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Article type : Original Article

Sensory nerve degeneration in a mouse model mimicking early manifestations of familial amyloid polyneuropathy due to transthyretin Ala97Ser

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/nan.12477

Key words

Familial amyloid polyneuropathy; Transthyretin; Intraepidermal nerve fibre; Nerve conduction studies; Knock-in mice

Running title: Sensory nerve degeneration in TTR-A97S mouse model

Abstract

Aims: Sensory nerve degeneration and consequent abnormal sensations are the earliest and most prevalent manifestations of familial amyloid polyneuropathy (FAP) due to amyloidogenic transthyretin (TTR). FAP is a relentlessly progressive degenerative disease of the peripheral nervous system. However, there is a lack of mouse models to replicate the early neuropathic manifestations of FAP.

Methods: We established human TTR knock-in mice by replacing one allele of the mouse *Ttr* locus with human wild-type *TTR* ($hTTR^{wt}$) or human *TTR* with the A97S mutation ($hTTR^{A97S}$). Given the late onset of neuropathic manifestations in A97S-FAP, we investigated nerve pathology, physiology, and behavioural tests in these mice at two age points: the adult group (8 – 56 weeks) and the aging group (> 104 weeks).

Results: In the adult group, nerve profiles, neurophysiology, and behaviour were similar between $hTTR^{wt}$ and $hTTR^{A97S}$ mice. By contrast, aging $hTTR^{A97S}$ mice showed small fibre neuropathy with decreased intraepidermal nerve fibre density and behavioural signs of mechanical allodynia. Furthermore, significant reductions in sural nerve myelinated nerve fibre density and sensory nerve action potential amplitudes in these mice indicated degeneration of large sensory fibres. The unaffected motor nerve physiology replicated the early symptoms of FAP patients, i.e., sensory nerves were more vulnerable to mutant TTR than motor nerves.

Conclusions: These results demonstrate that the hTTR^{A97S} mouse model develops sensory nerve pathology and corresponding physiology mimicking A97S-FAP and provides a platform to develop new therapies for the early stage of A97S-FAP.

Abbreviations

CMAP Compound muscle action potential

FAP Familial amyloid polyneuropathy

IENF Intraepidermal nerve fibre

NMJ Neuromuscular junction

PGP9.5 Protein gene product 9.5

RFLP Restriction fragment length polymorphism

SNAP Sensory nerve action potential

TTR Transthyretin

Introduction

Familial amyloid polyneuropathy (FAP) is a relentlessly progressive neurodegenerative disorder that affects all types of peripheral nerves starting from the distal parts; hence, it is classified as a length-dependent neuropathy [1-7]. Among proteins responsible for amyloid aggregates, transthyretin (TTR) mutations account for the majority of genetic aetiologies, particularly the most prevalent mutation, TTR-V30M [1,2,8]. The age of onset of FAP vary among different mutant TTRs. Early-onset FAP mainly occurs in individuals with the TTR-V30M mutation [9-11]. In contrast, TTR-A97S, the most prevalent TTR mutation in Taiwan, is responsible for late-onset polyneuropathy in the 6th to 7th decades with marked axonal degeneration [4,12]. Although TTR-FAP causes a

devastating polyneuropathy, symptoms of sensory nerve degeneration (sensory neuropathy) including neuropathic pain behaviours featuring mechanical allodynia (pain from innocuous stimuli) are the earliest and most prevalent manifestations in clinical studies of FAP [2,13,14].

Transgenic mice carrying TTR point mutations serve as excellent models to understand the functions of TTR and the pathophysiology of amyloid aggregates that mimic FAP [15-21]. In TTR-V30M transgenic mice carrying either mutant human TTR driven by the mouse metallothionein 1 (MT-1) promoter [20] or a 6-kb sequence upstream of mutant human TTR (6.0-hMet30) [16,19,22], amyloid deposits developed in the oesophagus and gastrointestinal regions starting at 6 months of age. TTR-L55P transgenic mice carrying a 19-kb 5' untranslated region including the transcriptional element of human TTR [23,24] or human TTR-L55P cDNA driven by the SMT-1 promoter [18] developed amyloid deposition as early as 1-3 months of age on a TTR-null background. Recently, a humanized mouse model has been established by knocking in the human *TTR* (h*TTR*) transgene in the mouse *Ttr* (m*Ttr*) locus on a TTR-null background [21]. While these TTR-transgenic mice reveal important mechanisms underlying TTR-mediated amyloid aggregation [25-28], the nerve pathology and consequent functional alterations remain elusive.

Clinically, sensory nerve degeneration is divided into a small-diameter nociceptive type (small fibre neuropathy) and a large-diameter proprioceptive type (large fibre neuropathy). In the past, there was a lack of objective, quantifiable methods to examine small-diameter nociceptive nerve degeneration at the light microscopic level [29]. To circumvent this obstacle, we and several other groups have developed skin biopsy as a novel approach [30-33] that has now become a standard technique for diagnosing small fibre sensory neuropathy [34]. This test is complementary to nerve conduction studies for large fibre neuropathy. Hence, integrated examinations of sensory nerves including skin biopsy for intraepidermal nerve fibre (IENF) density and nerve conduction studies examining sensory nerve action potential [35-37] are mandatory to establish a mouse model of FAP for (1) understanding the progression of FAP and (2) developing therapeutic strategies for treating FAP.

Given the lack of evidence on nerve pathology in previous TTR-FAP transgenic mouse models and potentially distinct phenotypes among them, in this study we investigate TTR-A97S knock-in mice through comprehensive neuropathy examinations including nerve pathology, physiology, and behavioural tests to understand whether the clinical symptoms of FAP could be replicated in these mice.

Material and methods

Study design and animal handling

Human TTR knock-in mice on the 129/sv background were generated by the Transgenic Mouse Core Facility of National Taiwan University. Knock-in mice carrying wild-type and A97S human TTR were generated by replacing m*Ttr* with the h*TTR* without altering the promoter and enhancer sequences of m*Ttr*. These mice were backcrossed to C57BL/6 females, and the heterozygotes with the genotypes hTTR^{wt}/mTtr^{wt} (abbreviated as $hTTR^{wt}$) and hTTR^{A97S}/mTtr^{wt} (abbreviated as $hTTR^{A97S}$) after N3 were used in the study. The generation of knock-in mice and all procedures were approved by the Institutional Animal Care and Use Committee, National Taiwan University College of Medicine, and were conducted according to the Guide for the Care and Use of Laboratory Animals from the National Research Council. The total numbers of $hTTR^{wt}$ and $hTTR^{A97S}$ mice used in this study were 29 and 39, respectively.

Generation of human wild-type TTR and mutant TTR-A97S knock-in mice

Since the exon 1 sequence of m*Ttr* and h*TTR* is highly conserved except for one amino acid difference (Ala > Thr), we first created a construct containing a point mutation (G > A) in m*Ttr* exon 1 followed by a *loxP*-flanked *Neo* cassette with homologous recombination and a further excision of the *Neo* cassette by Cre recombinase. Exons 2-4 of h*TTR* cloned from control subjects and FAP patients were inserted downstream of a *loxP-FRT-Neo* cassette-*FRT* fragment of plasmid PL451 to create human wild-type TTR and mutant TTR-A97S knock-in vectors, respectively. Final constructs

were generated by homologous recombination of vectors containing h*TTR* exons 2-4 and mutant m*Ttr* exon 1 and subsequent removal of the *FRT-Neo* cassette by Cre and FLP recombinases. PCR and sequencing were used to verify the sequences in all the above steps. The restriction enzymes NotI, BamHI, SalI, AvrI, SpeI, ScaI, and NdeI (New England Biolabs, Ipswich, MA) were used. ES cells (129X1/SvJ X 129S1/Sv) with human wild-type TTR and mutant TTR-A97S knock-in were produced and screened with Southern blotting followed by chimera mouse production via blastocyst injection.

Genotyping of human TTR knock-in mice

Successful knock-in mice were first confirmed by PCR for the remaining *loxP* site with the primer pair P1 (5'-ATAGCGGCCGCTTGGCAGGGATCAGCAGCCTG-3') and P2 (5'-TGGAGCGAAACAAGAATTACACACT-3'). Briefly, the *loxP* site was amplified with gradient PCR at a hybridization temperature starting at 65 °C. The hybridization temperature decreased 0.3 °C each cycle for 34 cycles. The genotypes of $hTTR^{wt}$ and $hTTR^{A97S}$ mice were further screened and confirmed by restriction fragment length polymorphism (RFLP) with the primer pair P3 (5'-TGACTCTGTACTCCTGCTC-3') and P4 (5'-TTCAGGTCCACTGGAGGA-3') at 55 °C for 35 cycles. The PCR products were digested with FokI (New England Biolabs) for 4 h at 37 °C to detect the site of the mutation A97S in exon 4 of h*TTR*. Genomic DNA from FAP patients was used as a positive control.

To measure m*Ttr* and h*TTR* mRNA expression in the human TTR knock-in mice, we extracted mRNA from liver tissues by NucleoSpin RNA II (MACHEREY-NAGEL, Bethlehem, PA) and reverse-transcribed it to cDNA with SuperScript III (Invitrogen, Carlsbad, CA). m*Ttr* and h*TTR* mRNA expression was detected with the following probes: m*Ttr* cDNA-F 5'-TTCGCGGATGTGGTTTCACA-3', m*Ttr* cDNA-R 5'-GACCAGGATCTTGCCAAAGC-3'; and h*TTR* cDNA-F 5'-TGCAGAGGTGGTSTTCACAG-3', h*TTR* cDNA-R 5'-TTCTCCTCCAGTGGACCTGA-3'.

Histological staining for amyloid deposits

We performed Congo red staining to detect amyloid deposits. For confirmation of amyloid, adjacent sections were stained with Congo red and thioflavin T or TTR immunohistochemistry. Mouse tissues were harvested after 2% paraformaldehyde-lysine-periodate (PLP) perfusion under anaesthesia. Congo red staining was conducted on paraffin-embedded sections (5 μm for the heart, liver, kidney, intestine, stomach, oesophagus, and colon tissues) and cryosections (10 μm for the sural nerve, proximal sciatic nerve, and dorsal root ganglia and 30 μm for the footpad and gastrocnemius muscle). We performed Congo red staining following a standard protocol [38]. Amyloid aggregates with birefringence were checked and photographed with a polarized microscope (model: 250P, Zoomkop Company, New Taipei, Taiwan). For thioflavin T staining, sections were incubated with 0.5% thioflavin T in 0.1 N HCl for 15 min. Thioflavin T showed a bright green fluorescence when binding to amyloid fibrils. In contrast, the dye fluorescence was faint in the absence of amyloid deposits.

TTR immunohistochemistry

Paraffin sections were pretreated with antigen retrieval by incubating in citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) for 10 min. Immunohistochemistry was performed on paraffin-embedded sections and cryosections with anti-human TTR (Abcam, Cambridge, UK) in Tris buffer containing 0.1% Triton X-100 and 0.5% nonfat milk. The immunoreactivities were detected with biotinylated goat anti-rabbit IgG followed by incubation with the avidin-biotin complex (ABC kit, Vector Laboratories, Burlingame, CA). The reaction product was developed with 3,3’-diaminobenzidine (Sigma).

Western blot analysis

The fresh mouse livers were homogenized with ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, NaCl 150 mM, 1% Nonidet P-40, 0.5% deoxycholate) containing proteinase inhibitor cocktail (Sigma, St. Louis, MO). The homogenates were centrifuged at 12,000 g

at 4 °C for 15 min, and the supernatants were collected. Mouse blood was obtained from the heart through 22 G needles under anaesthesia before sacrifice. The blood was incubated at room temperature for 30 min and at 4 °C for another 30 min. After centrifugation at 2,500 g at 4 °C for 10 min, the supernatants were collected and stored at -20 °C. Proteins (60 μg) and sera (6 μL of serum diluted to 1:50 in 0.9% NaCl) were separated by 10% SDS-PAGE and were transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Billerica, MA). Nonspecific binding sites were blocked with 5% (w/v) nonfat milk or T-Pro Fast Blocking Buffer (New Taipei, Taiwan), and the blots were incubated with rabbit anti-human TTR (Abcam) antibody suspended in blocking solution at 4 °C overnight. Horseradish peroxidase-linked anti-rabbit IgG (Promega, Madison, WI) was used for detection of signals. The intensity of the bands was quantified by densitometry with ImageJ v.1.47h (National Institutes of Health, Bethesda, MD).

ELISA

Mouse TTR and human TTR protein levels in the serum of $hTTR^{wt}$ and $hTTR^{A97S}$ mice were assessed by ELISA kits from Abnova (KA2070, Taipei, Taiwan) and Abcam (ab108895), respectively. Sera from human TTR knock-in mice were diluted to 1:2,000 for mouse TTR protein measurements and to 1:10,000 for human TTR protein measurements following the manufacturer's protocols. The serum from peripheral venous blood of FAP patients was used as a positive control.

Behavioural test

Mice at designated age points were individually placed in a dark plastic container on metal mesh and allowed to habituate to the new environment for 1 h. A dynamic plantar aesthesiometer (Ugo Basile, Comerio, Varese, Italy) was used to deliver mechanical stimuli with ascending forces of 0 to 15 g over a 30-s period on the plantar surface of the hindpaw from below the floor of the test chamber. Five tests were performed on each hindpaw, alternating between hindpaws with a minimal interval

of 5 min between measurements, and the stimulus force at which the animal withdrew its hindpaw was recorded. The median of the measurements was used for analysis.

Sural nerve pathology

The assessment of nerve pathology followed our established protocol [39]. The sural nerves were collected at the level of the trifurcation of sciatic nerve and then fixed in 5% glutaraldehyde in 0.1 M phosphate buffer (PB) at 4 °C overnight. The tissues were post-fixed in 2% osmic acid for 2 h at room temperature, dehydrated and embedded in Epon 812 resin (Polysciences, Philadelphia, PA). Semi-thin sections were cut on a Reichert Ultracut E (Leica, Wetzlar, Germany) and stained with toluidine blue. Myelinated nerve fibres were photographed at an original magnification of × 40 under a Leica DM2500 microscope. All myelinated nerve fibres in the entire fascicle were counted by using Image-Pro PLUS software (Media Cybernetics, Silver Spring, MD). Myelinated nerve fibre density was derived and expressed as the number of nerve fibres per square millimetre of nerve fascicle (nerves/mm^2). Ultrathin (70 nm) sections were stained with uranyl acetate and lead citrate and observed under a transmission electron microscope (H7100, Hitachi, Tokyo Japan).

The morphometry evaluation of myelinated nerve fibres with abnormal myelin sheaths was further defined as myelinated nerve fibres with (1) infolded myelin loop, (2) outfolded myelin loop, and (3) separation of lamellae or wide incisures according to previous studies [40,41]. Semi-thin sections were examined at an original magnification of × 40. The proportion of myelinated nerve fibres with abnormalities in myelin sheaths was calculated from the total number of myelinated nerve fibres counted in each nerve.

Quantification of skin innervation

Mice were sacrificed under anaesthesia and were fixed by 2% PLP perfusion. The footpad tissues were post-fixed with PLP for 2 h and cryosectioned into 30-μm sections perpendicular to the dermis. Immunohistochemistry was performed as previously described by using anti-protein gene product 9.5 (PGP9.5; UltraClone, Isle of Wight, UK).

Epidermal innervation was quantified following established protocols, and the slides were coded to ensure that measurements were blinded [42]. Any nerve fibre ascending from subepidermal plexus with branches inside the epidermis was counted as one, while branches in the dermis were counted as separate fibres. ImageJ was used to measure the total length of epidermis along the upper margin of the stratum corneum. Intraepidermal nerve fibre density was derived as the number of fibres per millimetre of epidermal length.

Neurophysiologic studies

Nerve conduction studies were performed before sacrifice according to our previously described standardized protocols [43]. Amplitudes of sensory nerve action potentials (SNAPs) and compound muscle action potentials (CMAPs) were recorded with a Nicolet Viking-Quest System (Nicolet Biomedical, Madison, WI). For the recording of CMAPs, the stimulating electrodes were inserted at the sciatic notch to stimulate the sciatic nerve, and the recording electrodes were placed on the plantar muscles. For the recording of SNAPs, the stimulating electrodes were placed on the lateral side of the ankle, and recording electrodes were placed on the sural nerve. A monopolar electrode was inserted into the tail as the ground electrode. Amplitudes calculated by baseline-to-peak values of CMAP and SNAP were analysed.

Neuromuscular junction (NMJ) innervation

Cholinesterase (ChE) histochemistry combined with PGP9.5 immunohistochemistry was performed on longitudinal cryosections (30 μm) of gastrocnemius muscles according to published protocols [43] to evaluate the innervation of NMJs. Slides were coded and ten fields were randomly photographed and examined at an original magnification of × 20 under a Leica DM2500 microscope. The proportions of innervated NMJs were calculated by the number of innervated NMJs divided by the number of total NMJs on all stained sections.

Morphometric analysis of muscle fibres

Medial parts of gastrocnemius muscle were embedded in paraffin. Transverse sections (5 μm) were stained with haematoxylin and eosin. Five fields for each slide were randomly photographed at an original magnification of × 20 under a Leica DM2500 microscope. The transverse area of each muscle fibre was measured with Image-Pro PLUS software.

Statistics

Data were expressed as the mean ± SD and were analysed in Prism (GraphPad Software, San Diego, CA). Student's *t*-test was used for the comparisons of $hTTR^{wt}$ and $hTTR^{A97S}$ mice. One-way ANOVA with Tukey's post hoc test was used in the analyses of TTR in the sera and myelinated nerve fibres with abnormal myelin sheaths. Pearson's correlation between SNAP and myelinated nerve fibre density was calculated along with the slope of the regression line, including the 95% CI (95% confidence interval). The results were considered statistically significant at $P < 0.05$.

Results

Establishment of human wild-type and A97S TTR knock-in mice

To explore the effect of TTR-A97S, we generated knock-in mice for human wild-type TTR and mutant TTR-A97S by replacing m*Ttr* with h*TTR* through homogenous recombination (Figure 1). We validated the human TTR knock-in mice with PCR for the remaining *loxP* site (Figure S1A). The mice containing a 700-bp *loxP* PCR product were further confirmed by RFLP for exon 4 of h*TTR*. FokI digestion left exon 4 of h*TTR* intact with a 347-bp band in $hTTR^{wt}$ mice, while there were an additional 2 bands of ~210 and ~130 bp in $hTTR^{A97S}$ mice, i.e., (210-, 137-bp) and (214-, 133-bp) (Figure S1B).

To confirm the expression of *TTR* mRNA in the hepatocytes of human TTR knock-in mice, mRNA from the liver was tested with primers specifically designed for m*Ttr* and h*TTR* mRNA. In this study, we used heterozygotes carrying one human wild-type *TTR* (hTTR^{wt} mice) or human *TTR*-A97S allele (hTTR^{A97S} mice). Mice of both genotypes expressed both m*Ttr* and h*TTR* mRNA (Figure S1C).

Expression of human wild-type and A97S TTR proteins

We evaluated human TTR protein expression in knock-in mice with western blotting. Human TTR proteins were detectable in the liver and serum of both hTTR^{wt} and hTTR^{A97S} mice but not in those of wild-type littermates (Figure 2A,B). Clinically, patients with TTR-A97S had late-onset neuropathy. Hence, this study examined 2 groups of transgenic mice, according to their age: the adult group (8 – 56 weeks) and the aging group (> 104 weeks).

We next quantified both mouse TTR and human TTR proteins in serum with ELISA (Figure 2C). The level of mouse TTR protein was similar between hTTR^{wt} and hTTR^{A97S} mice in both the adult and aging groups (adult: 164.4 ± 30.9 vs. 139.9 ± 27.4 μg/ml, adjusted P = 0.116; aging: 142.0 ± 26.7 vs. 147.1 ± 25.6 μg/ml, adjusted P = 0.967, by 1-way ANOVA with Tukey's post hoc test). In contrast, human TTR protein was significantly lower in hTTR^{A97S} mice than in hTTR^{wt} mice in both the adult and aging groups (adult: 75.8 ± 29.6 vs. 41.8 ± 11.2 μg/ml, adjusted P = 0.0011; aging: 66.5 ± 22.7 vs. 46.8 ± 15.6 μg/ml, adjusted P = 0.043, by 1-way ANOVA with Tukey's post hoc test).

We then visualized TTR-A97S aggregates in tissues with Congo red staining. Amyloid deposition with birefringence under polarized microscopy was detected in the interstium of distal convoluted tubule of the kidney, epineurium of the sural nerve, subperineurial space of the proximal part of sciatic nerve, and the ductal part of the sweat gland in the footpad skin of aging hTTR^{A97S} mice, but was absent in the adult group. This observation was further confirmed by thioflavin T staining and anti-TTR immunostaining. There were thioflavin T fluorescence profiles at the same location in the kidney and sural nerve comparable to the site of Congo red staining (Figure 3). For TTR

immunostaining, punctate TTR(+) profiles were detected at the same position of the proximal sciatic nerve and hindpaw pad skin comparable to the site of Congo red staining (Figure 4).

General status of hTTR^{A97S} mice

The general appearances of human TTR knock-in mice were similar between hTTR^{wt} and hTTR^{A97S}. Both genotypes developed and grew normally. There was no difference between genotypes in the body weight of adult and aging mice (adult: 34.7 ± 3.39 vs. 32.8 ± 4.75 g, P = 0.279; aging: 34.5 ± 3.18 vs. 37.0 ± 4.96 g, P = 0.143).

Neuropathic behaviours in hTTR^{A97S} mice

To examine neuropathic behavioural patterns in these mice, we measured withdrawal thresholds to mechanical stimuli. In the adult group, there was no difference between genotypes in the mechanical threshold of the hindpaw (4.99 ± 1.24 vs. 4.97 ± 1.14 g, P = 0.974). Aging hTTR^{A97S} mice became hypersensitive to mechanical stimuli as shown by a significant reduction (~9.4%) of the mechanical threshold (5.24 ± 0.47 vs. 4.75 ± 0.57 g, P = 0.041), a manifestation of neuropathic pain behaviours.

Small nerve fibre pathology of hTTR^{A97S} mice

Given the existence of neuropathic pain behaviours as a manifestation of small fibre neuropathy, we examined the IENFs in the hindpaw pad skin with PGP9.5 immunostaining (Figure 5) for pathological evidence. Continuous, dotted, unmyelinated nerves arose from the bundles of subepidermal nerve plexuses, which appeared linear with dense staining in both age groups of hTTR^{wt} mice and in adult hTTR^{A97S} mice. In aging hTTR^{A97S} mice, the abundance of IENFs was reduced and the length and staining intensity of subepidermal nerve plexus were decreased, indicating nerve degeneration (Figure 5A). Quantitatively, IENF densities were similar in the adult groups of both genotypes (13.92

± 4.93 vs. 10.54 ± 6.33 nerves/mm, *P* = 0.131). In the aging group, skin denervation was confirmed by a significant reduction of IENF density in hTTR^{A97S} mice compared with that in hTTR^{wt} mice (11.75 ± 4.85 vs. 7.21 ± 3.10 nerves/mm, *P* = 0.021, Figure 5B).

Large nerve fibre pathology of hTTR^{A97S} mice

To investigate whether A97S mutation resulted in structural abnormalities in large-diameter sensory nerves, we next examined semi-thin sections of sural nerves (Figure 6A). The myelin sheaths of the adult group were compact with circular profiles in both genotypes. In the aging group, myelinated nerve fibres became irregular with increased myelin abnormalities, i.e., (1) outfolded myelin loop, (2) infolded myelin loop, and (3) separation of lamellae, or wide incisure (Figure 6C); these findings were more obvious in hTTR^{A97S} mice than in hTTR^{wt} mice. Furthermore, there were profiles of degenerating nerves and macrophages engulfing myelinated and unmyelinated nerve debris in aging hTTR^{A97S} mice under electron microscopy (Figure S2). Only limited Remak bundles with collagen pockets were observed in both genotypes. These observations were validated by quantification. Myelinated nerve fibre density was comparable in the adult groups of both genotypes (25454 ± 2835 vs. 23351 ± 2062 nerves/mm^2, *P* = 0.217). In the aging groups, there was a significant reduction of myelinated nerve fibre density in hTTR^{A97S} mice (25232 ± 2395 vs. 20938 ± 4192 nerves/mm^2, *P* = 0.024, Figure 6B). The proportion of myelinated nerve fibres with abnormal myelin sheaths was not different between the 2 genotypes in the adult group (16.8 ± 3.8 vs. 20.3 ± 4.1 %, adjusted *P* = 0.716). In contrast, in the aging group, there was a significant increase in the proportion of myelinated nerve fibres with abnormal myelin sheaths in hTTR^{A97S} mice compared to that in hTTR^{wt} ones (23.8 ± 6.1 vs. 34.8 ± 6.0 %, adjusted *P* = 0.018, Figure 6C and Table S1), indicating the progression of nerve pathology was much more severe in aging hTTR^{A97S} mice.

To examine whether the nerve pathology affected the functions of myelinated nerves, we performed nerve conduction studies (Figure 7A). In the adult group, the amplitudes of SNAPs were similar in hTTR^{wt} and hTTR^{A97S} mice (817.1 ± 151.9 vs. 669.6 ± 242.0 μV, *P* = 0.473). The amplitudes of SNAPs in the aging hTTR^{A97S} mice were significantly lower than those of the aging hTTR^{wt} mice (966 ±

344 vs. 636 ± 198 μV, P = 0.007). Furthermore, there was a linear correlation between myelinated nerve fibre density and SNAP amplitude in aging mice (r = 0.67, P = 0.0049, Figure 7B).

For motor nerve physiology, amplitudes of CMAPs were similar between $hTTR^{wt}$ and $hTTR^{A97S}$ mice in both adult and aging groups (adult: 7.07 ± 1.64 vs. 8.01 ± 2.25 mV, P = 0.109; aging: 4.59 ± 1.49 vs. 4.00 ± 1.52 mV, P = 0.328, Figure S3). To further investigate the potential involvement of motor nerves, we performed 2 analyses: (1) innervation of neuromuscular junctions (NMJs) and (2) muscle fibre size in gastrocnemius muscles of both aging genotypes. Most NMJs were innervated by PGP9.5(+) axons in the aging group of both genotypes (Figure S4A). In addition, the cross-sectional areas of muscle fibres in both genotypes were similar (Figure S4B). The above observations were confirmed by quantitative analyses which showed similar (1) proportion of innervated NMJs ($hTTR^{wt}$: 81.5 ± 5.0 vs. $hTTR^{A97S}$: 78.1 ± 4.0 %, P = 0.453) and (2) transverse area of muscle fibres ($hTTR^{wt}$: 888.5 ± 66.4 vs. $hTTR^{A97S}$: 917.9 ± 86.2 μm^2, P = 0.666, Figure S4C) of the gastrocnemius muscle. Our findings demonstrated sensory nerves were more vulnerable than motor nerves in aging $hTTR^{A97S}$ mice.

Discussion

This study documents sensory nerve pathology and its consequent physiological and behavioural abnormalities as the major manifestations in human TTR-A97S knock-in mice (Table 1). The phenotype of the knock-in mice is reminiscent of what has been observed in the early stage of FAP [14,44], including (1) small fibre neuropathy with reduced intraepidermal nerve fibres of the pad skin and decreased mechanical thresholds, as well as (2) large fibre sensory neuropathy with reduced myelinated nerve fibre density in sural nerves accompanied by reduced sural sensory nerve action potential amplitudes.

Small fibre nociceptive neuropathy is the most prevalent manifestation of FAP and includes (1) degeneration of skin nerves and (2) neuropathic pain. Nociceptive nerve degeneration in the skin is readily examined by skin biopsy with quantification of IENF density, a standard technique in clinical

practice for the diagnosis of small fibre neuropathy [37,45,46]. This study demonstrated that IENF density was lower in aging hTTR^{A97S} mice than in hTTR^{wt} mice. The reduction of IENF density reflected cutaneous nerve degeneration at the electron microscopic level [30]. Neuropathic pain is the major positive sensory symptom of FAP [14,47]. For example, patients with FAP due to TTR-V30M mutation frequently perceive enhanced sensitivity or discomfort on exposure to mechanical stimuli that healthy subjects consider innocuous instead of painful, i.e., mechanical allodynia [2,14]. The observations on hTTR^{A97S} mice corroborate with what we have observed in human A97S-FAP [4]. In these hTTR^{A97S} mice, there was a significant reduction in mechanical threshold, indicating mechanical allodynia. Taken together, the current characterization of human TTR-A97S knock-in mice establishes a model of FAP with small fibre neuropathy.

In A97S-FAP patients, amyloid deposition appeared as amorphous aggregates surrounding vascular walls of sural nerves and perivascular area of the skin [4,12], similar to V30M-FAP in previous studies [8,38]. Furthermore, A97S-FAP and V30M-FAP patients showed distinct degeneration in the sensory and autonomic nerves [4,5,7,14,38]. Our current model of human TTR-A97S knock-in mice showed amyloid deposits, mainly in sensory nerves, and prominent degenerative signs in sural nerves and skin nerves, mimicking the clinical manifestations of A97S-FAP patients. These findings indicate that amyloid aggregates paralleled sensory nerve degeneration in hTTR^{A97S} mice, highlighting the importance of amyloid deposits in sensory nerve degeneration. While amyloid deposits due to mutant TTR are pathognomonic in FAP [8,5], the low prevalence and late appearance of amyloid in our study may be related to the interactions of mutant TTR with endogenous mouse TTR. TTR tetramers are much more stable in the presence of the mouse TTR protein [23,26], which may result in fewer monomeric amyloidogenic TTR intermediates. In addition, the effect of mouse TTR on TTR aggregation in TTR-FAP transgenic mouse models is distinct. For example, endogenous normal mouse TTR eliminated L55P-induced TTR amyloidosis but had no effect on V30M-derived amyloid deposition [16,23]. Furthermore, TTR aggregates were observed in the dorsal root ganglia of TTR-V30M transgenic mice, and the mutant TTR secreted from the Schwann cells was inhibitory to neurite outgrowth *in vitro* [48]. These observations raise the possibility that mutant TTR in the form of amyloid aggregates is neurotoxic. In this study, the

absence of amyloid deposition in the adult group contributed to the absence of early-onset symptoms and neurological disorders prior to TTR aggregate formation. Alternatively, the key cytotoxic or neurotoxic agent might be monomeric or low-molecular-mass TTR rather than amyloid aggregates [49-51]. Given that amyloid was only detected in a portion of tissues, this observation raises additional potential mechanisms and provides foundations for testing the hypothesis: the neurotoxicity of TTR-A97S may parallel amyloid deposits.

FAP patients with the TTR-A97S develop unique and characteristic clinical features compared to those with the most common genotype, TTR-V30M. A97S-FAP patients belong to the late-onset type and 36.8% of patients with TTR-A97S had episodes of rapid deterioration [12], while V30M-FAP patients had both early- and late-onset types and chronic progressive course [2,8,9]. The current TTR-A97S knock-in mouse model replicated the early phase of FAP due to TTR-A97S. These observations raise further research issues, i.e., whether the inherent nature of TTR-A97S or the existence of superimposed triggers induces neuropathy.

Given that sensory symptom phenotypes of neurological deficits are early manifestations of FAP and major targets of clinical trials, it is critical to develop an animal model that recapitulates sensory nerve degeneration along with its physiological and functional consequences. The current model of human TTR-A97S knock-in mice fulfilled these criteria: (1) small fibre sensory neuropathy with degeneration of nerve fibres in skin and mechanical allodynia and (2) large fibre sensory neuropathy with reduced myelinated nerve fibre density in sural nerves and corresponding reduction of sensory nerve action potential amplitudes. The late occurrence of these sensory phenotypes might be attributable to a difference in the processing of human TTR protein by mice. Further studies will target this issue to generate transgenic mice that develop sensory phenotypes at an early stage.

The major discrepancy between this model and clinical FAP patients is the lack of motor deficits. Reduced compound muscle action potential amplitude is an indicator of motor nerve degeneration and dysfunctions in the late stage of full-blown neuropathy in FAP [44,52]. In the natural course of FAP, sensory neuropathy is an early event in stage I. Motor deficits occur later, in

stage II and the advanced stages III and IV [47,52]. Hence, the hTTR^{A97S} mouse model represents the early phase of FAP stage I. Further modification of this model may replicate the entire manifestation of FAP either with homozygous human mutant TTR alleles or by a targeted TTR production with *Trpv1-Cre* mice, producing more prominent nerve pathology. Since current medications can only slow the progression of FAP [14,53,54], our human TTR-A97S knock-in mouse model provides a platform to develop new therapies targeting early stage of FAP.

Conclusions

In this report, we investigate the sensory nerve profiles, including behavioural response, neurophysiology, and nerve pathology, of a C57BL/6 mouse model with one mouse allele replaced by human wild-type *TTR* (hTTR^{wt}) or human *TTR* with A97S mutation (hTTR^{A97S}). Aging hTTR^{A97S} mice showed neuropathic pain behaviour, decreased nerve conduction, and reduced nerve fibre density, the major initial manifestations of FAP.

Conflict of interest

The authors declare that they have no conflict of interest

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee of National Taiwan University Hospital. All procedures performed in studies involving animals were in accordance with the ethical standards of the Institutional Animal Care and Use Committee, National Taiwan University College of Medicine.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Acknowledgements

We thank the staff of Transgenic Mouse Core Facility of National Taiwan University and Laboratory Animal Center, National Taiwan University College of Medicine, for technical assistance. This work was supported by grants from the Ministry of Science and Technology, Taiwan (104-2320-B-002-019-MY3 and 104-2321-B-002-070); National Taiwan University (106R881001); and the Translational Medicine Project of National Taiwan University College of Medicine and National Taiwan University Hospital (101C101-201) to ST Hsieh.

Author contributions

HWK and HC researched and analysed the data and wrote the manuscript. WML performed Congo red staining. HC, WML, ISY and SWL generated the knock-in mice. STH supervised the project and wrote and revised the manuscript.

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Table 1. Nerve pathology of knock-in mice expressing human TTR-A97S

	Adult	Aging
Small nerve fibre		
Mechanical threshold	no change	↓
IENF density	no change	↓
Large nerve fibre		
SNAP	no change	↓
CMAP	no change	no change

IENF: intraepidermal nerve fibre. SNAP: sensory nerve action potential. CMAP: compound muscle action potential. no change: compared with human wild-type TTR knock-in mice. ↓: reduced compared with human wild-type TTR knock-in mice.

Figure 1. Generation of human wild-type and A97S transthyretin (TTR) knock-in vectors. Homologous recombination between the mouse *Ttr* (m*Ttr*) vector and the targeting vector resulted in a mutant m*Ttr* vector containing an A23T (G > A) point mutation in exon 1. In the next step, the replacement vector was inserted into the targeted clone by homologous recombination. This vector contained either exons 2-4 of normal human *TTR* (h*TTR*) cloned from control subjects or exons 2-4 of h*TTR* with an A97S mutation cloned from FAP patients. The original exons 2-4 of m*Ttr* were removed by Cre and FLP recombinases to create human wild-type and A97S TTR knock-in vectors.

Figure 2. Expression of human TTR protein in knock-in mice. (**A,B**) The blots showed that human TTR proteins were detectable in the liver lysate (**A**) and serum (**B**) of human TTR knock-in mice, but not in those of littermate controls, by a specific anti-human TTR antibody. (**C**) The amounts of mouse TTR (mTtr) and human TTR (hTTR) protein in mice were further evaluated by ELISA. $hTTR^{wt}$ and $hTTR^{A97S}$ mice had similar amounts of mouse TTR protein in the serum. In contrast, the expression of human TTR protein in the serum of $hTTR^{A97S}$ mice was significantly lower than that of $hTTR^{wt}$ mice in both the adult and aging groups. The numbers of subjects are shown in bar graphs. $mTTR^{C}$: littermate controls. n.s.: not significant. *$P < 0.05$ and **$P < 0.01$ by 1-way ANOVA with Tukey's post hoc test.

Figure 3. Congo red- and thioflavin T-stained amyloid deposits in aging $hTTR^{A97S}$ mice. Amyloid deposition stained by Congo red exhibited apple-green birefringence under polarized microscopy (inserts in **A**,**C**). There were Congo red and thioflavin T staining at the interstice of distal convoluted tubule of the kidney (**A**,**B**) and epineurium of the sural nerve (SN) (**C**,**D**). Scale bars: 50 μm; inserts: 25 μm.

Figure 4. Congo red- and anti-human TTR-stained amyloid deposits in aging $hTTR^{A97S}$ mice. Amyloid deposition stained by Congo red exhibited apple-green birefringence under polarized microscopy (inserts in **A**,**C**). On the adjacent section, punctate TTR(+) profiles were observed at subperineurial space of the proximal part of the sciatic nerve (**A**,**B**) and the ductal part of the sweat gland (SG) in the footpad skin (**C**,**D**) of aging $hTTR^{A97S}$ mice. Scale bars: 50 μm; inserts: 25 μm.

Figure 5. Skin innervation in human TTR knock-in mice. (**A**) Mouse footpad sections were immunostained for protein gene product 9.5 (PGP9.5). PGP9.5(+) nerves in the epidermis appeared as continuous dots (inserts) that arose from the bundles of subepidermal nerve plexuses (SNP, arrowheads). SNP appeared as continuous linear bundles in adult $hTTR^{wt}$ and $hTTR^{A97S}$ mice and in aging $hTTR^{wt}$ mice. The length of SNP was reduced and the appearance became punctate and

fragmented (arrows), indicating nerve degeneration in aging hTTR^{A97S} mice. Scale bars: 50 µm; inserts: 25 µm. (**B**) Quantitatively, intraepidermal nerve fibre (IENF) density was similar in adult hTTR^{A97S} and hTTR^{wt} mice. In contrast, the IENF density was lower in aging hTTR^{A97S} mice than in hTTR^{wt} mice. The numbers of subjects are shown in bar graphs. n.s.: not significant. *P < 0.05 by a 2-tailed Student's *t*-test.

Figure 6. Pathology of myelinated nerve fibres in sural nerves of human TTR knock-in mice. (**A**) Myelinated nerve fibres in the sural nerve was revealed by toluidine blue staining on semi-thin sections. Knock-in mice showed abundant myelinated nerve fibres with proper nerve profiles of myelin sheaths in the adult group. In the aging group, there were apparent nerve degeneration profiles in hTTR^{A97S} mice compared with hTTR^{wt} mice. Scale bar: 50 µm. (**B**) Quantitatively, there was no significant difference between genotypes in the myelinated nerve fibre density of adult mice. In contrast, there was a significant reduction in the myelinated nerve fibre density of hTTR^{A97S} mice compared with that of hTTR^{wt} mice in the aging group. The numbers of subjects are shown in bar graphs. n.s.: not significant. *P < 0.05 by a 2-tailed Student's *t*-test. (**C**) Myelin sheath abnormalities including outfolded myelin (arrowhead), infolded myelin (arrows), and separation of lamellae or wide incisures (asterisks) were investigated in semi-thin sections. Quantitatively, there was a significant increase in the proportion of myelinated nerve fibres with abnormal myelin sheaths in aging hTTR^{A97S} mice compared with that in hTTR^{wt} mice. Scale bar: 10 µm. The numbers of subjects are shown in bar graphs. n.s.: not significant. *P < 0.05, **P < 0.005, and ***P < 0.001, by 1-way ANOVA with Tukey's post hoc test.

Figure 7. Neurophysiologic examinations of myelinated sensory nerves in human TTR knock-in mice. (**A**) The neurophysiology of myelinated sensory nerves was assessed with nerve conduction studies before sacrifice. The amplitudes of sensory nerve action potentials (SNAPs) were similar between adult hTTR^{wt} mice and hTTR^{A97S} mice. In the aging group, SNAP amplitudes were lower in hTTR^{A97S} mice than in hTTR^{wt} mice. The numbers of subjects are shown in bar graphs. n.s.: not significant. **P

< 0.01 by a 2-tailed Student's *t*-test. (**B**) In aging groups, SNAP amplitudes showed a significant correlation with the myelinated nerve fibre density of the sural nerves. Solid line: regression line. Dotted lines: 95% confidence bands.