**Review Article**

**Transthyretin and familial amyloidotic polyneuropathy**

**Recent progress in understanding the molecular mechanism of neurodegeneration**

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**Introduction**

The term amyloidosis refers to disorders that are caused by the extracellular deposition of insoluble amyloid fibrils, which are derived from the misfolding of proteins which, under normal conditions, are soluble. A large number (> 20) of unrelated proteins are known to form amyloid in vivo.

Familial amyloidotic polyneuropathy (FAP) was described more than 50 years ago in a group of patients in Portugal who had a fatal hereditary amyloidosis characterized by a sensorimotor peripheral polyneuropathy and autonomic dysfunction [1]. It is inherited in an autosomal dominant pattern [1–3]. It has a wide geographic distribution [4,5], with the affected countries including Portugal [6,7], Japan [3,8], Scandinavian countries [9,10] and the Americas [11,12].

Familial amyloidotic polyneuropathy (FAP) is an inherited autosomal dominant disease that is commonly caused by accumulation of deposits of transthyretin (TTR) amyloid around peripheral nerves. The only effective treatment for FAP is liver transplantation. However, recent studies on TTR aggregation provide clues to the mechanism of the molecular pathogenesis of FAP and suggest new avenues for therapeutic intervention. It is increasingly recognized that there are common features of a number of protein-misfolding diseases that can lead to neurodegeneration. As for other amyloidogenic proteins, the most toxic forms of aggregated TTR are likely to be the low-molecular-mass diffusible species, and there is increasing evidence that this toxicity is mediated by disturbances in calcium homeostasis. This article reviews what is already known about the mechanism of TTR aggregation in FAP and describes how recent discoveries in other areas of amyloid research, particularly Alzheimer’s disease, provide clues to the molecular pathogenesis of FAP.

**Abbreviations**

ER, endoplasmic reticulum; FAP, familial amyloidotic polyneuropathy; GAG, glycosaminoglycan; HS, heparan sulfate; MAP, mitogen-activated protein; RBP, retinol-binding protein; TTR, transthyretin

The age of onset varies from 20 to 70 years with a mean age of onset in the 30s [3,13,14].

The peripheral nervous system is the most commonly affected tissue in the majority of patients [5,15]. The initial symptom is usually a sensory peripheral neuropathy in the lower limbs, with pain and temperature sensation being the most severely affected, followed by motor impairments later in the course of the disease, causing wasting and weakness [1,16,17]. Most patients with FAP have early and severe impairment of the autonomic nervous system, commonly manifested by dyshidrosis, sexual impotence, alternating diarrhea and constipation, orthostatic hypotension, and urinary incontinence [18,19]. Cardiac and renal dysfunction may also be observed [3,20,21]. A less common oculoleptomeningeal form of FAP has also been described, characterized by cerebral infarction and...
hemorrhage, hydrocephalus, ataxia, spastic paralysis, seizures, convulsion, dementia, and visual deterioration [22–24]. In some cases, the primary clinical manifestation is carpal tunnel syndrome [25,26], whereas in others the eyes are the main affected organ, resulting in oculomotor impairment with vitreous opacity, keratoconjunctivitis sicca, glaucoma and papillary disorders [27–29]. In general, therefore FAP has a very heterogeneous clinical presentation [30,31].

Neuropathological studies have demonstrated that axonal degeneration and neuronal loss are associated with extensive endoneurial amyloid deposits commonly formed from transthyretin (TTR) [15,32]. FAP is associated with systemic extracellular amyloid deposition, particularly in the peripheral nervous system [33–36]. Biopsy and autopsy of patients with the common V30M TTR mutation, for example, show that amyloid deposition is present in nerve trunks, plexuses and sensory and autonomic ganglia [34,35]. Amyloid deposits are mainly present in the endoneurium, usually accompanied by destruction of the myelin sheath, degeneration of nerve fibers and neuronal loss [32,34,37]. Amyloid deposits have also been detected in the choroid plexus, cardiovascular system and kidneys [36,38]. The oculoleptomeningeal form of FAP is characterized by severe, diffuse amyloidosis of the leptomeninges and subarachnoid vessels associated with patchy fibrosis, obliteration of the subarachnoid space and widespread neuronal loss [22,39].

Genetics of FAP

Human TTR is encoded by a single-copy gene on the long arm of chromosome 18. The gene spans ≈7 kb and contains 4 exons, each with approximately 200 bases [40–42]. An 18-amino-acid signal peptide is encoded by the first exon. This sequence is cleaved before secretion of mature TTR. The sequence of the TTR gene is highly conserved over evolution, as there is more than 80% identity in the sequences of mammalian TTRs [43].

In 1984, V30M TTR was identified as a common underlying genetic variant of FAP [44]. Since then, a large number of mutations in TTR have been detected; many of them are associated with FAP and are evenly distributed over the TTR sequence [45–47] (Figs 1 and 2A). Among the amyloidogenic TTR mutations, V30M is the most common, and has been detected in many kindreds around the world [5,46,47]. The diagnosis of FAP is partly based on the detection of amyloidogenic TTR variants in the plasma [48–51] or cerebrospinal fluid [49,52]. Genetic examination can also be used to screen carriers of TTR mutations [57,58] and for prenatal diagnosis [56,59,60].

Structure and function of TTR

TTR was previously known as prealbumin because it was first identified in the cerebrospinal fluid [61] and later in the serum [62] as a component that migrated ahead of albumin in an electrical field. Subsequently, the name transthyretin became more accepted when the protein was shown to be a carrier of thyroxine [63,64]. In human plasma, TTR is present at a concentration of 0.25 g L⁻¹ [65,66].

The structure of a TTR dimer is shown in Fig. 2. Native TTR is a tetramer and contains two identical thyroxine-binding sites located in a channel at the center of the molecule [67]. The two binding sites display negative cooperativity which is due to an allosteric effect resulting from the occupancy of the first binding site [68]. TTR is also involved in the transportation of retinol by forming a complex with the smaller retinol-binding protein (RBP) [69,70]. The TTR–RBP–retinol complex is formed in the endoplasmic reticulum (ER) of hepatocytes, and the formation of this complex can prevent loss of holo-RBP from the plasma by filtration through the renal glomeruli [71]. Although four RBP-binding sites have been identified on one TTR molecule, steric hindrance prevents the binding of more than two RBP molecules per tetramer [72]. Most of the TTR in the circulation is not bound to RBP [73].

As TTR does not cross the blood–brain barrier to any significant extent, a different source of production, apart from the liver, must exist to account for the protein in the cerebrospinal fluid. Indeed, TTR synthesis has been detected in the choroid plexus [74,75]. However, TTR is not likely to be essential for life as a TTR knockout mouse has normal fetal development and a normal lifespan [76]. TTR has a fast turnover rate with a plasma half-life of 2 days [77].

Native TTR is a tetramer comprising four identical subunits each of which contains 127 amino-acid residues and has a molecular mass of ≈14 kDa [78]. Each monomer contains eight β-strands denoted A–H and a short helix between strands E and F [70,79] (Fig. 2). The β-strands are organized into a wedge-shaped β-barrel, which is formed by two antiparallel four-stranded β-sheets containing the DAGH and CBEH strands, respectively [79]. Two TTR monomers join edge-to-edge to form a dimer, stabilized by antiparallel hydrogen-bonding between adjacent H–H and F–F strands. Thus one TTR dimer is composed of two eight-stranded sheets with a pronounced concave shape [79,80]. The native tetrameric structure of TTR is then
formed from two dimers through hydrophobic interactions between the A–B loop of one monomer and the H strand of the opposite dimer, creating a 50 Å central channel that contains the two binding sites for thyroxine [81]. The four binding sites for RBP are located on the surface of a TTR molecule [72]. The overall 3D structure of TTR has been maintained over vertebrate evolution, and, notably, the amino-acid sequences in the thyroxine-binding site are identical in all species examined to date [82].

**Mechanism of TTR amyloidogenesis**

Several studies suggest that amyloidogenic mutations destabilize the native structure of TTR, thereby inducing conformational changes which lead to dissociation of the tetramers into partially unfolded species which can subsequently self-assemble into amyloid fibrils [83–89]. Under physiological conditions including temperature, pH, ionic strength, and protein concentration, mutant TTR molecules can dissociate into non-native monomers with a distinct compact structure capable of partially unfolding and forming high-molecular-mass soluble aggregates [90,91]. Indeed, there is a correlation between the thermodynamic stability of TTR variants and their potential to form partially unfolded monomers and soluble aggregates [92,93]. Amyloidogenic TTR variants have lower thermodynamic stability [94]. Furthermore, studies on wild-type TTR have shown that increased temperature...
can induce conformational changes, which enable normal TTR to assemble into fibrillar structures at physiological pH [95]. Similarly, at high hydrostatic pressure, native TTR can undergo partial misfolding to form amyloidogenic species [96]. There is an inverse correlation between the stability of TTR variants at high pressure and their amyloidogenic potential. Therefore, decreased stability is probably important for misfolding of the native structure and formation of amyloidogenic intermediates.

A hot spot for amyloidogenic mutations occurs in the region between residues 45 and 58. This region contains the C strand, CD loop, and D strand which are located at the edge of each monomer [97]. It has been suggested that the amyloidogenic intermediate has a modified monomeric structure consisting of six β-strands instead of eight, with the C and D strands and the intervening loop forming a large loop, exposing some hydrophobic residues in this region that are normally buried on the inside of the protein [98]. Dislocation of the C and D strands from their native edge region may result in the formation of a new interface involving A and B strands which is open for intermolecular interactions and consequently, a shift in strand register of subunit assembly [99]. The crystal structure of L55P TTR has revealed rearrangements in strands C and D, where the proline for leucine substitution disrupts the hydrogen bonds between strands D and A, destabilizing the monomer–monomer interface contacts [95,100]. Examination of the crystal structure of V30M TTR shows that the substitution of methionine for valine results in a slight conformational change that is transmitted through the protein core to Cys10, rendering the thiol group more exposed [101]. Another study using a high-resolution crystal structure of V30M TTR has found that the substitution forces the two β-sheets of each monomer to become more separated, resulting in a distortion of the thyroxine-binding cavity, and associated with a decreased affinity for thyroxine [102]. Increased susceptibility of TTR molecules to water infiltration may be critical for the formation of amyloidogenic intermediates [96]. Notwithstanding these results, however, the significance of observed conformational changes caused by amyloidogenic mutations has been questioned, as a comparison between 23 crystal structures of TTR variants, including a number of amyloidogenic and nonamyloidogenic TTR mutants, failed to find any obvious significant difference in their structure [100].

A study of heterozygous patients with Portugal-type FAP (V30M) showed that the wild-type and V30M TTR are present in a ratio of 2 : 1 and 1 : 2 in plasma and amyloid fibrils, respectively [9]. It has been proposed that the building block of amyloid fibrils is a TTR dimer containing at least one mutant subunit or tetramers containing two or more mutant subunits. After chemical cross-linking, TTR dimers can still form amyloid fibrils, and the subunit interfaces in amyloid fibrils are similar to the natural dimeric interchain association of native TTR [103]. After limited proteolysis, N-terminally truncated dimers can form amyloid fibrils [104]. TTR amyloid fibrils could also be formed from TTR tetramers linked by disulfide bridges, as the V30M mutation results in the exposure of

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**Fig. 2.** Structure of a human TTR dimer (protein data bank accession code 1THC) from Ciszak et al. [149] showing the location of amyloidogenic mutations and position of β-strands. (A) The structure of the polypeptide backbone of the two chains (purple and blue) is shown along with the location of the N-terminus and C-terminus. The location of residues where amyloidogenic mutations can be found is shown in yellow. (B) Secondary structure of the dimer complexed to 3',5'-dibromo-2',4,4',6-tetrahydroxyaurone, a flavone derivative, showing the location of the eight regions of β-strand labeled A–H.
C10 for disulfide bond formation [101]. There is evidence for disulfide bridges between subunits in the amyloid fibrils from homozygous and heterozygous patients with the V30M mutation [105]. However, this cannot be the only mechanism of aggregation, as a mutation at the critical position, C10R, is also amyloidogenic [106].

A study of amyloidogenesis using Y78F TTR, which destabilizes interface interactions by loosening the AB loop, identified an abnormal tetrameric structure, suggesting that a modified tetramer might be an early intermediate in the fibrillogenesis pathway [107]. To determine the structural change involved in amyloidogenesis, a highly amyloidogenic triple D-strand mutant (G53S/E54D/L55S) was designed, which resulted in a conformational change in the CD loop, D-strand and the DE loop, denoted as the β-slip [108]. It is suggested that the β-slip creates new interactions at a potential amyloid packing site, in which distorted but intact tetramers are the basic building blocks for TTR amyloid. It has also been suggested that regions with α-helical structure undergo an α to β transition and that the β-strands may then associate into a regular fibrillar structure [109].

TTR monomers may be the predominant building blocks of amyloid fibrils. When size-exclusion chromatography was used to monitor the amyloid formation of TTR variants including L55P and V30M TTR, a fraction of TTR monomers was detected preceding aggregation [92]. A similar observation was made in analytical ultracentrifugation studies [110]. The idea that monomers are the building blocks of fibrils is further supported by a detailed structural analysis of TTR amyloid fibrils [86]. In addition, in a study in which TTR variants designed with different quaternary stability were examined, similar conclusions were reached [111].

The kinetics of denaturation at acidic pH and fibril formation are much faster for monomeric TTR than for tetrameric TTR, suggesting that the rate-limiting step may be the formation of monomers [112]. The significance of tetramer dissociation into monomers has also been examined by means of an engineered TTR double mutant (F87M/L110M) that remains monomeric at physiological pH. A study on the aggregation of the monomeric TTR variant (F87M/L110M) found that the monomer forms amyloid fibrils by a multistep process which is not accelerated by seeding, suggesting that the formation of oligomeric nucleus is not required [113]. However, these results do not preclude the possibility that oligomeric TTR is the nucleus of polymerization; as the F87M/L110M double mutant TTR is not a native structure, it conceivably may not aggregate in a manner similar to that which occurs in vivo.

**TTR-induced neurotoxicity in FAP**

The mechanism of TTR-induced neurotoxicity in FAP is very poorly understood. A number of questions remain unanswered. It is unclear why TTR is preferentially deposited in certain regions such as peripheral nerve or cardiac muscle. The major neurotoxic forms of TTR are also unknown. In addition, the mechanism of TTR-induced neuropathy is far from clear.

It is well recognized that many different types of amyloid are toxic. For example, in the central nervous system, the build up of β-amyloid protein (Aβ) leads to neurodegeneration in Alzheimer’s disease [114]. Although less common, three other amyloidogenic proteins, prion protein [115], which causes Creutzfeldt–Jakob disease in humans, and the British and Danish dementia peptides (named ABri and ADan, respectively), which cause rare British and Danish dementias, also induce neurodegeneration [116]. Lessons learned from studies on these diseases, in particular Alzheimer’s disease, may help to explain some aspects of the pathogenesis of FAP. The idea is discussed further in the following sections.

**Tissue-specific pattern of TTR deposition**

Although TTR is synthesized in the liver, it is typically deposited in a number of tissues [5,36,38,74,117]. It is quite likely that endogenous factors may initiate TTR deposition within a tissue and that the distribution of TTR deposition reflects the presence of these endogenous factors. In the case of the Aβ protein of Alzheimer’s disease, a number of proteins and factors (pathological chaperones), such as apolipoprotein E, have been suggested to contribute to aggregation and deposition [118]. Although the ε4 allele of the apolipoprotein E gene is linked to increased Aβ deposition and an earlier age of onset in Alzheimer’s disease, there is no similar association with FAP [119]. However, there is evidence that glycosaminoglycans (GAGs) may be involved in TTR deposition. GAGs are a heterogeneous group of highly sulfated carbohydrates that regulate a number of important physiological processes [120]. A number of different GAGs are found including heparan sulfate (HS), dermatan sulfate, keratan sulfate and chondroitin sulfate, which differ in the structure of the carbohydrate backbone and in the extent of sulfation. They are commonly found in proteoglycans attached to a
variety of core proteins, which may be membrane-bound or secreted [120].

GAGs are commonly found in association with amyloid deposits including TTR amyloid [121–123]. In cardiac deposits, there is a close association between the presence of amyloid and the basement membrane around myocardial cells [117], and studies by Smeland et al. [124] have shown that TTR can bind to the basement membrane HS proteoglycan perlecan. In FAP, amyloid deposits commonly occur in the endoneurium [125], which is rich in extracellular matrix proteins including chondroitin sulfate proteoglycans [126].

A number of studies suggest that GAGs, in particular HS, influence amyloidogenesis in vivo. HS can bind to amyloid and promote fibrillogenesis [127]. Amyloid deposition is commonly seen in association with basement membranes [128], which are rich in HS proteoglycan. Overexpression of heparanase, which digests endogenous HS, can render mice resistant to amyloid protein A amyloidosis [129], and other studies suggest that low-molecular-mass HS analogues may inhibit amyloid deposition in transgenic mouse models of Alzheimer’s disease [130]. Although GAGs are found in association with TTR deposits in vivo, to date, there have been no studies on the effect of GAGs on TTR aggregation. This is potentially an important area of research because of the possibility that GAG analogues may be useful to prevent TTR amyloid deposition for the treatment of FAP.

Identification of toxic species

While most attention has been focused on the structure of amyloid fibrils, there is now increasing evidence, that in many protein-misfolding diseases, it is the lower-molecular-mass oligomeric species that are the most toxic. A number of studies [131–134] have provided strong evidence that oligomeric or low-molecular-mass diffusible species are the most toxic forms of Aβ. In general, low-molecular-mass oligomeric or prefibrillar species of amyloid proteins seem to be much more neurotoxic than larger amyloid fibrils [131,135]. The presence of oligomeric species that are not deposited as amyloid may explain why amyloid load correlates poorly with the severity of dementia in Alzheimer’s disease [136].

The formation of monomeric TTR may be a key step in the aggregation pathway. Studies by Lashuel et al. [110] and Reixach et al. [137] indicate that monomers or low-molecular-mass oligomers may be the most toxic forms. Using an assay of cell viability, Reixach et al. [137] found that TTR amyloid fibrils of > 100 kDa were not toxic, whereas monomeric or very low-molecular-mass TTR was cytotoxic. Dimeric or low-molecular-mass TTR has been reported to be neurotoxic [138,139]. Similar conclusions were reached by Hou et al. [140] using SH-SY5Y cells. In these experiments, atomic force microscopy and dynamic light scattering were used to characterize the oligomeric species of TTR. The presence of low-molecular-mass TTR aggregates was found to be correlated with the ability of TTR to induce calcium influx via voltage-gated calcium channels. High-molecular-mass (fibrillar) species were found to be much less effective in their ability to induce calcium influx.

The identification of toxic species is more than of academic interest. Ultimately, if therapies are to be aimed at inhibiting amyloid deposition, then it will be important to ensure that this strategy does not increase the concentrations of the more toxic low-molecular-mass species. If the amyloid deposits are less toxic than the oligomeric TTR species, decreasing the concentration of the amyloid deposits would only be a sensible strategy if the concentration of the oligomeric species were also decreased.

Mechanism of neurotoxicity in FAP: the lesson from other amyloidoses

A number of studies have examined the mechanism of neurotoxicity in FAP [32,140–143]. The biochemical events by which amyloidogenic proteins exert a neurotoxic effect are still unclear [114]. However, it seems increasingly likely that neurotoxicity is a common property of all types of amyloid. As proteins that do not normally form amyloid can be cytoxic, this suggests it is the amyloid conformation per se that is the toxic principle. Indeed, there is little evidence to suggest that there is any amino-acid sequence specificity to the toxic effect [135]. For example, although the amyloidogenic ABri protein associated with British dementia is quite unrelated in amino-acid sequence to the amyloid protein Aβ of Alzheimer’s disease, both peptides cause dementias, with some having common neuropathological features such as neurofibrillary tangle formation [143]. Similarly, the deposition of gelso lin and apolipoprotein AI, which have little or no amino-acid sequence similarity to TTR, can also cause FAP [5]. Therefore, toxicity is associated with specific conformational features of β-structure-rich protein aggregates, and does not seem to be related to the presence of specific sequences or patterns of amino-acid residues.

Amyloid proteins can influence similar biochemical pathways, providing further evidence for a common mechanism of causation. For example, Aβ is known to
cause decreased mitochondrial activity, increase apoptosis, activate caspases, induce ER stress, mobilize calcium, and alter mitogen-activated protein (MAP) kinase signaling [114]. Similar changes in mitochondrial activity, MAP kinase signaling, caspase activation, and ER stress have been reported for TTR [137,142,144]. As Aβ and TTR activate similar intracellular signaling mechanisms, this implies that the early biochemical events which trigger these mechanisms may also be similar. Nevertheless, the ‘receptor’ which mediates the neurotoxicity is unknown. Studies by Monteiro et al. [142] have implicated the receptor for advanced glycation end-products (RAGE), which has also been reported to bind Aβ. The RAGE plays an important role in a variety of physiological events and regulates nuclear factor k-B (NF-kB), mitogen activated protein kinase (MAPK), and Jun – N-terminal kinase (JUNK) signaling [145], all of which may be affected in FAP in vivo [142].

However, it is unclear whether all of the neurotoxic effects could be mediated through a single receptor. Indeed, many different types of cells, expressing a wide variety of different cell-surface receptors, have been shown to be susceptible to amyloid toxicity. Cecchi et al. [146] have shown that the susceptibility of cells to amyloid toxicity is related to the capacity of the cells to buffer the intracellular calcium concentration. This suggests that disruption of calcium homeostasis may be a key event in amyloid toxicity. In support of this idea, recent studies by Teixeira et al. [144] suggest that TTR may cause ER stress, resulting in the release of calcium from ER stores. Cecchi et al. [146] have also proposed that disruption of membrane structure may correlate with disturbances in calcium homeostasis.

In an attempt to identify the ‘receptor’ responsible for the toxic effect of TTR, Hou et al. [141] examined the binding of TTR to a plasma-membrane-enriched...
fraction isolated from neuroblastoma cells. In agreement with Cecchi et al. [146], Hou et al. [141] found that the binding of TTR to the membrane and the extent of disruption of membrane fluidity correlated with the degree of toxicity. In another study, Hou et al. [140] showed that TTR aggregates induce calcium influx in the same cell type. As calcium channels are localized to specific lipid raft domains within membranes [147] and as disruption of these domains has been shown to activate voltage-gated channels [148], this raises the possibility that TTR-mediated disruption of lipid raft organization may lead to calcium entry [140].

**An integrated approach to amyloidosis**

On the basis of the studies reported here, it is becoming clear that amyloidoses share common mechanisms of toxicity. Increasingly, it is recognized that low-molecular-mass oligomeric species are the most toxic, and that the higher-molecular-mass fibrils and large amyloid deposits are less toxic. Amyloid proteins share common features such as the ability to bind to lipid membranes and to activate specific intracellular pathways, particularly those involving calcium homeostasis.

A model of the mechanism of TTR-induced neurotoxicity is presented (Fig. 3). In this model, amyloidogenic mutations in TTR destabilize the native structure of the tetramer and induce dissociation of the tetramer into dimers and monomers. The gradual formation of a sufficiently high concentration of nuclei (possibly monomers) results in oligomerization and the formation of oligomers and protofibrillar species that are toxic. These low-molecular-mass aggregated forms interact with the membrane lipids or specific receptors to induce a toxic effect. Although, in this model, the larger amyloid deposits correlate with toxicity, these deposits are not as the most toxic form. However, they may provide a local pool of aggregated TTR, which can dissociate into lower-molecular-mass oligomeric forms and which thereby can contribute to the pool of toxic species.

It is clear that what is learnt from the study of one amyloidosis may have application to another amyloidosis. Although most studies have focused on the effects of one, or perhaps two, amyloidogenic peptides or proteins, it can be argued that a more integrated approach to the study of amyloid neurotoxicity is needed. In this regard, studies on other amyloidoses that cause neurodegeneration (Alzheimer’s disease, prion diseases, British and Danish familial dementias) may provide clues to understanding the pathogenesis and treatment of FAP.

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