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STUDIES OF AMYLOID TOXICITY IN DROSOPHILA MODELS AND EFFECTS OF THE BRICHOS DOMAIN

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Studies of amyloid toxicity in Drosophila models and effects of the BRICHOS domain
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

Amyloid diseases involve specific protein misfolding events and formation of fibrillar deposits. The symptoms of these diseases are broad and dependent on site of accumulation, with different amyloid proteins depositing in specific tissues or systematically. One such protein is transthyretin (TTR) associated with senile systemic amyloidosis, familial amyloid polyneuropathy and familial amyloid cardiomyopathy. We show that the glycosaminoglycan heparan sulfate (HS) can be co-localized with TTR in elder myopathic heart tissue and identify residue 24-35 of TTR as the binding site of HS. Moreover, we show that heparin, a HS homolog, can promote fibril formation and accumulation of TTR using cell cultures and a *Drosophila in vivo* model.

It has been shown that certain chaperones are associated with amyloid disease and can promote or inhibit the aggregation into amyloid. BRICHOS is an approximately 100 residue protein domain present in over a 1000 proteins divided into 10 families. BRICHOS containing proteins have been ascribed a wide variety of functions and some are associated with diseases such as respiratory distress syndrome, dementia and cancer. The BRICHOS domains of proSP-C, a precursor protein to lung surfactant protein C, and Bri2, a protein associated with familial British and Danish dementia, can act as chaperones and inhibit amyloid fibril formation of the amyloid-β peptide (Aβ) of Alzheimer's disease (AD). We show that both proSP-C and Bri2 BRICHOS can prevent aggregation of Aβ *in vivo* using *Drosophila melanogaster* as a model organism. Moreover, BRICHOS can inhibit the toxicity of Aβ, increasing the life span and locomotor activity of the flies.

We also identify expression of Bri2 in human pancreas and show that Bri2 co-localizes with the islet amyloid polypeptide (IAPP) linked to type 2 diabetes (T2D). Furthermore, Bri2 BRICHOS can inhibit the aggregation of IAPP *in vitro* and reduce the toxic effects of IAPP in cell cultures and *in vivo* in a *Drosophila* model.

These results show that the BRICHOS domain inhibits the aggregation and toxicity of both Aβ and IAPP. The BRICHOS domain, in particular the Bri2 BRICHOS domain, could be used as a potential pharmaceutical agent in treatment of amyloid diseases. Similar effects on both Aβ and IAPP suggest that the BRICHOS domain also could effect the amyloid formation and toxicity of other amyloid proteins, which would be an interesting area to further investigate.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to using their Roman numerals.

I. Noborn F, O'Callaghan P*, **Hermansson E***, Zhang X, Ancsin JB, Damas AM, Dacklin I, Presto J, Johansson J, Saraiva MJ, Lundgren E, Kisilevsky R, Westermark P, Li JP. (2011) Heparan sulfate/heparin promotes tranthyretin fibrillization through selective binding to a basic motif in the protein. Proc Natl Acad Sci U S A. 108(14):5584-9. *These authors contributed equally to this work.

II. **Hermansson E**, Schultz S, Crowther D, Linse S, Winblad B, Westermark G, Johansson J, Presto J. (2014) The chaperone domain BRICHOS prevents CNS toxicity of amyloid-β peptide in *Drosophila melanogaster*. Dis Model Mech. 7(6):659-65.

III. Poska H*, **Hermansson E***, Presto J, Johansson J. The BRICHOS domain of dementia related Bri2 protein is a potent inhibitor of Aβ42 toxicity *in vivo* (manuscript). *These authors contributed equally to this work.

IV. Oskarsson M, **Hermansson E**, Johansson J, Presto J, Westermark G. The BRICHOS domain of Bri2 inhibits IAPP aggregation and toxicity (manuscript)

Papers not included in this thesis:

Willander H, **Hermansson E**, Johansson J, Presto J. (2011). BRICHOS domain associated with lung fibrosis, dementia and cancer—a chaperone that prevents amyloid fibril formation? FEBS J. 278(20):3893-904.

Willander H, Askarieh G, Landreh M, Westermark P, Nordling K, Keränen H, **Hermansson E**, Hamvas A, Nogee LM, Bergman T, Saenz A, Casals C, Åqvistg J, Jörnvall H, Berglund H, Presto J, Knight SD, Johansson J. (2012). High-resolution strucutre of a BRICHOS domain and its implications for anti-amyloid chaperone activity on lung surfactant protein C. Proc Natl Acad Sci U S A. 109(7):2325-9.

Biverstål H, Dolfe L, **Hermansson E**, Leppert A, Reifenrath M, Winblad B, Presto J, Johansson J (2015). Dissociation of a BRICHOS trimer into monomers leads to increased inhibitory effect on Aβ42 fibril formation. Biochim Biophys Acta. S1570-9639(15)00101-6.

CONTENTS

LIST OF ABBREVIATIONS

Aß	Amyloid β peptide
AD	Alzheimer's disease
ApoE	Apolipoprotein E
APP	Amyloid β precursor protein
CNS	Central nervous system
Elav	Embryonic lethal abnormal vision
ER	Endoplasmic reticulum
FBD	Familial British Dementia
FDD	Familial Danish Dementia
GAGs	Glycoaminoglycans
GFP	Green fluorescent protein
GKN	Gastrokine
GMR	Glass multimer reporter
HS	Heparan sulfate
Hsp	Heath shock protein
IAPP	Islet amyloid polypeptide
ILD	Interstitial lung disease
PD	Parkinson's disease
PLA	Proximity ligation assay
proSP-C	Surfactant protein C precursor
PrP	Prion protein
SP-B	Surfactant protein B
SP-C	Surfactant protein C
ThT	Thioflavine T
TM	Transmembrane
TTR	Transthyretin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
UAS	Upstream activating sequence

1 INTRODUCTION

1.1 Amyloids and amyloid diseases

Amyloids are protein aggregates that arise from misfolding of specific proteins (Merlini and Bellotti 2003). There are more than 30 human diseases were proteins deposit as fibrillar material in organs and tissues, collectively known as amyloidosis (Table 1) (Westermark et al. 2007, Sipe et al. 2012, Sipe et al. 2014). Among those are Alzheimer´s disease (AD), Parkinson's disease (PD), Huntington's disease and type 2 diabetes (T2D), where each disease is characterized by a specific amyloid fibril forming protein (Westermark et al. 2007). In some amyloid diseases, the proteins deposit in a single organ (localized) such as the brain in AD while in e.g. inflammatory-associated amyloidosis deposits are found in multiple organs (systemic), such as the liver, spleen and kidneys (Westermark et al. 2007). The classic characterization of amyloids is that they exhibit a β-sheet structure and can be identified by exhibiting birefringence when stained with Congo red and viewed under polarized light, and as rigid fibrils of approximately 10 nm in diameter when viewed with electron microscopy (Sipe and Cohen 2000, Westermark et al. 2002). In some cases, the proteins can have an inherited ability to be destabilized and form amyloid fibrils, which becomes pronounced in elder individuals, like transthyretin (TTR) in patients with senile systemic amyloidosis (Saraiva 2001). In other cases, constant high levels of amyloid forming proteins can cause disease, which is the case with β_2-microglobulin in patients undergoing extended hemodialysis (Gejyo et al. 1985, Verdone et al. 2002). The misfolding of amyloidgenic proteins can also be caused by mutations in the gene encoding the protein such in e.g. the case of the arctic mutation in familial AD (Nilsberth et al. 2001) or the TTR_{V30M} mutation of TTR amyloidosis (Saraiva et al. 1984).

Table 1. Amyloid diseases, their respective fibril forming proteins and target organs. Adapted from Sipe et al, 2014 (Sipe et al. 2014). Variants indicate abnormality such as mutations or polymorphisms. ANS = Autonomic nervous system, CNS = Central nervous system, PNS = Peripheral nervous system.

Fibril protein	Amyloid forming protein	Target organ
AL	Immunoglubulin Light Chain	All organs except CNS
AH	Immunoglubin Heavy Chain	All organs except CNS
AA	(Apo) Serum Amyloid A	All organs except CNS
ATTR	Transthyretin, wild type	Heart, ligaments, tenosynvium
	Transthyretin, variants	PNS, ANS, heart, eye, leptomeninges
Aβ2M	β2-Microglobulin, wild type	Musculoskeletal system
AApoAI	Apolipoprotein A I, variants	Heart, liver kidney, PNS, testis larynx, skin
AApoAII	Apolipoprotein A II, variants	Kidney
AApoAIV	Apolipoprotein A IV, wild type	Kidney medulla and systemic
AGel	Gelsolin, variants	PNS, cornea
ALys	Lysozyme, variants	Kidney
ALECT2	Leukocyte Chemotactic Factor-2	Kidney, primarily
AFib	Fibrinogen α, variants	Kidney, primarily
ACys	Cystatin C, variants	PNS, skin
ABri	Bri2, variants	CNS
ADan	Bri2, variants	CNS
Aβ	Aβ Protein Precursor (APP), wild type, variants	CNS
APrP	Prion Protein, wild type, variants	Brain
ACal	(Pro) Calcitonin	C-cell thyroid tumors
AIAPP	Islet Amyloid Polypeptide	Islet of Langerhans
AANF	Atrial Natriuretic Factor	Cardiac atria
APro	Prolactin	Pituitary prolactinomas, aging pituitary
AIns	Insulin	Iatrogenic, local injection
ASPC	Surfactant Protein C Precursor, variants	Lung
AGal7	Galectin 7	Skin
ACor	Cornedesmosin	Cornified epithelia, hair follicles
AMed	Lactadherin	Senile aortic, media
Aker	Kerato-epithelin	Cornea
ALac	Lactoferrin	Cornea
AOAAP	Odontogenic Ameloblast-Associated protein	Odontogenic tumors
ASem1	Semenogelin 1	Vesicula seminalis
AEnf	Enfurvitide	Iatrogenic

Viewing amyloid fibrils using a transmission electron microscope reveals a characteristic structure of the fibrils (Fig 1) (Cohen and Calkins 1959, Shirahama and Cohen 1967). The structure of amyloid proteins consists of a cross-β sheet with β-strands running perpendicular to the fiber axis bound to neighboring strands through backbone hydrogen bonds (Fig 1). Analysis of microcrystals of amyloid peptides have revealed a double β-sheet where the side chains protruding from the two sheets form a tight steric-zipper (Nelson et al. 2005). Brain extracts from human AD patients analyzed by solid state nuclear magnetic resonance have shown that *in vivo* fibrils can be structurally different from *in vitro* fibrils (Lu et al. 2013), with differences in orientation of the side-chains, allowing for interaction with other molecules in the amyloid structure. Furthermore, it was shown that amyloid fibrils from patients with distinct clinical histories can be structurally different. Detailed knowledge of molecular structures and interactions further supports the generic nature of fibrils from different amyloid proteins (Wasmer et al. 2008, Fitzpatrick et al. 2013). The generic nature can be credited to common properties of the polypeptide backbone facilitating the hydrogen bonds of the fibril core, and differences in structure resulting from how side chains are incorporated into the common fibrillar architecture (Fandrich and Dobson 2002, Chiti and Dobson 2006).

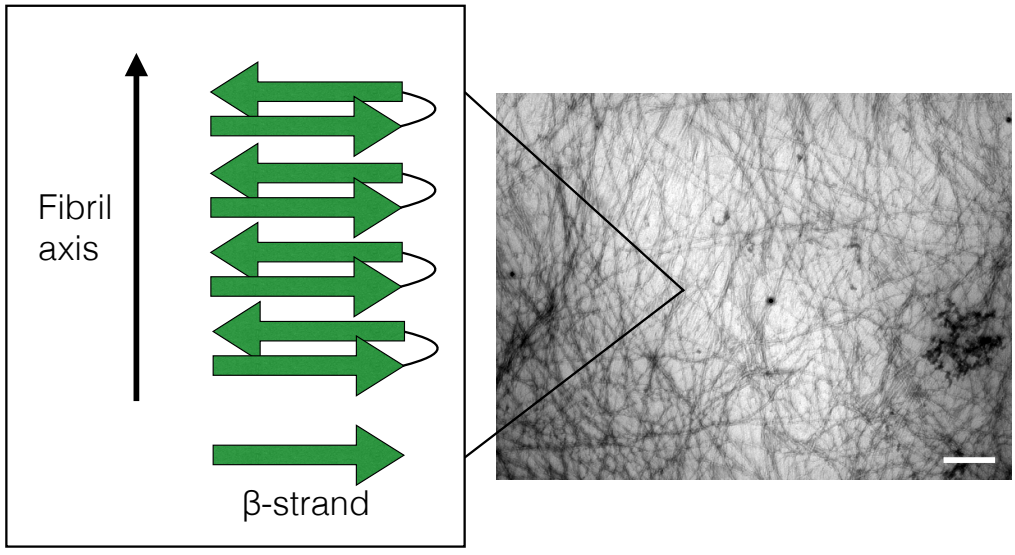

Figure 1. Amyloid fibrils are made up of β-sheets. The electron microscopy image on the right shows fibrils formed by islet amyloid polypeptide protein (IAPP) associated to type 2 diabetes. Photo by Marie Oskarsson. Scale bar represents 200 nm.

The formation of amyloid fibrils typically follows a sigmoidal reaction time course and is generally divided into three phases (Fig 2). The first phase is the lag phase were amyloid proteins form oligomers (nuclei) that can promote further fibrillization (Arosio et al. 2015).

Larger aggregates and amyloid fibrils are present during this phase, though in amounts low enough to not be detected using assays such as a thioflavine T (ThT), commonly used for studies of the aggregation process (Arosio et al. 2014). The lag phase is followed by the elongation phase were protofibrils are formed and rapidly aggregates, forming amyloid fibrils. This is detected as a rapid signal increase. Finally, the plateau phase is reached when equilibrium between fibrils and precursor is achieved and the signal increase levels out. Fibrils can work as templates for monomers, meaning that when monomers interact with fibrils the monomers adopt the same cross-β conformation as the preexisting peptides. Adding preformed fibrils to a solution during the lag phase promotes fibril formation as a result of seeding (Jucker and Walker 2011).

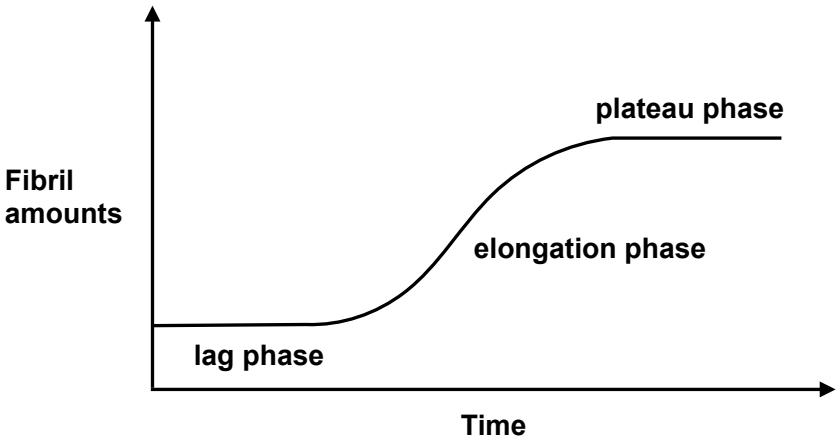

Figure 2. The process of fibril formation. The process starts with a lag phase, were the amount of aggregation and fibril formation is too low to be detected. This is followed by the elongation phase, were protofibrils form and aggregates into amyloid fibrils, which results in a rapid increase in signal. This is followed by the plateau phase, were equilibrium is reach and the signal pans out.

The typical reaction kinetics of amyloid formation is a feature of nucleated polymerization (Serio et al. 2000). In traditional nucleated polymerization, the initial formation of aggregates occur from soluble precursor peptides, followed by elongation of fibrils through incorporation of additional precursors. However, amyloid formation also includes secondary processes, which can be dominant in the contribution to amyloid growth (Knowles et al. 2009). The formation of new fibrils can be divided into three general processes (Fig 3) (Cohen et al. 2013). In the primary pathway, monomers interact, forming oligomers that can act as nuclei for the formation of amyloid fibrils. The secondary pathways are either monomer-dependent or monomer-independent. Fibril fragmentation is an important monomer-independent process, were fragmentation of fibrils increases the amount of fibril ends that can generate further growth. Surface catalyzed secondary nucleation is a

monomer-dependent process where monomer binding to fibrils catalyze oligomer formation. Recently it was shown, by using experimental ThT data and kinetic models that the proliferation of toxic oligomers and aggregates of AD mainly occurs through a secondary nucleation mechanism (Cohen et al. 2013).

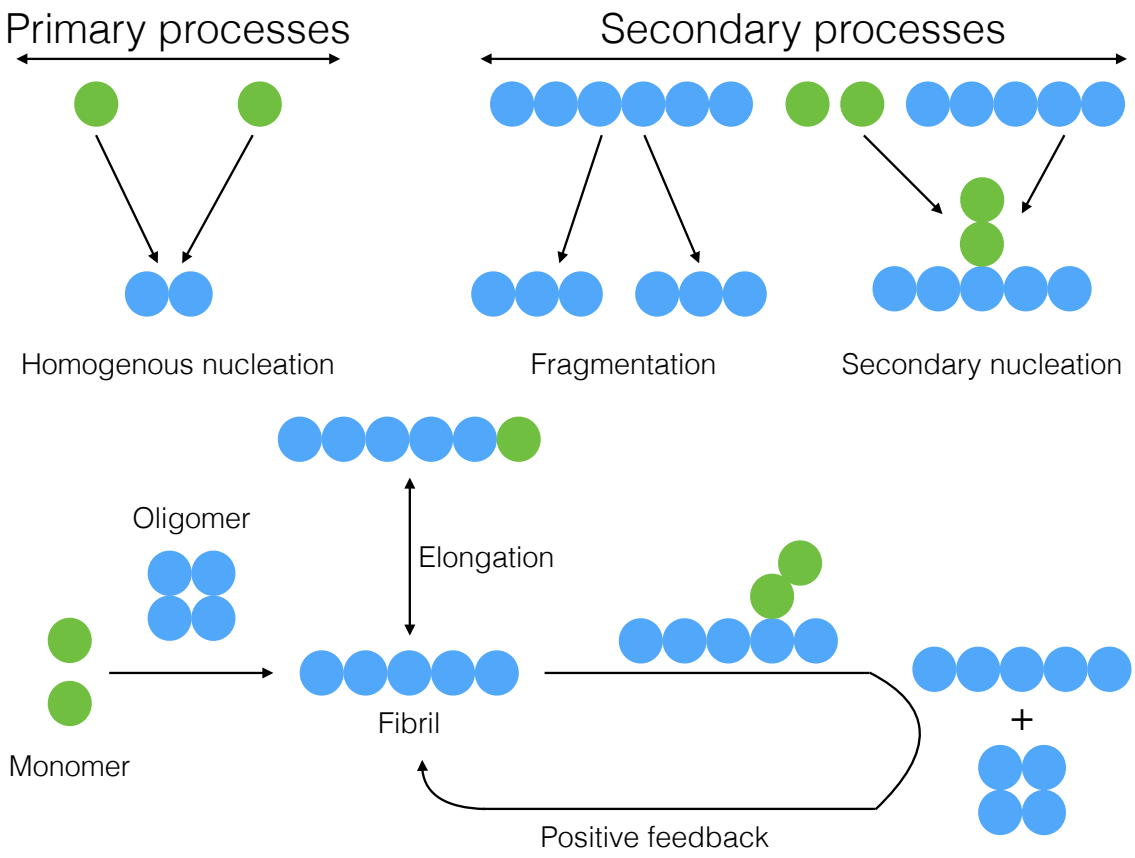

Figure 3. Major fibril formation processes and diagram of pathways. The formation of fibrils can be divided into different processes. In the primary nucleation pathway, monomers interact with each other to form oligomers and fibrils. Two major secondary pathways are fragmentation, were fibrils fragment and increase the number of fibril ends available for binding, and secondary nucleation, were monomers bind to the fibrillar surface. The lower part of the figure shows a schematic diagram of primary nucleation and secondary nucleation molecular pathways. Adapted from Cohen et al, 2013 (Cohen et al. 2013).

The amyloid forming proteins make up for the major part of amyloid deposits. However, other components can usually be found as well. Three of the more studied components are heparan sulfate (HS), serum amyloid P component and Apolipoprotein E (ApoE). HS is a glycosaminoglycan (GAG) composed of repeating disaccharide units and is believed to be an important player in amyloid fibril formation. HS has been associated with several amyloid diseases, affecting processing and promoting fibril formation of amyloid proteins (Leveugle et al. 1997). Serum amyloid P component is a protein that can bind to amyloid fibrils (Pepys et al. 1979), preventing proteolysis (Tennent et al. 1995) and can be radiolabeled to be used

for studying amyloid deposits in the human body (Hawkins 2002). Another protein that has been found in amyloid deposits is ApoE that has been associated with T2D (Charge et al. 1996) and AD (Strittmatter et al. 1993).

1.1.1 Alzheimer´s disease

AD is the most common form of dementia representing 60-80% of all cases (Alzheimer´s Association 2014). It is a neurodegenerative disease and symptoms include memory loss and cognitive problems such as disorientation and confusion. AD can be divided into two groups, sporadic and familial. Sporadic cases are by far the most prevalent form of AD, making up 95% of all cases, and familial forms makes up for only a small number of all cases (Blennow et al. 2006). There are two aggregated hallmark proteins in AD, Aß and tau (Hardy and Selkoe 2002).

The Aß peptide was first discovered in 1984 (Glenner and Wong 1984) and identified as the main component of extracellular amyloid plaques of AD (Masters et al. 1985). The amyloid hypothesis postulates that the accumulation of Aß is the primary cause of AD and the other parts of the disease process are secondary (Hardy and Selkoe 2002). The precursor protein of Aß is APP, a type 1 transmembrane (TM) protein, with the N-terminal located in the ER lumen. APP can be processed in two distinct pathways, the amyloidogenic and the non-amyloidogenic, the difference being the initial proteolytic cleavage. In the amyloidogenic pathway, APP is first cleaved by ß-secretase at the N-terminal side of Aß, releasing a soluble 100 kDa N-terminal fragment and a membrane-bound 12 kDa C-terminal fragment (Fig 4). Further cleavage of the C-terminal fragment by γ-secretase produces the Aß peptides (Haass et al. 1993). The dominant species of Aß are Aß40 (40 residues in length) and Aß42 (42 residues in length), although Aß43 has been shown to be more prevalent in amyloid plaque cores of AD patients than Aß40 and shows similar aggregation propensity as Aß42 (Welander et al. 2009). The non-amyloidogenic pathway instead starts with cleavage of APP by α-secretase (Sisodia 1992), generating a soluble N-terminal fragment and a 10 kDa membrane-bound C-terminal fragment. Cleavage by γ-secretase of the C-terminal fragment produces the p3 peptide (Fig 4) (Haass et al. 1993), which is nonpathogenic. The familial, early onset AD cases are linked to mutations in the APP gene, located on chromosome 21 (Tanzi et al. 1987, Korenberg et al. 1989) or presenilin genes (which are part of the γ-secretase complex). Several different mutations of the APP gene have been linked to the early onset of the disease (Chartier-Harlin et al. 1991, Goate et al. 1991, Mullan et al. 1992), affecting the aggregation propensity of the Aß peptide. The gene of presenilin 1 is located on

chromosome 14 (Sherrington et al. 1995) and presenilin 2 on chromosome 2 (Rogaev et al. 1995), and mutations in any of them are linked to early-onset AD. Mutations in presenilin 1 affect the conformation of presenilin 1 and interaction with APP (Berezovska et al. 2005), and mutations in both presenilin 1 and presenilin 2 increase the ratio of Aß42/Aß40 and lead to early onset AD (Borchelt et al. 1996, Scheuner et al. 1996). An isoform of ApoE, ApoE4, increases the likelihood of developing AD by, among other things, modulating trafficking of APP and Aß production (Bu 2009, Yu et al. 2014).

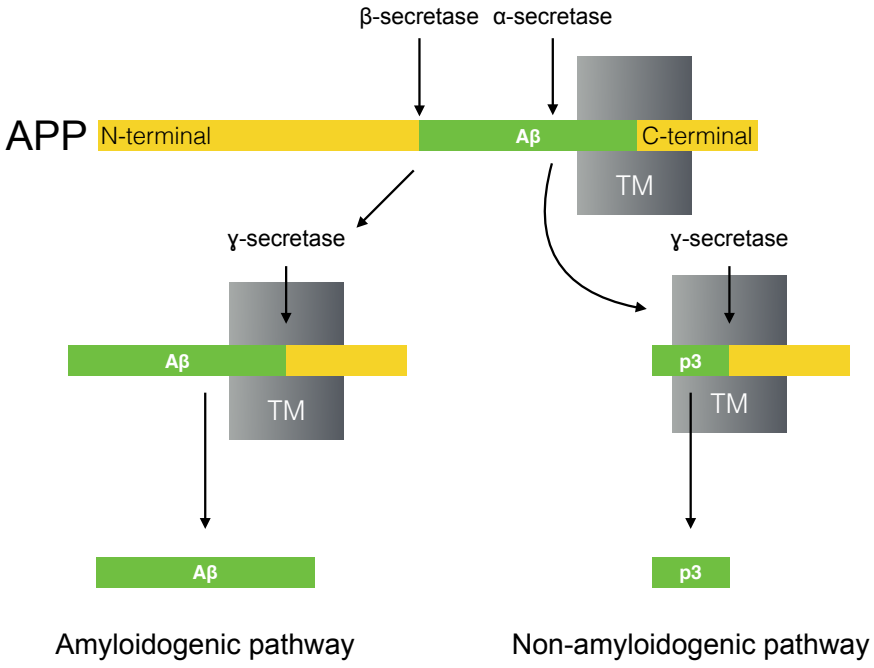

Figure 4. Different pathways in APP processing. APP cleavage by β- and γ-secretase result in the production of Aβ peptide, hence known as the amyloidogenic pathway. Cleavage by α- and γ-secretase results in the production of the nonpathogenic p3 peptide, known as the non-amyloidogenic pathway.

Aβ40 is the most abundant form of the Aβ, although research points to the fact that the main toxic component is Aβ42 (Younkin 1995). Previously, it was believed that Aβ toxicity was due to the plaques formed by aggregation and fibrillization of Aβ. In more recent years this theory has been revised and it is now believed that oligomers of Aβ formed during the aggregation process are the main reason for toxicity (Walsh and Selkoe 2004). This would also explain why certain findings indicate that there is no clear link between amount of plaques and clinical symptoms in AD patients (Braak and Braak 1991). The new theories about Aβ toxicity are further strengthened by the fact that certain Aβ mutations, which leads to early onset of AD, have been shown to produce increased numbers of oligomers (Nilsberth et al. 2001).

The second hallmark of AD is the presence of intracellular neurofibrillary tangles made of hyperphosphorylated tau (Grundke-Iqbal et al. 1986). In contrast to Aβ plaques, the amount of neurofibrillary tangles correlate with the clinical symptoms of AD (Braak and Braak 1991, Goedert and Spillantini 2006). Studies have suggested that synergistic effects of Aβ and tau can impair mitochondrial function and energy homeostasis (Rhein et al. 2009). It has also recently been suggested that the aggregation and toxicity of the Aβ and tau protein have similarities to that of the prion protein (PrP), in that toxic forms can be secreted and taken up by surrounding neurons, and misprocessed proteins can be used as a template for further aggregation of misfolded proteins (Jucker and Walker 2011, Hall and Patuto 2012).

Currently, there are no cures or treatments for stopping AD from progressing. Memory impairments as well as behavior changes can be treated to some extent with medication. However, no real cure has been found despite extensive clinical trials (Schneider et al. 2014). Almost 200 drugs have reached stage 2 clinical trials, however only cholinesterase inhibitors and memantine have been approved as treatments of AD over the past 30 years and both show limited effects. Lower synthesis of acetylcholine is associated with reduced cognition in dementia and AD (Perry et al. 1978, Francis et al. 1985) and raising the levels of acetylcholine increases the expression of acetylcholine receptors (Barnes et al. 2000) and is linked to the improvement of other neurotransmitter systems (Francis et al. 1993, Dijk et al. 1995). Therefore, by inhibiting the action of acetylcholine-hydrolyzing enzyme acetylcholinesterase, the levels of acetylcholine are raised and cholinergic neurotransmission can be improved (Giacobini 2003). Memantine is a uncompetitive, voltage-dependent glutamate receptor antagonist, with rapid blocking-unblocking kinetics and moderate binding affinity (Parsons et al. 1993). These properties allow memantine to interact with the glutaminergic signaling system and influence dysfunctional receptor activation in AD, which are otherwise overactivated (Danysz et al. 2000). As toxic Aβ oligomers have come more into focus as a main culprit behind AD (Hayden and Teplow 2013), strategizes that are directed to inhibit the aggregation of Aβ have emerged. In animal models it has been shown that antibodies against Aβ can improve memory and decrease the plaque load (Rasool et al. 2013). One Aβ antibody has been shown to decrease the amount of Aβ oligomers in the brain and CSF of a mouse model with no impact on the monomeric amounts (Tucker et al. 2015). However, several antibodies have been tested in clinical trials and have shown no benefits or even adverse effects in later phases (Schneider et al. 2014). Treatment strategizes also include indirectly targeting the Aβ aggregation by affecting the processing of APP with α-, β- or γ-modulators. Etazolate, a α-secretase activator have shown acceptable safety and tolerability (Vellas et al. 2011), with further clinical trials on-

going. β-secretase inhibitors have been tested, but development has been impaired by problems to cross the blood brain barrier. One β-secretase inhibitor, pioglitazone, showed acceptable tolerability, but no beneficial effects (Geldmacher et al. 2011). A γ-secretase inhibitor showed no improvement in cognition and was associated with adverse effects including skin cancer and infections (Doody et al. 2013). Tramiprosate, a compound reducing Aβ aggregation, gave negative results in a phase III trial (Aisen et al. 2011). There are currently several pharmaceutical candidates in various stages of clinical trials (Schneider et al. 2014).

1.1.2 Transthyretin amyloidosis

Transthyretin (TTR) is a protein present in blood and cerebrospinal fluid. It has a molecular weight of 14 kDa and appears as a homotetramer in its native state (Blake et al. 1978). Functionally, TTR has been shown to transport retinol (Vitamin A) and thyroxine and the name transthyretin is derived from thyroxine and retinol (Raz et al. 1970). TTR has also been suggested to be involved in development and regeneration of the nervous system (Soprano et al. 1985). In TTR amyloidosis the homotetrameric structure is destabilized, which causes the release of monomeric TTR subunits that are prone to misfold, aggregate and form amyloid fibrils (Quintas et al. 2001, Wiseman et al. 2005). The shift in equilibrium between monomers and homotetramers have been shown to be effected by high temperatures and low pH, resulting in an increased amount of monomers (Kelly et al. 1997). Over 100 point mutations have been associated with hereditary forms of disease (Connors et al. 2003) and these familial forms are often lethal. Several mutations are associated with destabilization of the homotetrameric form of TTR, leading to an increase in monomer content, resulting in amyloid formation (Hammarström et al. 2002). Mutations also affect where TTR is deposited in the body. Wild type TTR is mostly found as amyloid inclusions in the heart (Westermark et al. 1990), but is also found in liver and lungs (Pitkanen et al. 1984). The most common mutant TTR_{V30M} (Saraiva et al. 1984) is found as amyloid inclusion in the peripheral nervous system and gastrointestinal tract (Saraiva et al. 1984). The TTR_{V122I} mutation (Gorevic et al. 1989) is found exclusively as amyloid deposits in cardiac tissue and is carried by 4% of the African-American population (Jacobson et al. 1996). TTR is primarily produced in the liver and therefore a liver transplant can be used to replace the expression of mutated variants of TTR with wild type TTR, preventing neuropathy to some extent (Holmgren et al. 1991). In recent time, stabilizing the homotetramer of TTR with a small molecular compound has shown promise as a viable option to prevent amyloid inclusion and toxicity of TTR (Bulawa et al. 2012).

1.1.3 Diabetes mellitus

Diabetes mellitus is a group of diseases affecting over 380 million people (Guariguata et al. 2014) and is characterized by the lack of, or dysfunction of insulin and disturbed glucose and lipid metabolism. The two major forms are type 1 (T1D) diabetes and T2D. In T1D or juvenile diabetes the insulin producing β-cells are targeted through an autoimmune response, leading to decreased production of insulin (van Belle et al. 2011). The most common form of diabetes is T2D also known as adult-onset diabetes, which makes up for 90% of all diabetes cases. Development of T2D is affected by a number of genetic and environmental factors, such as age, obesity, diet and lifestyle. The disease is caused either by dysfunction of insulin producing β-cells or the failure of cells to respond to insulin secretion. Islet amyloid polypeptide (IAPP) is a 37 residue peptide (Westermark et al. 1987) naturally expressed in the β-cells of Langerhans islets and stored in secretory granules together with insulin (Lukinius et al. 1989). Several functions have been ascribed to IAPP, including effects on secretion of insulin (Gebre-Medhin et al. 1998), pain reduction (Huang et al. 2010) and involvement in calcium homeostasis (Dacquin et al. 2004). The insulin producing β-cells are found in the islet of Langerhans and in T2D patients amyloid deposits containing IAPP have been found within these islets (Westermark et al. 1986, Westermark et al. 1987). IAPP can form toxic oligomers, which can reduce the number of insulin producing β-cells (Gurlo et al. 2010). The working hypothesis of IAPPs toxicity is that the aggregation starts inside the cells and is then spread out from the cells, causing toxicity to surrounding cells (Paulsson et al. 2006). GAGs such as HS have also been shown to bind IAPP (Watson et al. 1997, Castillo et al. 1998), promoting fibril formation.

1.1.4 Other amyloid diseases

In Parkinson´s disease (PD), the protein α-synuclein is the major component of the abnormal aggregates called Lewy bodies (Spillantini et al. 1997). Although the Lewy bodies and α-synuclein fibrils are hallmarks of PD, recent research points to oligomers of α-synuclein having a significant toxicity and can cause cell death (Outeiro et al. 2008, Nasstrom et al. 2011, Winner et al. 2011).

Prion diseases are a group of diseases including Creutzfeldt-Jakob, bovine spongiform encephalopathy, scrapie, fatal familial insomnia and kuru (Liberski 2012) The prion diseases were first thought to be caused by a virus, but were later found to be caused by a specific protein that was named prion (from proteinaceous infectious particle) (Prusiner 1982). The prion is distinguished from other amyloid proteins because it can be transferred from one

individual to another by uptake of the misfolded PrP. The incubation periods of the prion diseases are usually long, but once symptoms appear, the disease can progress rapidly. Other amyloid proteins such as Aβ have also been suggested to have prion-like properties, such as seeding and cell-to-cell transmission of soluble oligomeric Aβ (Nath et al. 2012, Jucker and Walker 2013).

The most common localized amyloid is aortic medial amyloid and amyloid inclusions of this variant can be found in basically everyone over the age of 55 (Westermark and Westermark 2011). A cleavage product of the glycoprotein lactadherin, medin is the major component of amyloid inclusions in of the disease (Häggqvist et al. 1999). The 50 residue long peptide has been shown to form fibrils *in vitro* and the last 18-19 amino acids constitute the amyloid-promoting region (Larsson et al. 2007).

1.2 MOLECULAR CHAPERONES

Proteins need a certain three dimensional fold in order to function correctly, however proteins are in risk of misfolding and aggregating, which can lead to loss of function and toxic oligomers forming (Hartl et al. 2011). Proteins go through many different conformations, while they fold into their native state. The free energy landscape of protein folding is usually illustrated as a funnel that the protein travels down into to reach its native folded state (Fig 5) (Jahn and Radford 2005). However, the ruggedness of the energy landscape can result in proteins ending up locked in non-native, non-functional conformations. Aggregation and fibril formation are examples of this phenomenon and the amyloid fibril is considered to represent a global minimum of free energy.

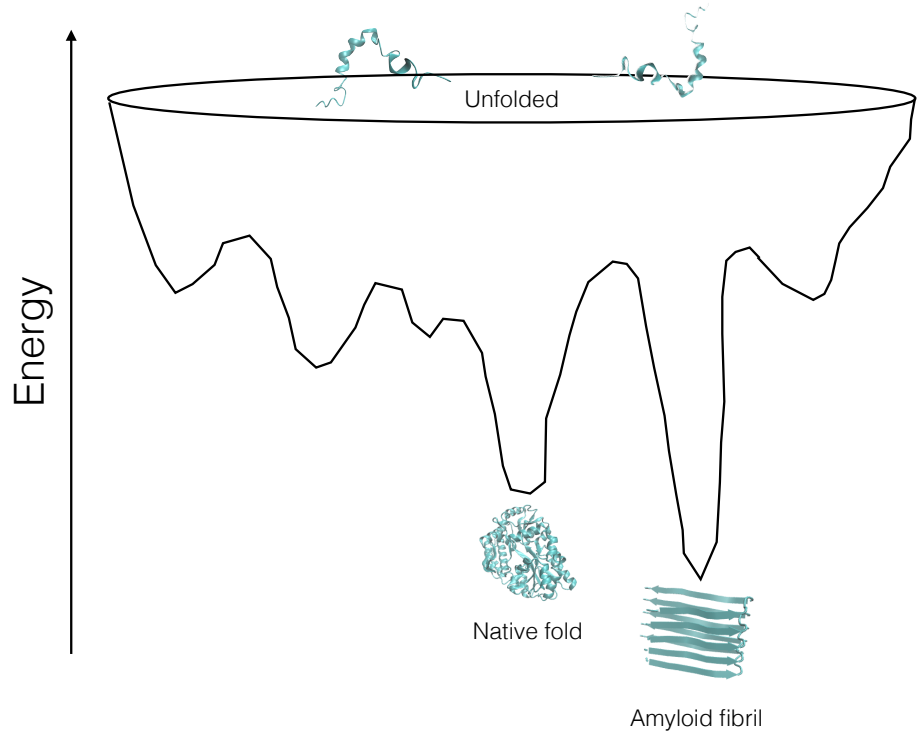

Fig 5. Schematic picture of funnel-shaped free energy landscape that proteins travel during folding. Images of native folded protein and amyloid fibrils were produced with VMD version 1.9.2 from structural data (www.pdb.org, PDB ID 1Z0Q (Tomaselli et al. 2006), 3G7W (Wiltzius et al. 2009) and 2BEG (Luhrs et al. 2005)). Adapted from Jahn and Radford, 2005 (Jahn and Radford 2005).

In order to prevent misfolding of proteins, cells need chaperones to assist in the folding of proteins (Hartl et al. 2011). The definition of a chaperone is a protein that can interact with, stabilize or help another protein to fold without being incorporated in its final structure (Hartl 1996). Proteins can misfold and aggregate due to cellular stress, including mutations, temperature changes, pH changes and ageing. Because several chaperone families are upregulated as a response to temperature increase, they are known as heat shock proteins (HSPs). There are six different families of HSPs, traditionally named after their molecular weight (Muchowski and Wacker 2005). The six families are HSPH (also known as HSP110), HSPC (HSP90), HSPA (HSP70), HSPD/E (HSP60/HSP10), DNAJ (HSP40) and HSPB (small HSP) (Kampinga et al. 2009). HSPA chaperones are important proteins in protein folding and proteostasis control and they contain an ATP-binding domain and a C-terminal substrate-binding site. HSPA, along with its co-chaperone DNAJ, have been shown to be involved in assembly of proteins into macromolecular complexes, prevention of protein aggregation and protein degradation (Broadley and Hartl 2009). Both HSPA and HSPC can inhibit aggregation of Aβ *in vitro*. HSPDs, also known as chaperonins, are large double-ring complexes that function by enclosing and folding substrate proteins up to sizes of around 60

kDa. The HSPD counterpart in *E.coli*, GroEL, has been shown to interact with at least 250 different proteins (Kerner et al. 2005). The human protein DNAJB6, belonging to the DNAJ family, was recently shown to inhibit the fibril formation of Aβ42 *in vitro* (Mansson et al. 2014). HSPBs are involved in several human diseases (Kampinga and Garrido 2012) and have been shown to reduce toxicity of Aβ oligomers in a transgenic mouse model (Ojha et al. 2011). There are also extracellular chaperones, such as clusterin (also known as Apolipoprotein J). Clusterin is a 75-80 kDa heterodimeric protein that can interact with a wide range of molecules and has shown to be involved in apoptotic disease states such as neurodegeneration (Jones and Jomary 2002). Clusterin can also inhibit the aggregation and fibril formation of Aβ and α-synuclein (Yerbury et al. 2007).

1.3 THE BRICHOS DOMAIN

The BRICHOS domain was discovered by Sanchez-Pulido et al in 2002. The name was derived from three of the protein families containing BRICHOS domains, Bri, Chondromodulin-1 and surfactant protein C (SP-C) (Sanchez-Pulido et al. 2002). The BRICHOS family was expanded in 2009, when it was found that BRICHOS domains are present in over 300 proteins (Hedlund et al. 2009) divided into 10 families (Fig 6).

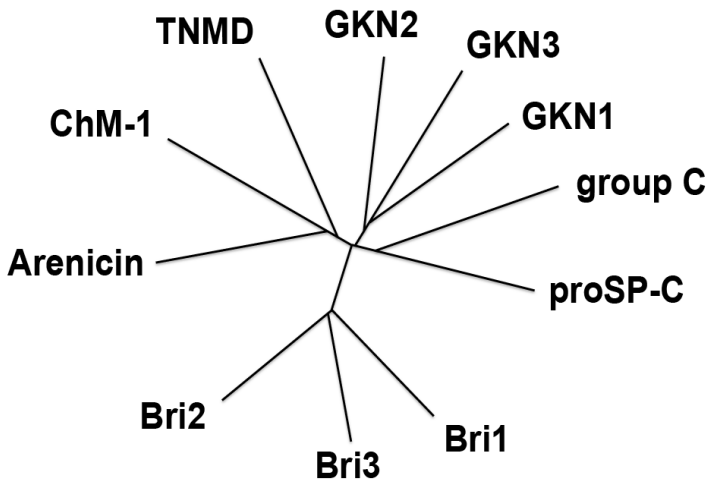

Figure 6. The BRICHOS family tree. The distance between the different families reflects their evolutionary separation. Image taken from Knight et al, 2013 (Knight et al. 2013) and reproduced with author's permission.

The BRICHOS domain is around 100 amino acids long and although the sequence similarity between different species is low, predicted structures of the BRICHOS domains are similar and they share some common motifs (Sanchez-Pulido et al. 2002, Hedlund et al. 2009). The N-terminal part of BRICHOS containing proteins is a cytosolic region, followed by a TM region, a linker, a BRICHOS domain and finally a C-terminal part (Fig 7). This is true for all

BRICHOS containing proteins with the exception of proSP-C, which lacks the C-terminal part. Moreover, all BRICHOS containing protein have a region of high ß-sheet propensity in the C-terminal part, whereas in proSP-C, the TM region instead has a high ß-sheet propensity (Willander et al. 2011). The overall sequence similarities are low between different BRICHOS domains, with only three amino acids being generally conserved, one Asp and two Cys (Sanchez-Pulido et al. 2002). The two Cys residues have been shown to form a disulphide bridge in proSP-C (Casals et al. 2008), which indicates that this is likely the case in other BRICHOS containing proteins as well.

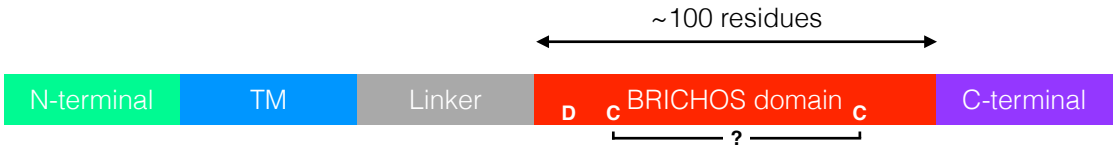

Figure 7. The general structure of a BRICHOS containing protein. The structure begins with an N-terminal region, followed by a TM region, a linker region, the BRICHOS domain and a C-terminal part. Most BRICHOS proteins share this structure with an exception being proSP-C, which lacks the C-terminal part.

The only structure of a BRICHOS domain currently available is the crystal structure of proSP-C BRICHOS (Willander et al. 2012a). The structure of proSP-Cs BRICHOS domain shows a unique folding motif (Willander et al. 2012a), where five β-strands form the central part. The central β-sheet is flanked by one α-helix on either side, one on face A of the protein and one on face B (Fig 8). Conserved residues and interstitial lung disease (ILD) associated mutations suggest that face A is involved in peptide binding. The hydrophobic surface of face A is normally blocked by α-helix 1, but molecular dynamic simulations showed that the movement of this α-helix makes the surface accessible for interaction with substrates (Willander et al. 2012a). Exposed side-chains of face A correlate with the assumed target peptides properties, which was first observed from comparison of proSP-C and Bri2. The postulated target region of the BRICHOS domain of proSP-C is the hydrophobic TM part and the face A of the proSP-C mainly consists of hydrophobic residues (Willander et al. 2012a). Bri2 BRICHOS on the other hand binds the C-terminally released peptide of Bri2, Bri23 (Peng et al. 2010), which is more polar and contains charged residues. In correlation with this, face A of Bri2 BRICHOS contains charged residues were proSP-C has hydrophobic residues. Other BRICHOS domains can also be modeled based on the proSP-C BRICHOS structure, and Bri family proteins and gastrokines show correlation between the target peptide and the proposed binding site (Knight et al. 2013).

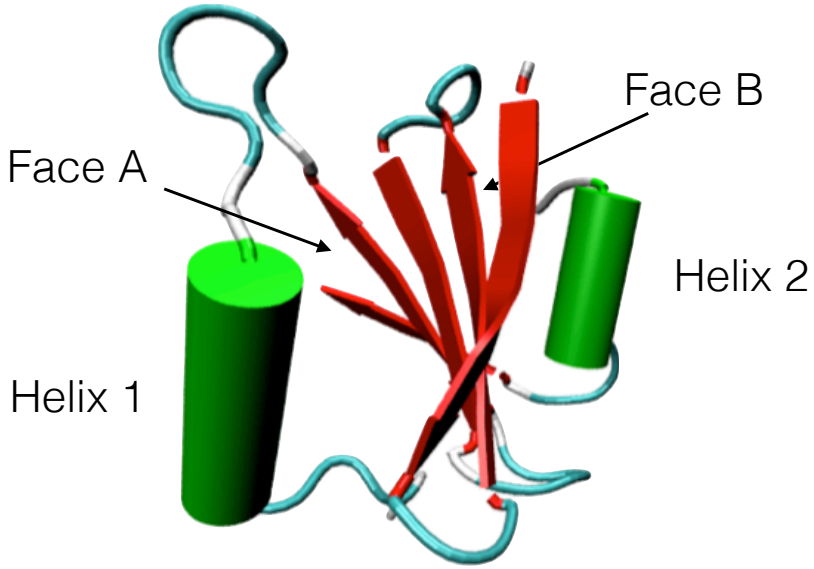

Figure 8. The structure of the BRICHOS domain of proSP-C. The structure has a unique fold and is composed of a five-stranded β-sheet and one α-helix on each side of the β-sheet. Image produced with VMD version 1.9.2 from the structural data (www.pdb.org, PDB ID 2YAD (Willander et al. 2012a)).

The BRICHOS domain has been suggested to have a chaperone-like function towards the ß-sheet propensity region in BRICHOS containing proteins (Willander et al. 2012a, Knight et al. 2013). In papers II-IV of this thesis, the effects of two BRICHOS domains, proSP-C and Bri2, on amyloid aggregation and toxicity have been studied.

1.3.1 ProSP-C

ProSP-C is the precursor protein to lung surfactant protein C (SP-C) and is a type 2 TM protein (N-terminal part located in the cytosol) (Keller et al. 1991). The function of lung surfactants is to lower the surface tension of the water-air interface and prevent lung collapse at the end of expiration (Whitsett and Weaver 2002). The precursor form is expressed as a 197 amino acid protein. A multistep cleavage process generates mature SP-C, starting with cleavage of the C-terminal ER luminal part, followed by cleavage of the N-terminal part (Beers et al. 1994, Johnson et al. 2001, Solarin et al. 2001). As a result, the 35 residue mature form of SP-C is produced. Mutations in the proSP-C gene (SFTPC) are associated with ILD (Nogee et al. 2001, Nogee et al. 2002), of which 55% are spontaneous mutations, while 45% are inherited (Hamvas 2006). The severity of disease and age of onset can vary between individuals with the same mutation (Hamvas 2006). Most mutations are located in the linker region or the BRICHOS domain, with I73T, a linker region point mutation, being the most common (Wert et al. 2009). It has been shown that I73T and Δ91-93 give rise to ILD with amyloid deposits in the lung tissue (Willander et al. 2012a).

The mature SP-C forms an α-helix and is inserted into the ER membrane. The α-helix structure of SP-C is stable when it is located in the membrane, but outside of the membrane the peptide can rapidly aggregate and form amyloid fibrils (Szyperski et al. 1998, Gustafsson et al. 1999). This is due to that the TM mature part of SP-C is primarily composed of Ile and Val, residues with a high β-sheet propensity (Kallberg et al. 2001) and therefor have high likelihood to form amyloid (Johansson et al. 2010). Replacing these residues in SP-C with a poly-Leu stretches with a higher α-helical propensity, results in a stable α-helical conformation (Nilsson et al. 1998) and this peptide that can be used to make a synthetic lung surfactant (Johansson et al. 2003). In order for the TM segment to fold correctly it has been suggested that the BRICHOS domain of proSP-C can act as molecular chaperone and facilitate formation of its α-helical fold (Willander et al. 2012a). This is supported by the fact that many mutations causing amyloid deposition and ILD are located in the BRICHOS domain. The crystal structure of proSP-C BRICHOS domain showed a trimer, which is also supported by analytical ultracentrifugation, size exclusion chromatography, native gel electrophoresis and electrospray mass spectrometry (Casals et al. 2008, Willander et al. 2012a). Molecular dynamics simulations suggests that the monomer is the active from, because helix 1 on face A needs to move away from the hydrophobic binding site to be exposed, which can not occur in the trimer (Willander et al. 2012a). Comparing the properties of face A of other BRICHOS families to their putative client peptides support that the trimer is an inactive storage form, while the monomer is the active form (Knight et al. 2013). Dissociation of trimeric proSP-C BRICHOS into monomers increases the capacity to inhibit Aβ42 fibril formation (Biverstal et al. 2015), supporting the theory that the monomer of BRICHOS is the active form.

The BRICHOS domain of proSP-C has been shown to prevent aggregation and fibril formation of the Aβ peptide of AD (Johansson et al. 2009, Nerelius et al. 2009a, Willander et al. 2012b). The BRICHOS domain interacts with Aβ, maintaining Aβ as an unstructured monomer for an extended time period thereby delaying fibrillization (Willander et al. 2012b). Recent data suggest that the proSP-C BRICHOS domain delays the fibril formation by binding to fibrils and blocking the secondary nucleation of Aβ42 (Fig 9) (Cohen et al. 2015). This alters the aggregation process of Aβ42, and the process proceeds through primary nucleation and elongation alone. Since the secondary nucleation has been shown to be a major source of oligomers during Aβ42 aggregation (Cohen et al. 2013), the effects of BRICHOS is a substantial reduction in the amounts of toxic oligomers created (Fig 9).

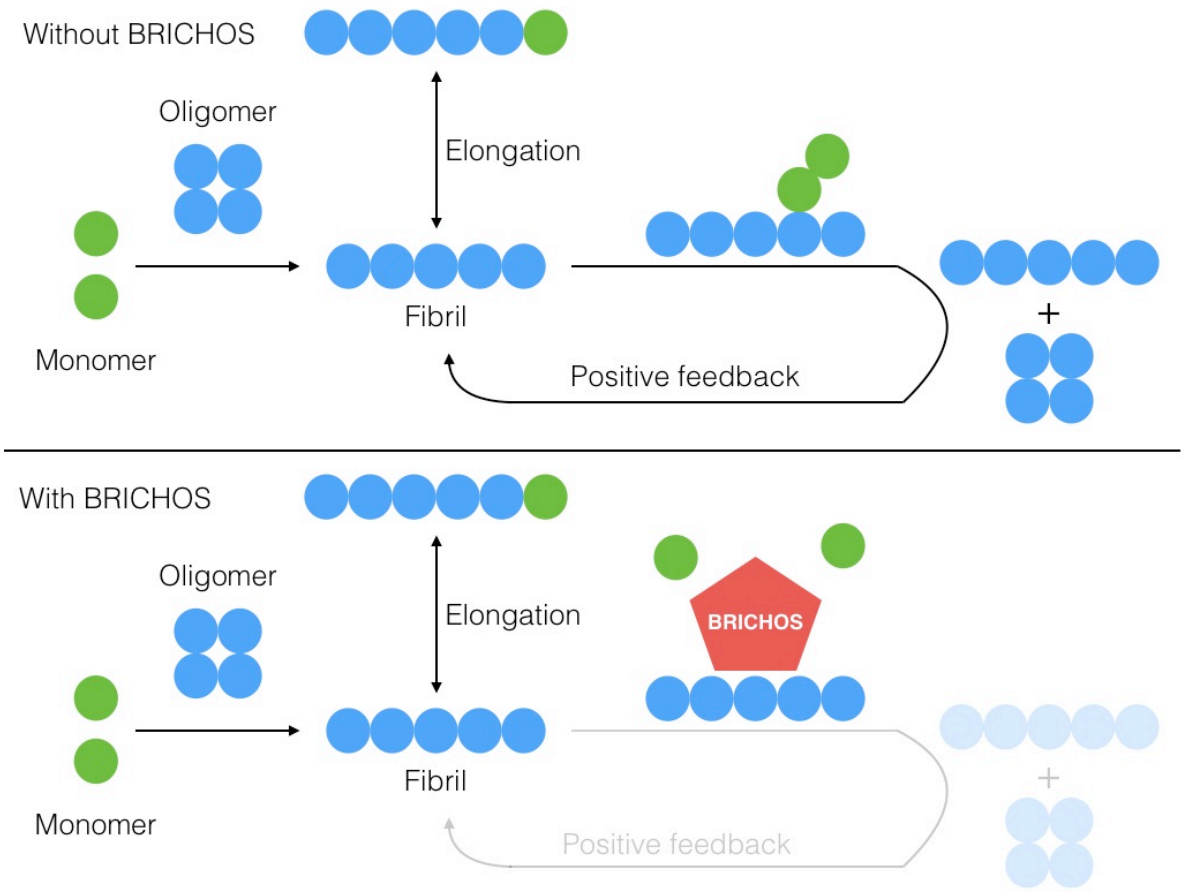

Figure 9. BRICHOS inhibits formation of toxic Aβ42 oligomers. Diagram of molecular pathways involved in Aβ42 fibril formation. BRICHOS binds to fibrils and inhibits the secondary nucleation, thereby preventing the major source of oligomer formation. Adapted from Cohen et al, 2015 (Cohen et al. 2015).

1.3.2 Bri2

Bri2 is a type 2 TM protein encoded by the gene integral transmembrane protein 2 B (ITM2B) (Deleersnijder et al. 1996). It belongs to the same family as Bri1 (ITM2A) (Pittois et al. 1999) and Bri3 (ITM2C) (Vidal et al. 2001) and is expressed both in the brain and several peripheral tissues. The initially produced, full length Bri2 is 266 amino acids long. Bri2 is cleaved in the secretory pathway by furin in the C-terminal part (Kim et al. 2002), which produces a 243 residue, mature Bri2 and a small, C-terminal peptide (Bri23) (Fig 10) (Kim et al. 1999). The mature Bri2 is cleaved by ADAM10 and the BRICHOS domain is subsequently secreted into the extracellular space. The remaining N-terminal part undergoes intramembrane proteolysis by SPPL2a/b (Martin et al. 2008). Mutations in the Bri2 gene can lead to the release of elongated C-terminal peptides, which can cause amyloidosis. In familial British dementia (FBD), a single base substitution leads to a 34 amino acid long amyloid peptide (ABri) being released (Vidal et al. 1999). In familial Danish dementia (FDD), a decamer duplication insertion causes a reading-frame shift and subsequent release of a 34 amino acid peptide (ADan) (Vidal et al. 2000). Both ABri and ADan can form fibrils and

deposit in the brain. However it has been suggested that ABri/ADan amyloid formation does not cause FBD/FDD, but rather the loss of function of Bri2 and concomitant effects on APP processing (Tamayev et al. 2010a, Tamayev et al. 2010b). Mouse models of both FBD and FDD have shown reduced expression of mature Bri2 and severe hippocampal memory deficits, but no signs of cerebral amyloidosis or taupathy. Moreover, $Bri2^{+/-}$ show similar deficits as the FBD/FDD mice, and memory loss in FDD mice can be prevented by co-expression of wild type Bri2. Bri2 has also been found to co-localize with Aβ in amyloid plaques, suggesting interaction during the misfolding and aggregation process (Tomidokoro et al. 2005, Del Campo et al. 2014). Mature Bri2 has also been shown to inhibit APP processing by interacting with secretases and reducing their access to APP in the intracellular compartments where APP is processed (Matsuda et al. 2011). Moreover, Bri2 has been recognized as one of the 20 most important mediators in ApoE4 carriers and late-onset AD patients (Rhinn et al. 2013).

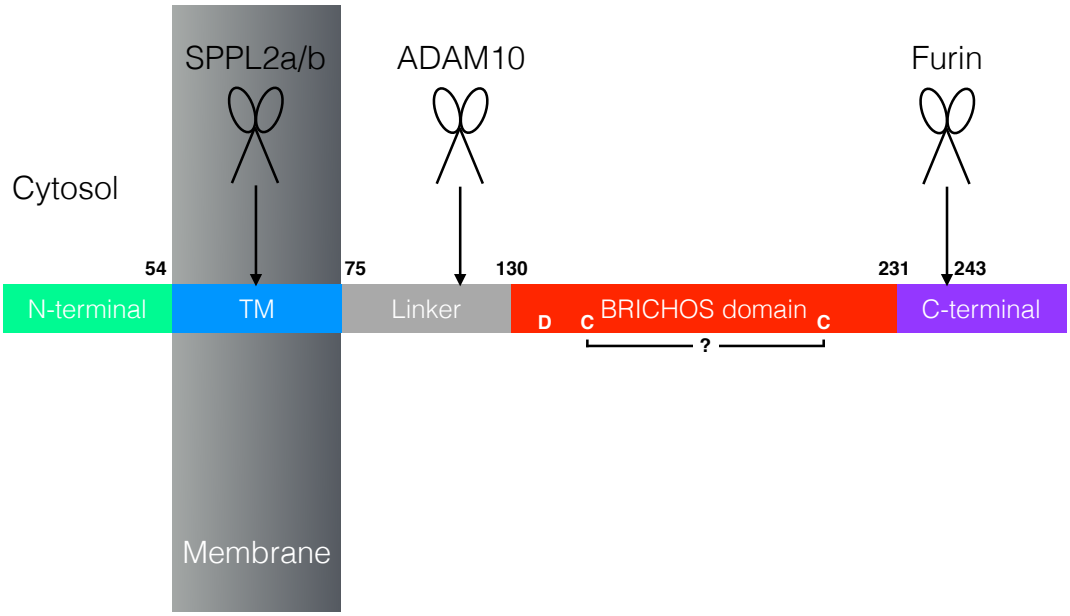

Figure 10. Processing of Bri2. The full length Bri2 is first cleaved by furin, producing the mature form and Bri23. Processing by ADAM10 releases the BRICHOS domain and the N-terminal part is subsequently cleaved by SPPL2a/b.

The structure of Bri2 BRICHOS has not been determined, however a homology model has been created based on data from the crystal structure of proSP-C BRICHOS (Willander et al. 2012b). This model suggests that the Bri2 BRICHOS has a similar structure as proSP-C BRICHOS, with a highly conserved central β-sheet core region.

Like proSP-C BRICHOS, Bri2 BRICHOS can interact with Aβ peptides, delaying fibril formation (Peng et al. 2010, Willander et al. 2012b). Moreover, Bri2 BRICHOS seems to be more effective in preventing Aβ aggregation than proSP-C BRICHOS (Willander et al. 2012b). Bri2 can also interact with APP and affect Aβ production (Fotinopoulou et al. 2005, Matsuda et al. 2005, Kim et al. 2008, Matsuda et al. 2008) and a fusion protein of Aβ and Bri2 expressed in a mouse model showed completely intact cognitive performance, in sharp contrast to APP expressing mice (Kim et al. 2013).

1.3.3 Other BRICHOS families

As previously mentioned, BRICHOS containing proteins can be divided into 10 distinct groups. Apart from Bri2, there are two more families of Bri proteins. Bri1 is a 263 residue long protein and has been linked to chondrogenic differentiation (Deleersnijder et al. 1996, Van den Plas and Merregaert 2004). Bri3 is mostly expressed in the brain (Vidal et al. 2001)

and proteolytically cleaved by furin in the same manner as Bri2 (Wickham et al. 2005). Furthermore, Bri3 has been shown to co-localize and co-immunoprecipitate with APP and overexpression of Bri3 reduces cleavage of APP by α- and β-secretase by blocking the target site of the secretases (Matsuda et al. 2009).

There are three families of gastrokines, GKN1, GKN2 and GKN3. GKN1 is expressed in gastric mucosa (Martin et al. 2003) and has been associated to gastric cancer. It is highly conserved and suggested to be important in mucosal protection (Oien et al. 2003, Oien et al. 2004). GKN1 has also been shown to inhibit amyloid formation of Aβ40 (Altieri et al. 2014). GKN2 is just like GKN1 expressed in gastric mucosa and associated to gastric cancer. It has been shown to interact with TFF1 and TFF2, members of trefoil factor family. GKN2 can form a heterodimer with TFF1 in humans (Westley et al. 2005) and the mouse homolog has been found to bind to TFF2 (Otto et al. 2006). GKN3 is a recently discovered group of gastrokines in mice, and associated to gastric atrophy (Otto et al. 2006). The human homolog seems to have become non-functional due to mutation resulting in a premature stop-codon.

Group C is a poorly characterized family of BRICHOS containing proteins that has been found in organism all the way down to *Drosophila melanogaster* and shows a high conservation in the C-terminal part (Hedlund et al. 2009). The protein is suggested to be 260 amino acids in humans and to have a N-glycosylating site. Chondromodulin-1 (ChM-1), also known as leukocyte cell-derived chemotaxin, is a 335 residues precursor protein. Processing through glycosylation and cleavage by furin renders a 28 kDa glycoprotein (Azizan et al. 2001). ChM-1 has been linked to chondrosarcoma and loss of ChM-1 expression effect suppression of tumor angiogenesis and growth (Hayami et al. 1999). Tenomodulin is a protein similar to ChM-1 and has found to be expressed in eye, skeletal muscle and whole rib of adult mouse. Elevated levels of expression have also been noted in mouse embryonic development (Shukunami et al. 2001, Yamana et al. 2001). Arenicin is an anti-microbial peptide found so far only in worms, for which the structure of the C-terminal peptide has been solved (Lee et al. 2007, Andra et al. 2008). It has been suggested that arenicin induces apoptosis through accumulation of intracellular reactive oxygen species and mitochondrial damage in fungal cells (Cho and Lee 2011).

1.4 DROSOPHILA MELANOGASTER

Drosophila melanogaster, commonly known as the fruit fly, is a fly species of the *Drosophilidae* family. It has been used extensively in research fields including genetics, physiology, biochemistry and evolution science. There are several advantages in using

Drosophila as a model system. Flies are easy to handle and don't require a lot of space, and costs are in general low. The flies only have four chromosomes, three autosomes and one sex chromosome. Female flies can produce up to 100 eggs per day, making generation of large numbers of flies easy. Males and females are also easily distinguishable under a light microscope. Meiotic recombination is not present in the males, which makes it easier to avoid unwanted recombination when crossing flies. A number of balancer chromosome fly lines can be used to avert unwanted recombination. A balancer chromosome is essentially a product of multiple chromosomal inversions. Homozygote balancer chromosomes lead to non-viable flies as do recombination of the inverted regions with non-balancer chromosomes. Balancer fly lines are usually also coupled to a specific phenotypical marker, which ensures that flies of different genotypes can be distinguished by external phenotypes such as curly wings, stubble hairs or eye color (Fig 11). Many analogs to human proteins exist in *Drosophila* and about 80% of disease related genes have a counterpart in the *Drosophila* (Reiter et al. 2001). Studying and using *Drosophila melanogaster* flies as a model system has a long scientific tradition and tracks back to the beginning of the 20th century. Many important scientific discoveries have been made using *Drosophila* as a model in research on hereditary effects, diseases, learning, and signaling pathways among others. In 1908, T.H Morgan selected *Drosophila* as a model of heredity. Among his findings were that the eye color was sex linked and the gene controlling this was located on the X chromosome (Morgan 1910), and this and other discoveries led to Morgan being awarded the Nobel Prize in 1933. In 1913, A.H. Sturtevant constructed the first genome map and was able to show that genes are arrange in a linear fashion (Sturtevant 1913). In 1917, H.J. Muller introduced balancers in *Drosophila* research (Muller 1918) and in 1927 he was able to show genetic damage and mutation in *Drosophila* using ionizing radiation (Muller 1927). The later discovery resulted in him winning the Nobel Prize in 1946. In 2000 the whole genome of *Drosophila* was sequenced (Adams et al. 2000). In 2011, the Nobel Prize in Physiology or Medicine was awarded to J. Hoffman for research about the innate immunity. Using a *Drosophila* model Hoffman demonstrated that the Toll gene was necessary for combating fungal infection by identifying pathogenic microorganism and triggering the immune response (Lemaitre et al. 1996).

Figure 11. Balancer phenotypes in *Drosophila* flies. The top row shows the wild type phenotypes and the bottom row the balancer phenotypes. The hairy shoulders phenotype adds additional hairs to the "shoulder" area. The stubble phenotype makes the hairs on the flies' backs shorter and thicker. The curly phenotype makes the wings curl instead of being straight like in wild type flies.

One of the more important developments in the *Drosophila* field is the introduction of the Gal4/UAS system (Brand and Perrimon 1993). The essential principle of this system is that a gene of interest is placed downstream of an upstream activating sequence (UAS), which is inserted into the fly genome by injection of a target vector into fly embryos. The gene of interest will not be expressed with this insert alone and to achieve expression, the transcription factor Gal4 is needed (Brand and Perrimon 1993). Expression of the transgene is promoted when Gal4 binds to the UAS. Gal4 is not naturally present in the *Drosophila*, as it is derived from *Saccharomyces cerevisiae.* Expression is achieved by crossing the responder line with a driver line, which has a cell or tissue specific expression of Gal4. As Gal4 is needed for expression of the gene of interest, the expression of the transgene will only occur in the cells with Gal4 (Brand and Perrimon 1993). Crossing the same responder line with different Gal4 driver lines can therefore generate flies with different expression levels and locations (Fig 12). This also allows for high toxicity genes to be studied, as the genes can be kept silent in the responder flies until crossed with driver flies. The expression of Gal4 is temperature dependent and higher temperatures results in higher expression levels (Duffy 2002). Several different UAS dependent genes can be combined in the same fly for studying co-expression of different proteins. Today, there are thousands of fly lines using the UAS/Gal4 system available. One of the most common drivers is the ElavC155 driver, which gives a pan-neuronal expression of Gal4 (Lin and Goodman 1994). Another commonly used driver is the glass multimer reporter (GMR) driver (Freeman 1996), which has a primary eye-

specific expression profile. The expression of a reporter such as green fluorescence protein (GFP) can also be incorporated into a driver line, which can be utilized to investigate which cells or tissues that express the gene of interest. The UAS system cannot only be used to induce expression, as several UAS controlled RNAi lines have been created and utilized. In this instance, the Gal4 is instead used to specifically suppress the expression of a certain gene (Brand and Perrimon 1993).

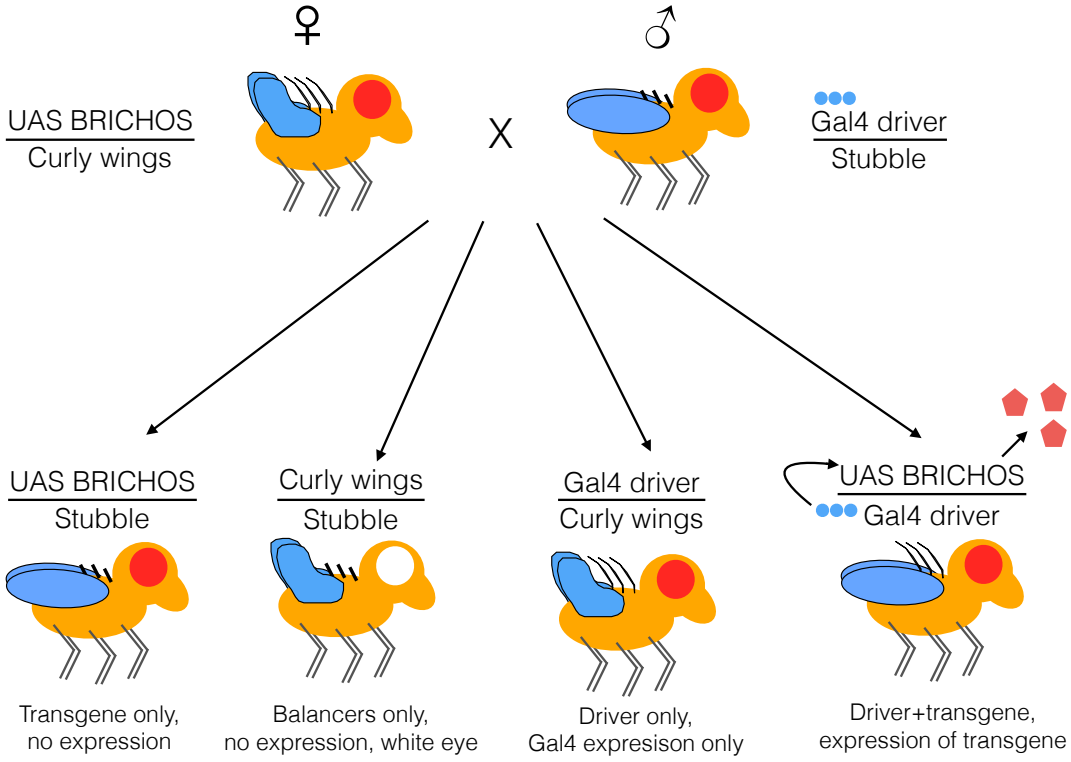

Figure 12. The UAS Gal4 system. When a female fly carrying the transgene of interest (in this case BRICHOS) is crossed with a Gal4 expression male fly, the resulting progeny containing both the UAS BRICHOS transgene and the Gal4 driver will express the BRICHOS domain. In other possible outcomes, the BRICHOS protein cannot be expressed. The white eye phenotype of the flies with only balancer results from this phenotype lacking transgene insert, which contains the element for red eye color.

1.4.1 Models of amyloid disease in *Drosophila*

1.4.1.1 Models of Alzheimer´s disease

There are several *Drosophila* models of amyloid diseases available. For Alzheimer´s disease, several models has been created and utilized in different manners. One strategy has been to create transgenic strains expressing human Aβ peptides with different length and mutations (Iijima et al. 2004, Crowther et al. 2005). In these models it has been shown that Aβ42 and $A\beta42_{E22G}$ (also known as the artic mutant) causes toxicity, reducing the locomotor activity and life span of the flies when expressed in the CNS. $A\beta42_{E22G}$ was also shown to be the

more toxic variant, with enhanced effects compared to the wild type Aβ42 (Crowther et al. 2005). Expression of Aβ42 in the eyes of the flies led to retinal degeneration. In another study, flies expressing two copies of Aβ42 fused together via a short linker (a tandem construct) were generated. Expression of the tandem constructs show highly reduced life span, malformation of eyes, increased deposition of Aβ42 and higher levels of soluble oligomers (Speretta et al. 2012). The malformation of the eyes in this model is shown in Fig 13. Another approach for studying Alzheimer´s disease and Aβ toxicity has been to develop flies expressing human APP together with BACE (β-secretase) to replicate the processing of APP into Aβ peptides. Constructing flies with targeted expression of APP, BACE and presenilins, the effects of APP with and without processing could be compared. It was found that APP with expression of BACE and presenilins lead to age-dependent neurodegeneration due to generation of toxic species, whereas expression of full length APP only did not have severe negative effects on the flies (Greeve et al. 2004). Aβ42 *Drosophila* models have been used in a number of studies aimed at finding inhibitors of Aβ aggregation and toxicity. One strategy includes co-expressing engineered proteins binding Aβ42 (Luheshi et al. 2010), which showed to be effective inhibitors for Aβ42 toxicity, increasing the life span and locomotor activity of the flies. Furthermore, the amount of insoluble Aβ was reduced. Another strategy is to feed the flies compounds mixed with their normal food. Curcumin was fed to flies in one study and the flies showed increased activity and life span when compared to flies only expressing Aβ42 or $A\beta42_{E22G}$ (Caesar et al. 2012). Interestingly, this study also showed that curcumin does not decrease the fibril formation, but rather accelerates the process. The reduced toxicity was therefore ascribed to curcumin reducing levels of toxic Aβ42 oligomers. In another study, synthetic ligands binding and stabilizing Aβ in a α-helical conformation were fed to Aβ42 expressing flies, which led to increased life span and reduced eye tissue destruction (Nerelius et al. 2009b).

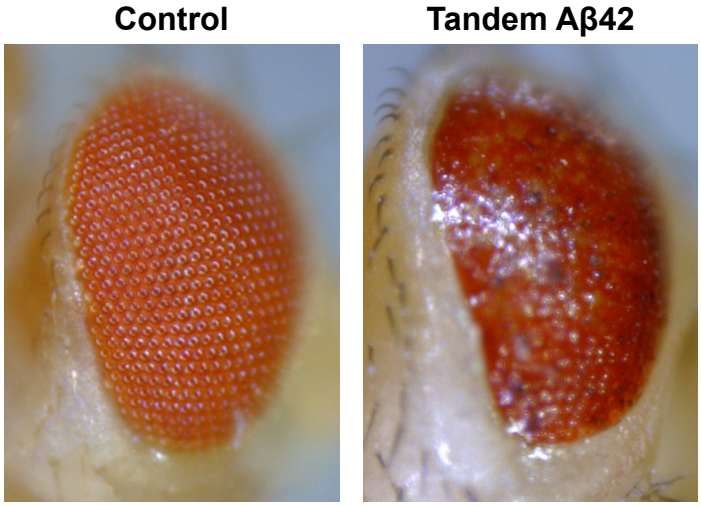

Figure 13. Aβ42 causes toxicity in flies. Expression of a tandem Aβ42 construct causes severe malformation of the eyes compared to the control flies (Speretta et al. 2012).

1.4.1.2 Transthyretin expressing flies

Drosophila models of familial amyloid polyneuropathy have been made by creating transthyretin expressing flies. Both wild type and mutants such as TTR_{V30M} have been created, along with an extra aggregation prone engineered double mutant variant $TTR_{V14N/V16E}$ (Pokrzywa et al. 2007). Expression of TTR_{V30M} and $TTR_{V14N/V16E}$ lead to decreased life span, neurodegeneration, reduced locomotor activity and eye tissue damage (Pokrzywa et al. 2007, Berg et al. 2009).

1.4.1.3 IAPP expressing flies

A model for studying T2D amyloidosis has been created (Schultz et al. 2011). In this work three different transgenic fly lines were created, expressing human proIAPP, human IAPP and non-amyloidogenic mouse IAPP. Only proIAPP reduced the life span of the flies, whereas human IAPP and mouse IAPP had no significant effects. Aggregates of both human proIAPP and human IAPP could be detected in the CNS and in fat bodies of the fly head (Schultz et al. 2011). The accumulation of human proIAPP and human IAPP in the fat bodies was further characterized and shown to result in structurally organized granules with a pentagonal rod-like structure (Schultz et al. 2011).

1.4.1.4 α-synuclein expressing flies

A *Drosophila* model of PD was published in 2000 (Feany and Bender 2000). In this model, wild type or mutant forms of α-synuclein are expressed leading to intraneuronal inclusions, decreased locomotor activity and eye degeneration (Feany and Bender 2000). All variants

showed similar toxicity. Co-expression of HSPA and α-synuclein in dopaminergic neurons in flies was later shown to protect against α-synuclein-induced neuronal degeneration (Auluck et al. 2002).

2 AIMS OF PRESENT INVESTIGATION

The overall aim of this thesis was to study amyloid diseases and investigate the effects of the BRICHOS domain on aggregation and toxicity of amyloid proteins. More specifically, we used *Drosophila* models to achieve these goals. The specific objectives were as follows:

Paper I: To characterize possible HS interactions with TTR and effects on aggregation.

Papers II and III: To study the effects of proSP-C and Bri2 BRICHOS on Aβ aggregation and toxicity *in vivo* using a *Drosophila* model system

Paper IV: To investigate possible association of Bri2 and IAPP in human pancreatic islets and study the effects of Bri2 BRICHOS on IAPP aggregation and toxicity *in vitro* and *in vivo*

3 MATERIALS AND METHODS

More detailed descriptions about the materials and methods used in this thesis can be found in papers I-IV. In this part I will go into some general concept of methods used in the different studies.

3.1 FLY GENERATION

We have generated our BRICHOS flies using a system called the φC31 (Bateman et al. 2006). The advantage of this system is that one can achieve site-specific expression, eliminating a number of problems related to random gene insertion such as variable expression levels or unwanted insertion into an essential gene. The φC31 integrase is derived from the bacteriophage φC31 and encodes a recombinase that mediates site-specific recombination between two attachment sites, attB and attP (Thorpe et al. 2000). Insertion of the gene of interest can be directed to a specific site by using the attB-sequence in a vector and a fly line that has an attP sequence already incorporated into a specific site in the genome.

For our fly lines we used the pUASTattB vector, a vector based on the pUAST vector (Brand and Perrimon 1993) containing five optimized Gal4 binding sites. Our constructs were inserted into the vector, which was then sent to BestGene Inc for injection and initial cultivation of fly lines. In study II or III we chose to incorporate our BRICHOS genes at site 86Fb, since this site have a high rate of successful integration (Bischof et al. 2007). The site is located on the third chromosome, which was important because most Aβ lines that we used have their transgene inserted in the second chromosome, making crossings easier.

3.2 LONGEVITY ASSAY

The longevity assays were performed by keeping 10 flies of a genotype per tube at constant temperature and humidity, changing the food every 2-3 days and counting the number of flies alive. Aβ42 expression in the CNS reduces the life span of the flies compared to control flies (Crowther et al. 2005). Variability can be caused by several factors, e.g. the food composition, temperature and humidity. If the food is too moist, the flies can get stuck and drown, if the food is to dry the flies and larval will be dehydrated. The relative temperature and humidity in the incubator also affects the flies' longevity. Higher temperature induces a higher expression of Gal4 (Duffy 2002), leading to increased expression of transgenes and possibly higher toxicity. Keeping constant temperatures, humidity levels and regular changing the food is essential to decrease the variability.

3.3 LOCOMOTOR ACTIVITY

The locomotor activities of groups of 5 flies were measured by counting the number of flies able to climb a certain height in a given time. It has previously been shown that flies expressing Aβ42 in the CNS develop age-dependent locomotor deficits compared to control flies (Crowther et al. 2005).

3.4 IMMUNOHISTOCHEMISTRY AND CONFOCAL MICROSCOPY

To visualize the expression and deposition of the various proteins used in these studies, antibody staining and visualization by confocal microscopy was used. Two different methods were used for visualization in the fly head and brain. In paper I, the flies were decapitated and the heads embedded and frozen in Tissue-Tek O.C.T compound followed by cryosectioning. In subsequent steps the sections were fixated and stained with antibodies for analysis by confocal microscopy. The advantage of this method is that the whole head can be studied, including the eyes and the brain, however due to sectioning the overall three dimensional structure is lost. The second method used was to dissect out the brain from the fly heads, fixating it and stained with antibodies followed by visualization of the whole brain. In doing so, the three dimensional structure of the brain is intact, but surrounding areas such as the fat bodies and the eyes cannot be studied.

3.5 PROXIMITY LIGATION ASSAY

In order to detect co-localization in tissue samples, we used proximity ligation assay (PLA, Olink) (Fredriksson et al. 2002, Soderberg et al. 2006). PLA, like conventional immunohistochemistry, uses antibodies, but with higher sensitivity and specificity. In order for a PLA signal to occur, dual binding events need to take place. In PLA, primary antibodies from two different hosts are used, binding to the same or different targets. Instead of a conventional fluorophore, the secondary antibodies are coupled with oligonucleotides, which are complementary. If these oligonucleotides are in close proximity, they can be ligated and amplified, giving a fluorescence signal. The amplification process results in high sensitivity.

3.6 THIOFLAVIN T ASSAY

Thioflavine T (ThT) is a benzothiazole salt that is used for measuring the fibril formation of amyloid proteins. When ThT binds to β-sheets its fluorescence emission increases and a characteristic red shift in wavelength of emission maximum is seen (LeVine 1999).

3.7 CELL APOPTOSIS ASSAY

Caspase-3 is a member of a group of proteases activated during cellular apoptosis (Taylor et al. 2008). The target for caspase-3 is a four amino acid (DXXD) motif. Stable cell lines transfected with a vector producing two fluorophores linked by a DXXD sequence can be used to monitor caspase-3 activity (Kohler et al. 2003). When the fluorophores are linked to each other, fluorescence energy transfer (FRET) occurs and the signal can be measured. When apoptosis occurs, caspase-3 cleavage of the linking region results in loss of the FRET signal.

4 RESULTS

4.1 PAPER I

Since wild type TTR is stable in homotetrameric configuration, one can argue that factors that can cause a shift into aggregation prone monomers will affect amyloid propensity. HS has been associated with several amyloid diseases and found to promote fibril formation. In this study we examined the association and effects of HS on TTR. Staining myopathic heart tissue from an elderly patient using Congo red showed the characteristic birefringence and staining with Alcian Blue, for detection of HS, revealed a similar pattern. TTR and HS therefore seem to co-localize in heart tissue, which was supported by using antibodies against TTR and HS.

The effect of HS on aggregation of TTR was investigated with a ThT assay under acidic conditions. The addition of HS to TTR promoted the fibril formation and high sulfated heparin increased the fibrillization more than lower sulfated forms of HS. The length of the polysaccharide chains also had an effect, and heparin was the overall most effective compound in increasing the fibril formation.

The interaction of TTR and HS was characterized using surface plasmon resonance. The binding of heparin to TTR was significantly higher at low pH than at higher pH, and higher sulfation degree increased the affinity of HS. By using peptide fragments and full length TTR incubated with heparin, the region of binding in TTR was localized to residues 24-35. This region contains three basic amino acids, which are likely involved in the interaction with HS. Since this region is buried in the native homotetramer of TTR, dissociation into monomers is needed for effective binding of HS.

Comparing wild type and HS-deficient cells incubated with TTR, showed significantly higher TTR fibrillization for wild type cells. Finally, we used a *Drosophila* model expressing $TTR_{V14N/V16E}$, an engineered, aggregation prone variant of TTR. Since the valines are buried in a hydrophobic environment in wild type TTR, the exchange to polar residues causes destabilization leading to heightened aggregation tendency. The flies were fed with either standard media, heparin supplemented food or low molecular weight heparin supplemented food. Lysates of the heads from flies fed with heparin supplemented food gave ThT and Alcian blue signal, whereas standard media and low molecular weight heparin supplemented food did not. Sectioning the heads and staining with antibodies for heparin and TTR revealed co-deposition in the retina, which was not present in control flies.

To summarize our findings, HS can be found co-localized with TTR in a myopathic heart tissue and the presence of HS seems to promote fibril formation of TTR both *in vitro* and *in vivo*. In addition, we identified that the region of residues 24-35 of TTR contains the binding site of HS and that low pH is necessary for effective binding.

4.2 PAPER II

It has previously been shown *in vitro* that the BRICHOS domain of proSP-C can interact with Aβ42 and inhibit its aggregation into fibrils (Johansson et al. 2009, Nerelius et al. 2009a, Willander et al. 2012b). To examine proSP-C BRICHOS effects on Aβ42 in a living organism, we generated a UAS/Gal4 dependent *Drosophila melanogaster* model expressing the linker region and BRICHOS domain of proSP-C coupled to a signal peptide from lung surfactant protein B. We used the φC31 system for site-specific insertion into the 3rd chromosome. These flies were crossed with Aβ42 expressing flies with either one or two copies of Aβ42 (Crowther et al. 2005). The expression levels were compared using qtPCR, which showed no effect on expression levels of Aβ when co-expressing proSP-C BRICHOS. Longevity and locomotor assays of non-expressing transgenic flies were also done to ensure that the transgenic insertion as such did not affect the flies.

Expressing Aβ42 in either one or two copies in the CNS of the flies using the Elav^{C155} driver resulted in decreased life span and locomotor activity when compared to control flies, with two copies of Aβ42 resulting in higher toxicity. Co-expressing Aβ42 and proSP-C BRICHOS showed increased life span and locomotor activity compared to flies only expressing Aβ42.

The effects of proSP-C BRICHOS on Aβ42 were further studied by dissecting out the brain of the flies and staining with antibodies against Aβ and proSP-C BRICHOS. Accumulation of Aβ42 was delayed when co-expressed with proSP-C BRICHOS, and proSP-C BRICHOS and Aβ42 could furthermore be co-localized in the central parts of the brain. An *in vitro* experiment using electron microscopy showed that proSP-C BRICHOS binds Aβ42 fibrils. Finally, we showed that the presence of proSP-C BRICHOS increases the ratio of soluble to insoluble Aβ42.

The results of the study show that proSP-C BRICHOS can have an inhibiting effect on Aβ42 aggregation and toxicity *in vivo*. Furthermore, proSP-C BRICHOS was shown to co-localize with Aβ fibrils and affect the levels of soluble/insoluble Aβ42. The presence of Aβ42 deposits at later time points, but without severe toxicity, indicate that proSP-C BRICHOS interferes with the formation of toxic species rather than preventing Aβ deposition altogether.

4.3 PAPER III

Having shown that the BRICHOS domain of proSP-C, a lung surfactant protein, could prevent toxicity and aggregation of Aβ42 (Paper II), we wanted to examine if the same was true for other BRICHOS domains. Previously, we have shown that the BRICHOS domain of Bri2 can inhibit fibril formation of Aβ42 in a more efficient way than proSP-C BRICHOS (Willander et al. 2012b).

We generated a fly strain with an insert of residue 90-236 of Bri2 together with a signal peptide from lung surfactant protein B. The flies were generated in the same manner as in paper II, although in this study, only flies with one copy of Aβ42 were used and different food composition, temperature and humidity levels were used. ProSP-C BRICHOS expressing flies were also generated to compare effects of two different BRICHOS proteins. qtPCR analysis of mRNA levels from fly heads showed that amounts of Aβ42 expressed was unaffected by the expression Bri2 BRICHOS.

Co-expression of Bri2 BRICHOS and Aβ42 showed improved survival over flies expressing Aβ42 alone. The expression of Aβ42 reduces the climbing ability of the flies when compared to control flies only expressing Gal4. Co-expression of either proSP-C BRICHOS or Bri2 BRICHOS causes the climbing ability to improve, with Bri2 BRICHOS showing the largest improvement.

Expressing Aβ42 in the eyes of the flies causes malformation and reduced diameter of the rhabdomeres of the flies' ommatidia. These effects can largely be inhibited by co-expression of Bri2 BRICHOS, while proSP-C BRICHOS seems to inhibit the reduction of the rhabdomers diameter to a somewhat smaller extent. Expression of Bri2 BRICHOS or proSP-C BRICHOS alone results in no phenotypical changes compared to control flies.

Localization of BRICHOS and Aβ42 was studied in the fly brain using immunofluorescence and confocal microscopy. Interestingly, the staining pattern of flies expressing Aβ42 alone and co-expressing Bri2 BRICHOS with Aβ42 was drastically different. In flies only expressing Aβ42, the Aβ is detected around the antennal lobes of the fly brain, while co-expression with Bri2 BRICHOS results in staining in the mushroom bodies, where Aβ42 co-localizes with Bri2 BRICHOS. Co-expression of proSP-C BRICHOS and Aβ42 instead leads to co-localized signal around the antennal lobes and the amount of Aβ42 deposits are reduced. Expressing Aβ42 together with GFP as a reporter protein results in no visible changes in the mushroom bodies structure compared to control flies.

Analyzing the amounts of soluble and insoluble Aβ42, it was seen that co-expression of Bri2 BRICHOS reduces the amounts of insoluble Aβ42. The soluble level seems largely unchanged by the co-expression of Bri2 BRICHOS.

In summary, Bri2 BRICHOS seems to be more efficient in inhibiting the toxic effects of Aβ42 than proSP-C BRICHOS. The difference in localization of Aβ42 in the fly brain indicates possible differences in the mechanism of action by the two BRICHOS domains. Bri2 BRICHOS ability to prevent Aβ42 toxicity and association to APP and Aβ (Matsuda et al. 2008) makes Bri2 BRICHOS a very interesting protein that could be harnessed for AD treatment.

4.4 PAPER IV

IAPP is a small peptide associated with T2D, which can aggregate and form amyloid fibrils. Bri2 is expressed in several tissues in the body and the anti-amyloid mechanism makes it an interesting candidate to study in conjunction with IAPP.

The presence of Bri2 in isolated human islets was shown using extracts and pancreatic sections and reactivity seems to appear in the β-cells and co-localize with insulin. However, comparing the staining of insulin and Bri2 indicates that Bri2 also is present in other cell types in the islets. Bri2 presence was furthermore shown by mRNA analysis, which showed that Bri2 is expressed in human islets. Culturing islets from donors in either normal or high glucose levels caused the mRNA levels of Bri2 to decrease, while levels of IAPP mRNA increased significantly.

Pancreatic sections from T2D patients were analyzed using PLA, which showed co-localization of IAPP and Bri2 in islet amyloid deposits. Sections from healthy individuals without amyloid deposits showed no co-localization of IAPP and Bri2, suggesting that co-localization and interaction occurs only in amyloid deposits and not in β-cells.

Using either ThT or pFTAA assays showed that Bri2 BRICHOS prevents IAPP from forming fibrils, even at substochiometric amounts and in the presence of cellular components.

Bri2 BRICHOS ability to prevent IAPP toxicity was studied using a caspase-3 fluorescence energy transfer (FRET) assay. In this assay, addition of IAPP induced apoptosis and this effect was concentration dependent. A 1:1, 2:1, 4:1 and 8:1 ratio of IAPP and Bri2 BRICHOS reduced the toxic effects. However a 10:1 ratio of IAPP and Bri2 BRICHOS increases the toxic effects of IAPP leading to more cell death, which indicates that substochimetric

additions of Bri2 BRICHOS can have a negative impact on cell survival, possibly due to formation of toxic species of IAPP.

The possible inhibition of IAPP toxicity by Bri2 BRICHOS was also evaluated in a *Drosophila melanogaster* model. By expressing IAPP and Bri2 BRICHOS in 8 lateral ventral neurons of each brain hemisphere of a fly, together with GFP as a reporter protein, the number cell nuclei could be counted. IAPP expression alone is toxic and causes a reduction in cell nuclei compared to control flies. Co-expression of IAPP and Bri2 BRICHOS increased the number of cell nuclei significantly, compared to IAPP only, showing that Bri2 BRICHOS can have a rescuing effect *in vivo*. Bri2 BRICHOS only expression gave no significant difference in cell numbers compared to control flies.

Our study suggests that Bri2 could be associated to IAPP in the amyloid fibril forming process in T2D. Bri2 is co-expressed with IAPP and insulin in β-cells and can be co-localized with IAPP in T2D patients. Bri2 BRICHOS can also prevent aggregation and fibril formation of IAPP *in vitro*, and also reduce the toxicity of IAPP in cell culture and *in vivo* in a *Drosophila* model. Taken together, this indicates that Bri2 could reduce aggregation and toxicity of IAPP and possibly be used as a novel treatment for T2D.

5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

HS has been implicated to play a vital role in several amyloid diseases and shown to promote fibril formation. In paper I, we showed that HS is co-localized with TTR in heart tissue of an elderly patient and promotes fibrillization *in vitro*. Moreover, the binding region of TTR was identified and we could show decreased fibril formation in HS-deficient cells and co-localization of heparin and TTR in a *Drosophila* model. The interactions, binding and co-localization of HS and TTR can be an important factor in TTR amyloidosis.

In paper II and III we show that the BRICHOS domain can prevent aggregation and toxicity *in vivo* by using a *Drosophila* model, reflecting previous *in vitro* results (Willander et al. 2012b), Bri2 BRICHOS is seemingly more potent than proSP-C BRICHOS in preventing aggregation and toxicity of Aβ42. Interestingly, as seen in the confocal images of the fly brain, the distribution pattern of Aβ42 is markedly different when co-expressed with proSP-C compared to Bri2 BRICHOS. This could be due to different mechanisms of action. It has been shown that proSP-C BRICHOS binds fibrils and prevents secondary nucleation specifically (Cohen et al. 2015), while the mechanism of Bri2 is currently being investigated. Co-expression of Aβ42 and Bri2 BRICHOS results in Aβ42 distribution in the mushroom bodies of the fly brain, which is interesting as this structure plays a important role in olfactory learning and memory (Davis 1993, Heisenberg 2003, Busto et al. 2010) and a *Drosophila* APP ortolog, essential for long-term memory (Goguel et al. 2011), is highly enriched in the mushroom bodies (Torroja et al. 1996).

The fibril formation inhibition by the BRICHOS domain has not been explored in detail for amyloid proteins other than Aβ, but the BRICHOS domain of proSP-C has also been shown to inhibit fibril formation of medin (Nerelius et al. 2009a). Bri2 is expressed in several tissues in humans and could therefore be associated with other amyloid diseases than AD. We investigated if Bri2 is expressed in pancreatic islets involved in T2D and examined possible interactions of Bri2 and IAPP. We found that Bri2 is expressed in the islets and co-localizes with IAPP in amyloid deposits. Interestingly no co-localization of IAPP and Bri2 could be detected in tissue lacking amyloid deposits, possibly indicating that Bri2 only binds to IAPP in aggregated and amyloid form. This is in line with recent findings about the interaction between proSP-C BRICHOS and Aβ (Cohen et al. 2015), indicating a possible common mechanism for BRICHOS domains in general. We also showed that Bri2 BRICHOS could prevent the fibril formation of IAPP for an extended time period in a similar, but more potent, manner as seen before with Aβ. Moreover, Bri2 BRICHOS can under certain conditions prevent toxicity of IAPP, as seen in cell toxicity assays and the *Drosophila* model. However,

using higher molar ratios of IAPP:Bri2 BRICHOS leads to increased toxicity. Further studies are required to determine the basis of this observation, but it could possibly be due to that Bri2 BRICHOS slows down the fibril formation process, and thereby extends the time under which toxic oligomers are present.

Utilizing a fly model for studying amyloid diseases is a viable option for looking at mechanisms and toxicity in an *in vivo* system. As mentioned previously, many important scientific insights and discoveries have come from the use of *Drosophila* as a model system. Comparing the results in papers I and IV obtained using fly models and human tissue sections, one can see that they agree well. In paper I, we showed that TTR can co-localize with HS in heart tissue from a myopathic patient. Using a fly model expressing TTR we show that by feeding the flies with heparin supplemented food, TTR and HS could be co-localized in the fly's heads. In paper IV we used human cell lines to study toxicity of IAPP and likewise, IAPP had a toxic effect on cells when expressed in lateral ventral neurons in a *Drosophila* model. Different amyloid proteins also have potentially different toxic effects, which is also reflected in the fly models. In paper II and III we use an Aβ42 fly model that in both studies decreases the longevity of the flies when expressed throughout the CNS. IAPP on the other hand, has previously been shown not to affect the longevity of the flies when expressed in the same way compared to control flies (Schultz et al. 2011). Interestingly, when expressing IAPP in the lateral ventral neurons using the pdf driver, IAPP expression decreases the number of cell nuclei. However, expression of Aβ42 with the same driver results in no significant difference from the control flies (Schultz 2011). As HS also has been linked to IAPP aggregation and shown to promote IAPP fibril formation (Watson et al. 1997, Castillo et al. 1998), the fly model of IAPP could be used to study this interaction *in vivo* with a similar strategy as used in paper I with TTR and HS. The field of *Drosophila* research is, as all other research fields, ever-evolving, and new genetic tools become available all the time. In 1993 the UAS/Gal4 system was introduced and further developed during the subsequent years. The last years have introduced techniques such as the CRISPR/Cas9 system (Gratz et al. 2013) in the *Drosophila*, facilitating precise deletion, insertion and sequence replacement in fly lines. These genetic tools, together with the many fly models of amyloid diseases available, makes *Drosophila* a viable choice for further studies of amyloidosis *in vivo*. There are of course drawbacks in using *Drosophila* as a model organism. The flies are small and fragile to environmental changes such as temperature or humidity, and due to their size the amounts of expressed protein is low, which in turn requires sensitive techniques for analysis. In studies with implications for human medicine one must also consider that the flies are

much less complex when compared to humans, e.g. their blood brain barrier is rudimentary compared to humans.

The BRICHOS domain models introduced in papers II, III and IV could be used to study other aspects of the BRICHOS domain *in vivo*, or to investigate effects of BRICHOS domains on other amyloid proteins, such as TTR or α-synuclein. We have also generated a model of proSP-C BRICHOS$_{L188Q}$, a mutant associated with ILD. This model could be used to study how mutations in the BRICHOS domain affect the functionality compared to the wild type counterpart *in vivo*.

The BRICHOS domain is far from the sole example of a chaperone inhibiting amyloid formation. As mentioned previously, HSPs have shown to exhibit similar effects on amyloid proteins (Auluck et al. 2002) and in a recent study the molecular chaperone DNAJB6, belonging to the DNAJ family, was shown to inhibit both primary and secondary nucleation of Aβ42 (Mansson et al. 2014). It has also been shown that non-chaperone proteins can exhibit chaperone-like inhibition of Aβ40 aggregation (Luo et al. 2014). The results of this thesis suggest that BRICHOS could in the future be used in treatment of AD. One general problem in AD treatment is getting a drug across the blood brain barrier. Getting a sizable protein domain to cross is challenging, although modern molecular engineering can be used to modify drugs for easier transport over the barrier (Pardridge 2012, Pardridge and Boado 2012). In the case of Bri2 BRICHOS, there could be alternative strategize like cell transplantation, or utilizing the endogenous protein, by e.g. upregulating its expression and/or modulate the processing of Bri2. As Bri2 is present in several other organs such as the pancreas, this could also be applicable for harnessing Bri2 in prevention of other amyloid diseases. A natural next step after showing inhibition of Aβ42 and IAPP in *Drosophila* is to continue research in another *in vivo* model, such as mice. Bri2 has already been shown to inhibit aggregation and toxicity of Aβ42 in a mouse model (Kim et al. 2013), however this was done with a fusion protein of Aβ42 and Bri2. Expressing Aβ42 in the mice and injecting BRICHOS to study possible treatment effects would give a more realistic situation, and could also be used to see if BRICHOS is transported across the blood brain barrier to any significant extent. Mice with Aβ42 and BRICHOS expressed individually could be constructed to study possible effects of upregulation of BRICHOS. There are a number of APP-mouse models which could be interesting to used instead of just mouse models expressing Aβ, especially seeing as Bri2 has been shown to modulate the processing of APP (Fotinopoulou et al. 2005, Matsuda et al. 2005, Matsuda et al. 2008). Several mouse models of APP have been created and shown behavioral and pathological characteristics similar to

AD (Hsiao et al. 1996, Sturchler-Pierrat et al. 1997). Knock-in models of human APP have led to overexpression and artificial phenotypes as a result of other APP fragments are overproduced alongside Aβ. A recent mouse model tried to solve this issue by humanizing the mouse APP sequence and introducing mutations to increase the ratio of Aβ40/Aβ42 or Aβ42/Aβ40 (Saito et al. 2014). The mice showed Aβ accumulation, neuroinflammation and memory impairment in an age-dependent manner reminiscent of AD.

As mentioned previously it was recently shown that increased dissociation of proSP-C BRICHOS trimers into monomers, either by substrate addition or mutation, increases the inhibition potential against Aβ42 (Biverstal et al. 2015). This further strengthens the possibility that the monomer is the active form, but also suggests that modification to BRICHOS structure could enhance its capacity for fibril formation inhibition and possibly prevention of toxicity. This could be tested *in vivo* by using a *Drosophila* model system with the same approach as we have used in papers II, III and IV, which would also give an important indication if increased dissociation into monomers would cause any side effects.

Summarizing our results from paper II, III and IV, we have shown that the BRICHOS domain can prevent toxicity of amyloid proteins in *in vivo* model systems. These results show that harnessing the effect of the BRICHOS domain against amyloid formation and toxicity could provide a novel candidate for pharmaceutical use against amyloid diseases.

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