SUBSTRATE SPECIFICITY OF TRANSTHYRETIN: IDENTIFICATION OF NATURAL SUBSTRATES IN THE NERVOUS SYSTEM


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Short Title: TTR has substrates in the nervous system

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Abbreviations: apolipoproteinA-I (apoA-I), knockout (KO), neuropeptide Y (NPY), norleucine (Nle, n), peptidylglycine α-amidating monooxygenase (PAM), proteolytically inactive TTR (TTRprot), retinol binding protein (RBP), thyroxine (T₄), transthyretin (TTR), wild type (wt).

Synopsis
Besides functioning as the plasma transporter of retinol and thyroxine, transthyretin (TTR) is a protease, cleaving apolipoproteinA-I (apoA-I) after a Phe residue. In this work we further investigated TTR substrate specificity. Both by using P-diverse libraries and a library of phosphonate inhibitors, a TTR preference for a Lys residue in P₁ was determined, suggesting that TTR might have a dual specificity and that, in addition to apoA-I, other TTR substrates might exist. Previous studies revealed that TTR is involved in the homeostasis of the nervous system, as it participates in neuropeptide maturation and enhances nerve regeneration. We followed by investigating whether TTR proteolytic activity is involved in these functions. Both wt TTR and proteolytically inactive TTR (TTRprot) had a similar effect in the expression of peptidylglycine α-amidating monooxygenase, the rate limiting enzyme in neuropeptide amidation, excluding the involvement of TTR proteolytic activity in neuropeptide maturation. However, TTR was able to cleave amidated neuropeptide Y (NPY), probably contributing for the increased NPY levels reported in TTR KO mice. To assess the involvement of TTR proteolytic activity in axonal regeneration, neurite outgrowth of cells cultivated with wt TTR or TTRprot, was measured. Cells grown with TTRprot displayed decreased neurite length, thereby suggesting that TTR proteolytic activity is important for its function as a regeneration enhancer. By showing that TTR is able to cleave NPY and that its proteolytic activity affects axonal growth, this study shows that TTR has natural substrates in the nervous system, further establishing its relevance in neurobiology.
Introduction

Transthyretin (TTR) is the plasma homotetrameric carrier of thyroxine (T4) and retinol, in the latter case through binding to retinol binding protein (RBP) [1]. In the plasma, a small TTR fraction is carried in high-density lipoproteins (HDL), through binding to apolipoprotein A-I (apoA-I) [2]. Besides being a transporter, TTR is able to cleave the C-terminus of apoA-I, being a novel cryptic protease [3]. The relevance of apoA-I cleavage by TTR in lipoprotein metabolism was determined; upon TTR cleavage, HDL display a reduced capacity to promote cholesterol efflux and cleaved apoA-I displays increased amyloidogenicity [4]. Several features including an optimum pH for activity of approximately 7, inhibition by serine protease inhibitors and cleavage in apoA-I after a Phe residue, strongly indicate that TTR is a serine protease [3]. However, its catalytic mechanism remains to be solved. The cryptic nature of TTR proteolytic activity derives not only from the fact that it lacks canonical structural protease determinants, but also because its physiological function is apparently unrelated to proteolysis [5].

The two major sites of TTR expression are the liver and the epithelial cells of the choroid plexus of the brain, which are the sources of the TTR protein in the plasma and in the cerebrospinal fluid (CSF), respectively [6]. The conservation of TTR expression in the choroid plexus from reptiles to mammals led to the hypothesis that the expression of this gene first arose in the brain of reptiles [7]. During human embryonic development, TTR is first expressed in the tela choroidea, the precursor of the choroid plexus, followed by expression in the liver [8, 9]. This pattern of TTR expression in the choroid plexus, conserved throughout evolution and starting early in embryonic development, points to a pivotal role for TTR in the brain. It has been suggested that TTR is involved in thyroid hormone homeostasis and hormone delivery to the brain [10]. However, this issue has been a subject of some controversy. Although studies with TTR KO mice revealed that TTR is not necessary for thyroid hormones to be normally distributed [11], an association between the lack of TTR and decreased apoptosis of neural stem cells of the subventricular zone [12] has been recently described [13]. Nevertheless, the pattern of TTR expression suggests that the relevance of TTR is more than that initially described in the literature, as a carrier of thyroid hormones.

A number of TTR mutations are related to a neurodegenerative disease, familial amyloid polyneuropathy (FAP) [13], characterized by the deposition of TTR fibrils particularly in peripheral nervous system (PNS) [14]. Under physiological conditions, TTR has access to the nerve through the blood and the cerebrospinal fluid (CSF). A function for TTR in nerve biology could explain its preferential deposition, when mutated, in the PNS. Several studies using TTR knockout (KO) mice [15] revealed new TTR functions specifically related to the nervous system. At the functional level, TTR KO mice present a sensorimotor impairment [16] and the absence of TTR is associated with reduced signs of depressive-like behavior [17]. At the molecular level, TTR was shown to regulate the overexpression of peptidylglycine α-amidating monoxygenase (PAM) mRNA. PAM, the rate-limiting enzyme in neuropeptide maturation, is overexpressed in the PNS and CNS of TTR KO, as well as in cell cultures grown in the absence of TTR [18]. PAM is the only enzyme that C-terminally amidates peptides and 50% of all known neuropeptides require amidation to be active. Neuropeptide Y (NPY) is the major neuropeptide present in the mammalian brain and its activation requires C-terminal α-amidation by PAM. As such, as a consequence of PAM mRNA overexpression, TTR KO mice, as well as cell cultures in which TTR is absent in the culture medium, present increased levels of amidated NPY [18]. Finally, after injury TTR enhances nerve regeneration: in vivo, following sciatic nerve crush TTR KO mice present a decreased regenerative capacity, and in vitro, cells grown in the presence of TTR display increased neurite outgrowth, namely an approximately 20% increase in the length of the longest neurite [16]. As it is possible that apoA-I may not be the major TTR substrate and given the phenotypes of TTR KO mice (which can be related to the absence of TTR proteolytic activity and not to the absence of the protein itself), it is important to analyse the physiological relevance of TTR proteolytic activity in the nervous system.

In this work we investigated the substrate specificity of TTR and assessed whether some of the phenotypes described in TTR KO mice, namely TTR involvement in PAM expression and nerve regeneration, might be related to the lack of TTR proteolytic activity. If so, this would indicate that a TTR
substrate should exist in the nervous system. The identification of novel TTR substrates would allow to further characterize the physiological relevance of TTR proteolytic activity, and identify its mechanism of action in the nervous system.

**Experimental Procedures**

**Proteins**
Recombinant wild type (wt) TTR and glutathionylated TTR were produced in BL-21 pLys Escherichia coli cells transformed with pET plasmids carrying TTR cDNA. Glutathionylated TTR was detected by MALDI-MS and was shown to be proteolytically inactive (TTRprot, unpublished data) using a TTR fluorogenic peptide as described below. After bacterial lysis, proteins were isolated and purified as previously described [3].

**Positional scanning synthetic combinatorial libraries**
To profile TTR preferences at substrate positions P4-P1, we used positional scanning synthetic combinatorial libraries (PS-SCLs) of fluorogenic peptides [19]. To determine the P1 preference of TTR, we used a P1-diverse tetrapeptide library of 160,000 substrates each containing the fluorogenic leaving group 7-amino-4-carbamoylmethylcoumarin (ACC). The P1-diverse library consists of 20 wells in which only the P1 residue remains constant at each one of the 20 proteinogenic amino acids, excluding Cys and including Nle. The P2, P3, and P4 positions consist of an equimolar mixture of the 20 amino acids, for a total of 8,000 substrate sequences per well. The extended P4–P2 specificity was profiled with tetrapeptide libraries in which the P1 position was held constant. Three P1-fixed sublibraries denoting the second fixed position (P4, P3, P2) and consisting of 19 wells addressing a fixed amino acid (Cys omitted and Nle substituted for Met) were screened (361 compounds per well and 6,859 compound per library). Assays were performed at 37 °C in 50 mM Tris pH 7.0. Final concentration for each substrate compound was 10 nM. Substrate hydrolysis was initiated by addition of enzyme (5 µM) and monitored fluorometrically with excitation at 380 nm and emission at 450 nm for 3 h.

**TTR screening with a library of phosphonate inhibitors**
A total number of 160 compounds from a library of peptide phosphonate inhibitors of serine proteases was used in the screening [20]. Library screening was carried out in a dose response manner with inhibitor concentrations ranging from 1 to 50 µM. TTR (5 µM) was incubated with the inhibitors for 30 min at 37°C, in 50 mM Tris pH 7. After enzyme-inhibitor incubation, 5 µM of the substrate Abz-ESFKVS-EDDnp was added and the kinetics of the reactions were followed at 37°C for 1 h as previously described [3]. The above substrate was designed in order to encompass the apoA-I sequence cleaved by TTR (cleavage occurs following the Phe residue in the hexapeptide) as previously reported [3]. Relative fluorescence values were converted to percentages of residual activity relative to uninhibited controls.

**Assessment of TTR proteolytic activity**
TTR proteolytic activity was tested with the fluorogenic peptide Abz-ESFKVS-EDDnp as described above. Specificity rate constants ($k_{cat}/K_m$) were determined under pseudo first-order conditions. Pseudo first order rate constants were obtained from the linear plots where the y axis corresponds to Ln ((Fmax–Ftime)/Fmax), where Fmax is the fluorescence corresponding to total degradation of 5 µM substrate and Ftime is the fluorescence measured at each time point, and the x axis corresponds to the time of reaction. The slope of the linear plots, corresponding to the first order rate constants, was divided by the total enzyme concentration to provide $k_{cat}/K_m$. 
**T₄ binding assays**

Binding of wt TTR and TTR⁰ in T₄ was assayed quantitatively by a gel filtration procedure as described previously [21]. Briefly, a 30 nM solution of TTR (either wt TTR or TTR⁰) in 0.1 M Tris, 0.1 M NaCl and 0.001 M EDTA buffer, pH 8.0, was incubated with a constant amount of labeled ¹²⁵I-T₄ (∼50,000 cpm; Perkin Elmer) and increasing concentrations of cold T₄ (0–1000 nM; Sigma) overnight at 4 °C. ¹²⁵I-T₄ bound to TTR was separated from unbound T₄ by gel filtration chromatography through a Bio-Gel P6-DG column (Bio-Rad). The eluates containing bound T₄ were collected and counted in a gamma counter (Wizard 1470, Wallac Oy). Binding was expressed as the ratio between bound T₄/total T₄. All samples were run in triplicate and the data were analysed using GraphPad Prism software.

**RBP binding assays**

Wild type TTR was iodinated according to the iodogen method. [2] Briefly, 15 µg of wt TTR were added to reaction tubes coated with 10 µg of iodogen (Sigma) and containing 100 µL of 0.25 M phosphate buffer and 1 mCi of Na⁻¹²⁵I (Perkin-Elmer). The reaction was allowed to proceed in an ice bath for 20 min. Labeled TTR was separated from free iodide in a 5 mL Sephadex G50 column (GE Healthcare). RBP was isolated from human serum as previously described (3). For binding assays, 96 well plates (Maxisorp, Nunc) were coated with rabbit polyclonal anti-RBP (The Binding Site; 1:500) in coating buffer (0.1M bicarbonate/carbonate buffer, pH 9.6) and incubated overnight at 4 °C. ¹²⁵I-wt TTR (500,000 cpm/well) previously mixed with RBP (400 nM/well) was incubated with the plates alone or with the unlabeled competitors, either wt TTR or TTR⁰ at concentrations ranging from 0 to 5 µM, in binding buffer (0.1% milk, 10 mM Hepes in minimal essential medium (Invitrogen)) for 2 h at 37°C with gentle shaking. Binding was determined after five washes in ice-cold PBS with 0.05% Tween 20 (0.2 mL/wash). Then, 0.1 mL elution buffer (NaCl 0.1 M containing 1% nonidet P40) was added for 5 min at 37°C, and the content of the wells was aspirated and counted in a gamma counter (Wizard 1470, Wallac Oy). All samples were run in triplicates and results were analyzed using GraphPad Prism software.

**Uptake of TTR by a human hepatoma cell line**

The SAHep cell line (human hepatoma without TTR expression) was kindly provided by Dr. João Monjardino (Imperial College, London, UK). Cells were grown to confluence in 24-well plates in Dulbecco’s modified Eagle’s media (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma), 50 units/mL penicillin, and 50 µg/mL streptomycin (Invitrogen). For the competition experiments, cells were incubated with ¹²⁵I-wt TTR (100,000 cpm/well) in the presence of increasing concentrations of cold TTR (0–50 µg/mL; either wt TTR or TTR⁰), in DMEM with 0.1% ovalbumin (Sigma) for 4 h at 4°C. Cell-associated radioactivity was determined by measuring radioactivity of the PBS washed cell layer solubilized in 0.1M NaOH. All samples were run in quadruplicates and results were analyzed using GraphPad Prism software.

**Differential fluorescence gel electrophoresis (DGE) proteome profiling**

In order to identify additional plasma substrates for TTR, two distinct DGE experiments were performed. In the first experiment, 50 µL of plasma from TTR KO mice were incubated with 75 µg of either wt TTR or proteolytically inactive TTR (TTR⁰) in 50 mM Tris pH 7, overnight at 37°C. The sample incubated with wt TTR was labeled with Cy3 and the one incubated with TTR⁰ was labeled with Cy5. In a second experiment, 50 µL of plasma from TTR KO mice were incubated with (labeled with Cy5) or without (labeled with Cy3) 75 µg of wt TTR in 50 mM Tris pH 7, overnight at 37°C. In both experiments, 50 µg of each sample were loaded in the 2D gel. DGE was performed as a service by the W.M. Keck Facility, Yale University, New Haven, USA, according to the Ettan™ DGE manual (Amersham) available at http://keck.med.yale.edu/pdfs/Ettan%20DGE%20User%20Manual.pdf. Briefly, one sample was labeled with Cy-3 and the other with Cy-5; an equal amount of each sample was labeled and run in the DIGE experiment. 24 cm, pH 3-10 Immobiline (IPG) Drystrips (GE Healthcare) were rehydrated overnight with the two samples and isoelectric focusing was carried out. SDS PAGE...
(12.5%) was run, and immediately after, gels were scanned on a GE Healthcare Typhoon 9410 Imager. Gels were then fixed and stained with Sypro Ruby. GE Healthcare DeCyder software was used to quantify and to identify the differentially expressed protein spots with a fold change higher than 1.5. These spots were excised, MALDI-MS/MS spectra were acquired on each target (using Applied Biosystems 4800 ToF/Tof instrument), and the resulting peptide masses were subjected to database searching using Mascot algorithms.

**Influence of TTR proteolytic activity in PAM expression**

Neuropeptide processing was assessed by quantification of PAM expression in PC12 cells (European Collection of Cell Cultures), as previously reported [18]. Briefly, cells were grown in 6-well plates in William’s medium E (Invitrogen) supplemented with 2mM L-glutamine (Sigma) and 10% FBS (Invitrogen). When cells reached 50% confluence, FBS was withdrawn and William’s medium E was supplemented with 100 ng/mL 2.5S nerve growth factor (Sigma) and 10% of TTR KO mouse serum, or 10% TTR KO mouse serum containing 300 µg/mL of recombinant TTR (either wt or TTRprot). Seventy-two hours later, total RNA was extracted and PAM levels were determined by RT-PCR.

**NPY cleavage by TTR**

Amidated NPY (Bachem, 2 µg) was incubated either with 5 µg of wt TTR or TTRprot. Analysis of the reaction samples was performed by MALDI-TOF MS, as a service performed at the IPATIMUP Proteomics Unit, Porto, Portugal. Assessment of NPY cleavage by TTR was performed by comparing the molecular mass of NPY either incubated with active wt TTR or with TTRprot. As a control, the molecular mass of NPY alone was also determined by MALDI-TOF MS.

**Influence of TTR proteolytic activity in nerve regeneration**

The effect of TTR proteolytic activity in nerve regeneration was assessed by performing neurite outgrowth assays [16]. PC12 cells were grown in 6-well plates in D-MEM medium (Invitrogen) supplemented with 10% FBS. When cells reached 50% confluence, FBS was withdrawn and D-MEM medium was supplemented with 10% of either wt or TTR KO mouse serum, or 10% TTR KO mouse serum containing 300 µg/mL of recombinant TTR (either wt or TTRprot), i.e., the physiological concentration of TTR in the plasma. Forty hours later, PC12 cells were fixed in 2% neutral buffered formalin for 30 min, washed with PBS and kept at 4°C until further analysis. Neurite size was determined from 20x magnified fields using the Imaje J software (http://rsbweb.nih.gov/ij/). For each cell, the average neurite length was determined and at least 100 cells were analysed for each condition.

**Results**

**Libraries of Combinatorial Substrates and Phosphonate Inhibitors revealed a preference for a Lys residue in P1 for TTR proteolysis**

In order to determine the P1-P4 specificity for TTR cleavage, we used combinatorial peptide libraries [19]. The P1 site preference for TTR cleavage was determined as being Lys>Ala>Arg>Leu>Met>Phe (Fig. 1a). To define the extended substrate specificities of TTR, we performed a screening for P2-P4 positions with libraries in which the P1 was fixed to a Lys residue (Fig. 1b-d). The P2, P3 and P4 cleavage preferences were broad, which is common for some serine proteases, and as a consequence we were unable to identify TTR specificity for the P2-P4 sites. Moreover, by using a library of 160 peptide phosphonate inhibitors, which are irreversible inhibitors of serine proteases [20], TTR preference for a Lys residue in P1 was confirmed (Table 1). In a first screening we tested the inhibitors at a concentration of 50 µM and selected the compounds presenting an inhibitory effect higher than 90% (40 compounds were selected). In a second screening we performed dose-dependent experiments using inhibitor concentrations ranging from 50 to 1 µM; the most potent inhibitors were
selected as being the ones that did not show a dose response effect as they presented a high inhibitory effect at all the concentrations tested. At a concentration of 1 µM, the lowest concentration tested, those compounds were able to block more than 80% of TTR activity (Table 1). Analysis of the structures of the most potent compounds showed that TTR was preferentially inhibited by compounds with a basic residue as Lys in the P1 position and a Pro residue in P2. Among the most potent inhibitors only one presented a Phe in P1; one of the compounds presented an amidine-modified phenylalanine which has a basic-like behavior, similarly to Lys (Table 1).

Regarding the results for the P1 preference, it is noteworthy that TTR cleaves apoA-I after a Phe residue and not after the preferred Lys residue. As such, it is possible that other unknown TTR substrates with P1-Lys, Ala, Arg, Leu and Met exist.

**ApoA-I is the sole plasma TTR substrate as confirmed by DIGE**

In order to identify other putative natural TTR substrates in the plasma, we performed DIGE analysis of plasma from TTR KO mice incubated with TTR. To determine the differences in proteome profiling derived from proteolytic cleavage of a possible TTR plasma substrate, we selected only the common spots between the experiment where TTR KO plasma was incubated with either wt TTR or TTR^prot^- and the experiment where TTR KO plasma was either run alone or following incubation with wt TTR. In both experiments, there was only one spot increased when TTR KO plasma was incubated with wt TTR, corresponding to a fragment of a plasma protein originated by TTR cleavage (data not shown). This spot was analysed by MS and was identified as C-terminally cleaved mouse apoA-I. In agreement with this finding, we had previously shown that human TTR is able to cleave mouse apoA-I [4]. As such, despite the fact that we show that DIGE enables the identification of TTR substrates, we were unable to identify new putative TTR substrates in the plasma.

**TTR^prot^- retains its ability to bind TTR, RBP and its specific hepatic receptor**

In DIGE, as well as in the following experiments, we used a preparation of glutathionylated TTR that was shown to be proteolytically inactive. TTR proteolytic activity was assessed by determining the kinetic constants for the cleavage of the peptide Abz-ESFKVS-EDDnp. Whereas we determined a $k_{cat}/K_m$ of $10.5 \times 10^{-4} \text{min}^{-1} \text{µM}^{-1}$ for wt TTR, in glutathionylated preparations of TTR we observed complete loss of proteolytic activity. The modification by glutathione in this preparation was detected by MALDI-MS, by the presence of a mass peak corresponding to the mass of TTR mass with an increment of 305 Da, which corresponds to the molecular mass of glutathione. To confirm whether the biological functions of TTR, namely its ability to act as a plasma transporter, were maintained in the presence of the modification with glutathione in TTR^prot-, we assessed the ability of this preparation to bind its major ligands TTR and RBP. No significant differences were observed between wt TTR and TTR^prot- in binding to TTR, as shown by the log EC50 = 1.6 ± 0.1 nM and 1.7 ± 0.1 nM, respectively (Fig. 2a); the same finding was observed for binding to RBP, as both TTR preparations competed similarly with $^{125}$I-wt TTR (Fig. 2b), with a log EC50 = 0.9 ± 0.1 nM for wt TTR and 0.9 ± 0.2 nM for TTR^prot-. TTR uptake by its hepatic receptor was also assessed in experiments performed with human hepatomas, which were shown to internalize TTR by a specific yet unidentified receptor [22]. We determined that both wt TTR and TTR^prot- bind similarly to the hepatic receptor, as no significant differences in $^{125}$I- wt TTR cell association were observed by the presence of either of the proteins as shown by the log EC50 = 2.8 ± 0.2 ng/mL for wt TTR and 3.0 ± 0.3 ng/mL for TTR^prot- (Fig. 2c).

**TTR proteolytic activity is not relevant for its ability to regulate PAM mRNA expression**

As the P1 preference of TTR suggests that it may have additional substrates besides apoA-I, we started to unravel the effect of TTR proteolytic activity in recently described TTR functions. To analyse the effect of TTR proteolytic activity in PAM expression, we assessed the expression levels of PAM in PC12 cells grown in the presence of TTR KO serum (KO) or TTR KO serum supplemented with either wt TTR (KO + wt TTR) or TTR^prot-(KO + TTR^prot-). Previous experiments performed with PC12 cells...
demonstrated that addition of recombinant wt TTR to the cell growth medium leads to a decrease in PAM mRNA expression, when compared to cells grown in the absence of TTR [18]. If TTR proteolytic activity was involved in regulating PAM expression, it was expected that similarly to cells grown in the absence of TTR, in the presence of TTR$^\text{prot}$, PAM expression would be increased. However, both wt TTR and TTR$^\text{prot}$ led to a similar decrease of PAM mRNA levels in PC12 cultures (Fig. 3), showing that TTR proteolytic activity does not influence PAM expression.

In vitro, TTR is able to cleave NPY

Although we did not observe an effect of TTR proteolytic activity in PAM expression, we investigated whether in vitro TTR was able to cleave amidated NPY, as increased NPY levels are observed in the absence of TTR [18]. For this, we analysed the MS spectra of full-length NPY and NPY incubated either with wt TTR or TTR$^\text{prot}$. We observed that both full-length (Fig. 4A) or TTR$^\text{prot}$-incubated NPY (Fig. 4B) presented only one mass peak, corresponding approximately to the molecular mass of intact NPY (4272 Da). In the case of NPY incubated with wt TTR, we observed the fragmentation of the neuropeptide as shown by the appearance of two additional mass peaks, one with 3826 Da and a second with 4111 Da, besides the peak corresponding to intact NPY with an observed mass of 4273 Da (Fig. 4C). By calculating the difference between the mass of intact NPY and the mass corresponding to each of the fragmentation products, we determined that the generated NPY fragments correspond to amino acid residues 1-33 and 1-35, showing that TTR cleaves NPY after Arg33 and Arg35 (Fig. 4D). The TTR cleavage site on NPY is in agreement with a P1-Arg specificity determined with the P1-diverse libraries (P1 site preference for TTR cleavage Lys>Ala>Arg>Leu>Met>Phe).

TTR proteolytic activity affects its ability to promote neurite outgrowth

Another TTR function in the nervous system is its participation in nerve regeneration [16]. In vitro, neurite outgrowth of PC12 cells is augmented in the presence of TTR, which probably underlies the increased regenerative capacity of wt mice when compared to TTR KO mice [16]. To assess the putative involvement of TTR proteolytic activity in neurite outgrowth, we determined the average neurite length of PC12 cells cultivated in the presence of either wt or TTR KO mouse serum (KO), or TTR KO mouse serum containing either wt TTR (KO + TTR WT) or TTR$^\text{prot}$ (KO + TTR$^\text{prot}$). PC12 cells exposed to TTR KO serum displayed approximately 20% decreased average neurite length when compared to cells grown with wt serum, as previously reported [16]. Addition of wt TTR to TTR KO serum was able to rescue the decrease in neurite length of PC12 cells. However, cells grown with TTR$^\text{prot}$ displayed a similar neurite length as cells grown in the absence of TTR (Fig. 5). These results suggest that lack of TTR proteolytic activity, and not only the absence of TTR itself, is responsible for a decreased neurite length in PC12 cells, thus demonstrating that the proteolytic activity of TTR is relevant for the ability of TTR to promote neurite outgrowth.

Discussion

TTR was described as a protease able to cleave the C-terminus of apoA-I after the residue Phe 225 [3]. In this work we investigated the existence of other putative natural TTR substrates rather than apoA-I. We started by assessing the P1 to P4 preference for TTR cleavage using P-diverse libraries [19], and observed the following P1 preference: Lys>Ala>Arg>Leu>Met>Phe. This was further confirmed by performing a screening of TTR inhibition pattern using a library of peptide phosphonates [20] as TTR was inhibited by several phosphonate compounds, where the most potent inhibitors had a Lys residue in the P1 position and a Pro in P2. TTR seems to follow a dual specificity preferring basic amino acids at P1, such as Lys and Arg (this latest residue was preferred over Lys in the case of NPY), but also being able to cleave after hydrophobic/aromatic amino acids (as determined for apoA-I). Similarly, cathepsin G (cag) also presents two opposite specificities [23]. Cag was initially thought to posses a strict chymotrypsin-like specificity, with preference for cleavage after Phe residues. However, kinetic studies
demonstrated that Phe could be substituted by Lys, giving rise to similar $k_{cat}/K_m$ values. Regarding TTR, cleavage in apoA-I occurs after a Phe a residue, and not after the preferred Lys determined in vitro. Although we cannot state that Lys would be the best P1 amino acid in a physiological context, our results strengthen the need to search for other natural TTR substrates. It is however noteworthy that results obtained using a substrate library composed by tetrapeptides must be interpreted with caution. The interaction of short peptides with a given enzyme will certainly differ widely from the one achieved in a particular physiological environment with its natural substrates, which may display a complex tridimensional structure and multiple binding and/or interaction sites with the protease.

New TTR functions related to nerve biology have been recently identified. In this work we assessed the involvement of TTR proteolytic activity in two of the described functions, namely TTR participation in regulating PAM mRNA expression [18] and TTR ability to promote nerve regeneration [3, 4]. Although we demonstrated that TTR proteolytic activity is not necessary for TTR regulation of PAM expression, we determined that in vitro TTR is able to cleave NPY. As such, it is possible that in addition to the decreased levels of PAM expression, cleavage of amidated NPY by TTR might also contribute to the lower NPY levels observed in wt mice when compared to TTR KO littermates [18]. Moreover, we determined that TTR cleavage of NPY occurs after an Arg residue; this P1-Arg preference is in accordance with the results obtained with the P-diverse libraries, which demonstrated a preference for basic residues at the P1 site, and further suggests a dual specificity of TTR observed both in vitro (using P-diverse libraries) and in vivo (cleavage of apoA-I and NPY occurs after a Phe and Lys residue, respectively).

To ascertain whether the proteolytic activity of TTR is related to its ability to enhance nerve regeneration, we measured neurite outgrowth in PC12 cells cultivated in the presence of wt TTR or TTR$^{prot}$. In these experiments TTR was used at 300 µg/mL, which is the physiological concentration of the protein in the plasma. It is however noteworthy that in the settings of nerve injury the blood nerve barrier is disrupted enabling plasma to gain direct contact with the endonemium. Cells grown with TTR$^{prot}$ displayed decreased neurite length as compared with cells grown with proteolytically active wt TTR. This observation suggests that the proteolytic activity of TTR is relevant for its ability to promote neurite outgrowth. Although one cannot exclude that glutathionylation itself might affect neurite outgrowth, this hypothesis is hard to sustain as here we show that glutathionylated TTR maintains its transport functions, being still able to bind T4 and RBP. Moreover, studies assessing TTR uptake by its hepatic receptor, showed no major differences between the glutathionylated and the unmodified forms of the protein. As such, our results suggest that a TTR substrate probably exists in the nerve, and that its cleavage should enhance nerve regeneration.

Apart from the data here presented, a putative effect of TTR proteolytic activity in the biology of the nervous system is further supported by the pattern of TTR expression throughout evolution and embryonic development. It is interesting to note that in addition to the functions inferred from the TTR KO phenotype, TTR has been suggested to protect against amyloid beta (Aβ) deposition, the key pathological feature in Alzheimer’s disease [24]. When the nature of TTR-Aβ interaction was further investigated, TTR was found to be able to cleave Aβ in multiple positions with some of the generated Aβ peptides displaying lower amyloidogenic potential than the full-length counterpart [25]. The multiplicity of cleavage sites reported does not allow a direct comparison with the results here presented. It is however interesting that cleavage in Aβ following Phe, Lys and Ala residues is shown, in accordance with TTR specificity here described. Additionally, the identification of Aβ as a TTR substrate further stresses the involvement of its proteolytic activity in neurobiology. Until recently, apoA-I was the only known TTR substrate [3, 4]. Considering that TTR proteolytic activity might have been conserved during evolution, this functional property was highly unlikely to be maintained to increase the propensity for pathological conditions, as is the case of apoA-I cleavage which impacts in the development of atherosclerosis and amyloidosis [4].

In summary, our results show that TTR proteolytic activity is related with the recently described TTR functions in the nerve: we identified NPY as a novel TTR substrate and determined that the
proteolytic activity of TTR is important for the ability of this protein to enhance regeneration. As such, future work should address the identification of other TTR substrates in the nerve.

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References
14 Andrade, C. (1952) A peculiar form of peripheral neuropathy; familiar atypical generalized amyloidosis with special involvement of the peripheral nerves. Brain. 75, 408-427
Table 1 - TTR screening with a library of phosphonate inhibitors.
Chemical structure of substrate-based phosphonate compounds that at 1µM concentration yielded more than 80% inhibition of TTR proteolytic activity.

<table>
<thead>
<tr>
<th>Structure</th>
<th>P1</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>homolsine</td>
<td>proline</td>
</tr>
<tr>
<td></td>
<td>lysine</td>
<td>proline</td>
</tr>
<tr>
<td></td>
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<td>proline</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
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<td>proline</td>
</tr>
<tr>
<td></td>
<td>phenylalanine</td>
<td>leucine</td>
</tr>
<tr>
<td></td>
<td>4-(amidine)-phenylalanine</td>
<td>proline</td>
</tr>
</tbody>
</table>
Figure Legends

Fig. 1. Subsite preferences of TTR as determined by combinatorial P-diverse libraries.

a) P1 preference; b) P2 preference; c) P3 preference and d) P4 preference. The y axis represents the substrate cleavage rates in picomolar concentrations of fluorophore released per second. The x axis indicates the amino acid held constant at each position, designated by the one-letter code; n= norleucine. Error bars indicate ± SD.

Fig. 2. Comparison of the biological functions of wt TTR and TTRprot.

a) T4 binding to wt TTR and TTRprot. Competition was performed with increasing concentrations of cold T4 (0-1000 nM). b) Binding of 125I-wt TTR to RBP. Competition was performed with either unlabeled wt TTR or unlabeled TTRprot at increasing concentrations ranging from 0 to 5 µM. c) Cell association of 125I-wt TTR in SAHep cells. Competition was performed with either unlabeled wt TTR or unlabeled TTRprot at increasing concentrations ranging from 0 to 50 µg/mL. Error bars indicate ± SD.

Fig. 3. PAM expression in PC12 cells. PAM semiquantitative RT-PCR analysis of PC12 cells grown in the presence of TTR KO mouse serum (KO), or TTR KO mouse serum containing either wt TTR (KO + wt TTR) or TTRprot (KO + TTRprot). Results are presented as average ± SD; * P<0.05.

Fig. 4. NPY cleavage by TTR. MS spectra of a) full-length NPY; b) NPY incubated with TTRprot; c) NPY incubated with wt TTR; FL- full-length NPY; Fr- NPY fragments. d) Full-length NPY sequence displaying TTR cleavage sites (arrows); calculated molecular mass of each NPY fragment is shown.

Fig. 5. Neurite outgrowth in PC12 cells. Average neurite length per cell of PC12 cells after exposure to either wt (WT) or TTR KO serum (KO), or TTR KO serum supplemented with either wt TTR (KO+TTR WT) or TTRprot (KO + TTRprot). Results are presented as average ± SEM; * P<0.05.
Fig. 1

(a) P1

(b) P2

(c) P3

(d) P4
Fig. 2abc

a

T₄ bound/Total T₄

Log competitor (nM)

TTR WT
TTRmut

b

cpm TTR wt, TTR

Log competitor (nM)

TTR WT
TTRmut

c

pmol 125I wt TTR

Log competitor (ng/mL)

TTR WT
TTRmut
Fig. 3

![Graph showing PAM/β-actin levels for KO, KO + wt TTR, and KO + TTRprot-](graph.png)

- KO
- KO + wt TTR
- KO + TTRprot-

The graph indicates a significant difference between the groups, as marked by asterisks.
**Fig. 4**

Mass (m/z)

| a | 4276.79 |
| b | 4271.27 |
| c | 3826.49 |

Mass (m/z)

| 292.8 |
| 292.8 |
| 292.8 |

% Intensity

| 100 |
| 100 |
| 100 |

% Intensity

| 100 |
| 100 |
| 100 |

**d**


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18


19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

3825.2 Da 4109.5 Da
Fig. 5

Average neurite length/cell (µm)

WT   KO   KO + TTR
     WT   KO + TTRprot

* * *