

Chapter 9

Mechanisms of Transthyretin Aggregation and Toxicity

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Abstract Amyloidoses are characterised by the deposition of insoluble protein that occurs in the extracellular compartment of various tissues. One form of amyloidosis is caused by transthyretin (TTR) misfolding and deposition in target tissues. It is clear that many amyloidoses share common features of fibrillogenesis and toxicity. This chapter examines the mechanisms of TTR aggregation with a view to understanding the possible therapeutic interventions in amyloid disease.

Keywords Amyloid · Familial amyloidotic polyneuropathy · Fibrillogenesis · Amyloidosis · Calcium · Glycosoaminoglycans

Abbreviations

TTR Transthyretin
AFM Atomic force microscopy

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DLS	Dynamic light scattering
FAP	Familial amyloidotic polyneuropathy
A β	Amyloid beta protein
Å	Angstrom
nm	Nanometer
kDa	Kilodalton
SSA	Senile systemic amyloidosis
IAPP	Diabetes-associated islet amyloid polypeptide
PrP	Prion protein
HSPG	Heparan sulphate proteoglycan
CSPG	Chondroitin sulphate proteoglycan
GAG	Glycosaminoglycan
VGCC	Voltage-gated calcium channel
TRPM8	Transient receptor potential (melastatin) channel

9.1 Introduction

The amyloidoses constitute a disparate group of diseases characterised by the aggregation and extracellular deposition of insoluble protein (amyloid) in a variety of tissues. Accumulation of amyloid can lead to dysfunction of the affected organs. The underlying pathophysiology of the amyloidoses is still not completely understood and a large body of evidence now exists implicating amyloid formation in the pathogenesis of diseases such as Alzheimer's disease, prion diseases and the British and Danish dementias. There is increasing evidence that the mechanisms of pathogenesis of amyloidoses are similar and that it is the conformation of the amyloid, rather than the specific amino-acid sequence that is the key determinant of disease progression and toxicity (Bucciantini et al. 2002). A striking, but common feature of all amyloids is their high β -sheet content and their fibrillar ultrastructure. These observations have strengthened the notion that amyloidogenic proteins cause neurotoxicity by a similar mechanism. Therefore, a more complete understanding of the mechanisms of one amyloidosis may provide insights into the biology of other diseases.

The familial amyloidotic polyneuropathies (FAPs) are a group of diseases characterised by deposition of amyloid in the peripheral nervous system. One FAP is transthyretin (TTR)-related amyloidosis. TTR FAP is a rare autosomal dominant disease characterised by the deposition of TTR in nerves that can cause peripheral pain, paresthesia and muscular weakness leading ultimately to cardiac and renal system dysfunction (Ando et al. 2005a). The underlying molecular mechanisms responsible for TTR amyloidosis and the subsequent pathophysiological responses in tissues are yet to be fully understood. This chapter will review our current understanding of the mechanisms of TTR aggregation and toxicity *in vitro*. It also explores how this knowledge may inform the process of designing therapeutic agents to treat FAP. The implication of these studies on FAP and other important neurodegenerative diseases of the central nervous system such as Alzheimer's disease will also be discussed.

9.2 Transthyretin in Health and Disease

9.2.1 Structure and Function of TTR

TTR is a thyroid hormone transport protein that is highly conserved in sequence similarity and biological function (Blake and Oatley 1977). The primary structure is highly conserved throughout eutherian, marsupial, bird, reptile and fish species (Fung et al. 1988). TTR possesses a conserved structural motif of four identical monomeric subunits arranged to form a central channel, or binding domain. In humans, TTR is a relatively abundant protein that is primarily produced in the liver, and to a lesser extent in extra-hepatic tissues such as the choroid plexus. TTR is also involved in the transportation of retinol by forming a complex with a smaller, retinol-binding protein (Hagen and Elliott 1973).

The crystal structure of human TTR has been determined (Blake et al. 1971), and shown to be a 55 kDa tetramer with four identical 127 amino-acid subunits exhibiting an unusually high β -sheet content. Almost 45 % of the residues in a TTR monomer are organised into eight β -strands, identified as A-H, connected by loops, or short helix, as occurs between strands E and F (Fig. 9.1a). Strands CBEF are oriented orthogonally to strands DAGH, forming a prominent β -barrel (Fig. 9.1b). Extensive hydrogen bonding at the contact region between two monomers results in strong dimeric interactions (Blake et al. 1974). The native tetrameric structure of TTR is formed by two TTR dimers that bind through relatively weak hydrophobic and hydrophilic interactions between the AB loop of one monomer and the H strand of the two primed monomers, creating a 50 Å central channel that contains two thyroid hormone binding sites. The strength and extent of the monomeric interactions suggest that the dimers, rather than monomers or tetramers are the basic unit of TTR structure.

9.2.2 Transthyretin Amyloidosis and FAP

TTR amyloidosis is a relatively common inherited amyloid disease. However, the amyloidosis also exists as a sporadic, but asymptomatic disease in the elderly known as senile systemic amyloidosis (SSA). In SSA, deposits of wild-type TTR accumulate in cardiac tissue and only infrequently cause complications such as cardiomegaly and congestive cardiac failure (Westermarck et al. 1990). FAP is associated with systemic extracellular amyloid deposition in the peripheral nervous system. It is now established that the inherited forms of TTR amyloidosis are associated with over 100 mutations in the TTR gene with affected individuals or single families having a single mutation. Significantly, a single mutation can exhibit a wide variety of clinical presentations, age of onset and organ involvement suggesting a complicated picture of amyloid pathogenesis. FAP was originally described in a small group of patients in Portugal (Andrade 1952b). The disease was initially described as a peripheral neuropathy with some involvement of the renal and cardiac systems. The Portuguese kindred has since been extensively studied (Ando 2005; Ando et al. 2005b; Lashuel

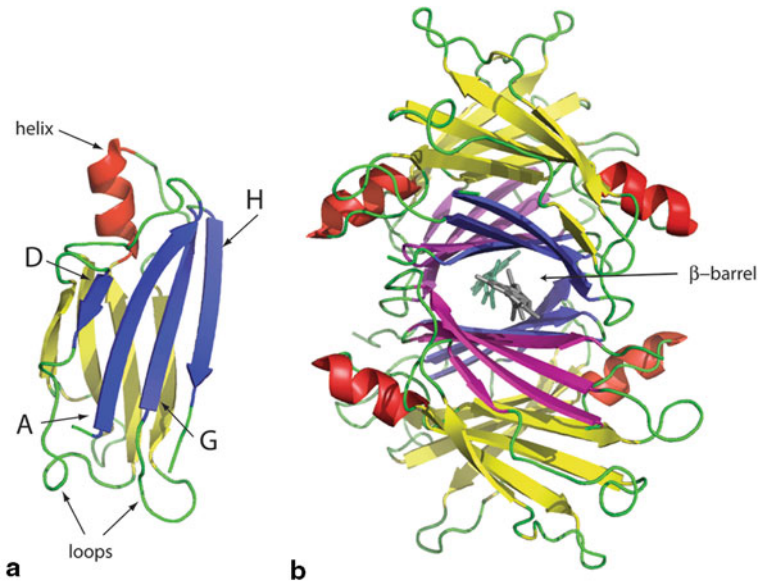


Fig. 9.1 Schematic representation of transthyretin monomer and tetramer structures. **a** TTR monomer with inner β -sheet (DAGH) shown in *blue* and the outer sheet (CBEF) shown by *yellow* shading. **b** Predicted structure of TTR tetramer complexed with 3',5'-dibromo-2',4,4',6-tetrahydroxyaurone, a flavone derivative. The diagram shows four inner, monomer β -sheets (shaded in magenta and blue) forming a prominent β -barrel. The diagrams were constructed using MacPyMOL (Schrodinger 2010) using coordinates from file PDB ID:1THC (Ciszak et al. 1992)

et al. 1998; Quintas et al. 1997a, 1999) and the typical presentation of this FAP involves small diameter fiber loss in the lower extremities, coupled with loss of temperature and touch sensation. Pain is common with a distal to proximal neuropathic presentation. Parathesias, lower limb motor impairment and generalised autonomic symptoms of constipation or diarrhoea can occur with further involvement of cardiac, renal and gastrointestinal systems and invariably, death of affected individuals.

Histopathological studies of TTR FAP have demonstrated that axonal degeneration and neuronal loss are associated with extensive endoneurial amyloid (Sousa 2003). Biopsy and autopsy material of patients with FAP show amyloid deposition in nerve trunks, plexuses and sensory and autonomic ganglia (Coimbra and Andrade 1971). Amyloid deposits are mainly present in the endoneurium, usually accompanied by destruction of the myelin sheath, degeneration of nerve fibers and neuronal loss (Takahashi et al. 1997). Amyloid deposits have also been detected in the choroid plexus, cardiovascular system and kidneys. The oculoleptomeningeal form of FAP is characterised by severe, diffuse amyloidosis of the leptomeninges and subarachnoid vessels associated with patchy fibrosis, obliteration of the subarachnoid space and widespread neuronal loss (Herrick et al. 1996; Uitti et al. 1988).

The most common (and extensively studied) variant, *Val30Met* (V30M) occurs in Portuguese, Swedish and Japanese kindreds, with age of presentation and severity of

disease symptoms varying considerably. Similar variability in phenotype occurs in other less frequent mutations such as *Leu58His*, *Thr60Ala*, *Ser77Tyr*, *Ile84Ser* and *Val22Ile* and the rare, but highly aggressive *Leu55Pro* (L55P) mutation which will be referred to extensively in this chapter.

9.3 Transthyretin Aggregation in vitro

The presence of amyloid in diseased tissue was originally documented in the mid 19th century (reviewed by Cohen and Calkins 1959). Advances in microscopy and histochemical staining techniques in the early part of the 20th century enabled researchers to apply the criteria of congophilic staining and birefringence under polarized light to positively identify amyloid deposits from biopsy and post-mortem tissue. Electron microscopic studies have since confirmed that amyloid deposits from diverse proteins all exhibit a similar, fibrillar structure. Generally, amyloid deposits are composed of rigid, unbranched fibrils ranging in width from 60–130 Å and 0.1–16 nm in length (Cohen and Calkins 1959; Sipe and Cohen 2000). These observations have formed the widely held view that the amyloidoses are a clinically diverse group of diseases that all involve proteins which have a common propensity to aggregate and produce insoluble fibrils. Proteins with little or no sequence similarity such as TTR, the A β protein of Alzheimer's disease, diabetes-associated islet amyloid protein (IAPP) and the prion proteins (PrP) can aggregate to form similar insoluble fibrillar deposits in vivo (Bucciantini et al. 2002; Dobson 2003; Stefani and Dobson 2003; Vendruscolo et al. 2003). Significantly, X-ray diffraction studies of protein fibrils has revealed a common structural theme of ordered secondary structure, with the peptide backbone of β -pleated sheets oriented perpendicular to the fibril axis (Jaroniec et al. 2004).

While many studies have examined the fibrillogenesis of TTR and other amyloidogenic proteins in vitro, there is little, if any, evidence correlating the aggregated species with cellular toxicity. Identification of toxic amyloidogenic aggregates would greatly accelerate the search for an effective treatment for a fatal disease such as FAP. Several studies have shown that mutant TTR aggregates to form high molecular weight oligomers more readily than wild-type TTR, and that further aggregation leads to the formation of amyloid fibrils (Kayed et al. 2003; Reixach et al. 2004). There is a good correlation between the rate of aggregation of TTR in vitro and the extent or severity of the disease phenotype (Hurshman et al. 2008; Lashuel et al. 1999; Quintas et al. 1997a). For example, the rare L55P mutation produces a significantly more aggressive amyloidosis than the more common V30M mutation, and in vitro studies have shown that L55P aggregates more much readily than V30M (Quintas et al. 1997a; Lashuel et al. 1998, 1999; Hammarström et al. 2002b; Hou et al. 2005; Pokrzywa et al. 2007; Hurshman et al. 2008). There is considerable scope therefore, to justify the use of TTR mutant proteins to study the pathogenesis and possible treatment of TTR FAP.

9.3.1 Mechanisms of TTR Aggregation

The mechanism by which TTR forms fibrils is not well understood. While it is generally accepted that some form of amyloidogenic intermediate species is important to initiate fibrillogenesis, the precise nature and sequence of molecular events that drives TTR association into fibrils are yet to be elucidated. Furthermore, it is not clear at what stage during fibrillogenesis that toxic species are formed. Over the last decade various groups have elucidated the crystal structure of over 20 mutant TTR variants. While these studies have shown structural differences that could be explained in terms of fibrillar structures, there is no apparent mechanism or conformational change that would explain fibril formation by all mutant forms of TTR (Hörnberg et al. 2000). Early attempts to understand TTR fibrillogenesis employed methods designed to solubilise mature fibrils and to determine their species composition (Costa et al. 1978), however the identity of the building blocks of TTR fibrils has remained elusive.

More recently, several studies have suggested mutations of wild-type TTR result in the destabilisation and dissociation of TTR tetramers into unfolded monomers and dimers which undergo further partial refolding, forming amyloidogenic intermediates (Cardoso et al. 2007; Colón et al. 1996; Lai et al. 1996; Lashuel et al. 1998, 1999). Significantly, there is a strong correlation between the thermodynamic stability of TTR variants and their propensity to form unfolded, soluble aggregates (Quintas et al. 2001; Shnyrov et al. 2000). The V30M mutation is the most frequently occurring variant and an examination of its crystal structure suggests the mutant substitution results in a conformational change in strand A, exposing Cys10, and rendering the thiol group more exposed (Terry et al. 1993). The formation of fibrils resulting from the association of TTR through disulphide bridges has been suggested (Thylén et al. 1993), however the existence of an amyloidogenic *Cys10Arg*(C10 A) variant would suggest that such a mechanism is not a significant factor in V30M fibrillogenesis. A “hot spot” for amyloidogenic mutations occurs in the region between residues 45 and 58. This region contains the C strand, C-D loop, and D strand which are located at the edge of each dimer (Serpell et al. 1995). The structure of the highly amyloidogenic and clinically aggressive L55P variant crystallizes in a different space to that of wild-type and other variant forms of TTR (Quintas et al. 1997b). Analysis of the structure suggests that strands C and D are disrupted, altering the hydrogen bonds between the AB loop of one dimer and strand H of the other dimer (Sebastião et al. 1998), in an area that defines weak, native dimer-dimer interactions. These observations suggest a significant destabilisation of the L55P tetramer with the formation of, as yet, undefined intermediate species that subsequently aggregate further to form fibrils. Whether these destabilised intermediates serve a dual purpose, namely, seeds for further polymerisation and aggregation or soluble, toxic oligomeric species responsible for the pathogenesis of FAP, has yet to be determined. Since the L55P variant is clinically aggressive it has been the focus of much of our own work investigating the mechanisms responsible for the toxicity of L55P in sensory neurons (Gasperini et al. 2011).

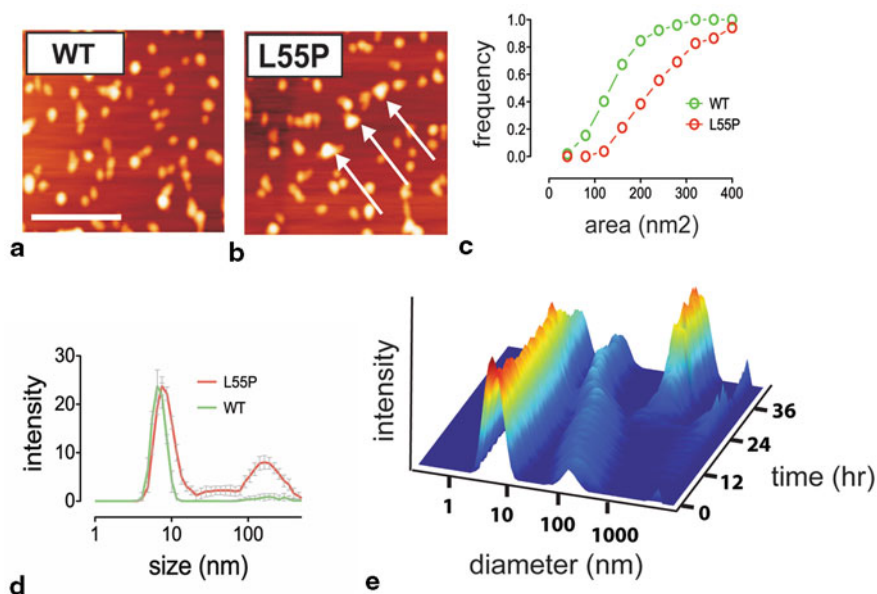


Fig. 9.2 Aggregation of transthyretin *in vitro*. AFM images of freshly prepared **a** Wild-type and **b** L55P transthyretin. *Arrows* denote large, oligomeric aggregates of L55P TTR (scale bar is 100 nm). **c** When particle cross-sectional areas from the AFM are quantitated, L55P (*red*) contain a higher proportion of large particles than wild-type preparation (*green*). **d** Using a dynamic light scattering technique, L55P (*red*) contains a population of large, soluble oligomeric aggregates ranging in size from 100–300 nm in diameter. **e** Qualitatively, when L55P is monitored by DLS at 37 °C over 36 h, these oligomeric species decrease in average size and appear to form much larger (1,000 nm) aggregates, probably protofibrils

9.3.2 Monitoring TTR Aggregation *in vitro*

We have compared the aggregation properties of the L55P variant with wild-type TTR protein using atomic force microscopy (AFM) and dynamic light scattering (DLS) techniques. Freshly prepared L55P and wild-type TTR in physiological buffers contains predominantly globular or amorphous particles ranging in apparent size from 10–50 nm in diameter (Fig. 9.2a, b). The smallest particles observed by AFM are approximately 10 nm in diameter and are probably tetramers. Significantly, using quantitative image analysis of particle cross-sectional area (Fig. 9.2c), the average AFM particle sizes of L55P are greater than those of wild-type preparations confirming that L55P is highly unstable at physiological pH and ionic strength (Quintas et al. 1997b).

Are these larger aggregates amyloidogenic intermediates or toxic oligomers? To answer this question, we have examined freshly prepared solutions of L55P more closely by measuring aggregate size distributions in real time by DLS (Fig. 9.3d).

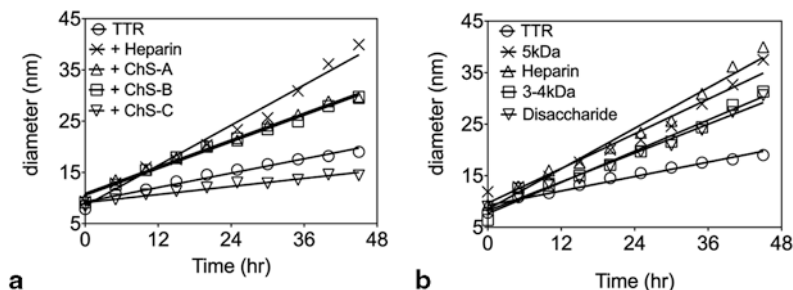


Fig. 9.3 Effect of glycosaminoglycans on TTR aggregation. **a** L55P TTR was incubated either without (*open circles*) or with heparin (*cross*), ChSA (*triangles*), ChSB (*squares*) or ChSC (*inverted triangles*) for 48 h and particle diameter measured using DLS. Heparin, ChSA and ChSB accelerated the increase TTR aggregation over time, while ChSC inhibited TTR aggregation. **b** L55P TTR was incubated either without (*circles*) or with heparin (*triangles*), 5 kDa heparin (*crosses*), 3–4 kDa heparin (*squares*) or heparin disaccharide (*inverted triangles*) for 48 h, and particle diameter measured using DLS. All heparin derivatives stimulated L55P TTR aggregation

This technique allows the measurement of size distribution (as measured by hydrodynamic radius) of protein preparations in real time. Using this technique, freshly prepared solutions of L55P at neutral pH show the presence of large molecular weight, pre-fibrillar aggregates in addition to the natively folded tetramer. Significantly monomers are not observed by DLS. However, the existence of small amounts of monomeric material cannot be excluded using this technique. Assuming a globular structure and a specific density of 1.39 g/cm^3 , which would be typical for many proteins, these intermediate aggregates would correspond to a molecular mass of $1810 \pm 160 \text{ kDa}$. These molecular species would therefore be predicted to contain approximately 130 TTR monomers. Interestingly, these intermediate species increase in size over a period of 12–36 h, during which a concomitant reduction in abundance of native tetramer occurs (Fig. 9.3e). After 36–48 h, oligomeric species are possibly converted into much larger, probably protofibril aggregates, in a nucleation-dependent manner. These observations are consistent, in part, with other studies (Hammarström et al. 2002a; Hurshman et al. 2008; Lai et al. 1996; Lashuel et al. 1998, 1999) that have demonstrated L55P dissociation into monomeric intermediates at neutral pH to form protofibrils $60\text{--}65 \text{ \AA}$ in diameter and approximately 50 nm in length. Another possible explanation for these observations would include the polymerization of natively folded, but conformationally distorted tetramers via a novel “ β -slip” mechanism (Eneqvist et al. 2000). This mechanism is not dependent on the formation of unfolded monomers as aggregation intermediates. A triple TTR variant, G53S/E54D/L55S has been generated to target the proposed amyloidogenic “hot-spot” discussed previously. This mutation induces a shift in strand B creating significantly extended C-D loops and shortened D-E loops. This “ β -slip” creates new interactions at a potential amyloid packing site, in which distorted but intact tetramers are the basic building blocks for TTR amyloidogenesis. Such a mechanism would be consistent with our experimental findings.

It is apparent that while DLS may lack the required sensitivity to detect monomeric TTR in appreciable quantities, the ability of DLS to monitor the self-assembly of products of tetramer disassociation into pre-fibrillar aggregates provides a valuable insight into TTR fibrillogenesis. The concept of partially unfolded monomers as quantal assemblies of intermediate aggregates *in vitro* has gained momentum in the field. Our current understanding of TTR fibrillogenesis would suggest that irrespective of the actual nature of the amyloidogenic intermediate(s), the *in-vitro* assembly of fibrils is probably a nucleation-dependent process. The precise mechanistic explanation for these observations remains unclear.

However, many crucial aspects of TTR fibrillogenesis remain unresolved. In particular, how does this complex picture of denaturation, refolding, aggregation and fibril formation at varying degrees of acidity and ionic strength drive the pathogenesis of TTR toxicity *in vivo*? In the context of an extracellular amyloidosis, is TTR fibrillogenesis a determinant of the severity of FAP? And finally, will a more complete understanding of TTR fibrillogenesis ultimately lead to specific, targeted therapeutic agents for FAP?

Using electron microscopy and image analysis techniques, Inoue et al. (1998) investigated the ultrastructure of mature and immature TTR fibrils in sural nerve biopsies of V30M FAP patients. Detailed analysis of averaged EM images showed mature TTR fibrils composed of a complex linear arrangement of TTR protofibrils, heparan sulphate (HSPG) and chondroitin sulphate proteoglycans (CSPG). While these studies have not been replicated, they suggest a mechanism for the regional and tissue-specific differences seen in amyloid deposits from different TTR variant FAP's.

Our group has recently investigated the effects of related molecules, the glycosaminoglycans (GAGs), on TTR aggregation *in-vitro*. Incubation of L55P TTR with heparin, chondroitin sulphate A (ChSA) or chondroitin sulphate B (ChSB) resulted in an acceleration of aggregation while incubation with chondroitin sulphate C (ChSC) resulted in inhibition of formation of soluble oligomeric species (Fig. 9.3a). Since inhibiting aggregation of proteins into toxic oligomers or fibrils would be a promising target for therapeutic intervention in FAP, the ability of small heparinoid molecules to influence aggregation of L55P was also examined. Three low molecular weight mucosal heparin fragments, a heparin disaccharide (MH di), a 3–4 kDa heparin fragment (MH 3–4 kDa) and a 5 kDa heparin fragment (MH 5 kDa) were tested. All the heparin derivatives tested stimulated aggregation of L55P, with the magnitude of aggregation dependent on the size of the fragment tested (Fig. 9.3b). Significantly, the differing effects of GAGs on TTR aggregation may have implications for our understanding of the pathogenesis of FAP and potential treatment strategies. There is evidence that GAGs can influence nucleation of other amyloidogenic proteins such as A β (McLaurin et al. 1999) and α -synuclein (Liu et al. 2005), suggesting that effects on nucleation are a common theme behind the effect of GAGs on aggregation of amyloidogenic proteins. If GAGs function through modification of nucleation-dependent aggregation processes they could be used as anti-amyloidogenic agents in FAP.

9.4 Mechanisms of Transthyretin Neurotoxicity in FAP

Understanding the mechanism of transthyretin toxicity may provide useful insights into the mechanism of amyloid toxicity in other significant amyloidoses such as Alzheimer's disease. TTR has been shown to be toxic to cells in culture (Hou et al. 2007; Sousa et al. 2001a, b). However, the complete biochemical mechanisms responsible for cellular toxicity and death are still unclear. There is good experimental evidence that misfolded proteins exert their toxic effects by interacting with cells by binding directly to lipid-rich areas of the cell membrane (Hou et al. 2005; Subasinghe et al. 2003). It has also been suggested that TTR-induced toxicity may be mediated by the receptor for advanced glycation end-products (RAGE) and that activation of RAGE leads to endoplasmic reticulum stress, activation of ERK1/2 and caspase-dependent apoptosis (Monteiro et al. 2006).

There is evidence suggesting the toxicity of amyloid is caused by an increase in cation permeability of neuronal cell membranes resulting in chronic cytosolic calcium dysregulation and subsequent cell death or dysfunction (Arispe et al. 1996; Koopmans et al. 1992; Mattson et al. 1992; Moe and Sprague 1992; Saito et al. 1993). However, the mechanism by which amyloid proteins induce calcium entry in cells is poorly understood. Some studies have suggested protofibrillar aggregates of interacting amyloidogenic proteins might embed in the membrane, leading to the formation of pore-like structures with consequent aberrant ion conductance (Tsigelny et al. 2008). Previously, we have shown TTR-induced calcium permeability in cell lines is primarily mediated by voltage-gated calcium channels (VGCC), with a small proportion ($\sim 20\%$) of the calcium influx through voltage-independent channels (Hou et al. 2007). Using embryonic sensory neurons from rodent dorsal root ganglia (DRG), we have demonstrated a novel mechanism of calcium entry requiring the coordinated activation of $\text{Na}_v 1.8$ voltage-gated sodium channels and transient receptor potential (TRP) M8 channels (Gasperini et al. 2011). The precise nature of the TTR aggregates that mediate this effect on sensory neurons is yet to be elucidated. One possibility is that TTR binds directly to cationic channels causing a conformational change leading to channel gating. However, there is no direct evidence for this. Another possibility is that TTR binds to lipid membrane components (Hou et al. 2005), thus altering cell membrane fluidity and triggering the opening of TRPM8 in DRG sensory neurons (Gasperini et al. 2011). TRPM8 channels have been described as the prototypic thermosensitive ion channel (McKemy et al. 2002; Peier et al. 2002), and are a prominent receptor subtype on small diameter (C and A δ fibre) sensory neurons (Staaf et al. 2010). Since FAP exhibits a variety of clinical manifestations, including progressive paresthesias involving the lower limbs and affecting thermosensation and nociception (Andrade 1952a), it is possible that TTR toxicity mediated through TRPM8 channels is a key molecular correlate of FAP pathogenesis.

9.5 Summary

At the present time, amyloidoses carry a significant economic burden to the health of our ageing society. It is generally accepted that this burden will continue to increase and overtake many other degenerative diseases. Our current understanding of the mechanisms responsible for aggregation and fibrillogenesis of many amyloidogenic proteins remains unclear. The design and delivery of effective therapeutic agents to inhibit or dissociate protein aggregates *in vivo* will require a complete understanding of how amyloid is deposited in target tissues and more importantly, how these aggregates mediate their toxic effects on cells. It is clear that what is learnt from studying TTR fibrillogenesis may have considerable application to other amyloidoses. In conclusion, TTR aggregation and its effects on a variety of cell types *in vitro* provides many testable hypotheses to study the pathogenesis of important diseases such as AD, prion diseases and the British and Danish dementias.

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