9629-37.
293. Kamal, A., et al., *Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I*. Neuron, 2000. **28**(2): p. 449-59.
294. Lazarov, O., et al., *Axonal transport, amyloid precursor protein, kinesin-1, and the processing apparatus: revisited*. J Neurosci, 2005. **25**(9): p. 2386-95.



295. Roberts, S.B., et al., *Non-amyloidogenic cleavage of the beta-amyloid precursor protein by an integral membrane metalloendopeptidase*. J Biol Chem, 1994. **269**(4): p. 3111-6.
296. Parvathy, S., et al., *Cleavage of Alzheimer's amyloid precursor protein by alpha-secretase occurs at the surface of neuronal cells*. Biochemistry, 1999. **38**(30): p. 9728-34.
297. Jolly-Tornetta, C. and B.A. Wolf, *Protein kinase C regulation of intracellular and cell surface amyloid precursor protein (APP) cleavage in CHO695 cells*. Biochemistry, 2000. **39**(49): p. 15282-90.
298. Skovronsky, D.M., et al., *Protein kinase C-dependent alpha-secretase competes with beta-secretase for cleavage of amyloid-beta precursor protein in the trans-golgi network*. J Biol Chem, 2000. **275**(4): p. 2568-75.
299. Kuentzel, S.L., et al., *The Alzheimer beta-amyloid protein precursor/protease nexin-II is cleaved by secretase in a trans-Golgi secretory compartment in human neuroglioma cells*. Biochem J, 1993. **295** ( Pt 2): p. 367-78.
300. Black, R.A., et al., *A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells*. Nature, 1997. **385**(6618): p. 729-33.
301. Moss, M.L., et al., *Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha*. Nature, 1997. **385**(6618): p. 733-6.
302. Buxbaum, J.D., et al., *Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid protein precursor*. J Biol Chem, 1998. **273**(43): p. 27765-7.
303. Lammich, S., et al., *Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease*. Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3922-7.

304. Koike, H., et al., *Membrane-anchored metalloprotease MDC9 has an alpha-secretase activity responsible for processing the amyloid precursor protein*. Biochem J, 1999. **343 Pt 2**: p. 371-5.
305. Postina, R., et al., *A disintegrin-metalloproteinase prevents amyloid plaque formation and hippocampal defects in an Alzheimer disease mouse model*. J Clin Invest, 2004. **113**(10): p. 1456-64.
306. Weskamp, G., et al., *Mice lacking the metalloprotease-disintegrin MDC9 (ADAM9) have no evident major abnormalities during development or adult life*. Mol Cell Biol, 2002. **22**(5): p. 1537-44.
307. Kuhn, P.H., et al., *ADAM10 is the physiologically relevant, constitutive alpha-secretase of the amyloid precursor protein in primary neurons*. EMBO J, 2010. **29**(17): p. 3020-32.
308. Roher, A.E., et al., *Structural alterations in the peptide backbone of beta-amyloid core protein may account for its deposition and stability in Alzheimer's disease*. J Biol Chem, 1993. **268**(5): p. 3072-83.
309. Gouras, G.K., et al., *Generation and regulation of beta-amyloid peptide variants by neurons*. J Neurochem, 1998. **71**(5): p. 1920-5.
310. Citron, M., et al., *Inhibition of amyloid beta-protein production in neural cells by the serine protease inhibitor AEBSF*. Neuron, 1996. **17**(1): p. 171-9.
311. Lin, X., et al., *Human aspartic protease memapsin 2 cleaves the beta-secretase site of beta-amyloid precursor protein*. Proc Natl Acad Sci U S A, 2000. **97**(4): p. 1456-60.
312. Hussain, I., et al., *Identification of a novel aspartic protease (Asp 2) as beta-secretase*. Mol Cell Neurosci, 1999. **14**(6): p. 419-27.

313. Fluhner, R., et al., *A non-amyloidogenic function of BACE-2 in the secretory pathway*. J Neurochem, 2002. **81**(5): p. 1011-20.
314. Dominguez, D., et al., *Phenotypic and biochemical analyses of BACE1- and BACE2-deficient mice*. J Biol Chem, 2005. **280**(35): p. 30797-806.
315. Bennett, B.D., et al., *A furin-like convertase mediates propeptide cleavage of BACE, the Alzheimer's beta -secretase*. J Biol Chem, 2000. **275**(48): p. 37712-7.
316. Capell, A., et al., *Maturation and pro-peptide cleavage of beta-secretase*. J Biol Chem, 2000. **275**(40): p. 30849-54.
317. Knops, J., et al., *Cell-type and amyloid precursor protein-type specific inhibition of A beta release by bafilomycin A1, a selective inhibitor of vacuolar ATPases*. J Biol Chem, 1995. **270**(6): p. 2419-22.
318. Fukumoto, H., et al., *Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease*. Arch Neurol, 2002. **59**(9): p. 1381-9.
319. Yang, L.B., et al., *Elevated beta-secretase expression and enzymatic activity detected in sporadic Alzheimer disease*. Nat Med, 2003. **9**(1): p. 3-4.
320. Roberds, S.L., et al., *BACE knockout mice are healthy despite lacking the primary beta-secretase activity in brain: implications for Alzheimer's disease therapeutics*. Hum Mol Genet, 2001. **10**(12): p. 1317-24.
321. Luo, Y., et al., *Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation*. Nat Neurosci, 2001. **4**(3): p. 231-2.
322. Ohno, M., et al., *BACE1 deficiency rescues memory deficits and cholinergic dysfunction in a mouse model of Alzheimer's disease*. Neuron, 2004. **41**(1): p. 27-33.

323. Ohno, M., et al., *Temporal memory deficits in Alzheimer's mouse models: rescue by genetic deletion of BACE1*. Eur J Neurosci, 2006. **23**(1): p. 251-60.
324. Laird, F.M., et al., *BACE1, a major determinant of selective vulnerability of the brain to amyloid-beta amyloidogenesis, is essential for cognitive, emotional, and synaptic functions*. J Neurosci, 2005. **25**(50): p. 11693-709.
325. Kobayashi, D., et al., *BACE1 gene deletion: impact on behavioral function in a model of Alzheimer's disease*. Neurobiol Aging, 2008. **29**(6): p. 861-73.
326. Hu, X., et al., *Bace1 modulates myelination in the central and peripheral nervous system*. Nat Neurosci, 2006. **9**(12): p. 1520-5.
327. Luo, Y., et al., *BACE1 (beta-secretase) knockout mice do not acquire compensatory gene expression changes or develop neural lesions over time*. Neurobiol Dis, 2003. **14**(1): p. 81-8.
328. Kimberly, W.T., et al., *Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2*. Proc Natl Acad Sci U S A, 2003. **100**(11): p. 6382-7.
329. Wolfe, M.S., et al., *Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity*. Nature, 1999. **398**(6727): p. 513-7.
330. Figueroa, D.J., et al., *Presenilin-dependent gamma-secretase activity modulates neurite outgrowth*. Neurobiol Dis, 2002. **9**(1): p. 49-60.
331. Thinakaran, G., et al., *Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo*. Neuron, 1996. **17**(1): p. 181-90.
332. Podlisny, M.B., et al., *Presenilin proteins undergo heterogeneous endoproteolysis between Thr291 and Ala299 and occur as stable N- and C-*

- terminal fragments in normal and Alzheimer brain tissue.* Neurobiol Dis, 1997. **3**(4): p. 325-37.
333. Levitan, D., et al., *PSI N- and C-terminal fragments form a complex that functions in APP processing and Notch signaling.* Proc Natl Acad Sci U S A, 2001. **98**(21): p. 12186-90.
  334. Laudon, H., et al., *Co-expressed presenilin 1 NTF and CTF form functional gamma-secretase complexes in cells devoid of full-length protein.* J Neurochem, 2004. **89**(1): p. 44-53.
  335. De Strooper, B., et al., *Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein.* Nature, 1998. **391**(6665): p. 387-90.
  336. Herreman, A., et al., *Total inactivation of gamma-secretase activity in presenilin-deficient embryonic stem cells.* Nat Cell Biol, 2000. **2**(7): p. 461-2.
  337. Area-Gomez, E., et al., *Presenilins are enriched in endoplasmic reticulum membranes associated with mitochondria.* Am J Pathol, 2009. **175**(5): p. 1810-6.
  338. Shirotani, K., et al., *Gamma-secretase activity is associated with a conformational change of nicastrin.* J Biol Chem, 2003. **278**(19): p. 16474-7.
  339. Yu, G., et al., *Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing.* Nature, 2000. **407**(6800): p. 48-54.
  340. Kaether, C., et al., *The presenilin C-terminus is required for ER-retention, nicastrin-binding and gamma-secretase activity.* EMBO J, 2004. **23**(24): p. 4738-48.
  341. Shah, S., et al., *Nicastrin functions as a gamma-secretase-substrate receptor.* Cell, 2005. **122**(3): p. 435-47.

342. Francis, R., et al., *aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation*. Dev Cell, 2002. **3**(1): p. 85-97.
343. Shirotani, K., et al., *Identification of distinct gamma-secretase complexes with different APH-1 variants*. J Biol Chem, 2004. **279**(40): p. 41340-5.
344. Lee, S.F., et al., *Mammalian APH-1 interacts with presenilin and nicastrin and is required for intramembrane proteolysis of amyloid-beta precursor protein and Notch*. J Biol Chem, 2002. **277**(47): p. 45013-9.
345. Takasugi, N., et al., *The role of presenilin cofactors in the gamma-secretase complex*. Nature, 2003. **422**(6930): p. 438-41.
346. Niimura, M., et al., *Aph-1 contributes to the stabilization and trafficking of the gamma-secretase complex through mechanisms involving intermolecular and intramolecular interactions*. J Biol Chem, 2005. **280**(13): p. 12967-75.
347. Lee, S.F., et al., *A conserved GXXXG motif in APH-1 is critical for assembly and activity of the gamma-secretase complex*. J Biol Chem, 2004. **279**(6): p. 4144-52.
348. LaVoie, M.J., et al., *Assembly of the gamma-secretase complex involves early formation of an intermediate subcomplex of Aph-1 and nicastrin*. J Biol Chem, 2003. **278**(39): p. 37213-22.
349. Luo, W.J., et al., *PEN-2 and APH-1 coordinately regulate proteolytic processing of presenilin 1*. J Biol Chem, 2003. **278**(10): p. 7850-4.
350. Steiner, H., et al., *PEN-2 is an integral component of the gamma-secretase complex required for coordinated expression of presenilin and nicastrin*. J Biol Chem, 2002. **277**(42): p. 39062-5.

351. Hu, Y. and M.E. Fortini, *Different cofactor activities in gamma-secretase assembly: evidence for a nicastrin-Aph-1 subcomplex*. J Cell Biol, 2003. **161**(4): p. 685-90.
352. Ahn, K., et al., *Activation and intrinsic gamma-secretase activity of presenilin 1*. Proc Natl Acad Sci U S A, 2010. **107**(50): p. 21435-40.
353. Lannfelt, L., *Genetics of Alzheimer's disease*. Acta Neurol Scand Suppl, 1996. **168**: p. 25-7.
354. Gomez-Isla, T., et al., *Clinical and pathological correlates of apolipoprotein E epsilon 4 in Alzheimer's disease*. Ann Neurol, 1996. **39**(1): p. 62-70.
355. Cummings, J.L., et al., *Alzheimer's disease: etiologies, pathophysiology, cognitive reserve, and treatment opportunities*. Neurology, 1998. **51**(1 Suppl 1): p. S2-17; discussion S65-7.
356. Chen, J.H., K.P. Lin, and Y.C. Chen, *Risk factors for dementia*. J Formos Med Assoc, 2009. **108**(10): p. 754-64.
357. Eckman, C.B., et al., *A new pathogenic mutation in the APP gene (I716V) increases the relative proportion of A beta 42(43)*. Hum Mol Genet, 1997. **6**(12): p. 2087-9.
358. Haass, C., et al., *Mutations associated with a locus for familial Alzheimer's disease result in alternative processing of amyloid beta-protein precursor*. J Biol Chem, 1994. **269**(26): p. 17741-8.
359. Mullan, M., et al., *A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid*. Nat Genet, 1992. **1**(5): p. 345-7.

360. Citron, M., et al., *Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production*. Nature, 1992. **360**(6405): p. 672-4.
361. Cai, X.D., T.E. Golde, and S.G. Younkin, *Release of excess amyloid beta protein from a mutant amyloid beta protein precursor*. Science, 1993. **259**(5094): p. 514-6.
362. Johnston, J.A., et al., *Increased beta-amyloid release and levels of amyloid precursor protein (APP) in fibroblast cell lines from family members with the Swedish Alzheimer's disease APP670/671 mutation*. FEBS Lett, 1994. **354**(3): p. 274-8.
363. Hendriks, L., et al., *Presenile dementia and cerebral haemorrhage linked to a mutation at codon 692 of the beta-amyloid precursor protein gene*. Nat Genet, 1992. **1**(3): p. 218-21.
364. Goate, A., et al., *Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease*. Nature, 1991. **349**(6311): p. 704-6.
365. Tanzi, R.E. and B.T. Hyman, *Alzheimer's mutation*. Nature, 1991. **350**(6319): p. 564.
366. Murrell, J., et al., *A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease*. Science, 1991. **254**(5028): p. 97-9.
367. Chartier-Harlin, M.C., et al., *Early-onset Alzheimer's disease caused by mutations at codon 717 of the beta-amyloid precursor protein gene*. Nature, 1991. **353**(6347): p. 844-6.



368. Suzuki, N., et al., *An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants.* Science, 1994. **264**(5163): p. 1336-40.
369. Kwok, J.B., et al., *Novel Leu723Pro amyloid precursor protein mutation increases amyloid beta42(43) peptide levels and induces apoptosis.* Ann Neurol, 2000. **47**(2): p. 249-53.
370. Ancolio, K., et al., *Unusual phenotypic alteration of beta amyloid precursor protein (betaAPP) maturation by a new Val-715 --> Met betaAPP-770 mutation responsible for probable early-onset Alzheimer's disease.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 4119-24.
371. Schellenberg, G.D., et al., *Genetic linkage evidence for a familial Alzheimer's disease locus on chromosome 14.* Science, 1992. **258**(5082): p. 668-71.
372. Sherrington, R., et al., *Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease.* Nature, 1995. **375**(6534): p. 754-60.
373. Rogaev, E.I., et al., *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, 1995. **376**(6543): p. 775-8.
374. Levy-Lahad, E., et al., *Candidate gene for the chromosome 1 familial Alzheimer's disease locus.* Science, 1995. **269**(5226): p. 973-7.
375. Levy-Lahad, E., et al., *A familial Alzheimer's disease locus on chromosome 1.* Science, 1995. **269**(5226): p. 970-3.
376. Sherrington, R., et al., *Alzheimer's disease associated with mutations in presenilin 2 is rare and variably penetrant.* Hum Mol Genet, 1996. **5**(7): p. 985-8.

377. Cruts, M., J. Theuns, and C. Van Broeckhoven, *Locus-specific mutation databases for neurodegenerative brain diseases*. Hum Mutat, 2012. **33**(9): p. 1340-4.
378. Borchelt, D.R., et al., *Familial Alzheimer's disease-linked presenilin 1 variants elevate Abeta1-42/1-40 ratio in vitro and in vivo*. Neuron, 1996. **17**(5): p. 1005-13.
379. Citron, M., et al., *Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice*. Nat Med, 1997. **3**(1): p. 67-72.
380. Scheuner, D., et al., *Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease*. Nat Med, 1996. **2**(8): p. 864-70.
381. Song, W., et al., *Proteolytic release and nuclear translocation of Notch-1 are induced by presenilin-1 and impaired by pathogenic presenilin-1 mutations*. Proc Natl Acad Sci U S A, 1999. **96**(12): p. 6959-63.
382. Lewis, P.A., et al., *The presenilin 1 C92S mutation increases abeta 42 production*. Biochem Biophys Res Commun, 2000. **277**(1): p. 261-3.
383. Bentahir, M., et al., *Presenilin clinical mutations can affect gamma-secretase activity by different mechanisms*. J Neurochem, 2006. **96**(3): p. 732-42.
384. Okochi, M., et al., *Presenilins mediate a dual intramembranous gamma-secretase cleavage of Notch-1*. EMBO J, 2002. **21**(20): p. 5408-16.
385. Funamoto, S., et al., *Truncated carboxyl-terminal fragments of beta-amyloid precursor protein are processed to amyloid beta-proteins 40 and 42*. Biochemistry, 2004. **43**(42): p. 13532-40.

386. Sato, T., et al., *Blocking the cleavage at midportion between gamma- and epsilon-sites remarkably suppresses the generation of amyloid beta-protein.* FEBS Lett, 2005. **579**(13): p. 2907-12.
387. Qi-Takahara, Y., et al., *Longer forms of amyloid beta protein: implications for the mechanism of intramembrane cleavage by gamma-secretase.* J Neurosci, 2005. **25**(2): p. 436-45.
388. Kakuda, N., et al., *Equimolar production of amyloid beta-protein and amyloid precursor protein intracellular domain from beta-carboxyl-terminal fragment by gamma-secretase.* J Biol Chem, 2006. **281**(21): p. 14776-86.
389. Coon, K.D., et al., *A high-density whole-genome association study reveals that APOE is the major susceptibility gene for sporadic late-onset Alzheimer's disease.* J Clin Psychiatry, 2007. **68**(4): p. 613-8.
390. Tsai, M.S., et al., *Apolipoprotein E: risk factor for Alzheimer disease.* Am J Hum Genet, 1994. **54**(4): p. 643-9.
391. Lucotte, G., J.C. Turpin, and P. Landais, *Apolipoprotein E-epsilon 4 allele doses in late-onset Alzheimer's disease.* Ann Neurol, 1994. **36**(4): p. 681-2.
392. Mayeux, R., et al., *The apolipoprotein epsilon 4 allele in patients with Alzheimer's disease.* Ann Neurol, 1993. **34**(5): p. 752-4.
393. Liddell, M., et al., *Confirmation of association between the e4 allele of apolipoprotein E and Alzheimer's disease.* J Med Genet, 1994. **31**(3): p. 197-200.
394. Brousseau, T., et al., *Confirmation of the epsilon 4 allele of the apolipoprotein E gene as a risk factor for late-onset Alzheimer's disease.* Neurology, 1994. **44**(2): p. 342-4.

395. Mahley, R.W., *Apolipoprotein E: cholesterol transport protein with expanding role in cell biology*. Science, 1988. **240**(4852): p. 622-30.
396. Pitas, R.E., et al., *Lipoproteins and their receptors in the central nervous system. Characterization of the lipoproteins in cerebrospinal fluid and identification of apolipoprotein B,E(LDL) receptors in the brain*. J Biol Chem, 1987. **262**(29): p. 14352-60.
397. Uchihara, T., et al., *ApoE immunoreactivity and microglial cells in Alzheimer's disease brain*. Neurosci Lett, 1995. **195**(1): p. 5-8.
398. Han, S.H., et al., *Apolipoprotein E is localized to the cytoplasm of human cortical neurons: a light and electron microscopic study*. J Neuropathol Exp Neurol, 1994. **53**(5): p. 535-44.
399. Xu, P.T., et al., *Specific regional transcription of apolipoprotein E in human brain neurons*. Am J Pathol, 1999. **154**(2): p. 601-11.
400. Metzger, R.E., et al., *Neurons of the human frontal cortex display apolipoprotein E immunoreactivity: implications for Alzheimer's disease*. J Neuropathol Exp Neurol, 1996. **55**(3): p. 372-80.
401. Xu, P.T., et al., *Regionally specific neuronal expression of human APOE gene in transgenic mice*. Neurosci Lett, 1998. **246**(2): p. 65-8.
402. Harris, F.M., et al., *Astroglial regulation of apolipoprotein E expression in neuronal cells. Implications for Alzheimer's disease*. J Biol Chem, 2004. **279**(5): p. 3862-8.
403. Mahley, R.W., K.H. Weisgraber, and Y. Huang, *Apolipoprotein E4: a causative factor and therapeutic target in neuropathology, including Alzheimer's disease*. Proc Natl Acad Sci U S A, 2006. **103**(15): p. 5644-51.

404. Mahley, R.W. and S.C. Rall, Jr., *Apolipoprotein E: far more than a lipid transport protein*. Annu Rev Genomics Hum Genet, 2000. **1**: p. 507-37.
405. Pfrieger, F.W., *Cholesterol homeostasis and function in neurons of the central nervous system*. Cell Mol Life Sci, 2003. **60**(6): p. 1158-71.
406. Nathan, B.P., et al., *Differential effects of apolipoproteins E3 and E4 on neuronal growth in vitro*. Science, 1994. **264**(5160): p. 850-2.
407. Bellosta, S., et al., *Stable expression and secretion of apolipoproteins E3 and E4 in mouse neuroblastoma cells produces differential effects on neurite outgrowth*. J Biol Chem, 1995. **270**(45): p. 27063-71.
408. Holtzman, D.M., et al., *Low density lipoprotein receptor-related protein mediates apolipoprotein E-dependent neurite outgrowth in a central nervous system-derived neuronal cell line*. Proc Natl Acad Sci U S A, 1995. **92**(21): p. 9480-4.
409. Sun, Y., et al., *Glial fibrillary acidic protein-apolipoprotein E (apoE) transgenic mice: astrocyte-specific expression and differing biological effects of astrocyte-secreted apoE3 and apoE4 lipoproteins*. J Neurosci, 1998. **18**(9): p. 3261-72.
410. Trommer, B.L., et al., *ApoE isoform affects LTP in human targeted replacement mice*. Neuroreport, 2004. **15**(17): p. 2655-8.
411. Corder, E.H., et al., *Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families*. Science, 1993. **261**(5123): p. 921-3.
412. Saunders, A.M., et al., *Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease*. Neurology, 1993. **43**(8): p. 1467-72.

413. Roses, A.D., *Apolipoprotein E alleles as risk factors in Alzheimer's disease*. Annu Rev Med, 1996. **47**: p. 387-400.
414. Bertram, L., et al., *Systematic meta-analyses of Alzheimer disease genetic association studies: the AlzGene database*. Nat Genet, 2007. **39**(1): p. 17-23.
415. West, H.L., G.W. Rebeck, and B.T. Hyman, *Frequency of the apolipoprotein E epsilon 2 allele is diminished in sporadic Alzheimer disease*. Neurosci Lett, 1994. **175**(1-2): p. 46-8.
416. Tiraboschi, P., et al., *Impact of APOE genotype on neuropathologic and neurochemical markers of Alzheimer disease*. Neurology, 2004. **62**(11): p. 1977-83.
417. Corder, E.H., et al., *Apolipoprotein E, survival in Alzheimer's disease patients, and the competing risks of death and Alzheimer's disease*. Neurology, 1995. **45**(7): p. 1323-8.
418. Meyer, M.R., et al., *APOE genotype predicts when--not whether--one is predisposed to develop Alzheimer disease*. Nat Genet, 1998. **19**(4): p. 321-2.
419. Farrer, L.A., et al., *Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium*. JAMA, 1997. **278**(16): p. 1349-56.
420. Tang, M.X., et al., *The APOE-epsilon4 allele and the risk of Alzheimer disease among African Americans, whites, and Hispanics*. JAMA, 1998. **279**(10): p. 751-5.
421. Murphy, G.M., Jr., et al., *No association between apolipoprotein E epsilon 4 allele and rate of decline in Alzheimer's disease*. Am J Psychiatry, 1997. **154**(5): p. 603-8.

422. Craft, S., et al., *Accelerated decline in apolipoprotein E-epsilon4 homozygotes with Alzheimer's disease*. Neurology, 1998. **51**(1): p. 149-53.
423. Growdon, J.H., et al., *Apolipoprotein E genotype does not influence rates of cognitive decline in Alzheimer's disease*. Neurology, 1996. **47**(2): p. 444-8.
424. Kurz, A., et al., *Apolipoprotein E epsilon 4 allele, cognitive decline, and deterioration of everyday performance in Alzheimer's disease*. Neurology, 1996. **47**(2): p. 440-3.
425. Wisniewski, T. and B. Frangione, *Apolipoprotein E: a pathological chaperone protein in patients with cerebral and systemic amyloid*. Neurosci Lett, 1992. **135**(2): p. 235-8.
426. Rebeck, G.W., et al., *Apolipoprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions*. Neuron, 1993. **11**(4): p. 575-80.
427. Schmechel, D.E., et al., *Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease*. Proc Natl Acad Sci U S A, 1993. **90**(20): p. 9649-53.
428. Reiman, E.M., et al., *Fibrillar amyloid-beta burden in cognitively normal people at 3 levels of genetic risk for Alzheimer's disease*. Proc Natl Acad Sci U S A, 2009. **106**(16): p. 6820-5.
429. Benjamin, R., et al., *Effects of apolipoprotein E genotype on cortical neuropathology in senile dementia of the Lewy body and Alzheimer's disease*. Neurodegeneration, 1995. **4**(4): p. 443-8.
430. Heinonen, O., et al., *Alzheimer pathology of patients carrying apolipoprotein E epsilon 4 allele*. Neurobiol Aging, 1995. **16**(4): p. 505-13.

431. Landen, M., et al., *The apolipoprotein E allele epsilon 4 does not correlate with the number of senile plaques or neurofibrillary tangles in patients with Alzheimer's disease*. J Neurol Neurosurg Psychiatry, 1996. **61**(4): p. 352-6.
432. Berlau, D.J., et al., *APOE epsilon2 is associated with intact cognition but increased Alzheimer pathology in the oldest old*. Neurology, 2009. **72**(9): p. 829-34.
433. Bales, K.R., et al., *Lack of apolipoprotein E dramatically reduces amyloid beta-peptide deposition*. Nat Genet, 1997. **17**(3): p. 263-4.
434. Bales, K.R., et al., *Apolipoprotein E is essential for amyloid deposition in the APP(V717F) transgenic mouse model of Alzheimer's disease*. Proc Natl Acad Sci U S A, 1999. **96**(26): p. 15233-8.
435. Fryer, J.D., et al., *Apolipoprotein E markedly facilitates age-dependent cerebral amyloid angiopathy and spontaneous hemorrhage in amyloid precursor protein transgenic mice*. J Neurosci, 2003. **23**(21): p. 7889-96.
436. Irizarry, M.C., et al., *Apolipoprotein E affects the amount, form, and anatomical distribution of amyloid beta-peptide deposition in homozygous APP(V717F) transgenic mice*. Acta Neuropathol, 2000. **100**(5): p. 451-8.
437. Holtzman, D.M., et al., *Apolipoprotein E isoform-dependent amyloid deposition and neuritic degeneration in a mouse model of Alzheimer's disease*. Proc Natl Acad Sci U S A, 2000. **97**(6): p. 2892-7.
438. Fagan, A.M., et al., *Human and murine ApoE markedly alters A beta metabolism before and after plaque formation in a mouse model of Alzheimer's disease*. Neurobiol Dis, 2002. **9**(3): p. 305-18.



439. Sanan, D.A., et al., *Apolipoprotein E associates with beta amyloid peptide of Alzheimer's disease to form novel monofibrils. Isoform apoE4 associates more efficiently than apoE3.* J Clin Invest, 1994. **94**(2): p. 860-9.
440. Strittmatter, W.J., et al., *Binding of human apolipoprotein E to synthetic amyloid beta peptide: isoform-specific effects and implications for late-onset Alzheimer disease.* Proc Natl Acad Sci U S A, 1993. **90**(17): p. 8098-102.
441. Strittmatter, W.J., et al., *Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease.* Proc Natl Acad Sci U S A, 1993. **90**(5): p. 1977-81.
442. LaDu, M.J., et al., *Isoform-specific binding of apolipoprotein E to beta-amyloid.* J Biol Chem, 1994. **269**(38): p. 23403-6.
443. Yang, D.S., et al., *Characterization of the binding of amyloid-beta peptide to cell culture-derived native apolipoprotein E2, E3, and E4 isoforms and to isoforms from human plasma.* J Neurochem, 1997. **68**(2): p. 721-5.
444. Aleshkov, S., C.R. Abraham, and V.I. Zannis, *Interaction of nascent ApoE2, ApoE3, and ApoE4 isoforms expressed in mammalian cells with amyloid peptide beta (1-40). Relevance to Alzheimer's disease.* Biochemistry, 1997. **36**(34): p. 10571-80.
445. Tokuda, T., et al., *Lipidation of apolipoprotein E influences its isoform-specific interaction with Alzheimer's amyloid beta peptides.* Biochem J, 2000. **348 Pt 2**: p. 359-65.
446. Ma, J., et al., *Amyloid-associated proteins alpha 1-antichymotrypsin and apolipoprotein E promote assembly of Alzheimer beta-protein into filaments.* Nature, 1994. **372**(6501): p. 92-4.

447. Wisniewski, T., et al., *Acceleration of Alzheimer's fibril formation by apolipoprotein E in vitro*. Am J Pathol, 1994. **145**(5): p. 1030-5.
448. Castano, E.M., et al., *Fibrillogenesis in Alzheimer's disease of amyloid beta peptides and apolipoprotein E*. Biochem J, 1995. **306** ( Pt 2): p. 599-604.
449. Beffert, U. and J. Poirier, *ApoE associated with lipid has a reduced capacity to inhibit beta-amyloid fibril formation*. Neuroreport, 1998. **9**(14): p. 3321-3.
450. Evans, K.C., et al., *Apolipoprotein E is a kinetic but not a thermodynamic inhibitor of amyloid formation: implications for the pathogenesis and treatment of Alzheimer disease*. Proc Natl Acad Sci U S A, 1995. **92**(3): p. 763-7.
451. Wood, S.J., W. Chan, and R. Wetzel, *Seeding of A beta fibril formation is inhibited by all three isotypes of apolipoprotein E*. Biochemistry, 1996. **35**(38): p. 12623-8.
452. Webster, S. and J. Rogers, *Relative efficacies of amyloid beta peptide (A beta) binding proteins in A beta aggregation*. J Neurosci Res, 1996. **46**(1): p. 58-66.
453. Hirsch-Reinshagen, V., et al., *The absence of ABCA1 decreases soluble ApoE levels but does not diminish amyloid deposition in two murine models of Alzheimer disease*. J Biol Chem, 2005. **280**(52): p. 43243-56.
454. Wahrle, S.E., et al., *Deletion of Abca1 increases Abeta deposition in the PDAPP transgenic mouse model of Alzheimer disease*. J Biol Chem, 2005. **280**(52): p. 43236-42.
455. Koldamova, R., M. Staufenbiel, and I. Lefterov, *Lack of ABCA1 considerably decreases brain ApoE level and increases amyloid deposition in APP23 mice*. J Biol Chem, 2005. **280**(52): p. 43224-35.

456. Wahrle, S.E., et al., *Overexpression of ABCA1 reduces amyloid deposition in the PDAPP mouse model of Alzheimer disease*. J Clin Invest, 2008. **118**(2): p. 671-82.
457. Mawuenyega, K.G., et al., *Decreased clearance of CNS beta-amyloid in Alzheimer's disease*. Science, 2010. **330**(6012): p. 1774.
458. Deane, R., et al., *apoE isoform-specific disruption of amyloid beta peptide clearance from mouse brain*. J Clin Invest, 2008. **118**(12): p. 4002-13.
459. Beffert, U., et al., *Beta-amyloid peptides increase the binding and internalization of apolipoprotein E to hippocampal neurons*. J Neurochem, 1998. **70**(4): p. 1458-66.
460. Yang, D.S., et al., *Apolipoprotein E promotes the binding and uptake of beta-amyloid into Chinese hamster ovary cells in an isoform-specific manner*. Neuroscience, 1999. **90**(4): p. 1217-26.
461. Cole, G.M. and M.D. Ard, *Influence of lipoproteins on microglial degradation of Alzheimer's amyloid beta-protein*. Microsc Res Tech, 2000. **50**(4): p. 316-24.
462. Yamauchi, K., et al., *Isoform-specific effect of apolipoprotein E on endocytosis of beta-amyloid in cultures of neuroblastoma cells*. Ann Clin Lab Sci, 2002. **32**(1): p. 65-74.
463. Jiang, Q., et al., *ApoE promotes the proteolytic degradation of Abeta*. Neuron, 2008. **58**(5): p. 681-93.
464. Bell, R.D., et al., *Transport pathways for clearance of human Alzheimer's amyloid beta-peptide and apolipoproteins E and J in the mouse central nervous system*. J Cereb Blood Flow Metab, 2007. **27**(5): p. 909-18.

465. Ito, S., et al., *Cerebral clearance of human amyloid-beta peptide (1-40) across the blood-brain barrier is reduced by self-aggregation and formation of low-density lipoprotein receptor-related protein-1 ligand complexes*. J Neurochem, 2007. **103**(6): p. 2482-90.
466. Ji, Y., et al., *Amyloid beta40/42 clearance across the blood-brain barrier following intra-ventricular injections in wild-type, apoE knock-out and human apoE3 or E4 expressing transgenic mice*. J Alzheimers Dis, 2001. **3**(1): p. 23-30.
467. Ye, S., et al., *Apolipoprotein (apo) E4 enhances amyloid beta peptide production in cultured neuronal cells: apoE structure as a potential therapeutic target*. Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18700-5.
468. He, X., et al., *Apolipoprotein receptor 2 and X11 alpha/beta mediate apolipoprotein E-induced endocytosis of amyloid-beta precursor protein and beta-secretase, leading to amyloid-beta production*. J Neurosci, 2007. **27**(15): p. 4052-60.
469. Biere, A.L., et al., *Co-expression of beta-amyloid precursor protein (betaAPP) and apolipoprotein E in cell culture: analysis of betaAPP processing*. Neurobiol Dis, 1995. **2**(3): p. 177-87.
470. Irizarry, M.C., et al., *Apolipoprotein E modulates gamma-secretase cleavage of the amyloid precursor protein*. J Neurochem, 2004. **90**(5): p. 1132-43.
471. Harris, F.M., et al., *Carboxyl-terminal-truncated apolipoprotein E4 causes Alzheimer's disease-like neurodegeneration and behavioral deficits in transgenic mice*. Proc Natl Acad Sci U S A, 2003. **100**(19): p. 10966-71.

472. Chang, S., et al., *Lipid- and receptor-binding regions of apolipoprotein E4 fragments act in concert to cause mitochondrial dysfunction and neurotoxicity.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18694-9.
473. Tolar, M., et al., *Truncated apolipoprotein E (ApoE) causes increased intracellular calcium and may mediate ApoE neurotoxicity.* J Neurosci, 1999. **19**(16): p. 7100-10.
474. Brecht, W.J., et al., *Neuron-specific apolipoprotein e4 proteolysis is associated with increased tau phosphorylation in brains of transgenic mice.* J Neurosci, 2004. **24**(10): p. 2527-34.
475. Drzezga, A., et al., *Cerebral glucose metabolism in patients with AD and different APOE genotypes.* Neurology, 2005. **64**(1): p. 102-7.
476. LaDu, M.J., et al., *Apolipoprotein E and apolipoprotein E receptors modulate A beta-induced glial neuroinflammatory responses.* Neurochem Int, 2001. **39**(5-6): p. 427-34.
477. Guo, L., M.J. LaDu, and L.J. Van Eldik, *A dual role for apolipoprotein e in neuroinflammation: anti- and pro-inflammatory activity.* J Mol Neurosci, 2004. **23**(3): p. 205-12.
478. May, P.C., et al., *Dynamics of gene expression for a hippocampal glycoprotein elevated in Alzheimer's disease and in response to experimental lesions in rat.* Neuron, 1990. **5**(6): p. 831-9.
479. Wong, P., et al., *Molecular characterization of human TRPM-2/clusterin, a gene associated with sperm maturation, apoptosis and neurodegeneration.* Eur J Biochem, 1994. **221**(3): p. 917-25.

480. Kirschbaum, L., S.E. Bozas, and I.D. Walker, *SP-40,40, a protein involved in the control of the complement pathway, possesses a unique array of disulphide bridges*. FEBS Lett, 1992. **297**(1-2): p. 70-6.
481. de Silva, H.V., et al., *Apolipoprotein J: structure and tissue distribution*. Biochemistry, 1990. **29**(22): p. 5380-9.
482. Pasinetti, G.M., et al., *Clusterin (SGP-2): a multifunctional glycoprotein with regional expression in astrocytes and neurons of the adult rat brain*. J Comp Neurol, 1994. **339**(3): p. 387-400.
483. May, P.C. and C.E. Finch, *Sulfated glycoprotein 2: new relationships of this multifunctional protein to neurodegeneration*. Trends Neurosci, 1992. **15**(10): p. 391-6.
484. Michel, D., et al., *Stress-induced transcription of the clusterin/apoJ gene*. Biochem J, 1997. **328** ( Pt 1): p. 45-50.
485. Loison, F., et al., *Up-regulation of the clusterin gene after proteotoxic stress: implication of HSF1-HSF2 heterocomplexes*. Biochem J, 2006. **395**(1): p. 223-31.
486. Bertrand, P., et al., *Association of apolipoprotein E genotype with brain levels of apolipoprotein E and apolipoprotein J (clusterin) in Alzheimer disease*. Brain Res Mol Brain Res, 1995. **33**(1): p. 174-8.
487. Giannakopoulos, P., et al., *Possible neuroprotective role of clusterin in Alzheimer's disease: a quantitative immunocytochemical study*. Acta Neuropathol, 1998. **95**(4): p. 387-94.
488. Lidstrom, A.M., et al., *Clusterin (apolipoprotein J) protein levels are increased in hippocampus and in frontal cortex in Alzheimer's disease*. Exp Neurol, 1998. **154**(2): p. 511-21.

489. Kida, E., N.H. Choi-Miura, and K.E. Wisniewski, *Deposition of apolipoproteins E and J in senile plaques is topographically determined in both Alzheimer's disease and Down's syndrome brain*. Brain Res, 1995. **685**(1-2): p. 211-6.
490. McGeer, P.L., T. Kawamata, and D.G. Walker, *Distribution of clusterin in Alzheimer brain tissue*. Brain Res, 1992. **579**(2): p. 337-41.
491. Nilselid, A.M., et al., *Clusterin in cerebrospinal fluid: analysis of carbohydrates and quantification of native and glycosylated forms*. Neurochem Int, 2006. **48**(8): p. 718-28.
492. Sihlbom, C., et al., *Structural and quantitative comparison of cerebrospinal fluid glycoproteins in Alzheimer's disease patients and healthy individuals*. Neurochem Res, 2008. **33**(7): p. 1332-40.
493. Thambisetty, M., et al., *Association of plasma clusterin concentration with severity, pathology, and progression in Alzheimer disease*. Arch Gen Psychiatry, 2010. **67**(7): p. 739-48.
494. Schrijvers, E.M., et al., *Plasma clusterin and the risk of Alzheimer disease*. JAMA, 2011. **305**(13): p. 1322-6.
495. Xing, Y.Y., et al., *Blood Clusterin Levels, rs9331888 Polymorphism, and the Risk of Alzheimer's Disease*. J Alzheimers Dis, 2012. **29**(3): p. 515-9.
496. Matsubara, E., B. Frangione, and J. Ghiso, *Characterization of apolipoprotein J-Alzheimer's A beta interaction*. J Biol Chem, 1995. **270**(13): p. 7563-7.
497. Ghiso, J., et al., *The cerebrospinal-fluid soluble form of Alzheimer's amyloid beta is complexed to SP-40,40 (apolipoprotein J), an inhibitor of the complement membrane-attack complex*. Biochem J, 1993. **293** ( Pt 1): p. 27-30.

498. Oda, T., et al., *Purification and characterization of brain clusterin*. Biochem Biophys Res Commun, 1994. **204**(3): p. 1131-6.
499. Oda, T., et al., *Clusterin (apoJ) alters the aggregation of amyloid beta-peptide (A beta 1-42) and forms slowly sedimenting A beta complexes that cause oxidative stress*. Exp Neurol, 1995. **136**(1): p. 22-31.
500. Wilson, M.R., J.J. Yerbury, and S. Poon, *Potential roles of abundant extracellular chaperones in the control of amyloid formation and toxicity*. Mol Biosyst, 2008. **4**(1): p. 42-52.
501. Acheson, A.G., et al., *L-arginine-induced relaxation of the internal anal sphincter is not mediated by nitric oxide*. Br J Surg, 2003. **90**(9): p. 1155-62.
502. DeMattos, R.B., et al., *Clusterin promotes amyloid plaque formation and is critical for neuritic toxicity in a mouse model of Alzheimer's disease*. Proc Natl Acad Sci U S A, 2002. **99**(16): p. 10843-8.
503. Nuutinen, T., et al., *Amyloid-beta 1-42 induced endocytosis and clusterin/apoJ protein accumulation in cultured human astrocytes*. Neurochem Int, 2007. **50**(3): p. 540-7.
504. Zlokovic, B.V., et al., *Glycoprotein 330/megalin: probable role in receptor-mediated transport of apolipoprotein J alone and in a complex with Alzheimer disease amyloid beta at the blood-brain and blood-cerebrospinal fluid barriers*. Proc Natl Acad Sci U S A, 1996. **93**(9): p. 4229-34.
505. Negorev, D., et al., *The Bin1 gene localizes to human chromosome 2q14 by PCR analysis of somatic cell hybrids and fluorescence in situ hybridization*. Genomics, 1996. **33**(2): p. 329-31.



506. Wechsler-Reya, R., et al., *Structural analysis of the human BIN1 gene. Evidence for tissue-specific transcriptional regulation and alternate RNA splicing*. J Biol Chem, 1997. **272**(50): p. 31453-8.
507. Wechsler-Reya, R., et al., *The putative tumor suppressor BIN1 is a short-lived nuclear phosphoprotein, the localization of which is altered in malignant cells*. Cancer Res, 1997. **57**(15): p. 3258-63.
508. Sakamuro, D., et al., *BIN1 is a novel MYC-interacting protein with features of a tumour suppressor*. Nat Genet, 1996. **14**(1): p. 69-77.
509. Wigge, P., et al., *Amphiphysin heterodimers: potential role in clathrin-mediated endocytosis*. Mol Biol Cell, 1997. **8**(10): p. 2003-15.
510. Pant, S., et al., *AMPH-1/Amphiphysin/Bin1 functions with RME-1/Ehd1 in endocytic recycling*. Nat Cell Biol, 2009. **11**(12): p. 1399-410.
511. Nicot, A.S., et al., *Mutations in amphiphysin 2 (BIN1) disrupt interaction with dynamin 2 and cause autosomal recessive centronuclear myopathy*. Nat Genet, 2007. **39**(9): p. 1134-9.
512. Zelhof, A.C., et al., *Drosophila Amphiphysin is implicated in protein localization and membrane morphogenesis but not in synaptic vesicle endocytosis*. Development, 2001. **128**(24): p. 5005-15.
513. Harold, D., et al., *Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease*. Nat Genet, 2009. **41**(10): p. 1088-93.
514. Seshadri, S., et al., *Genome-wide analysis of genetic loci associated with Alzheimer disease*. JAMA, 2010. **303**(18): p. 1832-40.

515. Carrasquillo, M.M., et al., *Replication of BIN1 association with Alzheimer's disease and evaluation of genetic interactions*. J Alzheimers Dis, 2011. **24**(4): p. 751-8.
516. Hu, X., et al., *Meta-analysis for genome-wide association study identifies multiple variants at the BIN1 locus associated with late-onset Alzheimer's disease*. PLoS One, 2011. **6**(2): p. e16616.
517. Wijsman, E.M., et al., *Genome-wide association of familial late-onset Alzheimer's disease replicates BIN1 and CLU and nominates CUGBP2 in interaction with APOE*. PLoS Genet, 2011. **7**(2): p. e1001308.
518. Lambert, J.C., et al., *Evidence of the association of BIN1 and PICALM with the AD risk in contrasting European populations*. Neurobiol Aging, 2011. **32**(4): p. 756 e11-5.
519. Lee, J.H., et al., *Identification of novel loci for Alzheimer disease and replication of CLU, PICALM, and BIN1 in Caribbean Hispanic individuals*. Arch Neurol, 2011. **68**(3): p. 320-8.
520. Kauwe, J.S., et al., *Fine mapping of genetic variants in BIN1, CLU, CR1 and PICALM for association with cerebrospinal fluid biomarkers for Alzheimer's disease*. PLoS One, 2011. **6**(2): p. e15918.
521. Kelly, B.L. and A. Ferreira, *Beta-amyloid disrupted synaptic vesicle endocytosis in cultured hippocampal neurons*. Neuroscience, 2007. **147**(1): p. 60-70.
522. Kaminski, W.E., et al., *Identification of a novel human sterol-sensitive ATP-binding cassette transporter (ABCA7)*. Biochem Biophys Res Commun, 2000. **273**(2): p. 532-8.

523. Ikeda, Y., et al., *Posttranscriptional regulation of human ABCA7 and its function for the apoA-I-dependent lipid release*. Biochem Biophys Res Commun, 2003. **311**(2): p. 313-8.
524. Kim, W.S., et al., *Abca7 null mice retain normal macrophage phosphatidylcholine and cholesterol efflux activity despite alterations in adipose mass and serum cholesterol levels*. J Biol Chem, 2005. **280**(5): p. 3989-95.
525. Kim, W.S., et al., *Quantitation of ATP-binding cassette subfamily-A transporter gene expression in primary human brain cells*. Neuroreport, 2006. **17**(9): p. 891-6.
526. Abe-Dohmae, S., et al., *Human ABCA7 supports apolipoprotein-mediated release of cellular cholesterol and phospholipid to generate high density lipoprotein*. J Biol Chem, 2004. **279**(1): p. 604-11.
527. Hayashi, M., et al., *Heterogeneity of high density lipoprotein generated by ABCA1 and ABCA7*. J Lipid Res, 2005. **46**(8): p. 1703-11.
528. Wang, N., et al., *ATP-binding cassette transporter A7 (ABCA7) binds apolipoprotein A-I and mediates cellular phospholipid but not cholesterol efflux*. J Biol Chem, 2003. **278**(44): p. 42906-12.
529. Linsel-Nitschke, P., et al., *Potential role of ABCA7 in cellular lipid efflux to apoA-I*. J Lipid Res, 2005. **46**(1): p. 86-92.
530. Jehle, A.W., et al., *ATP-binding cassette transporter A7 enhances phagocytosis of apoptotic cells and associated ERK signaling in macrophages*. J Cell Biol, 2006. **174**(4): p. 547-56.

531. Iwamoto, N., et al., *ABCA7 expression is regulated by cellular cholesterol through the SREBP2 pathway and associated with phagocytosis*. J Lipid Res, 2006. **47**(9): p. 1915-27.
532. Streit, W.J., *Microglia and Alzheimer's disease pathogenesis*. J Neurosci Res, 2004. **77**(1): p. 1-8.
533. Flanary, B.E. and W.J. Streit, *Progressive telomere shortening occurs in cultured rat microglia, but not astrocytes*. Glia, 2004. **45**(1): p. 75-88.
534. Naj, A.C., et al., *Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease*. Nat Genet, 2011. **43**(5): p. 436-41.
535. Logue, M.W., et al., *A comprehensive genetic association study of Alzheimer disease in African Americans*. Arch Neurol, 2011. **68**(12): p. 1569-79.
536. Duyckaerts, C., M.C. Potier, and B. Delatour, *Alzheimer disease models and human neuropathology: similarities and differences*. Acta Neuropathol, 2008. **115**(1): p. 5-38.
537. Kokjohn, T.A. and A.E. Roher, *Amyloid precursor protein transgenic mouse models and Alzheimer's disease: understanding the paradigms, limitations, and contributions*. Alzheimers Dement, 2009. **5**(4): p. 340-7.
538. Eriksen, J.L. and C.G. Janus, *Plaques, tangles, and memory loss in mouse models of neurodegeneration*. Behav Genet, 2007. **37**(1): p. 79-100.
539. Games, D., et al., *Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein*. Nature, 1995. **373**(6514): p. 523-7.

540. Masliah, E., et al., *Comparison of neurodegenerative pathology in transgenic mice overexpressing V717F beta-amyloid precursor protein and Alzheimer's disease*. J Neurosci, 1996. **16**(18): p. 5795-811.
541. Dodart, J.C., et al., *Behavioral disturbances in transgenic mice overexpressing the V717F beta-amyloid precursor protein*. Behav Neurosci, 1999. **113**(5): p. 982-90.
542. Chen, G., et al., *A learning deficit related to age and beta-amyloid plaques in a mouse model of Alzheimer's disease*. Nature, 2000. **408**(6815): p. 975-9.
543. Huitron-Resendiz, S., et al., *Age-independent and age-related deficits in visuospatial learning, sleep-wake states, thermoregulation and motor activity in PDAPP mice*. Brain Res, 2002. **928**(1-2): p. 126-37.
544. Ashe, K.H. and K.R. Zahs, *Probing the biology of Alzheimer's disease in mice*. Neuron, 2010. **66**(5): p. 631-45.
545. Hsiao, K., et al., *Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice*. Science, 1996. **274**(5284): p. 99-102.
546. Kawarabayashi, T., et al., *Age-dependent changes in brain, CSF, and plasma amyloid (beta) protein in the Tg2576 transgenic mouse model of Alzheimer's disease*. J Neurosci, 2001. **21**(2): p. 372-81.
547. Frautschy, S.A., et al., *Microglial response to amyloid plaques in APP<sup>sw</sup> transgenic mice*. Am J Pathol, 1998. **152**(1): p. 307-17.
548. Smith, M.A., et al., *Amyloid-beta deposition in Alzheimer transgenic mice is associated with oxidative stress*. J Neurochem, 1998. **70**(5): p. 2212-5.
549. Pappolla, M.A., et al., *Evidence of oxidative stress and in vivo neurotoxicity of beta-amyloid in a transgenic mouse model of Alzheimer's disease: a chronic*

- oxidative paradigm for testing antioxidant therapies in vivo*. Am J Pathol, 1998. **152**(4): p. 871-7.
550. Irizarry, M.C., et al., *APP<sup>Sw</sup> transgenic mice develop age-related A beta deposits and neuropil abnormalities, but no neuronal loss in CA1*. J Neuropathol Exp Neurol, 1997. **56**(9): p. 965-73.
  551. Duff, K., et al., *Increased amyloid-beta<sub>42</sub>(43) in brains of mice expressing mutant presenilin 1*. Nature, 1996. **383**(6602): p. 710-3.
  552. Holcomb, L., et al., *Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes*. Nat Med, 1998. **4**(1): p. 97-100.
  553. McGowan, E., et al., *Amyloid phenotype characterization of transgenic mice overexpressing both mutant amyloid precursor protein and mutant presenilin 1 transgenes*. Neurobiol Dis, 1999. **6**(4): p. 231-44.
  554. Radde, R., et al., *Abeta<sub>42</sub>-driven cerebral amyloidosis in transgenic mice reveals early and robust pathology*. EMBO Rep, 2006. **7**(9): p. 940-6.
  555. Arendash, G.W., et al., *Behavioral assessment of Alzheimer's transgenic mice following long-term Abeta vaccination: task specificity and correlations between Abeta deposition and spatial memory*. DNA Cell Biol, 2001. **20**(11): p. 737-44.
  556. Gordon, M.N., et al., *Correlation between cognitive deficits and Abeta deposits in transgenic APP+PS1 mice*. Neurobiol Aging, 2001. **22**(3): p. 377-85.
  557. Lewis, J., et al., *Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein*. Nat Genet, 2000. **25**(4): p. 402-5.

558. Santacruz, K., et al., *Tau suppression in a neurodegenerative mouse model improves memory function*. Science, 2005. **309**(5733): p. 476-81.
559. Lewis, J., et al., *Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP*. Science, 2001. **293**(5534): p. 1487-91.
560. Ribe, E.M., et al., *Accelerated amyloid deposition, neurofibrillary degeneration and neuronal loss in double mutant APP/tau transgenic mice*. Neurobiol Dis, 2005. **20**(3): p. 814-22.
561. Oddo, S., et al., *Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease*. Neurobiol Aging, 2003. **24**(8): p. 1063-70.
562. McGowan, E., et al., *Abeta42 is essential for parenchymal and vascular amyloid deposition in mice*. Neuron, 2005. **47**(2): p. 191-9.
563. Yankner, B.A., et al., *Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease*. Science, 1989. **245**(4916): p. 417-20.
564. Fukuchi, K., et al., *Overexpression of amyloid precursor protein alters its normal processing and is associated with neurotoxicity*. Biochem Biophys Res Commun, 1992. **182**(1): p. 165-73.
565. Neve, R.L., et al., *Transgenic mice expressing APP-C100 in the brain*. Neurobiol Aging, 1996. **17**(2): p. 191-203.
566. Fukuchi, K., et al., *High levels of circulating beta-amyloid peptide do not cause cerebral beta-amyloidosis in transgenic mice*. Am J Pathol, 1996. **149**(1): p. 219-27.
567. Ring, S., et al., *The secreted beta-amyloid precursor protein ectodomain APPs alpha is sufficient to rescue the anatomical, behavioral, and*

- electrophysiological abnormalities of APP-deficient mice.* J Neurosci, 2007. **27**(29): p. 7817-26.
568. Weyer, S.W., et al., *APP and APLP2 are essential at PNS and CNS synapses for transmission, spatial learning and LTP.* EMBO J, 2011. **30**(11): p. 2266-80.
  569. Li, H., et al., *Soluble amyloid precursor protein (APP) regulates transthyretin and Klotho gene expression without rescuing the essential function of APP.* Proc Natl Acad Sci U S A, 2010. **107**(40): p. 17362-7.
  570. Zheng, H., et al., *beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity.* Cell, 1995. **81**(4): p. 525-31.
  571. Magara, F., et al., *Genetic background changes the pattern of forebrain commissure defects in transgenic mice underexpressing the beta-amyloid-precursor protein.* Proc Natl Acad Sci U S A, 1999. **96**(8): p. 4656-61.
  572. Muller, U., et al., *Behavioral and anatomical deficits in mice homozygous for a modified beta-amyloid precursor protein gene.* Cell, 1994. **79**(5): p. 755-65.
  573. Freude, K.K., et al., *Soluble amyloid precursor protein induces rapid neural differentiation of human embryonic stem cells.* J Biol Chem, 2011. **286**(27): p. 24264-74.
  574. White, A.R., et al., *Copper levels are increased in the cerebral cortex and liver of APP and APLP2 knockout mice.* Brain Res, 1999. **842**(2): p. 439-44.
  575. Grimm, M.O., et al., *Regulation of cholesterol and sphingomyelin metabolism by amyloid-beta and presenilin.* Nat Cell Biol, 2005. **7**(11): p. 1118-23.
  576. Steinbach, J.P., et al., *Hypersensitivity to seizures in beta-amyloid precursor protein deficient mice.* Cell Death Differ, 1998. **5**(10): p. 858-66.



577. Phinney, A.L., et al., *No hippocampal neuron or synaptic bouton loss in learning-impaired aged beta-amyloid precursor protein-null mice.* Neuroscience, 1999. **90**(4): p. 1207-16.
578. Seabrook, G.R., et al., *Mechanisms contributing to the deficits in hippocampal synaptic plasticity in mice lacking amyloid precursor protein.* Neuropharmacology, 1999. **38**(3): p. 349-59.
579. Dawson, G.R., et al., *Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein.* Neuroscience, 1999. **90**(1): p. 1-13.
580. von Koch, C.S., et al., *Generation of APLP2 KO mice and early postnatal lethality in APLP2/APP double KO mice.* Neurobiol Aging, 1997. **18**(6): p. 661-9.
581. Heber, S., et al., *Mice with combined gene knock-outs reveal essential and partially redundant functions of amyloid precursor protein family members.* J Neurosci, 2000. **20**(21): p. 7951-63.
582. Klafki, H.W., et al., *Therapeutic approaches to Alzheimer's disease.* Brain, 2006. **129**(Pt 11): p. 2840-55.
583. Perry, E.K., et al., *Necropsy evidence of central cholinergic deficits in senile dementia.* Lancet, 1977. **1**(8004): p. 189.
584. Davies, P. and A.J. Maloney, *Selective loss of central cholinergic neurons in Alzheimer's disease.* Lancet, 1976. **2**(8000): p. 1403.
585. Kar, S., et al., *Interactions between beta-amyloid and central cholinergic neurons: implications for Alzheimer's disease.* J Psychiatry Neurosci, 2004. **29**(6): p. 427-41.

586. Bartus, R.T., et al., *The cholinergic hypothesis of geriatric memory dysfunction*. Science, 1982. **217**(4558): p. 408-14.
587. Frolkis, V.V., et al., *Acetylcholine metabolism and cholinergic regulation of functions in aging*. Gerontologia, 1973. **19**(1): p. 45-57.
588. Aisen, P.S., J. Cummings, and L.S. Schneider, *Symptomatic and nonamyloid/tau based pharmacologic treatment for Alzheimer disease*. Cold Spring Harb Perspect Med, 2012. **2**(3): p. a006395.
589. Tariot, P.N., et al., *A 5-month, randomized, placebo-controlled trial of galantamine in AD. The Galantamine USA-10 Study Group*. Neurology, 2000. **54**(12): p. 2269-76.
590. Winblad, B., et al., *A 1-year, randomized, placebo-controlled study of donepezil in patients with mild to moderate AD*. Neurology, 2001. **57**(3): p. 489-95.
591. Rosler, M., et al., *Efficacy and safety of rivastigmine in patients with Alzheimer's disease: international randomised controlled trial*. BMJ, 1999. **318**(7184): p. 633-8.
592. Rogers, S.L., et al., *A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease. Donepezil Study Group*. Neurology, 1998. **50**(1): p. 136-45.
593. Doody, R.S., et al., *Chronic donepezil treatment is associated with slowed cognitive decline in Alzheimer's disease*. Dement Geriatr Cogn Disord, 2001. **12**(4): p. 295-300.
594. Takeda, A., et al., *A systematic review of the clinical effectiveness of donepezil, rivastigmine and galantamine on cognition, quality of life and adverse events in Alzheimer's disease*. Int J Geriatr Psychiatry, 2006. **21**(1): p. 17-28.

595. Malaguarnera, M., et al., *Tacrine treatment of Alzheimer's disease: many expectations, few certainties*. Neuropsychobiology, 1998. **38**(4): p. 226-31.
596. Qizilbash, N., et al., *Tacrine for Alzheimer's disease*. Cochrane Database Syst Rev, 2000(3): p. CD000202.
597. Wong, W.J., et al., *A double-blind, placebo-controlled study of tacrine in Chinese patients with Alzheimer's disease*. Dement Geriatr Cogn Disord, 1999. **10**(4): p. 289-94.
598. Dodd, P.R., *Excited to death: different ways to lose your neurones*. Biogerontology, 2002. **3**(1-2): p. 51-6.
599. Hynd, M.R., H.L. Scott, and P.R. Dodd, *Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease*. Neurochem Int, 2004. **45**(5): p. 583-95.
600. Cotman, C.W., et al., *N-methyl-D-aspartate receptors and Alzheimer's disease*. Neurobiol Aging, 1989. **10**(5): p. 603-5; discussion 618-20.
601. Malenka, R.C. and R.A. Nicoll, *NMDA-receptor-dependent synaptic plasticity: multiple forms and mechanisms*. Trends Neurosci, 1993. **16**(12): p. 521-7.
602. Winblad, B. and N. Poritis, *Memantine in severe dementia: results of the 9M-Best Study (Benefit and efficacy in severely demented patients during treatment with memantine)*. Int J Geriatr Psychiatry, 1999. **14**(2): p. 135-46.
603. Tariot, P.N., et al., *Memantine treatment in patients with moderate to severe Alzheimer disease already receiving donepezil: a randomized controlled trial*. JAMA, 2004. **291**(3): p. 317-24.
604. Kornhuber, J. and G. Quack, *Cerebrospinal fluid and serum concentrations of the N-methyl-D-aspartate (NMDA) receptor antagonist memantine in man*. Neurosci Lett, 1995. **195**(2): p. 137-9.

605. Areosa, S.A., F. Sherriff, and R. McShane, *Memantine for dementia*. Cochrane Database Syst Rev, 2005(3): p. CD003154.
606. Martinez-Coria, H., et al., *Memantine improves cognition and reduces Alzheimer's-like neuropathology in transgenic mice*. Am J Pathol, 2010. **176**(2): p. 870-80.
607. Schwarzman, A.L., et al., *Transthyretin sequesters amyloid beta protein and prevents amyloid formation*. Proc Natl Acad Sci U S A, 1994. **91**(18): p. 8368-72.
608. Kerr, M.L., et al., *Inhibition of Abeta aggregation and neurotoxicity by the 39-kDa receptor-associated protein*. J Neurochem, 2010. **112**(5): p. 1199-209.
609. Castillo, G.M., et al., *Perlecan binds to the beta-amyloid proteins (A beta) of Alzheimer's disease, accelerates A beta fibril formation, and maintains A beta fibril stability*. J Neurochem, 1997. **69**(6): p. 2452-65.
610. Castillo, G.M., et al., *The sulfate moieties of glycosaminoglycans are critical for the enhancement of beta-amyloid protein fibril formation*. J Neurochem, 1999. **72**(4): p. 1681-7.
611. McLaurin, J., et al., *Interactions of Alzheimer amyloid-beta peptides with glycosaminoglycans effects on fibril nucleation and growth*. Eur J Biochem, 1999. **266**(3): p. 1101-10.
612. McLaurin, J., et al., *A sulfated proteoglycan aggregation factor mediates amyloid-beta peptide fibril formation and neurotoxicity*. Amyloid, 1999. **6**(4): p. 233-43.
613. Koppaka, V. and P.H. Axelsen, *Accelerated accumulation of amyloid beta proteins on oxidatively damaged lipid membranes*. Biochemistry, 2000. **39**(32): p. 10011-6.

614. Chauhan, A., I. Ray, and V.P. Chauhan, *Interaction of amyloid beta-protein with anionic phospholipids: possible involvement of Lys28 and C-terminus aliphatic amino acids*. Neurochem Res, 2000. **25**(3): p. 423-9.
615. Atwood, C.S., et al., *Dramatic aggregation of Alzheimer abeta by Cu(II) is induced by conditions representing physiological acidosis*. J Biol Chem, 1998. **273**(21): p. 12817-26.
616. House, E., et al., *Aluminium, iron, zinc and copper influence the in vitro formation of amyloid fibrils of Abeta42 in a manner which may have consequences for metal chelation therapy in Alzheimer's disease*. J Alzheimers Dis, 2004. **6**(3): p. 291-301.
617. Gervais, F., et al., *Targeting soluble Abeta peptide with Tramiprosate for the treatment of brain amyloidosis*. Neurobiol Aging, 2007. **28**(4): p. 537-47.
618. Aisen, P.S., et al., *Alzhemed: a potential treatment for Alzheimer's disease*. Curr Alzheimer Res, 2007. **4**(4): p. 473-8.
619. Koffie, R.M., et al., *Oligomeric amyloid beta associates with postsynaptic densities and correlates with excitatory synapse loss near senile plaques*. Proc Natl Acad Sci U S A, 2009. **106**(10): p. 4012-7.
620. Hartley, D.M., et al., *Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons*. J Neurosci, 1999. **19**(20): p. 8876-84.
621. Wang, H.W., et al., *Soluble oligomers of beta amyloid (1-42) inhibit long-term potentiation but not long-term depression in rat dentate gyrus*. Brain Res, 2002. **924**(2): p. 133-40.

622. Walsh, D.M., et al., *Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo*. *Nature*, 2002. **416**(6880): p. 535-9.
623. Schenk, D., et al., *Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse*. *Nature*, 1999. **400**(6740): p. 173-7.
624. Janus, C., et al., *A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease*. *Nature*, 2000. **408**(6815): p. 979-82.
625. Bayer, A.J., et al., *Evaluation of the safety and immunogenicity of synthetic Abeta42 (AN1792) in patients with AD*. *Neurology*, 2005. **64**(1): p. 94-101.
626. Gilman, S., et al., *Clinical effects of Abeta immunization (AN1792) in patients with AD in an interrupted trial*. *Neurology*, 2005. **64**(9): p. 1553-62.
627. Orgogozo, J.M., et al., *Subacute meningoencephalitis in a subset of patients with AD after Abeta42 immunization*. *Neurology*, 2003. **61**(1): p. 46-54.
628. Zurbriggen, R., et al., *Virosome-based active immunization targets soluble amyloid species rather than plaques in a transgenic mouse model of Alzheimer's disease*. *J Mol Neurosci*, 2005. **27**(2): p. 157-66.
629. Ryan, J.M. and M. Grundman, *Anti-amyloid-beta immunotherapy in Alzheimer's disease: ACC-001 clinical trials are ongoing*. *J Alzheimers Dis*, 2009. **17**(2): p. 243.
630. Solomon, B., *Generation of anti-beta-amyloid antibodies via phage display technology towards Alzheimer's disease vaccination*. *Vaccine*, 2005. **23**(17-18): p. 2327-30.

631. Salloway, S., et al., *A phase 2 multiple ascending dose trial of bapineuzumab in mild to moderate Alzheimer disease*. Neurology, 2009. **73**(24): p. 2061-70.
632. Rinne, J.O., et al., *11C-PiB PET assessment of change in fibrillar amyloid-beta load in patients with Alzheimer's disease treated with bapineuzumab: a phase 2, double-blind, placebo-controlled, ascending-dose study*. Lancet Neurol, 2010. **9**(4): p. 363-72.
633. Siemers, E.R., et al., *Safety and changes in plasma and cerebrospinal fluid amyloid beta after a single administration of an amyloid beta monoclonal antibody in subjects with Alzheimer disease*. Clin Neuropharmacol, 2010. **33**(2): p. 67-73.
634. Ferrer, I., et al., *Neuropathology and pathogenesis of encephalitis following amyloid-beta immunization in Alzheimer's disease*. Brain Pathol, 2004. **14**(1): p. 11-20.
635. Nicoll, J.A., et al., *Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report*. Nat Med, 2003. **9**(4): p. 448-52.
636. Bacskai, B.J., et al., *Non-Fc-mediated mechanisms are involved in clearance of amyloid-beta in vivo by immunotherapy*. J Neurosci, 2002. **22**(18): p. 7873-8.
637. DeMattos, R.B., et al., *Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease*. Proc Natl Acad Sci U S A, 2001. **98**(15): p. 8850-5.
638. Klaver, D.W., et al., *Is BACE1 a suitable therapeutic target for the treatment of Alzheimer's disease? Current strategies and future directions*. Biol Chem, 2010. **391**(8): p. 849-59.

639. Small, D.H., D.W. Klaver, and M. Beckman, *Regulation of proBACE1 by glycosaminoglycans*. Neurodegener Dis, 2008. **5**(3-4): p. 206-8.
640. Ghosh, A.K., et al., *Potent memapsin 2 (beta-secretase) inhibitors: design, synthesis, protein-ligand X-ray structure, and in vivo evaluation*. Bioorg Med Chem Lett, 2008. **18**(3): p. 1031-6.
641. Ghosh, A.K., M. Brindisi, and J. Tang, *Developing beta-secretase inhibitors for treatment of Alzheimer's disease*. J Neurochem, 2012. **120 Suppl 1**: p. 71-83.
642. Hu, X., et al., *Genetic deletion of BACE1 in mice affects remyelination of sciatic nerves*. FASEB J, 2008. **22**(8): p. 2970-80.
643. Eriksen, J.L., et al., *NSAIDs and enantiomers of flurbiprofen target gamma-secretase and lower Abeta 42 in vivo*. J Clin Invest, 2003. **112**(3): p. 440-9.
644. Wilcock, G.K., et al., *Efficacy and safety of tarenflurbil in mild to moderate Alzheimer's disease: a randomised phase II trial*. Lancet Neurol, 2008. **7**(6): p. 483-93.
645. Green, R.C., et al., *Effect of tarenflurbil on cognitive decline and activities of daily living in patients with mild Alzheimer disease: a randomized controlled trial*. JAMA, 2009. **302**(23): p. 2557-64.
646. Galasko, D.R., et al., *Safety, tolerability, pharmacokinetics, and Abeta levels after short-term administration of R-flurbiprofen in healthy elderly individuals*. Alzheimer Dis Assoc Disord, 2007. **21**(4): p. 292-9.
647. Fleisher, A.S., et al., *Phase 2 safety trial targeting amyloid beta production with a gamma-secretase inhibitor in Alzheimer disease*. Arch Neurol, 2008. **65**(8): p. 1031-8.



648. Henley, D.B., et al., *Development of semagacestat (LY450139), a functional gamma-secretase inhibitor, for the treatment of Alzheimer's disease*. Expert Opin Pharmacother, 2009. **10**(10): p. 1657-64.
649. Samson, K., *NerveCenter: Phase III Alzheimer trial halted: Search for therapeutic biomarkers continues*. Ann Neurol, 2010. **68**(4): p. A9-A12.
650. Schor, N.F., *What the halted phase III gamma-secretase inhibitor trial may (or may not) be telling us*. Ann Neurol, 2011. **69**(2): p. 237-9.
651. Louvi, A. and S. Artavanis-Tsakonas, *Notch signalling in vertebrate neural development*. Nat Rev Neurosci, 2006. **7**(2): p. 93-102.
652. Shih Ie, M. and T.L. Wang, *Notch signaling, gamma-secretase inhibitors, and cancer therapy*. Cancer Res, 2007. **67**(5): p. 1879-82.
653. Milano, J., et al., *Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation*. Toxicol Sci, 2004. **82**(1): p. 341-58.
654. Simons, M., et al., *Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons*. Proc Natl Acad Sci U S A, 1998. **95**(11): p. 6460-4.
655. Wahrle, S., et al., *Cholesterol-dependent gamma-secretase activity in buoyant cholesterol-rich membrane microdomains*. Neurobiol Dis, 2002. **9**(1): p. 11-23.
656. Refolo, L.M., et al., *A cholesterol-lowering drug reduces beta-amyloid pathology in a transgenic mouse model of Alzheimer's disease*. Neurobiol Dis, 2001. **8**(5): p. 890-9.

657. Fassbender, K., et al., *Simvastatin strongly reduces levels of Alzheimer's disease beta -amyloid peptides Abeta 42 and Abeta 40 in vitro and in vivo*. Proc Natl Acad Sci U S A, 2001. **98**(10): p. 5856-61.
658. Jick, H., et al., *Statins and the risk of dementia*. Lancet, 2000. **356**(9242): p. 1627-31.
659. Shie, F.S., et al., *Diet-induced hypercholesterolemia enhances brain A beta accumulation in transgenic mice*. Neuroreport, 2002. **13**(4): p. 455-9.
660. Refolo, L.M., et al., *Hypercholesterolemia accelerates the Alzheimer's amyloid pathology in a transgenic mouse model*. Neurobiol Dis, 2000. **7**(4): p. 321-31.
661. Trompet, S., et al., *Pravastatin and cognitive function in the elderly. Results of the PROSPER study*. J Neurol, 2010. **257**(1): p. 85-90.
662. McGuinness, B., et al., *Statins for the prevention of dementia*. Cochrane Database Syst Rev, 2009(2): p. CD003160.
663. Rafii, M.S. and P.S. Aisen, *Recent developments in Alzheimer's disease therapeutics*. BMC Med, 2009. **7**: p. 7.
664. Hardingham, T.E. and A.J. Fosang, *Proteoglycans: many forms and many functions*. FASEB J, 1992. **6**(3): p. 861-70.
665. Casu, B., *Structure and biological activity of heparin*. Adv Carbohydr Chem Biochem, 1985. **43**: p. 51-134.
666. Hileman, R.E., et al., *Glycosaminoglycan-protein interactions: definition of consensus sites in glycosaminoglycan binding proteins*. Bioessays, 1998. **20**(2): p. 156-67.
667. Linhardt, R.J., et al., *Isolation and characterization of human heparin*. Biochemistry, 1992. **31**(49): p. 12441-5.

668. Castillo, G.M., et al., *Sulfate content and specific glycosaminoglycan backbone of perlecan are critical for perlecan's enhancement of islet amyloid polypeptide (amylin) fibril formation*. Diabetes, 1998. **47**(4): p. 612-20.
669. Wedemeyer, J., M. Tsai, and S.J. Galli, *Roles of mast cells and basophils in innate and acquired immunity*. Curr Opin Immunol, 2000. **12**(6): p. 624-31.
670. Lindahl, U., M. Kusche-Gullberg, and L. Kjellen, *Regulated diversity of heparan sulfate*. J Biol Chem, 1998. **273**(39): p. 24979-82.
671. Silbert, J.E. and G. Sugumaran, *Biosynthesis of chondroitin/dermatan sulfate*. IUBMB Life, 2002. **54**(4): p. 177-86.
672. McDonald, J.A. and T.D. Camenisch, *Hyaluronan: genetic insights into the complex biology of a simple polysaccharide*. Glycoconj J, 2002. **19**(4-5): p. 331-9.
673. Meyer, K., et al., *The mucopolysaccharides of bovine cornea*. J Biol Chem, 1953. **205**(2): p. 611-6.
674. Krusius, T., et al., *Identification of an O-glycosidic mannose-linked sialylated tetrasaccharide and keratan sulfate oligosaccharides in the chondroitin sulfate proteoglycan of brain*. J Biol Chem, 1986. **261**(18): p. 8237-42.
675. Funderburgh, J.L., *Keratan sulfate: structure, biosynthesis, and function*. Glycobiology, 2000. **10**(10): p. 951-8.
676. Sugahara, K. and H. Kitagawa, *Recent advances in the study of the biosynthesis and functions of sulfated glycosaminoglycans*. Curr Opin Struct Biol, 2000. **10**(5): p. 518-27.
677. Lidholt, K., et al., *A single mutation affects both N-acetylglucosaminyltransferase and glucuronosyltransferase activities in a*

- Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis.*  
Proc Natl Acad Sci U S A, 1992. **89**(6): p. 2267-71.
678. Lidholt, K. and U. Lindahl, *Biosynthesis of heparin. The D-glucuronosyl- and N-acetyl-D-glucosaminyltransferase reactions and their relation to polymer modification.* Biochem J, 1992. **287** ( Pt 1): p. 21-9.
679. Izumikawa, T., et al., *Identification of chondroitin sulfate glucuronyltransferase as chondroitin synthase-3 involved in chondroitin polymerization: chondroitin polymerization is achieved by multiple enzyme complexes consisting of chondroitin synthase family members.* J Biol Chem, 2008. **283**(17): p. 11396-406.
680. Aikawa, J., et al., *Multiple isozymes of heparan sulfate/heparin GlcNAc N-deacetylase/GlcN N-sulfotransferase. Structure and activity of the fourth member, NDST4.* J Biol Chem, 2001. **276**(8): p. 5876-82.
681. Eriksson, I., et al., *cDNA cloning and sequencing of mouse mastocytoma glucosaminyl N-deacetylase/N-sulfotransferase, an enzyme involved in the biosynthesis of heparin.* J Biol Chem, 1994. **269**(14): p. 10438-43.
682. Li, J., et al., *Biosynthesis of heparin/heparan sulfate. cDNA cloning and expression of D-glucuronyl C5-epimerase from bovine lung.* J Biol Chem, 1997. **272**(44): p. 28158-63.
683. Doege, K.J., et al., *Complete coding sequence and deduced primary structure of the human cartilage large aggregating proteoglycan, aggrecan. Human-specific repeats, and additional alternatively spliced forms.* J Biol Chem, 1991. **266**(2): p. 894-902.
684. Zimmermann, D.R. and E. Ruoslahti, *Multiple domains of the large fibroblast proteoglycan, versican.* EMBO J, 1989. **8**(10): p. 2975-81.

685. Rauch, U., et al., *Cloning and primary structure of neurocan, a developmentally regulated, aggregating chondroitin sulfate proteoglycan of brain*. J Biol Chem, 1992. **267**(27): p. 19536-47.
686. Yamada, H., et al., *Molecular cloning of brevican, a novel brain proteoglycan of the aggrecan/versican family*. J Biol Chem, 1994. **269**(13): p. 10119-26.
687. Yamaguchi, Y., *Lecticans: organizers of the brain extracellular matrix*. Cell Mol Life Sci, 2000. **57**(2): p. 276-89.
688. Galtrey, C.M. and J.W. Fawcett, *The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system*. Brain Res Rev, 2007. **54**(1): p. 1-18.
689. Maurel, P., et al., *Phosphacan, a chondroitin sulfate proteoglycan of brain that interacts with neurons and neural cell-adhesion molecules, is an extracellular variant of a receptor-type protein tyrosine phosphatase*. Proc Natl Acad Sci U S A, 1994. **91**(7): p. 2512-6.
690. Hocking, A.M., T. Shinomura, and D.J. McQuillan, *Leucine-rich repeat glycoproteins of the extracellular matrix*. Matrix Biol, 1998. **17**(1): p. 1-19.
691. Nishiyama, A., et al., *The primary structure of NG2, a novel membrane-spanning proteoglycan*. J Cell Biol, 1991. **114**(2): p. 359-71.
692. Staub, E., B. Hinzmann, and A. Rosenthal, *A novel repeat in the melanoma-associated chondroitin sulfate proteoglycan defines a new protein family*. FEBS Lett, 2002. **527**(1-3): p. 114-8.
693. Watanabe, E., et al., *Neuroglycan C, a novel membrane-spanning chondroitin sulfate proteoglycan that is restricted to the brain*. J Biol Chem, 1995. **270**(45): p. 26876-82.

694. Aono, S., et al., *Genomic organization and expression pattern of mouse neuroglycan C in the cerebellar development*. J Biol Chem, 2000. **275**(1): p. 337-42.
695. Shioi, J., et al., *The Alzheimer amyloid precursor proteoglycan (appican) is present in brain and is produced by astrocytes but not by neurons in primary neural cultures*. J Biol Chem, 1995. **270**(20): p. 11839-44.
696. Pangalos, M.N., et al., *The chondroitin sulfate attachment site of appican is formed by splicing out exon 15 of the amyloid precursor gene*. J Biol Chem, 1995. **270**(18): p. 10388-91.
697. Shioi, J., et al., *Chondroitin sulfate proteoglycan form of the Alzheimer's beta-amyloid precursor*. J Biol Chem, 1992. **267**(20): p. 13819-22.
698. Dreyfuss, J.L., et al., *Heparan sulfate proteoglycans: structure, protein interactions and cell signaling*. An Acad Bras Cienc, 2009. **81**(3): p. 409-29.
699. Bernfield, M., et al., *Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans*. Annu Rev Cell Biol, 1992. **8**: p. 365-93.
700. Elenius, K. and M. Jalkanen, *Function of the syndecans--a family of cell surface proteoglycans*. J Cell Sci, 1994. **107** ( Pt 11): p. 2975-82.
701. David, G., *Integral membrane heparan sulfate proteoglycans*. FASEB J, 1993. **7**(11): p. 1023-30.
702. Schulz, J.G., et al., *Syndecan 3 intramembrane proteolysis is presenilin/gamma-secretase-dependent and modulates cytosolic signaling*. J Biol Chem, 2003. **278**(49): p. 48651-7.
703. Fitzgerald, M.L., et al., *Shedding of syndecan-1 and -4 ectodomains is regulated by multiple signaling pathways and mediated by a TIMP-3-sensitive metalloproteinase*. J Cell Biol, 2000. **148**(4): p. 811-24.

704. Filmus, J., M. Capurro, and J. Rast, *Glypicans*. Genome Biol, 2008. **9**(5): p. 224.
705. De Cat, B. and G. David, *Developmental roles of the glypicans*. Semin Cell Dev Biol, 2001. **12**(2): p. 117-25.
706. De Cat, B., et al., *Processing by proprotein convertases is required for glypican-3 modulation of cell survival, Wnt signaling, and gastrulation movements*. J Cell Biol, 2003. **163**(3): p. 625-35.
707. Iozzo, R.V., et al., *The biology of perlecan: the multifaceted heparan sulphate proteoglycan of basement membranes and pericellular matrices*. Biochem J, 1994. **302** ( Pt 3): p. 625-39.
708. Kirn-Safran, C., M.C. Farach-Carson, and D.D. Carson, *Multifunctionality of extracellular and cell surface heparan sulfate proteoglycans*. Cell Mol Life Sci, 2009. **66**(21): p. 3421-34.
709. Winzen, U., G.J. Cole, and W. Halfter, *Agrin is a chimeric proteoglycan with the attachment sites for heparan sulfate/chondroitin sulfate located in two multiple serine-glycine clusters*. J Biol Chem, 2003. **278**(32): p. 30106-14.
710. Iozzo, R.V., *Basement membrane proteoglycans: from cellar to ceiling*. Nat Rev Mol Cell Biol, 2005. **6**(8): p. 646-56.
711. Alliel, P.M., et al., *Testican, a multidomain testicular proteoglycan resembling modulators of cell social behaviour*. Eur J Biochem, 1993. **214**(1): p. 347-50.
712. Maeda, N., et al., *Functions of chondroitin sulfate and heparan sulfate in the developing brain*. Neurochem Res, 2011. **36**(7): p. 1228-40.
713. Properzi, F. and J.W. Fawcett, *Proteoglycans and brain repair*. News Physiol Sci, 2004. **19**: p. 33-8.

714. Carulli, D., et al., *Chondroitin sulfate proteoglycans in neural development and regeneration*. Curr Opin Neurobiol, 2005. **15**(1): p. 116-20.
715. Meyer-Puttlitz, B., et al., *Chondroitin sulfate and chondroitin/keratan sulfate proteoglycans of nervous tissue: developmental changes of neurocan and phosphacan*. J Neurochem, 1995. **65**(5): p. 2327-37.
716. Perris, R. and D. Perissinotto, *Role of the extracellular matrix during neural crest cell migration*. Mech Dev, 2000. **95**(1-2): p. 3-21.
717. Kubota, Y., et al., *Spatial and temporal changes in chondroitin sulfate distribution in the sclerotome play an essential role in the formation of migration patterns of mouse neural crest cells*. Dev Dyn, 1999. **214**(1): p. 55-65.
718. Perissinotto, D., et al., *Avian neural crest cell migration is diversely regulated by the two major hyaluronan-binding proteoglycans PG-M/versican and aggrecan*. Development, 2000. **127**(13): p. 2823-42.
719. Landolt, R.M., et al., *Versican is selectively expressed in embryonic tissues that act as barriers to neural crest cell migration and axon outgrowth*. Development, 1995. **121**(8): p. 2303-12.
720. Oakley, R.A. and K.W. Tosney, *Peanut agglutinin and chondroitin-6-sulfate are molecular markers for tissues that act as barriers to axon advance in the avian embryo*. Dev Biol, 1991. **147**(1): p. 187-206.
721. Oakley, R.A., et al., *Glycoconjugates mark a transient barrier to neural crest migration in the chicken embryo*. Development, 1994. **120**(1): p. 103-14.
722. Ring, C., J. Hassell, and W. Halfter, *Expression pattern of collagen IX and potential role in the segmentation of the peripheral nervous system*. Dev Biol, 1996. **180**(1): p. 41-53.



723. Snow, D.M., et al., *Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro*. Exp Neurol, 1990. **109**(1): p. 111-30.
724. Fichard, A., et al., *Involvement of a chondroitin sulfate proteoglycan in the avoidance of chick epidermis by dorsal root ganglia fibers: a study using beta-D-xyloside*. Dev Biol, 1991. **148**(1): p. 1-9.
725. Verna, J.M., A. Fichard, and R. Saxod, *Influence of glycosaminoglycans on neurite morphology and outgrowth patterns in vitro*. Int J Dev Neurosci, 1989. **7**(4): p. 389-99.
726. Dou, C.L. and J.M. Levine, *Inhibition of neurite growth by the NG2 chondroitin sulfate proteoglycan*. J Neurosci, 1994. **14**(12): p. 7616-28.
727. Wang, H., et al., *Chondroitin-4-sulfation negatively regulates axonal guidance and growth*. J Cell Sci, 2008. **121**(Pt 18): p. 3083-91.
728. Brittis, P.A., D.R. Canning, and J. Silver, *Chondroitin sulfate as a regulator of neuronal patterning in the retina*. Science, 1992. **255**(5045): p. 733-6.
729. Snow, D.M., et al., *A chondroitin sulfate proteoglycan may influence the direction of retinal ganglion cell outgrowth*. Development, 1991. **113**(4): p. 1473-85.
730. Engel, M., et al., *Chondroitin sulfate proteoglycans in the developing central nervous system. I. cellular sites of synthesis of neurocan and phosphacan*. J Comp Neurol, 1996. **366**(1): p. 34-43.
731. Ida, M., et al., *Identification and functions of chondroitin sulfate in the milieu of neural stem cells*. J Biol Chem, 2006. **281**(9): p. 5982-91.
732. Ogawa, T., et al., *Brevican in the developing hippocampal fimbria: differential expression in myelinating oligodendrocytes and adult astrocytes suggests a*

- dual role for brevican in central nervous system fiber tract development. J Comp Neurol*, 2001. **432**(3): p. 285-95.
733. Bekku, Y., et al., *Brevican distinctively assembles extracellular components at the large diameter nodes of Ranvier in the CNS. J Neurochem*, 2009. **108**(5): p. 1266-76.
  734. Holt, C.E. and B.J. Dickson, *Sugar codes for axons? Neuron*, 2005. **46**(2): p. 169-72.
  735. Wang, L. and J.L. Denburg, *A role for proteoglycans in the guidance of a subset of pioneer axons in cultured embryos of the cockroach. Neuron*, 1992. **8**(4): p. 701-14.
  736. Gurwitz, D. and D.D. Cunningham, *Neurite outgrowth activity of protease nexin-1 on neuroblastoma cells requires thrombin inhibition. J Cell Physiol*, 1990. **142**(1): p. 155-62.
  737. Williamson, T.G., et al., *Affinity purification of proteoglycans that bind to the amyloid protein precursor of Alzheimer's disease. J Neurochem*, 1995. **65**(5): p. 2201-8.
  738. Bronner-Fraser, M. and T. Lallier, *A monoclonal antibody against a laminin-heparan sulfate proteoglycan complex perturbs cranial neural crest migration in vivo. J Cell Biol*, 1988. **106**(4): p. 1321-9.
  739. Walicke, P.A., *Interactions between basic fibroblast growth factor (FGF) and glycosaminoglycans in promoting neurite outgrowth. Exp Neurol*, 1988. **102**(1): p. 144-8.
  740. Walz, A., et al., *Essential role of heparan sulfates in axon navigation and targeting in the developing visual system. Development*, 1997. **124**(12): p. 2421-30.

741. Irie, A., et al., *Specific heparan sulfate structures involved in retinal axon targeting*. Development, 2002. **129**(1): p. 61-70.
742. Lee, J.S., et al., *Axon sorting in the optic tract requires HSPG synthesis by ext2 (dackel) and extl3 (boxer)*. Neuron, 2004. **44**(6): p. 947-60.
743. Rhiner, C., et al., *Syndecan regulates cell migration and axon guidance in C. elegans*. Development, 2005. **132**(20): p. 4621-33.
744. Pratt, T., et al., *Heparan sulphation patterns generated by specific heparan sulfotransferase enzymes direct distinct aspects of retinal axon guidance at the optic chiasm*. J Neurosci, 2006. **26**(26): p. 6911-23.
745. Ford-Perriss, M., et al., *Localisation of specific heparan sulfate proteoglycans during the proliferative phase of brain development*. Dev Dyn, 2003. **227**(2): p. 170-84.
746. McLaughlin, D., et al., *Specific modification of heparan sulphate is required for normal cerebral cortical development*. Mech Dev, 2003. **120**(12): p. 1481-8.
747. Berardi, N., T. Pizzorusso, and L. Maffei, *Critical periods during sensory development*. Curr Opin Neurobiol, 2000. **10**(1): p. 138-45.
748. Deepa, S.S., et al., *Composition of perineuronal net extracellular matrix in rat brain: a different disaccharide composition for the net-associated proteoglycans*. J Biol Chem, 2006. **281**(26): p. 17789-800.
749. Kwok, J.C., D. Carulli, and J.W. Fawcett, *In vitro modeling of perineuronal nets: hyaluronan synthase and link protein are necessary for their formation and integrity*. J Neurochem, 2010. **114**(5): p. 1447-59.
750. Wang, D. and J. Fawcett, *The perineuronal net and the control of CNS plasticity*. Cell Tissue Res, 2012. **349**(1): p. 147-60.

751. Pizzorusso, T., et al., *Reactivation of ocular dominance plasticity in the adult visual cortex*. Science, 2002. **298**(5596): p. 1248-51.
752. McRae, P.A., et al., *Sensory deprivation alters aggrecan and perineuronal net expression in the mouse barrel cortex*. J Neurosci, 2007. **27**(20): p. 5405-13.
753. Bukalo, O., M. Schachner, and A. Dityatev, *Modification of extracellular matrix by enzymatic removal of chondroitin sulfate and by lack of tenascin-R differentially affects several forms of synaptic plasticity in the hippocampus*. Neuroscience, 2001. **104**(2): p. 359-69.
754. Zhou, X.H., et al., *Neurocan is dispensable for brain development*. Mol Cell Biol, 2001. **21**(17): p. 5970-8.
755. Brakebusch, C., et al., *Brevican-deficient mice display impaired hippocampal CA1 long-term potentiation but show no obvious deficits in learning and memory*. Mol Cell Biol, 2002. **22**(21): p. 7417-27.
756. Nitkin, R.M., et al., *Identification of agrin, a synaptic organizing protein from Torpedo electric organ*. J Cell Biol, 1987. **105**(6 Pt 1): p. 2471-8.
757. Reist, N.E., C. Magill, and U.J. McMahan, *Agrin-like molecules at synaptic sites in normal, denervated, and damaged skeletal muscles*. J Cell Biol, 1987. **105**(6 Pt 1): p. 2457-69.
758. Herbst, R. and S.J. Burden, *The juxtamembrane region of MuSK has a critical role in agrin-mediated signaling*. EMBO J, 2000. **19**(1): p. 67-77.
759. Ferreira, A., *Abnormal synapse formation in agrin-depleted hippocampal neurons*. J Cell Sci, 1999. **112** ( Pt 24): p. 4729-38.
760. Bose, C.M., et al., *Agrin controls synaptic differentiation in hippocampal neurons*. J Neurosci, 2000. **20**(24): p. 9086-95.

761. Gingras, J., et al., *Agrin plays an organizing role in the formation of sympathetic synapses*. J Cell Biol, 2002. **158**(6): p. 1109-18.
762. Ksiazek, I., et al., *Synapse loss in cortex of agrin-deficient mice after genetic rescue of perinatal death*. J Neurosci, 2007. **27**(27): p. 7183-95.
763. Tournell, C.E., R.A. Bergstrom, and A. Ferreira, *Progesterone-induced agrin expression in astrocytes modulates glia-neuron interactions leading to synapse formation*. Neuroscience, 2006. **141**(3): p. 1327-38.
764. Lin, Y.L., et al., *Syndecan-2 induces filopodia and dendritic spine formation via the neurofibromin-PKA-Ena/VASP pathway*. J Cell Biol, 2007. **177**(5): p. 829-41.
765. Rolls, A., R. Shechter, and M. Schwartz, *The bright side of the glial scar in CNS repair*. Nat Rev Neurosci, 2009. **10**(3): p. 235-41.
766. Davies, S.J., et al., *Robust regeneration of adult sensory axons in degenerating white matter of the adult rat spinal cord*. J Neurosci, 1999. **19**(14): p. 5810-22.
767. Asher, R.A., et al., *Chondroitin sulphate proteoglycans: inhibitory components of the glial scar*. Prog Brain Res, 2001. **132**: p. 611-9.
768. Levine, J.M., *Increased expression of the NG2 chondroitin-sulfate proteoglycan after brain injury*. J Neurosci, 1994. **14**(8): p. 4716-30.
769. Harris, N.G., et al., *Traumatic brain injury results in disparate regions of chondroitin sulfate proteoglycan expression that are temporally limited*. J Neurosci Res, 2009. **87**(13): p. 2937-50.
770. McKeon, R.J., A. Hoke, and J. Silver, *Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars*. Exp Neurol, 1995. **136**(1): p. 32-43.

771. Smith-Thomas, L.C., et al., *Increased axon regeneration in astrocytes grown in the presence of proteoglycan synthesis inhibitors*. J Cell Sci, 1995. **108** ( Pt 3): p. 1307-15.
772. Moon, L.D., et al., *Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC*. Nat Neurosci, 2001. **4**(5): p. 465-6.
773. Bradbury, E.J., et al., *Chondroitinase ABC promotes functional recovery after spinal cord injury*. Nature, 2002. **416**(6881): p. 636-40.
774. Yick, L.W., et al., *Axonal regeneration of Clarke's neurons beyond the spinal cord injury scar after treatment with chondroitinase ABC*. Exp Neurol, 2003. **182**(1): p. 160-8.
775. Moon, L.D., et al., *Relationship between sprouting axons, proteoglycans and glial cells following unilateral nigrostriatal axotomy in the adult rat*. Neuroscience, 2002. **109**(1): p. 101-17.
776. Iseki, K., et al., *Increased syndecan expression by pleiotrophin and FGF receptor-expressing astrocytes in injured brain tissue*. Glia, 2002. **39**(1): p. 1-9.
777. Hagino, S., et al., *Expression pattern of glypican-1 mRNA after brain injury in mice*. Neurosci Lett, 2003. **349**(1): p. 29-32.
778. Properzi, F., et al., *Heparan sulphate proteoglycans in glia and in the normal and injured CNS: expression of sulphotransferases and changes in sulphation*. Eur J Neurosci, 2008. **27**(3): p. 593-604.
779. Leadbeater, W.E., et al., *Intracellular trafficking in neurones and glia of fibroblast growth factor-2, fibroblast growth factor receptor 1 and heparan*

- sulphate proteoglycans in the injured adult rat cerebral cortex*. J Neurochem, 2006. **96**(4): p. 1189-200.
780. Snow, A.D., et al., *The presence of heparan sulfate proteoglycans in the neuritic plaques and congophilic angiopathy in Alzheimer's disease*. Am J Pathol, 1988. **133**(3): p. 456-63.
781. Perry, G., et al., *Association of heparan sulfate proteoglycan with the neurofibrillary tangles of Alzheimer's disease*. J Neurosci, 1991. **11**(11): p. 3679-83.
782. Cotman, S.L., W. Halfter, and G.J. Cole, *Agrin binds to beta-amyloid (Abeta), accelerates abeta fibril formation, and is localized to Abeta deposits in Alzheimer's disease brain*. Mol Cell Neurosci, 2000. **15**(2): p. 183-98.
783. Goedert, M., et al., *Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans*. Nature, 1996. **383**(6600): p. 550-3.
784. Snow, A.D., et al., *Sulfated glycosaminoglycans in amyloid plaques of prion diseases*. Acta Neuropathol, 1989. **77**(4): p. 337-42.
785. Snow, A.D., et al., *Immunolocalization of heparan sulfate proteoglycans to the prion protein amyloid plaques of Gerstmann-Straussler syndrome, Creutzfeldt-Jakob disease and scrapie*. Lab Invest, 1990. **63**(5): p. 601-11.
786. Liu, I.H., et al., *Agrin binds alpha-synuclein and modulates alpha-synuclein fibrillation*. Glycobiology, 2005. **15**(12): p. 1320-31.
787. DeWitt, D.A., et al., *Chondroitin sulfate proteoglycans are a common component of neuronal inclusions and astrocytic reaction in neurodegenerative diseases*. Brain Res, 1994. **656**(1): p. 205-9.

788. Sobel, R.A. and A.S. Ahmed, *White matter extracellular matrix chondroitin sulfate/dermatan sulfate proteoglycans in multiple sclerosis*. J Neuropathol Exp Neurol, 2001. **60**(12): p. 1198-207.
789. Mohan, H., et al., *Extracellular matrix in multiple sclerosis lesions: Fibrillar collagens, biglycan and decorin are upregulated and associated with infiltrating immune cells*. Brain Pathol, 2010. **20**(5): p. 966-75.
790. Ginsberg, S.D., et al., *Accumulation of intracellular amyloid-beta peptide (A beta 1-40) in mucopolysaccharidosis brains*. J Neuropathol Exp Neurol, 1999. **58**(8): p. 815-24.
791. Peat, R.A., et al., *Exclusion of biglycan mutations in a cohort of patients with neuromuscular disorders*. Neuromuscul Disord, 2008. **18**(8): p. 606-9.
792. Snow, A.D., et al., *Early accumulation of heparan sulfate in neurons and in the beta-amyloid protein-containing lesions of Alzheimer's disease and Down's syndrome*. Am J Pathol, 1990. **137**(5): p. 1253-70.
793. Su, J.H., B.J. Cummings, and C.W. Cotman, *Localization of heparan sulfate glycosaminoglycan and proteoglycan core protein in aged brain and Alzheimer's disease*. Neuroscience, 1992. **51**(4): p. 801-13.
794. Perlmuter, L.S., et al., *Microangiopathy and the colocalization of heparan sulfate proteoglycan with amyloid in senile plaques of Alzheimer's disease*. Brain Res, 1990. **508**(1): p. 13-9.
795. Snow, A.D., et al., *Heparan sulfate proteoglycan in diffuse plaques of hippocampus but not of cerebellum in Alzheimer's disease brain*. Am J Pathol, 1994. **144**(2): p. 337-47.



796. Verbeek, M.M., et al., *Agrin is a major heparan sulfate proteoglycan accumulating in Alzheimer's disease brain*. Am J Pathol, 1999. **155**(6): p. 2115-25.
797. DeWitt, D.A., et al., *Chondroitin sulfate proteoglycans are associated with the lesions of Alzheimer's disease*. Exp Neurol, 1993. **121**(2): p. 149-52.
798. Snow, A.D., et al., *Peripheral distribution of dermatan sulfate proteoglycans (decorin) in amyloid-containing plaques and their presence in neurofibrillary tangles of Alzheimer's disease*. J Histochem Cytochem, 1992. **40**(1): p. 105-13.
799. O'Callaghan, P., et al., *Heparan sulfate accumulation with Abeta deposits in Alzheimer's disease and Tg2576 mice is contributed by glial cells*. Brain Pathol, 2008. **18**(4): p. 548-61.
800. van Horssen, J., et al., *Heparan sulfate proteoglycan expression in cerebrovascular amyloid beta deposits in Alzheimer's disease and hereditary cerebral hemorrhage with amyloidosis (Dutch) brains*. Acta Neuropathol, 2001. **102**(6): p. 604-14.
801. van Horssen, J., et al., *Collagen XVIII: a novel heparan sulfate proteoglycan associated with vascular amyloid depositions and senile plaques in Alzheimer's disease brains*. Brain Pathol, 2002. **12**(4): p. 456-62.
802. Shimizu, H., et al., *Interaction between beta-amyloid protein and heparan sulfate proteoglycans from the cerebral capillary basement membrane in Alzheimer's disease*. J Clin Neurosci, 2009. **16**(2): p. 277-82.
803. Miller, J.D., et al., *Localization of perlecan (or a perlecan-related macromolecule) to isolated microglia in vitro and to microglia/macrophages following infusion of beta-amyloid protein into rodent hippocampus*. Glia, 1997. **21**(2): p. 228-43.

804. Gupta-Bansal, R., R.C. Frederickson, and K.R. Brunden, *Proteoglycan-mediated inhibition of A beta proteolysis. A potential cause of senile plaque accumulation.* J Biol Chem, 1995. **270**(31): p. 18666-71.
805. Shaffer, L.M., et al., *Amyloid beta protein (A beta) removal by neuroglial cells in culture.* Neurobiol Aging, 1995. **16**(5): p. 737-45.
806. Small, D.H., et al., *Association and release of the amyloid protein precursor of Alzheimer's disease from chick brain extracellular matrix.* J Neurosci, 1992. **12**(11): p. 4143-50.
807. Narindrasorasak, S., et al., *High affinity interactions between the Alzheimer's beta-amyloid precursor proteins and the basement membrane form of heparan sulfate proteoglycan.* J Biol Chem, 1991. **266**(20): p. 12878-83.
808. Schubert, D., et al., *Characterization of an amyloid beta precursor protein that binds heparin and contains tyrosine sulfate.* Proc Natl Acad Sci U S A, 1989. **86**(6): p. 2066-9.
809. Williamson, T.G., et al., *Secreted glypican binds to the amyloid precursor protein of Alzheimer's disease (APP) and inhibits APP-induced neurite outgrowth.* J Biol Chem, 1996. **271**(49): p. 31215-21.
810. Buee, L., et al., *Binding of secreted human neuroblastoma proteoglycans to the Alzheimer's amyloid A4 peptide.* Brain Res, 1993. **601**(1-2): p. 154-63.
811. Buee, L., et al., *Binding of vascular heparan sulfate proteoglycan to Alzheimer's amyloid precursor protein is mediated in part by the N-terminal region of A4 peptide.* Brain Res, 1993. **627**(2): p. 199-204.
812. Fraser, P.E., et al., *Effects of sulfate ions on Alzheimer beta/A4 peptide assemblies: implications for amyloid fibril-proteoglycan interactions.* J Neurochem, 1992. **59**(4): p. 1531-40.

813. McLaurin, J. and P.E. Fraser, *Effect of amino-acid substitutions on Alzheimer's amyloid-beta peptide-glycosaminoglycan interactions*. Eur J Biochem, 2000. **267**(21): p. 6353-61.
814. Brunden, K.R., et al., *pH-dependent binding of synthetic beta-amyloid peptides to glycosaminoglycans*. J Neurochem, 1993. **61**(6): p. 2147-54.
815. Watson, D.J., A.D. Lander, and D.J. Selkoe, *Heparin-binding properties of the amyloidogenic peptides Abeta and amylin. Dependence on aggregation state and inhibition by Congo red*. J Biol Chem, 1997. **272**(50): p. 31617-24.
816. Fraser, P.E., A.A. Darabie, and J.A. McLaurin, *Amyloid-beta interactions with chondroitin sulfate-derived monosaccharides and disaccharides. implications for drug development*. J Biol Chem, 2001. **276**(9): p. 6412-9.
817. Bravo, R., et al., *Sulfated polysaccharides promote the assembly of amyloid beta(1-42) peptide into stable fibrils of reduced cytotoxicity*. J Biol Chem, 2008. **283**(47): p. 32471-83.
818. Perez, M., et al., *Polymerization of tau into filaments in the presence of heparin: the minimal sequence required for tau-tau interaction*. J Neurochem, 1996. **67**(3): p. 1183-90.
819. Hasegawa, M., et al., *Alzheimer-like changes in microtubule-associated protein Tau induced by sulfated glycosaminoglycans. Inhibition of microtubule binding, stimulation of phosphorylation, and filament assembly depend on the degree of sulfation*. J Biol Chem, 1997. **272**(52): p. 33118-24.
820. Hernandez, F., et al., *Sulfo-glycosaminoglycan content affects PHF-tau solubility and allows the identification of different types of PHFs*. Brain Res, 2002. **935**(1-2): p. 65-72.

821. Paudel, H.K. and W. Li, *Heparin-induced conformational change in microtubule-associated protein Tau as detected by chemical cross-linking and phosphopeptide mapping*. J Biol Chem, 1999. **274**(12): p. 8029-38.
822. Dudas, B., et al., *Neuroprotective properties of glycosaminoglycans: potential treatment for neurodegenerative disorders*. Neurodegener Dis, 2008. **5**(3-4): p. 200-5.
823. Ma, Q., et al., *The blood-brain barrier accessibility of a heparin-derived oligosaccharides C3*. Thromb Res, 2002. **105**(5): p. 447-53.
824. Dudas, B., et al., *Oral and subcutaneous administration of the glycosaminoglycan C3 attenuates Abeta(25-35)-induced abnormal tau protein immunoreactivity in rat brain*. Neurobiol Aging, 2002. **23**(1): p. 97-104.
825. Rose, M., et al., *Protective effect of the heparin-derived oligosaccharide C3, on AF64A-induced cholinergic lesion in rats*. Neurobiol Aging, 2003. **24**(3): p. 481-90.
826. Rose, M., et al., *Glycosaminoglycan C3 protects against AF64A-induced cholinotoxicity in a dose-dependent and time-dependent manner*. Brain Res, 2004. **1015**(1-2): p. 96-102.
827. Zhu, H., J. Yu, and M.S. Kindy, *Inhibition of amyloidosis using low-molecular-weight heparins*. Mol Med, 2001. **7**(8): p. 517-22.
828. Kisilevsky, R., et al., *Arresting amyloidosis in vivo using small-molecule anionic sulphonates or sulphates: implications for Alzheimer's disease*. Nat Med, 1995. **1**(2): p. 143-8.
829. Walzer, M., et al., *Low molecular weight glycosaminoglycan blockade of beta-amyloid induced neuropathology*. Eur J Pharmacol, 2002. **445**(3): p. 211-20.

830. Bergamaschini, L., et al., *Peripheral treatment with enoxaparin, a low molecular weight heparin, reduces plaques and beta-amyloid accumulation in a mouse model of Alzheimer's disease*. J Neurosci, 2004. **24**(17): p. 4181-6.
831. Timmer, N.M., et al., *Enoxaparin treatment administered at both early and late stages of amyloid beta deposition improves cognition of APP<sup>swe</sup>/PS1<sup>dE9</sup> mice with differential effects on brain A beta levels*. Neurobiol Dis, 2010. **40**(1): p. 340-7.
832. Leveugle, B., et al., *Heparin promotes beta-secretase cleavage of the Alzheimer's amyloid precursor protein*. Neurochem Int, 1997. **30**(6): p. 543-8.
833. Scholefield, Z., et al., *Heparan sulfate regulates amyloid precursor protein processing by BACE1, the Alzheimer's beta-secretase*. J Cell Biol, 2003. **163**(1): p. 97-107.
834. Patey, S.J., et al., *Heparin derivatives as inhibitors of BACE-1, the Alzheimer's beta-secretase, with reduced activity against factor Xa and other proteases*. J Med Chem, 2006. **49**(20): p. 6129-32.
835. Patey, S.J., E.A. Yates, and J.E. Turnbull, *Novel heparan sulphate analogues: inhibition of beta-secretase cleavage of amyloid precursor protein*. Biochem Soc Trans, 2005. **33**(Pt 5): p. 1116-8.
836. Beckman, M., R.M. Holsinger, and D.H. Small, *Heparin activates beta-secretase (BACE1) of Alzheimer's disease and increases autocatalysis of the enzyme*. Biochemistry, 2006. **45**(21): p. 6703-14.
837. Klaver, D.W., et al., *Glycosaminoglycan-induced activation of the beta-secretase (BACE1) of Alzheimer's disease*. J Neurochem, 2010. **112**(6): p. 1552-61.

838. Bergamaschini, L., et al., *Heparin attenuates cytotoxic and inflammatory activity of Alzheimer amyloid-beta in vitro*. Neurobiol Aging, 2002. **23**(4): p. 531-6.
839. Cui, H., et al., *Effects of heparin and enoxaparin on APP processing and Abeta production in primary cortical neurons from Tg2576 mice*. PLoS One, 2011. **6**(7): p. e23007.
840. Sandwall, E., et al., *Heparan sulfate mediates amyloid-beta internalization and cytotoxicity*. Glycobiology, 2010. **20**(5): p. 533-41.
841. Kanekiyo, T., et al., *Heparan sulphate proteoglycan and the low-density lipoprotein receptor-related protein 1 constitute major pathways for neuronal amyloid-beta uptake*. J Neurosci, 2011. **31**(5): p. 1644-51.
842. Leveugle, B., et al., *Heparin oligosaccharides that pass the blood-brain barrier inhibit beta-amyloid precursor protein secretion and heparin binding to beta-amyloid peptide*. J Neurochem, 1998. **70**(2): p. 736-44.
843. Endres, K. and F. Fahrenholz, *The Role of the Anti-Amyloidogenic Secretase ADAM10 in Shedding the APP-Like Proteins*. Curr Alzheimer Res, 2011.
844. Lambert, M.P., et al., *Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins*. Proc Natl Acad Sci U S A, 1998. **95**(11): p. 6448-53.
845. Haass, C. and D.J. Selkoe, *Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide*. Nat Rev Mol Cell Biol, 2007. **8**(2): p. 101-12.
846. Kim, H.J., et al., *Selective neuronal degeneration induced by soluble oligomeric amyloid beta protein*. FASEB J, 2003. **17**(1): p. 118-20.

847. Small, D.H., et al., *Alzheimer's disease therapeutics: new approaches to an ageing problem*. IUBMB Life, 2004. **56**(4): p. 203-8.
848. Hirsh, J., *Heparin*. N Engl J Med, 1991. **324**(22): p. 1565-74.
849. Weitz, J.I., *Low-molecular-weight heparins*. N Engl J Med, 1997. **337**(10): p. 688-98.
850. Kadusevicius, E., et al., *Low-molecular-weight heparins: pharmacoeconomic decision modeling based on meta-analysis data*. Int J Technol Assess Health Care, 2010. **26**(3): p. 272-9.
851. Pollack, S.J., et al., *Sulfonated dyes attenuate the toxic effects of beta-amyloid in a structure-specific fashion*. Neurosci Lett, 1995. **197**(3): p. 211-4.
852. Pollack, S.J., et al., *Sulfated glycosaminoglycans and dyes attenuate the neurotoxic effects of beta-amyloid in rat PC12 cells*. Neurosci Lett, 1995. **184**(2): p. 113-6.
853. Ando, K., et al., *Role of phosphorylation of Alzheimer's amyloid precursor protein during neuronal differentiation*. J Neurosci, 1999. **19**(11): p. 4421-7.
854. Klafki, H.W., J. Wiltfang, and M. Staufenbiel, *Electrophoretic separation of betaA4 peptides (1-40) and (1-42)*. Anal Biochem, 1996. **237**(1): p. 24-9.
855. Dovey, H.F., et al., *Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain*. J Neurochem, 2001. **76**(1): p. 173-81.
856. Sahin, U., et al., *Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands*. J Cell Biol, 2004. **164**(5): p. 769-79.
857. Kveiborg, M., et al., *PKCalpha and PKCdelta regulate ADAM17-mediated ectodomain shedding of heparin binding-EGF through separate pathways*. PLoS One, 2011. **6**(2): p. e17168.

858. Mendelson, K., et al., *Stimulation of platelet-derived growth factor receptor beta (PDGFRbeta) activates ADAM17 and promotes metalloproteinase-dependent cross-talk between the PDGFRbeta and epidermal growth factor receptor (EGFR) signaling pathways.* J Biol Chem, 2010. **285**(32): p. 25024-32.
859. Racchi, M., et al., *Role of protein kinase Calpha in the regulated secretion of the amyloid precursor protein.* Mol Psychiatry, 2003. **8**(2): p. 209-16.
860. Klaver, D., et al., *Effect of heparin on APP metabolism and Abeta production in cortical neurons.* Neurodegener Dis, 2010. **7**(1-3): p. 187-9.
861. Xia, W., et al., *Presenilin complexes with the C-terminal fragments of amyloid precursor protein at the sites of amyloid beta-protein generation.* Proc Natl Acad Sci U S A, 2000. **97**(16): p. 9299-304.
862. Hoey, S.E., R.J. Williams, and M.S. Perkinson, *Synaptic NMDA receptor activation stimulates alpha-secretase amyloid precursor protein processing and inhibits amyloid-beta production.* J Neurosci, 2009. **29**(14): p. 4442-60.
863. Barten, D.M., et al., *Dynamics of {beta}-amyloid reductions in brain, cerebrospinal fluid, and plasma of {beta}-amyloid precursor protein transgenic mice treated with a {gamma}-secretase inhibitor.* J Pharmacol Exp Ther, 2005. **312**(2): p. 635-43.
864. Abramowski, D., et al., *Dynamics of Abeta turnover and deposition in different beta-amyloid precursor protein transgenic mouse models following gamma-secretase inhibition.* J Pharmacol Exp Ther, 2008. **327**(2): p. 411-24.
865. Johnston, J., et al., *The significance of the Swedish APP670/671 mutation for the development of Alzheimer's disease amyloidosis.* Neurochem Int, 1994. **25**(1): p. 73-80.



866. Nurcombe, V., et al., *Developmental regulation of neural response to FGF-1 and FGF-2 by heparan sulfate proteoglycan*. Science, 1993. **260**(5104): p. 103-6.
867. Sanderson, R.D., et al., *Fine structure of heparan sulfate regulates syndecan-1 function and cell behavior*. J Biol Chem, 1994. **269**(18): p. 13100-6.
868. Islam, T., et al., *Further evidence that periodate cleavage of heparin occurs primarily through the antithrombin binding site*. Carbohydr Res, 2002. **337**(21-23): p. 2239-43.
869. Matsuo, M., et al., *A novel regioselective desulfation of polysaccharide sulfates: Specific 6-O-desulfation with N,O-bis(trimethylsilyl)acetamide*. Carbohydr Res, 1993. **241**: p. 209-15.
870. Degenhardt, M., P. Ghosh, and H. Watzig, *Studies on the structural variations of pentosan polysulfate sodium (NaPPS) from different sources by capillary electrophoresis*. Arch Pharm (Weinheim), 2001. **334**(1): p. 27-9.
871. Li, B., et al., *Fucoidan: structure and bioactivity*. Molecules, 2008. **13**(8): p. 1671-95.
872. Agarwal, A. and I. Danishefsky, *Requirement of free carboxyl groups for the anticoagulant activity of heparin*. Thromb Res, 1986. **42**(5): p. 673-80.
873. Paredes-Gamero, E.J., et al., *Chemical reduction of carboxyl groups in heparin abolishes its vasodilatory activity*. J Cell Biochem, 2011.
874. Petitou, M., et al., *Synthesis of thrombin-inhibiting heparin mimetics without side effects*. Nature, 1999. **398**(6726): p. 417-22.
875. Delehedde, M., et al., *Fibroblast growth factor-2 binds to small heparin-derived oligosaccharides and stimulates a sustained phosphorylation of*

- p42/44 mitogen-activated protein kinase and proliferation of rat mammary fibroblasts*. Biochem J, 2002. **366**(Pt 1): p. 235-44.
876. Smith-Swintosky, V.L., et al., *Secreted forms of beta-amyloid precursor protein protect against ischemic brain injury*. J Neurochem, 1994. **63**(2): p. 781-4.
  877. Mattson, M.P., *Secreted forms of beta-amyloid precursor protein modulate dendrite outgrowth and calcium responses to glutamate in cultured embryonic hippocampal neurons*. J Neurobiol, 1994. **25**(4): p. 439-50.
  878. Kim, J., et al., *Abeta40 inhibits amyloid deposition in vivo*. J Neurosci, 2007. **27**(3): p. 627-33.
  879. Klinger, M.M., R.U. Margolis, and R.K. Margolis, *Isolation and characterization of the heparan sulfate proteoglycans of brain. Use of affinity chromatography on lipoprotein lipase-agarose*. J Biol Chem, 1985. **260**(7): p. 4082-90.
  880. Margolis, R.K., et al., *Effects of beta-xylosides on proteoglycan biosynthesis and morphology of PC12 pheochromocytoma cells and primary cultures of rat cerebellum*. J Cell Sci, 1991. **99** ( Pt 2): p. 237-46.
  881. Hotoda, N., et al., *A secreted form of human ADAM9 has an alpha-secretase activity for APP*. Biochem Biophys Res Commun, 2002. **293**(2): p. 800-5.
  882. Tanabe, C., et al., *ADAM19 is tightly associated with constitutive Alzheimer's disease APP alpha-secretase in A172 cells*. Biochem Biophys Res Commun, 2007. **352**(1): p. 111-7.
  883. Asai, M., et al., *Putative function of ADAM9, ADAM10, and ADAM17 as APP alpha-secretase*. Biochem Biophys Res Commun, 2003. **301**(1): p. 231-5.

884. Hardy, J. and D.J. Selkoe, *The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics*. Science, 2002. **297**(5580): p. 353-6.
885. Snow, A.D., et al., *An important role of heparan sulfate proteoglycan (Perlecan) in a model system for the deposition and persistence of fibrillar A beta-amyloid in rat brain*. Neuron, 1994. **12**(1): p. 219-34.
886. Panza, F., et al., *REVIEW: gamma-Secretase inhibitors for the treatment of Alzheimer's disease: The current state*. CNS Neurosci Ther, 2010. **16**(5): p. 272-84.
887. Saftig, P. and K. Reiss, *The "A Disintegrin And Metalloproteases" ADAM10 and ADAM17: novel drug targets with therapeutic potential?* Eur J Cell Biol, 2011. **90**(6-7): p. 527-35.
888. Asai, M., et al., *An alternative metabolic pathway of amyloid precursor protein C-terminal fragments via cathepsin B in a human neuroglioma model*. FASEB J, 2011. **25**(10): p. 3720-30.
889. Siman, R., J.P. Card, and L.G. Davis, *Proteolytic processing of beta-amyloid precursor by calpain I*. J Neurosci, 1990. **10**(7): p. 2400-11.
890. Vella, L.J. and R. Cappai, *Identification of a novel amyloid precursor protein processing pathway that generates secreted N-terminal fragments*. FASEB J, 2012. **26**(7): p. 2930-40.
891. Selkoe, D.J., *Resolving controversies on the path to Alzheimer's therapeutics*. Nat Med, 2011. **17**(9): p. 1060-5.
892. Gandhi, N.S. and R.L. Mancera, *The structure of glycosaminoglycans and their interactions with proteins*. Chem Biol Drug Des, 2008. **72**(6): p. 455-82.

893. Guerrini, M., et al., *Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events*. Nat Biotechnol, 2008. **26**(6): p. 669-75.
894. Anderson, V.R. and C.M. Perry, *Pentosan polysulfate: a review of its use in the relief of bladder pain or discomfort in interstitial cystitis*. Drugs, 2006. **66**(6): p. 821-35.
895. Savonenko, A.V., et al., *Alteration of BACE1-dependent NRG1/ErbB4 signaling and schizophrenia-like phenotypes in BACE1-null mice*. Proc Natl Acad Sci U S A, 2008. **105**(14): p. 5585-90.