Modeling Amyloid Disease in Drosophila melanogaster

Ina Berg
Abstract

Amyloid diseases are caused by protein misfolding and aggregation. To date there are 27 known proteins causing amyloid disorders involving brain and peripheral protein deposition. The proteins involved in this mechanism do not share sequence homology, but the amyloid fibrils share biophysical properties and possibly a common pathogenic mechanism. Amyloid deposits are known to be involved in a broad range of neurodegenerative diseases, such as Alzheimer’s disease and Creutzfeldt-Jakob disease, as well as in non-neuropathic diseases, such as senile systemic amyloidosis and type II diabetes.

During the last decade the fruit fly, *Drosophila melanogaster* (*Drosophila*), have increasingly been used as a model for neurodegenerative disease, such as Alzheimer’s disease, Huntington’s disease, amyotrophic lateral sclerosis, and familial amyloidotic polyneuropathy. The advantages of using the *Drosophila* model are the well-defined genetic characteristics, the quantity, short life span, simplicity in genetic manipulation and the powerful binary UAS-Gal4 transgenic system. The UAS-Gal4 system allows for rapid generation of individual strains in which expression of a specific gene of interest can be directed to different tissues or cell types. The system allows the target gene to be activated in different cell- and tissue-types by altering the activator-expressing lines.

This thesis has been focused on modeling amyloid diseases in *Drosophila*. This has been performed by:

- Creating new model systems of senile systemic amyloidosis and familial amyloidotic polyneuropathy in *Drosophila*
- Developing a new staining protocol for detection of amyloid in *Drosophila*
- Initiate a compound screen of Alzheimer’s disease modeled in *Drosophila*
During the course of the research underlying this thesis, Ina Berg was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.
List of papers

This thesis is based upon the following papers, which are referred to in the text by their roman numerals:

**Paper I**
Berg, I., S. Thor, and P. Hammarström  
Modeling familial amyloidotic polyneuropathy (Transthyretin V30M) in Drosophila melanogaster.  

**Paper II**
Berg, I., K.P.R., Nilsson, S. Thor, and P. Hammarström  
Efficient Imaging of Amyloid Deposits in Drosophila Models of Human Amyloidoses.  

**Paper III**
Berg, I., K.P.R., Nilsson, S. Thor, and P. Hammarström  
Curcumin alleviates Aβ induced neurotoxicity and vice versa without removing amyloid deposits in transgenic Drosophila.  
Manuscript.
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Background

Amyloid: its origin, structure and associated diseases

Introduction
There are currently 27 known proteins that can form amyloid deposits causing brain disorders and other diseases [1]. Although the proteins involved in these disorders do not share sequence homology, the amyloid fibrils do share biophysical properties and possibly a common pathogenic mechanism [2]. Amyloid deposits are known to be involved in a broad range of neurodegenerative diseases such as Alzheimer’s disease and Creutzfeldt-Jakob disease, which is one form of the spongiform encephalopathies, as well as some non-neuropathic diseases such as senile systemic amyloidosis and type II diabetes. Other neurodegenerative diseases, including Parkinson’s disease, amyotrophic lateral sclerosis, and Huntington’s disease, are also associated with amyloid-like fibril formation (Table 1). However, since the protein fibrils causing these diseases lack some of the characteristic hallmarks of amyloid classification, they are classified as amyloid-like diseases [1, 3]. We still lack an effective treatment to prevent protein misfolding in these amyloid and amyloid-like diseases. The protein aggregates that cause neurodegeneration may be cytoplasmic, nuclear, or extracellular. These deposits contain the culprit amyloid protein and other auxiliary proteins, which are diagnostically useful pathogenic features of these disorders [4].

It is not known exactly how amyloid fibrils develop in vivo, although it is known that the proteins interact with each other through the formation of hydrogen bonds between the backbones of the polypeptides. The deposits may occur at one or several sites in the body. A single type of protein forms amyloid fibrils, causing an amyloid-associated disease. In rare cases, such as in Alzheimer’s disease, multiple forms of amyloid deposits (neurofibrillary tangles and senile plaques) can cause the disease.
The primary risk factor for amyloid formation is advanced age [5]. However, destabilizing mutations within the amyloidogenic protein enhance the risk of earlier onset of the disease [3].

Table 1: Amyloid and amyloid-like disorders and their associated precursor proteins

<table>
<thead>
<tr>
<th>Disease</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NEURODEGENERATIVE DISEASES</strong></td>
<td></td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>Aβ1, Tau1</td>
</tr>
<tr>
<td>Spongiform encephalopathies</td>
<td>Prion protein1, or fragments thereof</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>α-Synuclein2</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>Superoxide dismutase12</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>Huntingtin2 with poly-glutamine expansion</td>
</tr>
<tr>
<td><strong>NON-NEUROPATHIC DISEASES</strong></td>
<td></td>
</tr>
<tr>
<td>Senile systemic amyloidosis</td>
<td>Wildtype Transthyretin1</td>
</tr>
<tr>
<td>Familial amyloidotic polyneuropathy</td>
<td>Mutant Transthyretin1</td>
</tr>
<tr>
<td>Diabetes, type II</td>
<td>Islet amyloid polypeptide1</td>
</tr>
</tbody>
</table>

1 True amyloid protein, 2 amyloid-like protein

History

Rudolf Virchow first coined the term “Corpora amylacea” in 1854 [6] to describe a macroscopic tissue abnormality (which he described as a physical beast) in nervous tissue. The amyloid found was thought to consist of a cellulose and starch analog, since iodine stained it purple or blue instead of red [7]. However the structure had previously been observed in post-mortem tissue dissections of what today we believe to have been systemic amyloidosis, but which then went under the name of “lardaceous or coleserin disease” and “wax-spleen” [8]. The fact that amyloid consists of proteins, and not of a cellulose or starch analog, was discovered some years later [9], although the name amyloid is derived from the Latin amyllum and the Greek amylon, both of which translate as starch [10]. At first, systemic amyloidosis was believed to be a response during inflammation associated with chronic disorders, and that all types of amyloidosis were composed of the same components. Even when different forms of amyloidosis were found, the possibility that the deposits might contain different forms of proteins was not considered [8].
The 'gold standard' used today for amyloid detection is Congo red, which was introduced in 1922 for amyloid and cellulose, although it had previously been used for dying textiles and paper. In 1927 it was discovered that when Congo red binds to amyloid deposits, an apple-green birefringence is emitted under crossed plane-polarized light. This was a major step forward for amyloid detection in tissue [8] and is still one of the criteria used in amyloid classification [1].

The modern history of amyloid began in 1959, when Alan S. Cohen and Evan Calkins presented the first picture of an amyloid protein fibril structure under high magnification [11]. This initiated the structural investigation of the amyloid fibril, and resulted in the discovery of the protofibrils that give rise to the amyloid specific X-ray diffraction pattern of a well-ordered cross-β-sheet pattern [8], which forms another important criterion for amyloid classification [1].

**Definition of amyloid**

Today, the definition of amyloid follows the consensus reached at the meeting of the Nomenclature Committee of the International Society of Amyloidosis, in November of 2006. Amyloid is defined as protein deposits found in vivo, which can be distinguished from non-amyloid protein deposits by: a characteristic fibril appearance under electron microscopy; a typical X-ray diffraction pattern; and an affinity of histological samples for the dye Congo red, which results in an apple-green birefringence under plane-polarized light [1].

The term amyloid was originally restricted to extracellular deposits. However, many types of amyloid have since been reported to start intracellularly, which then give rise to characteristic extracellular amyloid deposits found upon cell death. This has led to the definition of amyloid being updated such that some intracellular inclusions with a typically amyloid appearance, are now also classified as amyloid [1].

Many non-disease related peptides and proteins, as well as some artificial peptides and proteins, have also been reported to form fibrils with typical amyloid properties in vitro. This means that intra- or extracellular protein deposits that lack any of the biophysical properties, together with non-disease related proteins or peptides, artificial proteins or peptides, as well as fibrils produced in vitro, should not be classified as amyloid according to the International Society of Amyloidosis. [1] These types of protein fibrils are generally called intracellular protein inclusions or amyloid-like proteins or fibrils.

A disease caused by amyloid is called amyloidosis (plural, amyloidoses). An amyloidosis affecting one site or one type of tissue is
termed localized, while an amyloidosis affecting several organs is termed systemic. Although this is usually a logical definition, it is sometimes difficult to follow where amyloid deposits are found in blood vessels, and where they affect many organs and tissues of the body [1]. A protein that has been converted into its amyloid form is designated by its protein name prefixed with ‘A’, and appended with a suffix if any mutation is implicated [1], e.g. ATTRV30M.

**Structure of amyloid**

Amyloidogenic proteins may be either natively unfolded or natively folded proteins *in vivo*. Examples of natively unfolded proteins are α-synuclein, which causes Parkinson’s disease, the Aβ-peptide, which causes Alzheimer’s disease, and the islet amyloid polypeptide involved in type II diabetes. Other proteins retain their native conformation before going through a conformational change rendering them into forms that assemble into amyloid deposits such as the prion protein that causes Creutzfeldt-Jakob disease, the β2-microglobulin that causes hemodialysis-associated amyloidosis, or transthyretin that causes senile systemic amyloidosis.

For many years, the only available structural information on amyloid was from low resolution imaging of amyloid plaques taken from human tissue samples stained with iodine, followed later by information from high-resolution images and transmission electron microscopy [11]. To date, amyloid and amyloid-like fibrils are too large to be resolved in solution by nuclear magnetic resonance, and they do not crystallize to afford structures that can be determined by X-ray crystallography [3]. However, new techniques including atomic force microscopy [12], cryo-electron microscopy [13], solid-state nuclear magnetic resonance [14, 15], and even X-ray crystallography of small peptides [16] have now been able to give some structural information on amyloid and amyloid-like fibrils.

Although the precursor proteins of amyloid or amyloid-like fibrils may differ in size and in their primary and secondary structures, they still assemble into similar fibril structures [17]. These fibrils are composed of homogenous protein in an ordered structure of cross-β sheets measuring 75 Å – 100 Å in diameter [18]. The fibrils are un-branched and range from 0.1 µm to 10 µm in length [19]. Amyloid fibrils usually consist of 2 to 6 protofilaments, each about 2 nm – 5 nm in diameter. The protofilaments form a rope-like twisted structure that is 7 nm – 13 nm wide depending on the protein. The protofilaments can also associate
laterally to form long ribbons, 2 nm – 5 nm thick and up to 30 nm wide [3]. They can also assemble into fibrous nanocrystals in vitro [20].

X-ray diffraction data show that the β-sheets from the protein or peptide run perpendicular to the fiber axis in the protofilament. The number of β-sheets, as well as the number of residues involved in the β-sheets of the protofilament, may vary for different types of proteins [3]. The β-sheets of the protofilaments can, depending on the protein involved, be either parallel or antiparallel. The same protein or peptide can also differ in β-sheet arrangement depending on factors such as truncation or the physical environment where the amyloid or amyloid-like fibrils are formed. This is the case for the Aβ peptide (see Page 25 et seq.), which forms parallel β-sheets as variants Aβ1-40 and Aβ1-42, while the truncated Aβ34-42 forms anti-parallel protofilaments in vitro [21].

The X-ray diffraction pattern derived from amyloid fibrils is one criterion for amyloid classification. It is characterized by two distinct signals: a sharp refraction at 4.7 Å corresponding to the separation distance between the β-sheets in the protofilament, and a more diffuse reflection at 10 Å – 11 Å, corresponding to the distance between the protofilaments [22].

If stained with Congo red, the amyloid fibrils display an apple-green birefringence under plane-polarized light. This is most likely due to the ordered structure of the β-sheets. But some amyloid fibrils produced in vitro, even though they may fulfill all the other biophysical criteria of amyloid (e.g. lysozyme variants), do not stain with Congo red [23], which has led to the use of Congo red as the absolute marker for amyloid being questioned [24].

The observation that many non disease-related proteins also form amyloid fibrils under optimal conditions, has led to discussions concerning whether the amyloid fibril structure, with its many hydrogen bonds, is the lowest thermodynamic state that the protein backbone can obtain [25, 26]. Ex vivo fibrils isolated from patients display a structure similar to that of fibrils produced in vitro [3]. Protein aggregation may result from a mutation in a disease related gene, making the protein unstable in its normal fold and more aggregation prone; or aggregation may arise from environmental stress or aging, changing the equilibrium between properly folded and unfolded proteins in the cellular environment [4]. High resolution analysis of structures of short synthetic amyloidogenic peptides have shown that aromatic residues in the primary sequence can contribute to the stability of the core structure of the fibril [27].
Mechanism of amyloid fibril formation

Depending on the physical conditions, amyloidogenic proteins can assemble into multiple forms of amyloid-like structures \textit{in vitro} in a process known as ‘protein fibril polymorphism’ [28, 29] suggesting that several mechanisms may be involved in amyloid formation. Many potential mechanisms of amyloid fibril formation have been proposed. The mechanism generally accepted at present is the nucleation dependent mechanism model, although other models have also been proposed [30].

The nucleation-dependent mechanism model of \textit{in vitro} protein fibrillation under supersaturated conditions follows three phases (Figure 1) in which the formation of amyloid can be measured by Thioflavine T fluorescence or turbidity. First, the thermodynamically unstable nucleus is formed during the lag phase, when no signal of the process is measurable. Within the lag phase the misfolded protein assembles into an oligomeric nucleus. This is the rate-limited step of the fibrillation process in which the protein is forced to leave its normal folding and adopt the misfolded state. The lag phase is followed by the exponential growth phase (or elongation phase), where monomeric protein molecules start to elongate the newly built nucleus. The fibrillation process ends with the stationary phase, which has a strong signal, when no additional fibril growth occurs [30]. The lag phase of the reaction can be shortened by seeding with pre-formed amyloid species, which removes the need for nucleation as a limiting factor in the reaction [3]. Fibril elongation may occur at both ends during the fibrillation process, leading to a bidirectional growth that might be a common feature of amyloid fibrillation [31, 32].

![Figure 1. The three phases of a nucleation dependent mechanism of amyloid fibril formation performed \textit{in vitro}. The amount of amyloid formed is measured by Thioflavine T fluorescence or by turbidity.](image-url)
Diseases caused by Amyloid

Amyloid and amyloid-like deposits are present in several known diseases including: Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, Creutzfeldt-Jakob disease, senile systemic amyloidosis, familial amyloidotic polyneuropathy, and type II diabetes [3]. Most forms of amyloidoses are neurodegenerative disorders with unknown toxic species in which the formation of amyloid may either cause the disease and neurodegeneration, or have been produced as a side reaction during the process of neurodegeneration.

Amyloidoses can be broadly grouped into three classes: (i) neurodegenerative conditions, where protein aggregates appear in the central or peripheral nervous system; (ii) non-neuropathic localized amyloidoses, with aggregate formation in a specific tissue other than the brain; and (iii) non-neuropathic systemic amyloidoses, where aggregates occur in multiple tissues. Most forms of amyloidoses are sporadic; but hereditary, and transmissible amyloidoses also occur [3]. The diagnosis of any type of amyloidosis is usually performed by tissue biopsies of the heart, peripheral nerves, kidney, liver, or skin [33].

The risk of proteins misfolding during protein expression, folding and transport has driven the evolution of a defense mechanism against misfolded proteins. The cellular defense mechanism involves protein degradation by proteasomes, molecular chaperones, autophagy, or the formation of aggresomes (microtubule-mediated transport that collects the misfolded proteins as inclusion bodies close to the centriole and prevents the misfolded protein reaching the daughter cell during mitoses) [4]. Misfolded hyperphosphorylated tau (see Page 33 et seq.) does not tend to be packed in aggresomes, possibly because tau is a microtubule associated protein and thereby less displaceable [4]. Proteins involved in the secretory pathway are over-represented in protein misfolding diseases. These types of proteins fold within the endoplasmic reticulum and are later transported to their proper destination where the physical environment may differ. This makes the protein folding process complex since the protein must find its right conformation in the endoplasmatic reticulum, then escape degradation by the control mechanism and maintain its correct folded form until it arrives at its proper destination with its active conformation and function intact. Many amyloidogenic proteins fold properly in the endoplasmic reticulum and escape degradation, but following secretion they can partially unfold which facilitates amyloidogenesis in the extracellular space [34].

Several mechanisms of amyloid toxicity have been proposed. One mechanism suggests that amyloid forms a physical barrier in the extracellular space or in the tissue where it is aggregated, which destroys
the normal function of the cell or organ by blocking communication [30]. Amyloid fibrils are known to be toxic [35, 36] and it has been recently suggested that the pre-fibrillar species are more toxic than the mature amyloid itself [26]. Toxic mechanisms that have been suggested include the incorporation of oligomeric species into membranes, which then promotes membrane leakage [37]. This mechanism implies that the mature fibrils can act as a detoxification product capable of collecting the smaller, more toxic species. Several studies indicate that cellular deposits, such as inclusion bodies, are not toxic, but act like a cell protection response [4]. The toxicity of early species in the aggregation process could derive from their unbound, exposed amino- and carboxyl-groups acting as hydrogen donors or acceptors in interactions with other proteins in the cell. If these groups are buried in a properly folded protein, they will not interact incorrectly [4]. Amyloid plaques are known to bind large amounts of metal ions, especially copper, zinc, and iron [38]. The metal containing plaques could work as a pro-oxidant species by producing peroxides that affect cell viability by increasing oxidative stress [38, 39]. A simple model for amyloid toxicity is based on the collection of proteins essential for cell viability and cell survival, such as the amyloidogenic protein itself, or other proteins that can become trapped within the amyloid plaques, so causing a loss-of-function disease [30].

In some amyloidoses, e.g. Alzheimer’s disease, there is only a weak correlation between the plaque load of amyloid in post-mortem tissue and the clinical severity of the disease [40]. The same pattern is shown for other neurodegenerative disorders associated with protein misfolding, such as Parkinson’s disease, where there is a low correlation between the amounts of Lewy bodies composed by aggregated \(\alpha\)-synuclein, and cell death in the substantia nigra of the brain. However, a correlation does exist between the severity of Alzheimer’s disease and the levels of soluble, low molecular weight species. This makes the low molecular weight assemblages a potential diagnostic marker for Alzheimer’s disease in patients [41].

**Senile systemic amyloidosis**

The most common form of transthyretin (TTR) (see Page 19 et seq.) associated amyloidosis is senile systemic amyloidosis, a sporadic disorder with a late onset that primarily affects patients over 60 years of age. Senile systemic amyloidosis (SSA) is caused by the aggregation of wildtype TTR protein in the heart [42]. The disease affects more than 25% of the population over the age of 80 years [43]. Analysis of the
amyloid deposits in the SSA patients has shown that the deposits are mainly composed of truncated TTR proteins [44].

Restrictive cardiomyopathy is the major cause of morbidity and mortality in patients with TTR amyloidosis. Late stages of cardiomyopathy usually involve arterial fibrillation. The major challenge is to maintain a proper fluid balance in the patients. Cardiac arrhythmias by arterial ventricular block or sinus exits are common features [33].

Even though senile systemic amyloidosis affects approximately 25% of the population over the age of 80 years, it is still a fairly unknown disease. In a study of 32 super centenarians (people over the age of 110 years), who are usually very healthy, only 6% had suffered heart attacks and only 13% strokes. The common forms of disease affecting elderly people, namely Alzheimer’s disease and Parkinson’s disease, were also very uncommon in this group, affecting only 3%. Almost half of the super centenarians suffered from osteoporosis, and almost 90% had cataracts; but 41% still lived on their own and only needed help with small tasks in their everyday lives. The final mortality of the super centenarians was usually related to senile systemic amyloidosis [45].

**Familial amyloidotic polyneuropathy**

The most common autosomal-dominant inherited neurodegenerative disease is familial amyloidotic polyneuropathy. The disease can be caused by any one of over 70 different mutations in the TTR gene, with TTR V30M being by far the most prevalent. Familial amyloidotic polyneuropathy has an earlier age of onset than senile systemic amyloidosis, and displays a range of effects, from peripheral neuropathy to the impairment of autonomic organ function [46]. The development of familial TTR amyloidosis is most likely due to a change in the primary sequence of the protein by an inherited mutation, but the disease is also modulated by environmental factors as well as other genetic factors [33].

The different TTR mutations give rise to a variety of amyloidoses with different clinical symptoms [46]. The familial amyloidotic polyneuropathy starts with a dysfunction in the lower extremities. Proper motor function tends to be maintained until the sensory neurons are affected. The disease spreads from the feet to the ankles, and progresses during the disease to the upper part of the legs. Familial amyloidotic polyneuropathy is often related to amyloid cardiomyopathy [33]. In its later stages patients often complain of disturbed temperature sensation and hyperalgesia [46]. Vitreous opacities commonly occur in familial amyloidotic polyneuropathy patients (approximately 20% of the cases), caused by amyloid deposits in the retina or the ciliary nerve of the eye.
Deposits in the peripheral nerves start sporadically around arterioles. In more severe cases, the deposits affect the nerve fibers, resulting in severe demyelination and nerve fiber loss. The peripheral nervous system is the most common site of amyloid deposits in familial amyloidotic polyneuropathy patients, but the amyloid deposits in the heart, causing cardiomyopathy, is probably the main cause of death [33]. Patients are diagnosed for amyloid deposits by biopsies from the skin, rectal mucosa, and subcutaneous fat [46].

The only specific therapy for TTR amyloidosis is liver transplantation. TTR is mainly synthesized in the liver and the transplant is done in order to remove all traces of the mutated form [47]. Liver transplants started in Sweden 1990, and by 2004, 1200 people worldwide had undergone a transplant [48].

The stability of tetrameric TTR protein correlates with disease onset, supporting the notion that tetrameric instability is an amyloidogenic factor. No genetic factor other than the TTR gene has yet been identified that influences penetrance, disease onset, or the progression rate of the disease [46].

Familial mutations associated with Transthyretin amyloidosis

Most carriers of a mutated TTR gene are heterozygotic for the mutation, making both the native tetrameric protein and the amyloid fibrils as a mixture of both wildtype and mutant subunits [46]. However, familial amyloidotic polyneuropathy is not a specific disease of only TTR variants since mutated apolipoprotein A1 has also been found to cause the disease [49].

The mutants associated with familial amyloidotic polyneuropathy are commonly only found all over the world in single families; but endemic areas are present in Portugal, Sweden and Japan [33], which makes the disease fairly unusual. The prevalence of the mutation is between 1 in 100 000 and 1 in 1 000 000 [46]. The age of onset varies for the different mutations. The Swedish kindred carrying the TTRV30M has an age of onset between 55 and 60 years, with less than 50 % penetrance of the disease [50]. The same mutation in Portugal and Japan causes a much earlier onset of disease at between 30 to 35 years of age, and shows a much higher penetrance [46, 51]. The heredity patterns in Swedish patients with familial amyloidotic polyneuropathy reveal that the age of onset seems to be lower in the following generation than in the preceding one, particularly when the affected parent is the mother [52].
phenomenon of genetic anticipation is seen in most forms of familial amyloidotic polyneuropathy.

Transthyretin amyloidosis is the most common form of familial autosomal-dominant systemic amyloidosis. In most cases, only one nucleotide substitution in the TTR gene is required to develop the disease. There are over 100 known familial mutations reported to occur in the TTR gene and the majority are connected to amyloidosis [33]. In one case, an entire codon deletion has been found, which resulted in the loss of valine at position 122 [53]. Many of the mutations correspond to amino acid shifts at the end of the c-d β-strands, but mutations have been found throughout the TTR sequence [54].

Most individuals with family associated TTR disease are heterozygous, expressing both normal and variant TTR protein [46]. This makes the circulating TTR tetramer a combination of both wildtype and variant TTR [55]. The protein levels of the wildtype TTR are usually higher than the protein levels of the variant TTR in plasma [56]. This is probably due to the clearance of unstable variants of TTR by the control mechanism in the endoplasmic reticulum. The variant TTR protein is probably sent for degradation by the proteasome, so preventing it from reaching the circulation to the same extent as the wildtype TTR protein [57]. This makes the most unstable variants of TTR familial mutations an undesirable target for treatment with small molecule stabilizers, since these will allow more proteins to reach the circulation where they will be able to misfold into amyloid in tissues [58]. Amyloid deposits in patients suffering from a familial TTR amyloidosis usually contain 65% - 75% of variant TTR, the rest being composed of wildtype TTR [33]. Since there exists a wide variety of TTR mutations, the expression levels, as well as the protein composition within the amyloid, varies depending on the mutation. Some familial mutations associated with TTR amyloidosis, which are either common or have been used during the work of this thesis, are described below.

V122I

The most common TTR mutation has valine replaced at position 122 by isoleucine. This mutation is carried by 3.9% of the African-American population. The variant causes amyloid deposition mainly in the heart, which results in the disease familial amyloid cardiomyopathy [59].

V30M

The most common TTR mutation that causes familial amyloidotic polyneuropathy is a substitution of valine by methionine at position 30. This mutation is found in the north of Portugal [60], Japan [61], and in
BACKGROUND

the north of Sweden [46]. In Sweden, the specific disease form associated with this mutation is Skellefteåsjukan (the disease of Skellefteå), named after the small town of Skellefteå in the north of Sweden, where there is a high prevalence (2.6%) of the mutation [46]. The TTR<sub>V30M</sub> thermodynamically destabilizes the native state of the protein and facilitates tetramer dissociation and the formation of the monomeric amyloidogenic intermediate (see Page 22) [62, 63]. The age of onset for the same mutation varies with location. The age of onset among families in Portugal and Japan is 36 years, while the age of onset among the Swedish kindred is between 55 and 60 years of age [46].

L55P

Another familial amyloidotic polyneuropathy results from a mutation in which a leucine is substituted by a proline at position 55 in the TTR gene. This type of familial amyloidotic polyneuropathy has been reported in West Virginia [64] and in Taiwan [65]. Patients with this disease present with early-onset and rapid progression associated with autonomic neuropathy and amyloid deposition in the heart and eye [33]. In the West Virginian family, the age at onset decreased from the fourth to the second decade over four generations, which is a clear display of genetic anticipation commonly associated with familial amyloidotic polyneuropathy [66].

D18G

The most destabilized TTR variant characterized to date is a substitution of an aspartic acid to a glycine at position 18 [58]. This mutation is found in Hungary and Japan. The disease onset occurs in the fifth decade with the manifestation of disease clinically restricted to the central nervous system [67], where extensive amyloid deposits are mainly found in the leptomeningeal vessels and in the subarachnoid membrane [68].

In vitro analyses of ATTR<sub>D18G</sub> show a fibrillation rate 1 000-fold faster than for the wildtype TTR. The data also indicate that thyroxine binding facilitates tetramerization of TTR<sub>D18G</sub>. Analyses of serum and cerebrospinal fluid from patients are only able to detect wildtype TTR. The low stability of the protein combined with the inability to detect TTR<sub>D18G</sub> in serum and cerebrospinal fluid make indicates that the protein is probably rapidly degraded by the cellular defense mechanism, which might also explain the absence of an early onset of this potentially systemic disease caused by this highly destabilized mutant. It may be the thyroxine in the choroid plexus that prevents the degradation of TTR<sub>D18G</sub> by binding and stabilization, thereby allowing it to escape the cellular defense mechanism. This would result in the variant protein being present
in the cerebrospinal fluid, so facilitating amyloid formation in the central nervous system [58].

ALZHEIMER’S DISEASE
On November 3rd 1906, at the 37th meeting of the Society of Southwest Germany Psychiatrists in Tübingen, Alois Alzheimer presented a case of what is today recognized as the most common neurodegenerative disease, Alzheimer’s disease. Today, more than 20 million people worldwide suffer from Alzheimer’s disease with clinical symptoms which include progressive memory loss, focal symptoms, delusions, and hallucinations [5]. The histopathological hallmark of Alzheimer’s disease is the presence of extracellular senile plaques, intracellular neurofibrillary tangles, dystrophic neurites, degenerating neurons, and activated astrocytes and microglia, especially around the senile plaques [69]. Cerebral amyloid angiopathy caused by the degeneration of vessel walls and hemorrhages is found in approximately 80% of the Alzheimer’s disease patients, but is not a diagnostic criterion [70].

Alzheimer’s disease is a neurodegenerative disorder that currently affects approximately 2% of the population in industrialized countries. The risk of Alzheimer’s disease dramatically increases in individuals beyond the age of 70, and it is predicted that the disease will increase threefold within the next 50 years [71]. Most cases of Alzheimer’s disease are sporadic, and the familial form represents less than 1% of the total cases of Alzheimer’s disease. Age is a major risk factor for sporadic Alzheimer’s disease [5], while the family history of the disease, cardiovascular disease, diabetes, hypertension, heart disease, prior head injury, high alcohol intake, and stroke are factors that are associated with an increased risk of developing the disease [72]. Oxidative stress occurs early in the progression of Alzheimer’s disease [5]. Clinical studies indicate that elevated cholesterol may be a risk factor for the development of Alzheimer’s disease [73]. Small numbers of senile plaques and neurofibrillary tangles are found in most individuals in older age groups. The neurofibrillary tangles first appear in the transentorhinal region and then spread to the hippocampus, the amygdala, and to the cortex of the brain. Senile plaques first appear in the cortex and the two types of deposit seem to form independently; but in advanced stages of the disease, extensive deposits of senile plaques accelerate the formation of neurofibrillary tangles in the cortex of the brain [5].

Since 1992 the amyloid cascade mechanism has been the dominant hypothesis for the pathological hallmarks of Alzheimer’s disease. The mechanism suggests that the Aβ deposits is the causative agent of
Alzheimer’s pathology, and that the neurofibrillary tangles, neurodegeneration, vascular damage, and dementia follow as a direct result of these deposits [74]. However, it is not clear how this occurs. Neurodegeneration is estimated to start 20 to 30 years before the first clinical symptoms appear. In the initial early clinical phase characterized by amnestic mild cognitive impairment, the degree of cognitive impairment correlates with the amount of neurofibrillary tangles better than with the amount of senile plaques. Propagation of the disease is thought to be related to the spreading of the neurofibrillary tangles (Figure 2) [5]. In amorphous or diffuse senile plaques formed early in the disease, most Aβ peptides are composed of full-length Aβ [75]. Investigations have shown that these initial deposits are able to seed the growth of both full-length and truncated versions of the Aβ peptide into larger assemblies [69]. In sporadic Alzheimer’s disease, the Aβ1-42 concentration in cerebrospinal fluid increases at an early stage of the disease, but the concentration decreases with disease progression [76]. This makes Aβ1-42 alone an unreliable biomarker for the early diagnosis of Alzheimer’s disease [72]. Variation among individuals in the absolute concentrations of different lengths of Aβ, also makes it difficult to form diagnostic conclusions based on the quantification of total Aβ concentration. Instead, the ratio of Aβ1-42/Aβ1-40 is a more useful measure to confirm the diagnosis of probable Alzheimer’s disease. An increase of the Aβ1-42/Aβ1-40 ratio in both cerebrospinal fluid [76] and in plasma [77] correlates with an increased risk, onset, and progression of Alzheimer’s disease (Figure 2) [72]. The major sources of Aβ and phosphorylated tau in the cerebrospinal fluid of patients suffering of Alzheimer’s disease are assumed to correlate with neuronal injury or neurodegeneration. Aβ and phosphorylated tau in cerebrospinal fluid are thus important biomarkers for the diagnosis of Alzheimer’s disease. The increased concentration of Aβ and phosphorylated tau with disease progression appears to correlate with the conversion from cognitive normalcy or mild cognitive impairment to dementia (Figure 2) [77].

Treatments for Alzheimer’s disease include two classes of drugs: the acetyl cholinesterase inhibitors (tacrine, donepezil, rivastigmine, galantamine), and the N-methyl-D-aspartate receptor antagonist (memantine). These drugs enhance the remaining cognitive function, but they do not delay the disease progression by preventing senile plaque or neurofibrillary tangle formation. The drugs aimed to treat Alzheimer’s disease modulate the fibrillation pathway of Aβ by targeting molecular sites in order to prevent Aβ production, prevent the formation of toxic forms of Aβ, or prevent toxic effects of Aβ [72]. Vaccination with Aβ as an immunotherapy has been tested in vivo. Immunotherapy aims to bind
Figure 2. The amounts of senile plaques (black), neurofibrillary tangles (light gray) and the neuronal integrity (medium gray) in a relation to the time course of pathological and clinical stages of Alzheimer’s disease (AD). Modified from [77].

Amyloid, facilitate its degradation and block the toxic effect. Immunotherapy of transgenic mice models of Alzheimer’s disease reduces extracellular Aβ plaques, reduces intracellular Aβ accumulation, and leads to a clearance of early tau deposits. In the transgenic mice, the Aβ deposits were cleared before the tau deposits, with the tau clearance being mediated by proteasomes and being dependent on the phosphorylation grade of the tau protein. The hyperphosphorylated tau aggregates were unaffected by the immunotherapy. [78] Passive vaccination has been shown to improve the cognitive function of transgenic mice, and to reduce the concentration of both soluble Aβ and tau. The passive immunotherapy also reduced the amyloid plaques and neurofibrillary tangles [79]. Passive vaccination has now entered the clinical trials stage as a treatment for Alzheimer’s disease.

Familial mutations associated with Alzheimer’s disease

The major genetic risk factor for developing late-onset Alzheimer’s disease is the presence of the apolipoprotein E, type 4 allele [80]. One type 4 allele doubles or triples the risk of developing Alzheimer’s disease, while two type 4 alleles increase the risk up to 12 times. Apolipoprotein E binds to Aβ and becomes a component of senile plaques [81]. Mutation in the presenilin-1 gene is the most common cause of familial Alzheimer’s disease, but a mutation in the presenilin-2 gene is also connected to Alzheimer’s disease. More than 160 mutations in the
presenilin-1 and 2 genes have been identified. Mutations in the presenilin-1 and 2 genes increase the ratio of Aβ1-42/Aβ1-40 [5].

To date, there are 20 known disease-causing mutations in the AβPP gene (see Page 25 et seq.) (Figure 3) [5]. Mutations in the Aβ peptide leads to an increased amount of vascular deposits and senile plaques. Familial AβPP mutations, which are associated with early-onset Alzheimer’s disease, increase the Aβ1-42 concentration in cerebrospinal fluid by a factor of 1.5 – 1.9 [72]. The mutations are broadly divided into two groups: those situated within the Aβ peptide, and those situated at the flanking sequence of the Aβ peptide. The flanking mutants usually alter the ratio between the Aβ1-40 and Aβ1-42, by altering the cleavage position in the C-terminal part of the peptide, or they increase the total Aβ concentration by facilitating cleavage by β-secretase (see Page 29). The mutations may also be situated in the central part of the Aβ peptide, within residue numbers 21 – 25. These positions change the aggregation properties of the peptide [21].

The mutations in the tau gene (see Page 33 et seq.) are divided into two categories: those that influence the alternative splicing of the pre-mRNA, and those that primarily affect the protein properties. These mutations reduce the ability of tau to interact with microtubule, while some other mutations also promote aggregation into filaments [5].

Tau mutations that have been linked to Parkinsonism linked to chromosome 17 are missense, deletion, or silent mutations. These mutations change the relative isoform ratio of the tau protein by reducing
microtubule binding, and/or increasing tau aggregation [5]. Some mutations associated with familial Alzheimer’s disease are described below. The described mutations are either commonly used in models of Alzheimer’s disease or have been used during the work of the present thesis.

**London mutation**

The London mutation involves a change from valine to isoleucine at position 717 in the AβPP sequence, and was the first genetic mutation to be discovered in familial Alzheimer’s disease [82]. This mutation increases the Aβ1-42/Aβ1-40 ratio by altering the γ-secretase cleavage position in the C-terminal part of the peptide (see Page 26) [21].

**Swedish mutation**

The Swedish mutation involves two substitutions: lysine to aspargine, and methionine to leucine at positions 670 – 671 in the AβPP sequence, which is located just outside the N-terminus of the Aβ domain in the AβPP protein [83]. The Swedish mutation increases Aβ levels in plasma by six to eight times [84]. Research into the Swedish mutation has increased our understanding of AβPP processing, and has contributed to the development of a more sensitive ELISA (enzyme-linked immunosorbent assay) for measuring Aβ1-40 and Aβ1-42 concentrations, which is now used to diagnose Alzheimer’s disease in humans [70]. Research on the Swedish mutation has also contributed to the identification and characterization of the secretase BACE-1 [85].

**Dutch and Iowa mutations**

Mutations at positions 21 – 23 in the Aβ domain of AβPP, near the hydrophobic cluster, are a heterogeneous group of genetic mutations. They affect Aβ aggregation and degradation, but also AβPP processing. In the Dutch mutation, glutamic acid is substituted by glutamine at position 693 in the AβPP sequence [86]; in the Iowa mutant, aspartic acid is substituted by aspargine at position 694 in the AβPP sequence [87]. Both of these mutations are associated with cerebrovascular amyloid angiopathy and diffuse Aβ plaques, degenerating neurites and neurofibrillary tangles, resulting in hemorrhagic strokes, infarcts and dementia [86, 87]. The Dutch mutation increases the Aβ1-40 concentration in the cerebral spinal fluid, but the Aβ1-42 concentration remains unaffected. In *in vitro* experiments on Aβ1-42 E22Q fibrillation was found to increase both the amount of mature fibrils and the presence of oligomeric species. The Iowa mutation does not alter the concentrations of either
BACKGROUND

Aβ_{1-40} or Aβ_{1-42} in the cerebrospinal fluid, but in in vitro experiments on Aβ_{1-42 D23N} fibrillation was found to increase the amount of mature fibrils [21].

**Flemish**

The Flemish mutation, in which alanine is substituted by glycine at position 693 in the AβPP sequence, is often associated with pre-senile dementia or cerebrovascular amyloid angiopathy. The Flemish mutation decreases α-secretase cleaving, resulting in an increased level of total Aβ. In in vitro experiments on Aβ_{1-42 A21G} fibrillation was found to increase the amount of mature fibrils but to decrease the amount of oligomeric species [21].

**Arctic**

The Arctic mutation, in which glutamic acid is substituted by glycine at position 693 in the AβPP sequence, is associated with early-onset Alzheimer’s disease with onset occurring at 52 – 62 years of age [88]. The disease is associated with a large amount of neurofibrillary tangles, severe cerebrovascular amyloid angiopathy, and diffuse senile plaques in post-mortem brain tissue [89]. The Arctic mutation also increases β-secretase processing of AβPP by rendering the amyloid precursor protein less available to α-secretase (see Page 26) [90]. The Arctic mutation reduces the total concentration of circulating Aβ, but increases the formation of protofibrils in vitro [88].
Transthyretin

Introduction
Transthyretin (TTR) is an amyloidogenic protein involved in several amyloid diseases, both inherited and sporadic [91]. TTR is one of the most naturally mutated proteins, with over 100 known familial mutations [33]. TTR was discovered 1942 in human serum [92] and in cerebrospinal fluid [93]. TTR was first named Prealbumin since it was the only human plasma protein that migrated faster than albumin on an electrophoresis gel [94]. In 1958 TTR was identified to be a thyroid binding hormone, and the name was change to thyroxid-binding prealbumin [94]. Later, TTR was also identified to be a binder and transporter of retinol-binding protein [95] and the name was changed to Transthyretin which stands for transports thyroxine and retinol [96]; however, Prealbumin remains a commonly used name for TTR.

The gene corresponding to human TTR is situated on chromosome 18 of the human genome at position 18q11.2 – q12.1 [97]. The gene has a size of approximately 7 kbp and is composed of four exons [98]. TTR is widely distributed among vertebrates, indicating a conserved function for the protein [92].

Biological function
The biological function of TTR is to deliver thyroid hormones to cells and to keep the plasma storage of thyroid hormones in a non-degradable form. TTR is also responsible for binding to and transporting retinol (vitamin A) from the liver to the target cells by binding with retinol-binding protein [55]. Other thyroid hormone distributing proteins in humans are albumin and thyroxine-binding globulin. TTR is mainly synthesized in the liver by the hepatocytes and secreted into the bloodstream where it circulates [99]. Smaller amounts of TTR are also produced in the epithelial cells in the choroid plexus [100], in the retina and ciliary pigment epithelia of the eye [101], in the placenta, in the visceral yolk sac, in the intestine, and in the pancreas [92]. TTR is present at high concentrations in the circulation, 0.2 – 0.25 mg/ml [102]. The synthesis in the choroid plexus is significantly lower where TTR is secreted into the cerebrospinal fluid to a concentration of 0.02 – 0.04 mg/ml [100]. Low levels of TTR have also been detected in the skin, heart, skeletal muscles, kidneys, pituitary gland and testes [92].
TTR is responsible for the transportation of all retinol-binding protein in the plasma, but of only approximately 20% of the thyroid hormones (thyroxin and triiodothyronine), with the remaining 70% and 10% of the thyroid hormones being transported by thyroxine-binding globulin and serum albumin, respectively [103]. The syntheses of albumin and thyroxine-binding globulin have only been detected in the liver [92]. Due to the blood-brain barrier, TTR is the only thyroid-binding protein present in the cerebrospinal fluid [104]. The thyroid hormones are delivered to stem cells and progenitor cells in the subventricular zone of the brain, and are essential for cell cycle regulation [105]. All thyroid hormone-binding proteins have a higher affinity for thyroxine than triiodothyronine, but triiodothyronine has a higher affinity for the thyroid hormone nuclear receptor than thyroxine, making thyroxine the transported substance (or prohormone) and the triiodothyronine the active substance for thyroid hormone signaling. Thyoxine is converted to triiodothyronine in the tissue by deidinases [92].

TTR also binds the retionol-binding protein in a complex with retinol. This binding is believed to prevent the loss of retinol and retinol-binding protein during glomerular filtration in the kidneys by making the complex too big to be filtered out in the urine. The TTR-complex has a molecular weight of about 80 kD or 100 kD depending on whether TTR binds with one or two of the retinol-binding proteins. The retinol-binding protein alone has a molecular size of approximately 21 kD, which is small enough for it to be filtered out by the kidneys in its unbound state [95].

Since TTR only binds small amounts of thyroxine in the plasma and has a high binding capacity to numerous different aromatic compounds in vitro [106], it has been speculated that TTR, in addition to its classical function of transporting thyroxine and retinol-binding protein, may have other functions connected to nerve cell development [92]. TTR was recently reported to have proteolytic activity on Aβ (in both the aggregated and soluble forms) [107], apolipoprotein A1 [108], and amidated neuropeptide Y [109] in vitro. In the co-expression of TTR and Aβ it has been shown that TTR decreases the toxicity and prevents amyloid fibril formation in transgenic mice, possibly through a chaperone mechanism [110]. Other studies of TTR knockout mice have shown that TTR may also have roles in sensorimotor function and in nerve regeneration [111].

Structure
Under normal physiological conditions, human TTR exists as a homotetrameric protein, in which each monomer is composed of 127
amino acids. The protein is synthesized with a 20 amino acid long signal peptide, which renders TTR secretion into the extracellular space [112]. The TTR structure is dominated by β-sheets, where each subunit consists of eight β-strands and a short α-helix. This high β-strand content is believed to contribute to the extraordinary stability of the protein. The tetramerization of TTR can be described as a dimer of a dimer, where two monomers share the same β-sheet and the two dimers form the binding site for the two thyroxine molecules (Figure 4) [113].

![Figure 4. Ribbon drawing of the tetramer of human transthyretin. Produced in Jmol by pdb structure 1F41.](image)

There are two identical thyroxine-binding sites in the native TTR tetramer, but due to negative cooperativity, only one thyroxine molecule can bind to the TTR tetramer. TTR also has four binding sites for retinol-binding protein, but sterical hindrance of the proteins only allows two units to bind simultaneously [92].

The primary sequence has been highly conserved during evolution, with any changes predominantly occurring in the N-terminal region and not in the core structure or in the thyroxine binding sites [113]. A comparison of 23 naturally occurring familial mutations of the TTR protein revealed no structural differences in the TTR protein upon mutation [114], but significant changes in the stability of several disease-associated TTR mutants have been detected [58, 63].
**Amyloid formation**

Both wildtype TTR and single amino acid substituted variants form amyloid *in vivo* [115]. Why TTR forms amyloid in tissue is not currently known, although it may depend on the high levels of β-sheet already present in the native protein. TTR is also present in high concentrations in plasma, but a high metabolic processing rate only allows TTR proteins to circulate for approximately 1 – 2 days before they are degraded. The high rate of metabolic processing could be a factor in the amyloidogenic process [33], since ATTR is found in amyloid in both the full length and in the truncated form [44]. The importance of TTR fragmentation as a factor for amyloidogenesis is still largely unexplored.

Fibrillation of soluble TTR *in vitro* requires partial acidic denaturation or refolding of denatured proteins [116]. When TTR misfolds into fibrils *in vitro* the TTR tetramer must first dissociate into monomers, which then undergo partial unfolding into the monomeric amyloidogenic intermediate. A structural model of the monomeric amyloidogenic intermediate is structurally very similar to the monomeric structure within the TTR tetramer. The dissociation of the tetramer is the rate-limiting step of the misfolding process. The monomeric amyloidogenic intermediate is highly aggregation-prone *in vitro* and assembles into oligomers and protofilaments, which later grow into mature fibrils (Figure 5). Most investigations of the TTR fibrillation process are done during acidic conditions *in vitro* [117].

![Fibrillation mechanism of TTR](image)

**Figure 5.** The fibrillation mechanism of TTR *in vitro*. The native folded monomer dissociates into non-native structured monomers, which partially unfold to form the monomeric amyloidogenic intermediate. The intermediate assembles into soluble aggregates and grow into mature fibrils.

A large number of small molecules have been reported to bind TTR *in vitro*, such as diclofenac, diflunisal, and fluenamic acid. These substances shift the aggregation equilibrium towards the native state and prevent fibrillation by increasing the kinetic barrier for tetramer dissociation [106].
Stabilization of the native tetramer structure of TTR by the anti-inflammatory drug diflunisal has been tested both in *in vitro* and in clinical trials. Since diflunisal is a non-steroidal anti-inflammatory drug already present on the market in over 40 countries worldwide, it has already passed many of the safety requirements for a new drug approval. Diflunisal has the ability to stabilize the TTR tetramer and prevent it from dissociating into the monomeric amyloidogenic intermediate [106, 118]. Gene therapy has been proposed as a treatment for familial amyloidotic polyneuropathy in order to clear the mutated TTR version, and so leave only the wildtype TTR synthesized in the liver [119].
Amyloid-β Precursor Protein and the Amyloid-β Peptide

Introduction

The amyloid-β (Aβ) peptide is the major component of senile plaques, a histological hallmark of Alzheimer’s disease. The precursor protein of the Aβ peptide is amyloid-β precursor protein (AβPP). AβPP belongs to a conserved gene family that also includes the mammalian homologous proteins AβPP-like protein-1 and -2 (AβPLP1 and AβPLP2), the Drosophila melanogaster (Drosophila) AβPP-like protein (dAβPPL), and the Caenorhabditis elegans (C. elegans) AβPP-like protein-1 (AβPLP-1). The AβPP is the most studied protein in the AβPP gene family, due to its association with Alzheimer’s disease [120]. The gene coding for AβPP is situated on chromosome 21 and a triplicate of chromosome 21: the genetic cause of Down’s syndrome. Down’s syndrome patients above the age of 40 years show pathological hallmarks that resemble early-onset Alzheimer’s disease [5]. There are three splicing forms of mammalian AβPP: AβPP_{695}, AβPP_{751}, and AβPP_{770}, where the 695 amino acid long splicing form is found in neuronal cell membranes [120].

Biological function

The biological function of AβPP and AβPP-like proteins is still unknown. However, several in vitro and in vivo studies have indicated that AβPP could have a function in development of the adult nervous system, cell adhesion, neuronal survival, neurite outgrowth, synaptogenesis, vesicular transport, neuronal migration, modulation of synaptic plasticity, insulin and glucose homeostasis [120], and be involved in axonal outgrowth after traumatic brain injury [121]. AβPP_{751} and AβPP_{751} have been found to be involved in blood clotting, suggesting that Aβ might have a role as a sealant for the vascular system during bleeding [122], although it is still unknown whether this function is due to AβPP itself or any of its proteolytic cleavage products.
Proteolytic processing of Amyloid-β Precursor Protein

The AβPP protein has two cleavage pathways: the nonamyloidogenic pathway and the amyloidogenic pathway. AβPP has six cleavage sites in the region of the Aβ peptide, or close to it. The cleavage sites are named α, β, γ, δ, ε, and ζ, which correspond to cleavage by α-, β-, γ-, δ-, ε-, and ζ-secretase, respectively (Figure 6). The α, β and γ products are the most studied due to their correlation with Alzheimer’s disease. One of the cleavage products is the Aβ peptide, which is 39 to 43 amino acids long, depending on the cleavage position and which are usually named Aβ1-x or Aβx, where x stands for the number of residues in the peptide [21].

The nonamyloidogenic pathway

About 90% of the AβPP cleavage derives from the nonamyloidogenic pathway [34]. The α-secretase cleaves the AβPP protein in the central region of the Aβ peptide, near the ectoplasmic side of the plasma membrane, between residue 16 and 17 of the Aβ peptide. The cleavage releases the extracellular sAPPα fragment and leaves an 83 amino acid long peptide, C83, still bound to the membrane. The C83 fragment can then be further processed by γ-secretase, to give rise to the small peptide, P3, and the AβPP intracellular domain as cleavage products (Figure 7) [21, 120]. The AβPP intracellular domain has been found in the cell nuclei, and although the functions of P3 and AβPP intracellular domain are not known, their possible function as neuropeptides has been discussed, and a function for AβPP intracellular domain as a transcription factor has been proposed [123].
The amyloidogenic pathway

About 10% of the AβPP cleavage derives from the amyloidogenic pathway, from which the Aβ peptide is released as one of its cleavage products [34]. Two β-secretases have been identified in humans: the β-site AβPP-cleaving enzymes 1 and 2 (BACE-1 and BACE-2). Both β-secretases are more widely expressed in the human brain than the AβPP protein [120]. The amyloidogenic pathway starts with a cleavage by BACE-1, which releases the extracellular sAPPβ fragment and leaves a 99 amino acid long peptide, C99, still bound to the membrane. The N-terminal part of the C99 corresponds to the Aβ peptide. The C99 fragment is further processed by γ-secretase, resulting in the Aβ peptide and the AβPP intracellular domain as cleavage products (Figure 7). The γ-secretase has two alternative positions for cleavage in the C-terminal part of the Aβ peptide, which results in different lengths of the peptide: Aβ_{1-40} or Aβ_{1-42}. Aβ peptides have been found in lengths of from 39 to 45 amino acids, but the 40 and 42 amino acid long versions are the most prevalent [21]. In a healthy individual, the majority of the Aβ produced is of the Aβ_{1-40} and only about 5% – 15% of the total Aβ is composed of Aβ_{1-42} [72]. The alternative lengths of the Aβ peptide could be due to cleavage of ε- or ζ-secretase in a combination with γ-secretase [120].

Figure 7. Schematic illustration of the AβPP processing. The initiation cleavage of the AβPP occurs in the ectoplasmic domain by the α- or β-secretases, resulting in the sAPPα or sAPPβ fragments. The remaining membrane-bound fragments are continuously cleaved within the transmembrane region by the γ-secretase, releasing p3 or Aβ in combination with AβPP intracellular domain (AICD).
Structure

AβPP

All AβPP family proteins are type 1 integral membrane proteins, containing a large extracellular N-terminal region, a single transmembrane-spanning domain, and a small cytoplasmic C-terminal region. The Aβ peptide sequence is only found within the AβPP protein. The Aβ sequence within the AβPP is partly inserted into the cell membrane [120]. The complete structure of AβPP has not yet been determined, but the structures of separate domains have been resolved individually.

All AβPP proteins are subjected to several different forms of post-translational modification. All three mammalian splicing forms, as well as AβPPL and AβPL-1, undergo N-glycosylations. Both AβPP and AβPPL-2 have also been shown to be O-linked glycosylated in vivo. AβPP is also phosphorylated at several positions. The phosphorylation of a threonine at position 668 has been proposed to affect AβPP processing [120].

dAβPPL and AβPP695 share about 30 % sequence homology, even if dβAPPL lacks the Aβ-peptide [124]. Expression of a β-secretase-like protein in Drosophila has been shown to produce an Aβ-like peptide from the dAβPPL that creates amyloid-like fibrils and causes neurotoxicity [125].

Aβ-peptide

The Aβ peptide is a natively unstructured peptide in monomeric form. The first structure of the AAβ-peptide in an amyloid fibril came from solid-state nuclear magnetic resonance analysis of the 40 amino acid long variant. In this model, the AAβ1-40 peptide forms two β-strands from residues 12 – 24 and 30 – 40, creating the core region of the protofilament. The N-terminal sequence is composed of residues 1 to 8 and is unstructured. Residues 25 – 29 form a sharp bend that brings the two β-sheets into contact through sidechain-sidechain interactions. A salt bridge between aspartic acid at position 23, and lysine at position 28, stabilizes the turn. The peptides are stacked on top of each other to form the protofilament involving two β-sheets. The spectral resolution of solid-state nuclear magnetic resonance is generally lower than spectral resolution obtained with solution nuclear magnetic resonance, due to lower tumbling of the molecules [14].

The structural model of the fibrils composed of AAβ1-42 was obtained by hydrogen/deuterium-exchange nuclear magnetic resonance. In this model residues 1 – 17 are disordered, residues 18 – 26 and 31 – 42 form
two β-sheets connected by a small turn of residues 27 – 30. At least two molecules of Aβ1-42 are required to adopt the structure and form a protofilament (Figure 8). The protofilament has partially unpaired β-strands at the fibrillar ends, allowing new peptides to bind at both ends of the fibril [15].

Analyses of both AAβ1-40 [14] and AAβ1-42 [15] provide similar conclusions: that the fibrils contain a protofilament structure of two anti-parallel β-sheets, which stack with parallel β-strands between peptides.

Amyloid formation

Aggregation of Aβ peptides into amyloid fibrils does not occur in a linear pathway. Rather, distinct aggregation intermediates or oligomers are formed, which give rise to fibril formation. The intermediate states could either be on-pathway or off-pathway to make different forms of oligomers to be accumulated at different stages in the fibrillation process [126]. The fibrillation process, i.e. the folding of the Aβ peptide into amyloid fibrils in vitro, commences with a structural transition of the unfolded monomers. This step has been shown to involve the formation of a pre-α-helical state, which later assembles into a β-sheet rich aggregate. The fibrillation process thus makes Aβ change its conformation from being unstructured, to an α-helix, and finally to a β-sheet [21]. The structure of the smallest molecular weight oligomers of
the Aβ peptide have been characterized by atomic force microscopy. The oligomers are relatively compact with significant order, a width of approximately 1 nm – 3 nm, and a various lengths from between 5 nm – 10 nm. Larger oligomers with a length of 15 nm – 25 nm and a width ranging between 2 nm and 8 nm, are common at high concentrations of Aβ [127].

Several types of metastable nonfibrillar intermediates have been identified in vitro. Many types of intermediates are made up of spherical beads that range between 2 nm and 5 nm in diameter, and are composed of 20 peptides or more. The intermediates can be arranged linearly or in curly chains. The species are usually named protofibrils, and should not be confused with protofilaments. Protofibrils usually bind Congo red and Thioflavine T, indicating a well-ordered structure of β-sheets, and suggesting that they are on-pathway intermediates to mature fibrils [3]. The assembly of monomers into an ordered nucleus is thermodynamically unfavorable, and is supposed to be the rate-limiting step of the fibrillation process. The nucleus contains multiple sites for monomer addition, allowing a rapid growth of higher-ordered oligomers to develop [126].

Small soluble oligomers of both Aβ1-40 and Aβ1-42, composed of dimers to tetramers and pentamers to hexamers of peptides have been identified. Measurements by circular dichroism indicate that the oligomers are fairly disorganized and consist of high amounts of β-sheets, which may facilitate early assembly into larger oligomers, protofibrils or protofilaments [128]. Aggregation of Aβ42 correlates with cellular toxicity [35, 36]. Oligomeric Aβ42 is far more toxic than monomeric Aβ42 and fibrillar Aβ42 [72]. Decreasing the concentration of Aβ below the critical concentration in vitro prevents aggregation [34].

The C-terminal part of Aβ is composed of hydrophobic amino acids, and the hydrophobic stretch increases with increased length. This makes Aβ42 more amyloidogenic than Aβ40, but it does not change the thermodynamic stability of the peptide in solution. This makes the relative concentration of the two C-terminal variants, rather than the total concentration of the peptide, rate dependent for amyloid formation [129]. Investigations of amyloid fibrils from post-mortem brains of patients with Alzheimer’s disease show that only a fraction of the Aβ peptides belong to full length Aβ40 and Aβ42. The peptides are predominantly N- or C-terminally truncated variants [130]. The different Aβ variants in senile plaques and their significance to the pathology in Alzheimer’s disease are, as yet, poorly understood [70].
Polyphenols

Polyphenols are a large group of natural and synthetic molecules (over 8,000 molecules), composed of one or more aromatic phenol rings. Polyphenols have anti-\(\beta\)-aggregation, anti-oxidant, and anti-inflammatory properties. The most studied polyphenols with respect to their anti-\(\beta\)-aggregation properties are: (-)-Epigallocatechin-3-gallate (EGCG), curcumin, reservatrol, and polyphenolic grape seed extract.

EGCG is the major polyphenolic compound of green tea. EGCG binds natively unfolded \(\beta\), reduces Thioflavin T fluorescence, and promotes formation of unstructured, nontoxic off-pathway \(\beta\) oligomers, instead of on-pathway \(\beta\) oligomers and fibrils [131]. EGCG decreased \(\beta\) levels and reduced plaque load by as much as 50% in transgenic mice, which may due to its anti-\(\beta\)-aggregation properties [132].

Curcumin is the main content of the spice, turmeric. Many studies, in vitro and in vivo, have shown that curcumin inhibits \(\beta\) oligomers [133] and fibrils [133-136]. Curcumin is known to have an anti-inflammatory and anti-oxidative effect in vivo and studies indicate amyloid clearance [133, 137-139], reduction of \(\beta\)-induced toxicity [140], as well as reduce microglia activation [141]. Curcumin has also been shown to inhibit \(\beta\) toxicity in vivo, through inhibiting tau phosphorylation [139, 142], as well as regulating \(\beta\)PP and BACE-1 transcription by interfering with copper ions [143] in cell cultures. Curcumin has also been tested in humans as a candidate drug for Alzheimer’s disease [144]. Recent studies of curcumin have shown that it inhibits the oligomeric forms of \(\beta\), but accelerates the fibril form of \(\beta\) [145]. Curcumin binds strongly to amyloid deposits, making it a molecular candidate for histological staining in pathology [133, 146, 147], or as a useful derivative in combination with near-infrared imaging in living subjects [148]. Curcumin has also been shown to have effect, mainly due to its anti-inflammatory properties, on other diseases like, rheumatoid arthritis, pancreatitis, cancer, osteoarthritis, and in some ocular as well as gastrointestinal conditions such as ulcerative colitis [149]. The ability of curcumin to bind \(\beta\) and inhibit its aggregation is due to three structural features: a hydroxyl substitution on the aromatic end group, a rigid linker region between 8 Å and 16 Å in length, and a second terminal phenyl group [150]. Current strategies include the development of curcumin analogues with similar biological activity to curcumin, but with improved pharmacokinetic characteristics, including increased bioavailability and water solubility [151]. It has been speculated that, due to dietary preferences i.e. a high curry intake, curcumin may play a role in the significantly lower prevalence of Alzheimer’s disease in the Asian Indian
population [152].

Many studies have suggested that moderate red wine consumption may protect against the development of Alzheimer’s disease. This could be due to resveratrol, a component of red wine, that reduces Aβ production and toxicity [153]. Similarly, grape seed polyphenolic extract has been shown to inhibit Aβ aggregation and temporarily reduce cognitive impairment in a mouse model of Alzheimer’s disease [154].
Tau

Introduction
Tau was first discovered as a heat stable protein involved in microtubule assembly and it is now known to be one of the most important microtubule stabilizing proteins [5]. Neurofibrillary tangles (also called neuritic plaques, or paired helical filaments) are intracellular protein inclusions composed of hyperphosphorylated tau protein [155]. Neurofibrillary tangles are, together with senile plaques the histological hallmark of Alzheimer’s disease [5].

Filamentous tau deposits are present in a number of other neurodegenerative disorders, called tauopathies, including: corticobasal degeneration, Pick’s disease, progressive supranuclear palsy, frontotemporal dementia, and Parkinsonism linked to chromosome 17 [124, 156].

Because the tangles, at least initially, occur intracellularly in neurons they were not previously regarded as amyloid, which by definition was considered to be an extracellular substance. However, tau is now classified as an amyloidogenic protein. Neurofibrillary tangles are composed of paired helical filaments and the morphology of its fibrillar structure displays the amyloid specific X-ray diffraction pattern, and they bind Congo red. Neurofibrillary tangles occur not only in human but also in several other mammalian species [1].

Wildtype Drosophila has a single endogenous tau gene. The Drosophila tau protein can accumulate in axons in a similar manner as the mammalian tau [157].

Biological function
Tau is a microtubule stabilizing protein that is expressed in neurons and which localizes predominantly in axons [156]. Tau is also expressed at lower levels in astrocytes and oligodendrocytes [158]. The major function of tau is to regulate the assembly and stability of microtubules [156]. The mechanisms by which tau toxicity mediates neuronal dysfunction and neurodegeneration in Alzheimer’s disease and other tauopathies are at present unknown.

Structure
Human tau has six isoforms produced by alternative mRNA splicing of
exons 2, 3 and 10. The number of microtubule binding domains denotes the isoforms, as well as the presence or absence of two N-terminal domains (Figure 9). Three isoforms of tau have three microtubule-binding domains (3R); the other three isoforms have four such domains (4R). The microtubule-binding domains are located in the C-terminal of the protein and are positively charged, which allows tau to bind to the negatively charged microtubule. The isoforms with four microtubule-binding domains are approximately 40-times more efficient at stabilizing microtubules than those with three binding domains. However, they are also more prone to form neurofibrillary tangles than the isoforms with three microtubule-binding domains [124, 156].

![Figure 9](image)

**Figure 9.** A schematic illustration of the alternative splicing of human tau into six isoforms. Grey arrows indicate the splicing positions.

**Formation of Neurofibrillary Tangles**

The neurofibrillary tangles found in patients suffering from Alzheimer’s disease and other tauopathies are composed of hyperphosphorylated and aggregated tau protein. The distribution of neurofibrillary tangles correlates well with neurodegeneration and clinical symptoms [5]. There are many sites for tau phosphorylation, at least 30 of which are phosphorylated in neurofibrillary tangles [159]. Little is known about the role of the phosphorylation and dephosphorylation of specific sites with respect to aggregation and toxicity *in vivo* [124].

Unlike most other amyloid forming proteins, recombinant full-length tau proteins do not fibrillate spontaneously *in vitro*. The first step in the fibrillation process involves native tau protein being converted into the unfolded monomer. The unfolded monomer then forms into a partially folded intermediate, a process which is time-dependent but does not display a lag phase *in vitro*. The intermediate appears before filament
nucleation, and the intermediates can either aggregate or form a nucleus. The fibrillation process proceeds through an exponential growth phase and the filaments are finally produced (Figure 10) [160].

Figure 10. Fibrillation mechanism of tau. Native tau is phosphorylated and dissociates from microtubules. Excessive phosphorylation (hyperphosphorylation) renders misfolding into an intermediate that self assembles into a nucleus for further fibrillation growth forming paired helical filaments and ultimately neurofibrillary tangles.
Transgenic animal models of neurodegenerative diseases

Introduction

Genetically modified animal models come in a multitude of ‘flavors’, such as overexpressors, knockouts, knockins or regulatable transgenes. Such modified animals may increase the understanding of pathogenic mechanisms of human disorders and allow therapeutic approaches to be tested [70]. When expressed in a transgenic animal the gene should preferably be expressed at levels corresponding to that seen in vivo. This is particularly important in models used in therapeutic assays, since overexpression might require a much higher dose for the same effect than a normal expressor would. Hence, a therapeutic agent that might be effective in patients might be ineffective or toxic in a model with aberrant expression levels. The model should display both clinical and pathological phenotypes that are commonly observed in human patients. The phenotype for an amyloidosis should display progressive disease with increasing age, display amyloid deposits, and be correctly distributed in tissues [161].

The expression of a transgene can differ across generations and give rise to unstable phenotypes due to a loss of transgene copies or more complex genetic mechanisms. AβPP transgenic mice models commonly suffer from spontaneous death due to unknown mechanisms, which makes therapeutic investigations difficult since a candidate drug should affect the pathology and not the mechanism driving spontaneous death [70].

Animal modeling of Alzheimer’s disease has been experimentally assessed in C. elegans, Drosophila, mouse, rat, rabbit, dog, and non-human primates. Each model displays different aspects of the disease [162]. In the most frequently used mouse model, mice overproduce Aβ so giving rise to several pathological lesions including extracellular amyloid deposits, behavioral deficits and memory defects, but they do not exhibit global neuronal loss [163]. Progressive neurodegeneration in combination with extensive accumulation of Aβ have been seen in transgenic Aβ expressing Drosophila [164, 165]. Another interfering feature of the Drosophila model is that the toxicity of amyloidogenic peptides or proteins can be displayed in single or in combination in vivo, by simple crossings [124]. One mouse strain having a naturally occurring amyloidosis has been found with a naturally occurring mutation in the apolipoprotein A2 gene, which causes the disease [55].
Mouse models of TTR related diseases

No naturally occurring TTR-associated amyloidosis have been reported in mice [166]. Expression of TTR has been shown to have a protective role in transgenic mice models of Alzheimer’s disease [110, 167] as well as cell cultures [168].

Transgenic mouse models of familial amyloidotic polyneuropathy

Mouse model expressing low levels of TTRV30M
The first transgenic mouse model of human TTR was made in 1986 of the familial amyloidotic polyneuropathy associated TTRV30M. The transgenic mouse produced human TTRV30M protein at low concentrations in the liver, but no amyloid was found in the animals [169].

Mouse model overexpressing TTRV30M
The first transgenic mice overexpressing TTRV30M were produced by a distant enhancer element that increased the protein production in the liver 10-fold that of the previous model. No amyloid deposits were found in animals aged 12 months or less, but deposits were found in older animals aged 15 – 18 months [170].

Mouse model overexpressing TTRV30M in multiple tissues
A widely expressing TTRV30M transgenic mouse was produced, which expressed human TTRV30M in liver, heart, brain, skeletal muscle, kidney, and lung tissues. Amyloid deposits were detected in the mucosa of the small intestine in animals aged 6 months. Amyloid deposits were also observed in the renal glomeruli of animals aged 12 months. Patients with human familial amyloidotic polyneuropathy associated with the TTRV30M mutant, have amyloid deposits in the peripheral nervous system, the eye, and sometimes in the choroid plexus and in the heart. These deposits were not seen in this animal model [171].
Another widely expressing transgenic TTRV30M mouse model has been produced, but no amyloid deposits have been detected in the peripheral nervous systems of transgenic animals aged up to 24 months. In animals aged over 24 months, amyloid deposits were found in the stomach, intestine, glomeruli, heart, vascular system, and thyroid gland. The mice showed an increased level of amyloid deposits with increased age, which is consistent with human familial amyloidotic polyneuropathy. Deposits were prominent in tissues with a rich blood supply (kidneys, heart, and
thyroid gland) but not in the organs where the protein was expressed (liver and choroid plexus) [172].

**Mouse model overexpressing the intact human gene of TTR_{V30M}**

The intact human gene was reconstructed with most of its known regulatory sequences and used to generate several transgenic mice strains. These mice displayed a tissue specific protein deposit in the same tissues as those in which the protein was synthesized: viz. in the gut, heart, skin, and kidney. The animals overexpressed TTR_{V30M} to a large degree, but they still did not display peripheral or autonomic neuropathy [173].

**Mouse model overexpressing TTR_{L55P}**

Wildtype TTR and TTR_{L55P} overexpressing animals have been produced which contain all the known regulatory sequences of the human TTR gene. The wildtype TTR strain exhibited amyloid deposits in kidney and heart, at the age of 18 months. Both strains showed tissue expression in the same pattern as seen in human subjects. Young animals showed diffuse deposits while older animals had rigid deposits that could be stained by Congo red [174]. A TTR_{L55P} transgene crossed onto the murine TTR knockout background has also been generated. Some of these animals exhibited more extensive amyloid deposits suggesting that the murine TTR protein inhibits the aggregation and deposition seen in other transgenic animals. mRNA of TTR_{L55P} was mainly detected in brain, liver and eye, but also at low concentrations in heart, kidney, tongue, stomach, and skeletal muscles. Animals older than 18 months displayed protein deposits, but these were not detected by Congo red [170].
Table 2: Neuropathological characteristics of some transgenic mouse models of transthyretin associated diseases.

<table>
<thead>
<tr>
<th>Model</th>
<th>Age</th>
<th>CR_{OT}</th>
<th>CR_{N}</th>
<th>mTTR</th>
<th>Liver</th>
<th>OT</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTR_{V30M} [169]</td>
<td>&gt; 24</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TTR_{V30M} [170]</td>
<td>15–18</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>TTR_{V30M} [171]</td>
<td>6</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>TTR_{V30M} [172]</td>
<td>&gt; 24</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>TTR_{wt}, TTR_{V30M} [173]</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>TTR_{V30M} [174]</td>
<td>18</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TTR_{L55P} [170]</td>
<td>18</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

++ : extensive phenotype; + : detectable phenotype; - : no detected phenotype; nr: not reported. Age: age of plaque onset (months); CR_{OT}: presence of protein deposits in tissues other than neuronal tissues detected by Congo red; CR_{N}: presence of protein depositions in neuronal tissue detected by Congo red; mTTR: expression of murine TTR; Liver: expression specific to the liver; OT: expression found in tissues other than liver.

**Mouse models of Alzheimer’s disease**

Mouse models that are transgenic for mutant AβPP, mutant presenilin 1 and mutant tau have shown pathological lesions including both senile plaque and neurofibrillary tangles (Table 3) [165]. However, the behavioral phenotypes are mild and are often developed after several months. Neurodegeneration is not a feature of the majority of mouse models of Alzheimer’s disease [165].

AβPP knockout mice have been generated, and are viable and fertile, but obtain a lower weight and show reduced locomotor activity [72].

**Transgenic mouse models of Alzheimer’s disease**

*Aβ expressing mice*

Animal models expressing wildtype human AβPP are often of greater interest than mice expressing mutated forms of AβPP, because the majority of patients suffer from sporadic forms of Alzheimer’s disease. In early transgenic mice models, the human Aβ peptide was directly expressed under a promoter [70], in the same manner as the Alzheimer’s disease models in *Drosophila* [175]. These animals did not produce any
Aβ deposits in the brain, but amyloid deposits were found in the pancreas, intestine or skeletal muscles [70].

**Py.8.9**
Wildtype human AβPP overexpressing mice have been produced, showing AβPP synthesis and alternative splicing of the gene. The level of Aβ was low and no neurodegeneration was observed [176]. The mouse strain was redesigned with another promoter, resulting in an increased level of AβPP production. These mice displayed vascular amyloid deposits [177].

**BRI-wt-Ab42**
A transgenic mouse strain has been produced that expresses wildtype Aβ1-42 as a fused protein together with BRI, a transmembrane protein that is involved in amyloid deposits seen in cases of British familial dementia. The transgene produced high levels of Aβ in the brain of the mice. Extensive vascular and amyloid deposits, dystrophic neurons and an increased number of activated astrocytes were detected in the mice. The amyloid deposits in BRI-wt-Ab42 mice started in the cerebellum, but no neuropathology was found in aged animals [70].

**Transgenic mouse models of familial Alzheimer’s disease**

**PDAPP**
A transgenic strain producing all three isoforms of AβPPv717F by alternative splicing has been produced. The PDAPP mice produced increased levels of Aβ1-42 in the brain, up to 18 months of age [178]. These mice developed Aβ aggregates in the brain from the age of 8 months. The amount of aggregates increased and spread in 18-month old animals, but no neurodegeneration was found [179].

**APP-London**
Transgenic mice models expressing human AβPPv717F, the London mutation, have been produced. An increased level of Aβ1-42 was found in young mice and diffuse plaque and neurofibrillary tangles were found in aged animals. Learning and memory deficiency, using the Morris water maze test, were reported in the APP-London mice [70].
Tg2576
Tg2576 mice are the most frequently used AβPP transgenic models. Tg2576 mice carry the Swedish mutation, AβPP<sub>K670N/M671L</sub>, in the human AβPP gene. These mice display cognitive deficiencies and amyloid plaques [180]. A substantial amount of cerebral amyloid angiopathy is also often found in Tg2576 mice [70].

APP23
The APP23 model is another model of the Swedish mutation, expressed in the human AβPP<sub>751</sub> isofrom. At the age of 7 months these animals have a strong and highly specific expression in postmitotic neurons and they develop Congo red detectable senile plaques. APP23 mice have often been used to study cerebral amyloid angiopathy pathogenesis [70]. The APP23 mice crossed with TTR overexpressing mice have been shown to have decreased amounts of senile plaques and lower neurotoxicity, compared to APP23 mice of the same age. The suggested mechanism is that TTR binds toxic or pre-toxic Aβ aggregates in a chaperone-like manner [167].

Tg-ArcSwe
Tg-ArcSwe models expressing both the Swedish (AβPP<sub>K670N/M671L</sub>) and Arctic (AβPP<sub>E693G</sub>) mutation were developed by two independent groups [181, 182]. Small intracellular Aβ deposits were observed at the same time as the mice displayed a cognitive impairment. Amyloid plaques were observed to start surrounding the blood vessels six months after the disease onset [181].

Tg-APP<sub>arc</sub>
The Tg-APP<sub>arc</sub> mouse model expresses human AβPP with the Arctic mutation (AβPP<sub>E693G</sub>). The mice show an increased level of Aβ in the brain with increased age. Amyloid deposits, detected by Congo red, were found to occur in the hippocampus and spread to the thalamus with increased age. Cognitive behavioral testing revealed deficiencies in learning and memory [183].

3xTg-AD
The triple transgene, 3xTg-AD, has been produced by inserting AβPP<sub>K670N/M671L</sub> and tau<sub>P301L</sub> into a presenilin-1<sub>M146V</sub> knock-in background. Small intracellular Aβ deposits were visible in mice aged 3 months and extracellular senile plaques were visible in mice aged
between 6 and 12 months. The formation of neurofibrillary tangles was not seen until the mice were aged 18 months [184]. These mice have been used to develop immunotherapy as a treatment for Alzheimer’s disease [78, 79].

5xFAD

The first transgenic mouse model having neuronal degeneration was the 5xFAD transgenic model. The 5xFAD strains express five familial mutations linked with Alzheimer’s disease, the AβPP<sub>K670N/M671L/I716V/V717I</sub>, in a combination with presenilin-1<sub>M146L/L286V</sub>. Young mice express high concentrations of Aβ<sub>1-42</sub> and have an Aβ<sub>1-42</sub>/Aβ<sub>1-40</sub> ratio of 25:1 (compared with 0.1:1 – 0.2:1 in Tg2576). Amyloid deposits are detectable in mice only 2 months old [185].

Table 3: Neuropathological characteristics of some transgenic mouse models of Alzheimer’s disease. Modified from [70].

<table>
<thead>
<tr>
<th>Model</th>
<th>Age</th>
<th>SP</th>
<th>DP</th>
<th>CAA</th>
<th>iAβ</th>
<th>ND</th>
<th>CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bri-wt-Aβ42</td>
<td>3</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>nr</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PDAPP [179]</td>
<td>6–8</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>nr</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>APP-London [70]</td>
<td>&gt;12</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>nr</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Tg2576 [70]</td>
<td>9–11</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>APP23 [70]</td>
<td>6</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>nr</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Tg-ArcSwe [181, 182]</td>
<td>6</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Tg-APP&lt;sub&gt;ac&lt;/sub&gt; [183]</td>
<td>&gt;12</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>3xTg-AD [184]</td>
<td>6</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>++</td>
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<tr>
<td>5xFAD [185]</td>
<td>2</td>
<td>++</td>
<td>+</td>
<td>nr</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

+++ : extensive phenotype; + : detectable phenotype; - : no detected phenotype; nr: not reported. Age: age of plaque onset (months); SP: presence of senile plaques; DP: presence of diffuse plaques; CAA: cerebral amyloid angiopathy; iAβ: intraneuronal Aβ accumulation; ND: neurodegeneration; CNS: expression specific to the central nervous system.

**Drosophila as a model in neurodegenerative diseases**

During the last decade, the fruit fly *Drosophila melanogaster* (*Drosophila*), has increasingly been used as a model for neurodegenerative diseases, such as Alzheimer’s disease [164, 175, 186], Huntington’s disease [187], amyotrophic lateral sclerosis [188], and familial amyloidotic polyneuropathy [189, 190]. The advantages of using
a *Drosophila* model are their extremely well-defined genetic characteristics, their availability in large quantities, their short lifespan, and the simplicity with which they can be genetically manipulated [191, 192]. A drug-screen in *Drosophila* can be performed by mixing the drug in the food, by exposing the flies to vaporized chemicals, or by drug delivery to individual headless *Drosophila* [193]. Mixing the drug into the flies' food is by far the most used drug delivery route. Expression of amyloidogenic peptides into *Drosophila* have linked the phenotype of the flies' lifespan and behavior to the predicted aggregation propensity of the expressed peptides [194]. Analysis of the *Drosophila* genome has revealed that 77% of human disease-related genes have a homologue in *Drosophila* [195]. This led to the formation of the database “Homophila” in 2001, which lists all human disease related genes in *Drosophila* [196].

The UAS-Gal4 system

One of the major advantages with the *Drosophila* model is the UAS-Gal4 system [197]. This system allows the rapid generation of individual strains in which expression of a specific gene of interest can be directed to different tissues or cell types. The method separates the target gene from its transcriptional activator in two distinct transgenic lines, resulting in a silent target gene in the absence of its activator. This ensures that the parental lines are viable and when the target gene is turned on in the progeny, the phenotypic consequences of misexpression, including lethality, can be studied. The system allows the target gene to be activated in different cell- and tissue-types by altering the activator-expressing lines [197]. A library of different activator-expressing lines generating the target gene to be expressed in numerous distinct patterns has been generated at Bloomington *Drosophila* Stock Center at Indiana University, where almost 500 different Gal4-lines are available for purchase [198].

An activator from yeast, the transcription factor Gal4, which has no endogenous targets in *Drosophila*, is the most commonly used activator. The Gal4 binding site has been mutated, generating an optimized site to which Gal4 binds with high affinity to an upstream activating sequence (UAS), coupled to the target gene (Figure 11) [197]. Gal4 is more active at 29 °C than at 19 °C, giving a temperature dependent expression. Gal4 has also been introduced as a hormone-induced Gal4-system and a temperature-sensitive Gal80 TARGET system [199].
**BACKGROUND**

**Figure 11.** Schematic illustration of the function of the UAS-Gal4 system, allowing cell- or tissue-specific expression of the target gene (Gene X) in the offspring.

**Drosophila models of neurodegenerative diseases**

**Aβ producing Drosophila**

Triple transgenic *Drosophila* expressing human AβPP, human β-secretase, and *Drosophila* presenilin have been generated. The flies produced low levels of Aβ1-40 and Aβ1-42. The flies develop Aβ deposits and display age-dependent neurodegeneration [200].

**Aβ expressing Drosophila**

Aβ expressing flies have been produced simultaneously by two groups. UAS genes were inserted corresponding to the peptides Aβ1-40, Aβ1-42, a double inserted Aβ1-42, and Aβ1-42 E22G (the Arctic mutation), which also contained a secretion signal peptide at the N-terminus causing the peptides to be secreted towards the extracellular space [164, 175]. Both the Aβ1-40 and the Aβ1-42 peptides accumulate during aging in the fly brain [164], but only the Aβ1-42, the double inserted Aβ1-42, and the Aβ1-42 E22G formed protein deposits in the tissue [164, 175]. The Aβ1-42, the double inserted Aβ1-42, and the Aβ1-42 E22G caused reduced lifespan, locomotor defects, and age-dependent neurodegeneration in the brain and in the eye [164, 175]. Micrographs from the electron microscope revealed that most degenerating neurons showed necrotic cell death in the Aβ1-42 expressing fly [164].

45
**BACKGROUND**

**Tau expressing Drosophila**

*Drosophila* expressing human wildtype tau, tau<sub>R406W</sub>, have been created as models of human tauopathies as well as tau<sub>V337M</sub> associated with Parkinsonism linked to chromosome 17 [186, 201]. The expression of human wildtype or mutant tau resulted in a reduced lifespan and an age-dependent progressive neurodegeneration, characterized by nuclear fragmentation and vacuole formation in the eyes, which appears as a “rough”-eye phenotype [186].

**SSA and FAP models in Drosophila**

*Drosophila* expressing wildtype TTR, have been produced as well as mutants associated with familial amyloidotic polyneuropathy, TTR<sub>V30M</sub> and TTR<sub>L55P</sub> [189, 190]. Expression of TTR<sub>V30M</sub> in the nervous system resulted in a reduced lifespan and a reduced climbing ability indicating neurological impairment. Expression of wildtype TTR showed a milder phenotype. Congo red staining of the *Drosophila* brain shows positive amyloid binding in aged TTR<sub>V30M</sub> flies. Extensive brain vacuole formation was evident for the aged TTR<sub>V30M</sub> flies, whereas the wildtype TTR flies showed a milder phenotype. In addition, expression of TTR<sub>V30M</sub> in the eye leads to tissue damage, including the ‘rough-eye’ phenotype, morphological changes and fibrous deposition [190]. The TTR<sub>L55P</sub> expressing *Drosophila* exhibit a protein aggregation phenotype that increased with increasing age, a decreased lifespan, a change in wing posture, and attenuation of locomotor activity including compromised flight ability [189].

**Drosophila models of Parkinson’s disease**

A Parkinson’s disease *Drosophila* model, expressing human wildtype α-synuclein, as well as α-synuclein<sub>A30P</sub> and α-synuclein<sub>A53T</sub>, has been generated. Expression of α-synuclein in the nervous system produces a loss of dopaminergic neurons, filamentous intraneuronal inclusions containing α-synuclein and locomotor dysfunction [202].
Drosophila models of Huntington’s disease

A Huntington's disease Drosophila model that expresses polyglutamine-expanded huntingtin has been generated. As in human neurons, polyglutamine-expanded huntingtin induced neuronal degeneration and nuclear inclusions. The age of onset decreased and the severity of neuronal degeneration increased with increased length of the glutamine residues [187]. Other poly-glutamine associated proteins have also been modeled in Drosophila, also leading to neurodegeneration [203].

Drosophila model of Gerstmann-Sträussler-Scheinker syndrome

Drosophila expressing either wildtype mouse prion protein or Gerstmann-Sträussler-Scheinker syndrome-associated mouse prion protein, PrP_{101L}, have been generated. The PrP_{101L} flies show severe locomotor dysfunction and decreased lifespan. An age-dependent accumulation of misfolded prion protein and neuronal vacuolization has been observed. Flies expressing wildtype prion protein displayed no phenotype [204].

Drosophila model of amyotrophic lateral sclerosis

The human wildtype superoxide dismutase and five familial amyotrophic lateral sclerosis associated mutants have been generated in a Drosophila superoxide dismutase null background. The familial amyotrophic lateral sclerosis associated mutants had reduced lifespans compared with wildtype superoxide dismutase expressing flies. The decreased lifespan was probably due to an increased oxidative stress and decreased motor performance in the Drosophila [188].
This thesis is the result of approximately 5 years of work as a graduate student within the research group of Prof. Per Hammarström in collaboration with Prof. Stefan Thor. The work has focused on modeling amyloid diseases in *Drosophila melanogaster*. This has been performed by:

- Creating new model systems of senile systemic amyloidosis and familial amyloidotic polyneuropathy in *Drosophila melanogaster*
- Developing a new staining protocol for the detection of amyloid in *Drosophila melanogaster*
- Initiating a compound screen of Alzheimer’s disease modeled in *Drosophila melanogaster*

The work has resulted in three papers. The methods used in this work are briefly summarized in the methodology section of this thesis. This is followed by a short summary of the papers.
Generating transgenic Drosophila melanogaster

To generate transgenic animals, the new gene needs to fuse with the chromosomal DNA in the host cell. In making transgenic Drosophila, the new gene must be inserted into a specific cloning vector under the control of a consensus translational start site (CAAAATG). The gene is inserted into a Drosophila embryo by a P-element coupled transposase system. The transgene is inserted into the germ line by injection into the syncytial blastoderm stage, making a backcross necessary for developing the transgenic line (Protocol 2 in [205]).

Survival Assay

A survival assay is a robust way of estimating the general health of transgenic Drosophila expressing an amyloidogenic protein or peptide into the central nervous system by the post-mitotic C155-Gal4-driver. The Kaplan-Meier estimation of unfinished data estimates the proportion of a population surviving and median lifetime ($T_{50\%}$) of a whole population [206]. By comparing lifespan trajectory plots of different transgenic animals to an out-crossed wildtype line, the toxic effect of an expressed gene can be calculated (Protocol 4 in [205]). In this way, the toxicity of a protein or peptide has been shown to correlate with the aggregation propensity of the expressed protein or peptide [194].

External circumstances must be taken into consideration and controlled when conducting these types of experiments. Factors such as temperature, humidity, food, number of flies in the vial, the type of incubator, and laboratory procedures, may all affect the outcome of an experiment, which may lead to lab-specific results for all lifespan experiments, so yielding a different $T_{50\%}$ for the same cross in different environments. This effect must be taken into account when comparing lifespan trajectories published by different labs.
**Activity Assay**

**Climbing**

Climbing assays are used to measure locomotor function in *Drosophila* with neurodegenerative diseases induced via the postmitotic driver C155-Gal4 driver. After being gently shaken to the bottom of a vial, the number of flies that are able to climb to the top of a vial during 20 seconds are counted. The procedure should be repeated at least three times for every data point for flies of different ages, in order to achieve a stable average measure (Protocol 5 in [205]). Although flies naturally display a decreased ability to climb as a function of increased age, the expression of neurodegenerative peptides or proteins into the central nervous system exacerbates any decrease in flies’ ability to climb.

**DAM2 System**

A new technique for measuring locomotory behavior in *Drosophila* that has been recently established on the market is the DAM2 *Drosophila* Activity Monitor (TriKinetics Inc, Waltham, MA, USA). The flies are kept in 5 mm or 7 mm plastic tubes sealed with a lid and a cotton plug. As the flies walk horizontally back and forth within the tube they break an infrared beam across the center of the tube. Associated software counts every time the beam is broken to give a measure of the activity of flies in a tube. The DAM2 system contains 32 channels per unit and several units can be measured simultaneously.

**Tissue Staining**

**Mayer’s Haematoxylin**

Haematoxylin is a popular histological stain often used in combination with Eosin. Haematoxylin, which is extracted from the wood of the longwood tree, stains basophilic structures such as the cell nuclei, ribosomes, and regions in the cytoplasm rich in RNA. Haemotoxylin stains purple, while Eosin stains a bright pink color. Mayer’s Haematoxylin is commonly used for morphological studies investigated by bright-field microscopy.

**Immunohistochemistry**

Immunohistochemistry is used for localizing proteins by binding antibodies to specific antigens. Tissues are first fixed to prevent degradation and
then blocked to reduce non-specific binding. A primary antibody is used to detect and bind to a specific protein, followed by a secondary antibody, linked to a fluorescent dye or an enzyme producing color in the presence of a substrate, which binds to the primary antibody.

**Congo red and Thioflavine S**
The ‘gold standards’ for amyloid detection in tissues are Congo red and Thioflavine S. However, Congo red and Thioflavine S do not have sufficient specificity, and have emission fluorescence that is too weak compared to background staining, to allow amyloid imaging of *Drosophila* brain sections, such that only a few successful results with these stains have been reported in the literature [189, 190, 200]. To obtain the Congo red specific apple-green birefringence under crossed-polarized light in a *Drosophila* sample is nearly impossible, due to the presence of high background signals and the small size of deposits in *Drosophila* brains. In addition, Thioflavine S has a fluorescence emission spectrum that significantly overlaps background fluorescence, mainly from the tracheas and connective tissue of *Drosophila*.

**Luminescent Conjugated Oligothiophenes**
The amyloid specific probes, Luminescent Conjugated Polythiophenes (LCPs) and Luminescent Conjugated Oligothiophenes (LCOs), have recently been reported to be conformation-sensitive probes that emit distinctive fluorescence light upon binding to aggregated proteins [207-209]. The LCO undergoes a structural restriction upon protein binding and shifts the spectrum of emitted light, depending on the target protein and its conformation. LCOs bound to amyloid are very stable and highly resistant to photobleaching [210].
Results and discussion

Paper I

Aim
The aim of this project was to generate a new model of senile systemic amyloidoses and of familial amyloidotic polyneuropathy in *Drosophila*.

Results

Expression of human TTR in adult transgenic *Drosophila*

Transgenic *Drosophila* were generated that expressed a single gene of extracellular wildtype TTR (TTR<sub>wt</sub>) and TTR<sub>V30M</sub>, under the control of the Gal4/UAS system. Gene expression was controlled by immunohistochemistry with an anti-human TTR antibody of cryosections (Figure 1C in Paper I), and by SDS-PAGE followed by Western blotting (Figure 1D in Paper I) of brain sections corresponding to TTR<sub>V30M</sub> and TTR<sub>wt</sub> expressing flies. The protein concentration was equal in both transgenes and was estimated to correspond to 0.23 mg/ml of TTR expressed in the flies.

Lifespan assay

The effect of expressing amyloidogenic proteins in the central nervous system on the lifespans of transgenic flies, in comparison with a control group, was used as a marker of terminal disease. Expression of TTR<sub>V30M</sub> in the central nervous system resulted in reduced lifespan when compared to control flies (Figure 12B, and D). TTR<sub>wt</sub> showed a milder phenotype (Figure 12C, and D).
Climbing

The effect of expressing amyloidogenic proteins in the central nervous system on the climbing behavior of transgenic flies, in comparison with a control group, was used as a marker of neurological impairment. No significant tendency of reduced climbing before death was seen for the control flies. The TTR_{V30M} expressing flies showed a 4-day period of inactivity prior to death in the climbing assay compared to 2 days for TTR_{wt} expressing flies. This indicated that the mutant protein induced neurodegeneration that precedes death (Figure 12).

Figure 12. Survival assay and climbing assay of transgenic Drosophila A: C155-Gal4/+; B: C155-Gal4/UAS-TTR_{wt}; and C: C155-Gal4/UAS-TTR_{V30M}. The dashed lines indicate the climbing trajectory compared with the lifespan trajectory indicated by the solid line. D. Median day of survival (solid bar) compared to the median day of successful climbing (striped bar) for Gal4/+, C155-Gal4/UAS-TTR_{wt} and C155-Gal4/UAS-TTR_{V30M}. *** p<0.0001
Amyloid detection by staining with Congo red

Fluorescence micrographs of the *Drosophila* brain showed positive Congo red binding in aged transgenic flies expressing TTR in the central nervous system. The control flies, aged in parallel with the transgenic flies, showed very modest binding of Congo red whereas the TTR<sub>wt</sub> expressing flies showed a moderate binding indicating the presence of some amyloid deposits. The TTR<sub>V30M</sub> expressing flies showed extensive staining by Congo red, indicating amyloid deposits that were most prevalent in areas surrounding the cell bodies (Figure 13).

Vacuole formation

The morphological structure of the *Drosophila* brain was analyzed by bright field images of Mayer’s Hematoxylin stained cryostat sections of aged flies. The total number of vacuoles was calculated as well as the number of large vacuoles (> 10 µm in diameter). The control flies were found to have few vacuoles, virtually none of which were large. The TTR<sub>wt</sub> expressing flies had an increased number of vacuoles compared to the control, some of which were large. The TTR<sub>V30M</sub> expressing flies
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showed extensive vacuolation and a massive amount of large vacuoles (Figure 14). A concurrent feature of the TTRV30M expressing flies was the extensive vacuolation rendering a spongiform change throughout the brain, showing that the integrity of the brain had been severely compromised (Figure 5E-F in Paper I).

Figure 14. Number of vacuoles disclosed by Mayer’s Hematoxylin staining of sections of Drosophila heads. (●): Total number of vacuoles; and (▲): number of vacuoles > 10 µm plotted for Gal4/+, C155-Gal4/UAS-TTRwt and C155-Gal4/UAS-TTRV30M. *** p<0.0001

Eye degeneration

Although the expression of amyloidogenic TTR in the Drosophila eye does not affect its normal development, it does cause a progressive degeneration of the eye. Expression of TTRV30M leads to a more dramatic degeneration than does expression of TTRwt. After 25 days the eye has lost most of its pigmentation and is severely degenerated. Morphological changes included eye collapse and loss of symmetry of the compound eye, accompanied by widespread fibril deposits on the surface of the eye (Figure 15).
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Figure 15. Eye morphology of transgenic Drosophila. Scanning electron micrographs showing eye morphology of transgenic Drosophila. A-C: GMR-Gal4/+ displayed normal eye morphology. The arrow in C indicates small specks of dust or yeast cells (from food) adsorbed to the eye. E-F: GMR-Gal4/UAS-TTRwt showed normal eye morphology. Small amounts of fibril deposits were seen at higher magnification as indicated by the arrow in G. I-K: GMR-Gal4/UAS-TTRV30M showed obvious evidence of degeneration. The rough-eye phenotype included collapse of the eye (in J) and evident morphological change in the compound eye. Areas showing large amounts of fibril deposition were visible. Cryostat sections of eyes from flies stained with Mayer’s Hematoxyline. D: GMR-Gal4/+ eyes were intact with a normal ommatidia structure. H: GMR-Gal4/UAS-TTRwt shows a mild curly morphology in the ommatidia, but no vacuolization. L: GMR-Gal4/UAS-TTRV30M showed some vacuolation and the ommatidia showed a curly morphology. Black, gray and white scale bars in the micrographs indicate 100 µm, 20 µm and 10µm respectively.
Conclusions

To establish a *Drosophila* model for TTR-related amyloid diseases, transgenic *Drosophila* expressing human extracellular TTR\textsubscript{wt} and TTR\textsubscript{V30M}, under the control of the Gal4/UAS system were generated. Expression of TTR\textsubscript{V30M} in the nervous system resulted in brain lesions, reduced lifespan and impaired climbing ability, when compared to normal flies. TTR\textsubscript{wt} expressing flies exhibited milder changes in the phenotype. Expression of TTR\textsubscript{V30M} in the eye led to tissue degeneration including rough-eye, morphological changes and fibrous deposition. These results demonstrate that *Drosophila* is a useful system in the study of TTR-related human neurodegenerative diseases.
Paper II

Aim
The aim of this project was to generate a better staining procedure to detect amyloid deposits in *Drosophila*. This is of great importance since the conventional markers for amyloid, Congo red and Thioflavine S, do not allow high magnification of amyloid fibril deposits in *Drosophila*. The new protocol also makes it possible to distinguish between different forms of amyloid composed of different proteins or peptides, with the same amyloid specific probe.

Results
In this study we have used three different Aβ expressing *Drosophila* lines (Aβ1-42 single transgene, Aβ1-42 double transgene, and Aβ1-42 E22G single transgene) [175], one tau expressing *Drosophila* line [186], and one TTRV30M expressing *Drosophila* line [190], all expressed in the *Drosophila* central nervous system by the Gal4/UAS system [197].

P-FTAA staining of Aβ1-42 amyloid in *Drosophila* sections compared to Thioflavine S and Congo red

Staining of *Drosophila* cryo-sections to detect amyloid was performed with the benchmarked Congo red and Thioflavine S as amyloid specific probes, and compared with the results from staining performed by the LCO, pFTAA [209]. Staining with Congo red gave a low signal to noise ratio (Figure 16) and background binding was increased in the area of the eyes (Figure 1A in paper II). Staining with Thioflavine S gave better results than were achieved with Congo red. However, although small Thioflavine S-positive species were detected (Figure 16), background staining was even more severe than for Congo red (Figure 1B in paper II). A highly specific and intense staining was obtained from the LCO, which revealed a heavy amyloid load in the brain. Besides a general binding to the retina and exoskeleton, almost no background fluorescence was obtained from the actual brain tissue in the LCO stained section (Figure 1C in paper II).
RESULTS AND DISCUSSION

Figure 16. Comparing staining of double inserted Aβ1-42 expressed Drosophila performed by the three amyloid specific probes A: Congo red; B: Thioflavine S; and C: the LCO pFTAA. Scale bars indicate 200 µm. Arrows indicates amyloid deposits and arrowheads indicates areas with strong background binding.

Double staining of protein aggregates in Drosophila sections with p-FTAA and antibodies

Figure 17. Staining of protein aggregates in transgenic Drosophila stained by protein specific antibody, the LCO pFTAA and DAPI. Scale bars indicate 20 µm. Arrows indicate small aggregates and open arrowheads indicate long extended fibrillar structures.
The three different Aβ expressing *Drosophila* lines [175] showed extensive LCO positive aggregates, but in different patterns depending on genotype. The single Aβ1-42 genotype showed most LCO binding species surrounding the cell nuclei. Very few extended species with fibril structure were obtained (Figure 17A). The double inserted Aβ1-42 genotype showed as extensive amyloid staining from LCO as the single insert Aβ variant, but with several long extended amyloid deposits with fibril morphology (Figure 17B). The Aβ1-42 E2G genotype showed a spot-like staining from both LCO and antibodies. The antibody staining showed a near perfect co-localization with the LCO, but the antibodies did not stain the fibril structure to the same extent as the LCO (Figure 17C). The DAPI staining from regions with a high amount of LCO positive species was decreased, indicating neuronal loss. The tau expressing *Drosophila* [186] showed small LCO positive and antibody binding aggregates that were mostly found outside the regions of the cell nuclei (Figure 17D). The TTRV30M expressing *Drosophila* [190] showed LCO staining of small aggregates which were TTR positive mainly in the area close to the cell nuclei, but extracellular aggregates were also visible (Figure 17E). In the control flies no aggregated protein was detected (Figure 17F).

**Spectral analysis of p-FTAA stained amyloid in *Drosophila* sections by microspectroscopy**

Spectral analyses of LCO-stained double inserted Aβ1-42 *Drosophila* sections revealed a distinct double emission peak at 520 nm and 545 nm (Figure 18A). Tau expressing *Drosophila* showed more red shifted spectra than the spectra corresponding to Aβ expressing *Drosophila*, with a single broad emission peak at 558 nm (Figure 18B). These emission spectra are similar to those previously reported for mouse and human tissue samples of Alzheimer’s disease [209]. The emission spectra correspond well with those from senile plaques (Figure 18C) and neurofibrillary tangles (Figure 18D) of tissue samples from a patient with Alzheimer’s disease [209].
RESULTS AND DISCUSSION

Figure 18. Spectral analyses of p-FTAA stained amyloid in Drosophila sections compared to human tissue samples of Alzheimer’s disease. A: transgenic Drosophila expressing double inserted Aβ1-42; B: transgenic Drosophila expressing tau; and C–D: human tissue samples of Alzheimer’s disease, corresponding to senile plaque in C and neurofibrillary tangles in D. E: Comparative spectra of extended fibril structure of double inserted Aβ1-42 (solid line) and tau (dotted line) of Drosophila. F: Comparative spectra of human tissue samples of Alzheimer’s disease showing senile plaque (solid line) and neurofibrillary tangles (dotted line).

WHOLE DROSOPHILA BRAIN AMYLOID IMAGING BY P-FTAA

Due to the high specificity and low background binding of the LCO, the protocol can also be used to detect amyloid in dissected whole Drosophila brain (see Figure 4 in Paper II). The high specificity, low background, stability against photobleaching, and the opportunity for spectral analyses, make the LCO the most potent amyloid probe for Drosophila samples reported to date.

CONCLUSIONS

This protocol resulted in the robust detection of protein fibrils, with very low background staining in the Drosophila brain from using a Conjugated Luminescent Oligothiophene (LCO), p-FTAA probe. When compared with standard amyloid-specific probes, the LCO makes co-staining with antibodies feasible, and enables high resolution imaging of several different protein aggregates in the Drosophila brain, such as Aβ1.
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42. Aβ_{1-42} E22G, TTR_{V30M} and human tau. Additionally, Aβ and tau aggregates could be distinguished from each other due to their distinctive LCO emission spectra. Furthermore, this protocol enables 3D-brain mapping of amyloid distribution in whole-mount Drosophila brains. The use of p-FTAA combined with other probes, antibodies and/or dyes, will aid in the rapid characterization of various amyloid deposits in the rapidly growing number of Drosophila models of neurodegenerative diseases.
Paper III

Aim
The aim of this project was to test a well-documented candidate drug (curcumin) for Alzheimer’s disease in Drosophila in order to gain a deeper understanding of the molecular mechanisms of the disease.

Results
In this study we used four different $\alpha\beta$ expressing Drosophila lines ($\alpha\beta_{1-40}$ single transgene, $\alpha\beta_{1-42}$ single transgene, $\alpha\beta_{1-42}$ double transgene, and $\alpha\beta_{1-42}\, E22G$ single transgene) [175], and one tau expressing Drosophila line [186], all expressed in the Drosophila central nervous system and eyes by the Gal4/UAS system [197]. The C155-Gal4 driver [211] was used for all experiments, except for the eye degeneration assays, in which the GMR-Gal4 driver [212] was employed.

Lifespan and climbing assay
The lifespan and climbing behavior of flies treated with curcumin displayed a rescue effect on genotypes having the strongest degenerative phenotype. The increased lifespan following curcumin treatments was striking for flies expressing $\alpha\beta_{1-42}\, E22G$. The median survival among flies treated with the medium curcumin concentration increased to 75 %. However, the control flies suffered a toxic effect from curcumin treatments that increased with increasing curcumin concentrations. The toxic effect of the curcumin treatment among the control group evened out the rescue effect on genotypes with the mild phenotype (Figure 19). The climbing assay results display the same trend as the lifespan results, with only a few days of delay (see supplementary figure in Paper III).
RESULTS AND DISCUSSION

Figure 19. Lifespan trajectories of transgenic *Drosophila* for A: Control flies; B: Aβ₁₋₄₀ expressing flies; C: single inserted Aβ₁₋₄₂ expressing flies; D: Double inserted Aβ₁₋₄₂ expressing flies; E: Aβ₁₋₄₂ E₂₂G expressing flies; F: Tau expressing flies. The black, yellow, orange, and red lines represent the quantities of added curcumin as 0, 1, 10, and 100 µg curcumin per g yeast paste, respectively.

Activity assay

The activity of flies was measured every 5 days over a 24 h period with the DAM2 system to compare with results from the conventional climbing assay. Total activity decreased with age, but the number of beam breaks per hour was almost consistent during the first few hours. However, the decrease in the number of total beam breaks with increasing age was due to an overall decrease in the number of hours when flies were active. All flies expressing Aβ and tau showed a higher number of beam breaks and a continuation of activity over a longer time following curcumin treatment. The effect of curcumin generally decreased with increased age. Flies expressing the tau protein showed the largest increase in activity of all genotypes following curcumin treatment, but their lifespans or climbing behavior were not affected (Figure 20).
RESULTS AND DISCUSSION

Figure 20. Activity assays of Control flies, Aβ\(_{1-40}\) expressing flies, Aβ\(_{1-42}\) expressing flies, Double inserted Aβ\(_{1-42}\) expressing flies, Aβ\(_{1-42}\) E22G expressing flies, Tau expressing flies, and the total number of beam breaks by all genotypes. Activity assays in the absence (solid line and bars) and presence (dotted line and bar) of curcumin treatments performed 5, 10, 15, and 20 days after eclosion are represented by black, red, blue, and green graphs and bars, respectively, for each genotype.
Amyloid detection in histological samples

Histological samples of sections stained with protein specific antibody, the amyloid specific LCO, and the nuclear marker DAPI revealed no change in amyloid deposits following curcumin treatment. All genotypes displayed different amyloid staining pathology (Figures 3 and 4 in Paper III), but no differences were observed between curcumin treated and untreated flies.

In vitro fibrillation followed by Western blot and transmission electron microscopy

Since the flies exhibited increased lifespans and activity following curcumin treatment, but did not show any effect in the amyloid staining pathology, a fibrillation assay of synthetic Aβ1-42 peptide in absence and presence of curcumin was performed. The fibrillation of recombinant Aβ1-42 peptide in the absence and presence of curcumin was followed by Western blot of native PAGE gels and by transmission electron microscopy. Western blot of freshly dissolved Aβ1-42 in the presence and absence of curcumin showed bands corresponding to monomeric Aβ1-42 peptide, as well as an oligomer smear. After 60 minutes of fibrillation, fibrils that were unable to penetrate the gel were detected in reactions containing low and medium concentrations of curcumin with a decreased population of oligomers. After 180 minutes of fibrillation there were no differences in the amount of large aggregates among any of the samples (Figure 21A). Transmission electron micrographs of samples taken at different times during the fibrillation assay indicated an increased tendency for Aβ1-42 fibril formation in the presence of curcumin. Taken together curcumin appeared to enhance the fibrillation into large aggregates and decreased the residence time of soluble oligomers (Figure 21B and C)
Figure 21. A: Western Blot of aliquots from *in vitro* fibrillization of Aβ₁-₄₂ taken at time point 0, 60, and 180 minutes. Fibrillation was performed in the absence and presence of 0.0001 %, 0.001 %, and 0.01 % of curcumin. Transmission electron micrographs from *in vitro* fibrillatization of synthetic Aβ₁-₄₂ taken after B: 60 minutes, and C: 180 minutes.
Conclusions

The lifespan and climbing behavior of Alzheimer’s disease Drosophila models treated with curcumin displayed a positive pharmacological effect in terms of survival. Importantly, the pharmacological effect was directly dependent on genotype, rendering the strongest mitigative effect by curcumin for Aβ expressing flies exhibiting the worst phenotype. The increased lifespan upon curcumin treatment was especially striking for flies expressing the Aβ peptide with the Arctic mutant Aβ1-42 E22G.

Flies assayed for their locomotor activity using the DAM2 system displayed another tendency in curcumin-induced effects on activity compared to the climbing assay. All flies expressing Aβ and tau showed a higher number of beam breaks and a continuation of activity during a larger number of hours upon curcumin treatment. The effect of curcumin was usually decreased with increased age. Unexpectedly, the flies expressing the tau protein showed the largest increase in activity of all genotypes upon curcumin treatment.

Visual inspection in the fluorescence microscope of histological sections of aged fly brains and eyes stained with a specific antibody, the amyloid specific LCO, and the nuclear marker DAPI, displayed no change in amyloid deposition upon curcumin treatment. All genotypes display different staining pathology.

The in vitro fibrillation assays of recombinant Aβ1-42 peptide showed that the presence of curcumin decreased the population of soluble oligomers and appeared to accelerate formation of large amyloid fibrils.

It is plausible that the apparent toxicity of curcumin within Drosophila, which appears to be absent for mammalian cells, does suggest that the neuroprotective effect of curcumin is even stronger than that reported here. The main drawback for curcumin for use as a drug for treatment of Alzheimer’s disease appears to be the poor bioavailability and stability in solution. With that in mind it is encouraging that curcumin analogues are synthesized as candidate drugs towards Alzheimer’s disease.
1. Drug screens

It has previously been shown that small molecule stabilization of tetrameric transthyretin can lock the native state of TTR, so preventing dissociation and hence blocking amyloidogenesis [106, 118]. The TTR transgenic flies modeling human TTR amyloid diseases (Paper I) allow small molecule screens to suppress degeneration, increase lifespan, and alleviate amyloid deposition. The decreased lifespan of the generated TTR transgenic flies was minor when compared with other models such as the Aβ expressing flies [175], but the neurodegeneration of the TTR\textsubscript{V30M} expressing flies was considerable. The neurodegeneration, together with activity measurements performed in the same manner as described in Paper III, would enable experimental investigations to be performed in the absence or presence of small molecule stabilizations in order to study the interplay between these hallmarks of the disease.

The Aβ expressing flies [175] should also be investigated in the presence of other candidate drugs in a similar manner to that described in Paper III. Curcumin is well-documented as having anti-inflammatory and anti-aggregation properties, but other candidate molecular drugs have also been reported [213]. The short lifespan of \textit{Drosophila} and the simplicity with which they can be genetically manipulated, means that the \textit{Drosophila} model could be used for screening large numbers of potential drugs for Alzheimer’s disease.

2. Molecular chaperone and anti-chaperone theories

When TTR and Aβ are co-expressed, it has been shown that TTR decreases the toxicity and prevents amyloid fibril formation in transgenic mice, possibly through a chaperone mechanism [110]. The expression of amyloidogenic peptides in \textit{Drosophila} has linked the phenotype of the flies’ lifespan and behavior to the predicted aggregation propensity of the expressed peptides [194]. This makes it possible to investigate the aggregation of multiple proteins in combination. Expression of a small engineered Aβ-binding protein has recently been reported to reduce Aβ
toxicity, in the same transgenic Aβ expressing *Drosophila* strain used in this thesis [214]. This is of great interest, not only for a combination of TTR and Aβ, but also for mimicking human Alzheimer’s disease by expressing Aβ and tau in combination, or by expressing different ratios of Aβ<sub>1-40</sub> and Aβ<sub>1-42</sub> in transgenic *Drosophila*.

Since treating Aβ expressing *Drosophila* with curcumin increased lifespan and activity, but did not remove amyloid deposits from their tissue we hypothesized that soluble Aβ<sub>1-42</sub> oligomers were responsible for neurotoxicity (Paper III). However it would be of interest to investigate if other factors may explain why curcumin increased lifespan and activity. This could be performed by a gene array of *Drosophila* expressing Aβ in the presence and absence of curcumin to identify possible molecular mechanisms that underlie its effects. Curcumin treatment may alter the expression of those genes responsible for the pharmacological effect of curcumin.

3. Histological staining screens

The main problem with working with *Drosophila* has been the difficulty of detecting amyloid deposits, and different forms of amyloid (polymorphs or various proteins), in high magnification micrographs of *Drosophila* tissue. The new staining protocol of *Drosophila* amyloid deposits (Paper II) should facilitate investigating other *Drosophila* models of amyloidoses. Employing micro spectroscopy to analyze differential emission spectra also make it possible to distinguish between different forms of amyloid. By employing this method, different forms of amyloid could also be monitored during drug delivery to the flies.

Work on the new amyloid specific probes, (LCOs) have demonstrated the capacity of these dyes to function as conformation-sensitive probes, emitting distinct fluorescence light upon binding to aggregated proteins [207-209]. New LCO-probes with different properties are constantly being developed. The *Drosophila* models of different neurodegenerative diseases could play an important role in the development of new and better LCOs, thus facilitating the detection of amyloid diseases in humans. The *Drosophila* model system can provide a tissue specific expression of a single amyloidogenic protein or different amyloidogenic proteins in combination in a controlled manner. *Drosophila* could efficiently be used for differentiating between intra- and extracellular deposits that cannot easily be quantified by imaging in cell cultures. Thus, the initial screening of new probes for characterizing amyloid aggregates can suggestively be performed using *Drosophila*.
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