



INFLUENCE OF TRANSTHYRETIN ON NEUROPEPTIDE PROCESSING

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**INFLUENCE OF TRANSTHYRETIN ON NEUROPEPTIDE
PROCESSING**

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TABLE OF CONTENTS

SUMMARY	i
SUMÁRIO	iii
SOMMAIRE	v
PART I – GENERAL INTRODUCTION.....	1
1 TRANSTHYRETIN.....	3
1.1 TTR gene structure	3
1.2 TTR gene regulation	4
1.3 TTR expression	5
1.4 TTR structure.....	6
1.5 TTR physiological functions.....	7
1.5.1 Transport of T ₄	8
1.5.2 Transport of retinol	11
1.5.3 TTR proteolytic activity	12
1.6 TTR metabolism	13
2 TTR KO MICE.....	15
2.1 Generation of the TTR KO mice.....	15
2.2 Characterization of the TTR KO mice	15
2.2.1 Thyroid hormone metabolism in TTR KO mice	16
2.2.2 Retinol metabolism in TTR KO mice	17
2.2.3 Neural stem cell niche in the brain of TTR KO mice	18
2.2.4 Sensorimotor performance of TTR KO mice.....	18
2.2.5 Lipid and glucose metabolism in TTR KO mice	19
3 TTR IN DISORDERS OF THE NERVOUS SYSTEM	20
3.1 Familial amyloid polyneuropathy (FAP)	20
3.2 Nerve injury	22
3.3 Alzheimer’s disease.....	22
3.4 Memory impairment.....	23
3.5 Depression.....	24

3.6	Psychosis.....	25
3.6.1	Schizophrenia.....	26
3.6.2	Bipolar disorder	27
3.7	Other neurodegenerative disorders.....	28
3.7.1	Frontotemporal dementia	28
3.7.2	Parkinson's disease.....	28
3.7.3	Amyotrophic lateral sclerosis.....	28
4	NEUROPEPTIDES	29
4.1	Biosynthesis, processing and secretion of neuropeptides.....	29
4.2	Amidation of neuropeptides	30
4.3	Neuropeptide Y.....	32
4.3.1	NPY synthesis and processing.....	32
4.3.2	NPY localization	33
4.3.3	NPY functions.....	33
4.3.3.1	Energy homeostasis	34
4.3.3.2	Depression and anxiety	34
4.3.3.3	Memory.....	35
4.3.3.4	Epilepsy and glutamate excitotoxicity	35
4.3.3.5	Neurogenesis	36
4.3.3.6	Alzheimer's disease.....	37
4.3.3.7	Parkinson's disease.....	37
4.3.4	NPY receptors	38
4.3.5	NPY metabolism.....	39
4.3.6	NPY models.....	40
4.3.6.1	NPY overexpressing models	40
4.3.6.2	NPY knockout model	42
5	CONCLUDING REMARKS.....	44
	PART II – RESEARCH PROJECT	45
	OBJECTIVES.....	47
	CHAPTER 1 – TTR IS RELATED TO DIFFERENTIAL GENE EXPRESSION IN THE NERVOUS SYTEM: PAM UPREGULATION IN THE ABSENCE OF TTR.....	49
	CHAPTER 2 – SEARCHING FOR UPSTREAM AND DOWNSTREAM MOLECULES PARTICIPATING IN TTR REGULATION OF PAM EXPRESSION	67

CHAPTER 3 – PART I - TRANSTHYRETIN KNOCKOUTS ARE A NEW MOUSE MODEL FOR INCREASED NEUROPEPTIDE Y.....	95
CHAPTER 3 – PART II - INCREASED NEUROPROTECTION AND DECREASED NEUROPROLIFERATION IN TTR KO MICE	123
CHAPTER 3 – PART III - NPY EXPRESSION IN THE OSTEOLASTIC LINEAGE REVEALS A POSSIBLE ROLE IN THE REGULATION OF BONE FORMATION.....	145
CHAPTER 4 - INCREASED LIPOPROTEIN LIPASE IS RELATED TO INCREASED SPHINGOLIPID CONTENT FOLLOWING NERVE CRUSH	169
CONCLUSIONS AND FUTURE PERSPECTIVES.....	191
PART III – APPENDIX.....	195
List of abbreviations	197
References	199

Summary

Transthyretin (TTR) is a plasma protein that acts physiologically as the transporter of thyroxine and retinol. The two major sites of TTR synthesis are the liver and the choroid plexus of the brain, which are respectively the sources of TTR found in the plasma and in the cerebrospinal fluid (CSF). Several point mutations in TTR are associated with familial amyloid polyneuropathy (FAP), an autosomal dominant lethal disorder characterized by systemic extracellular deposition of TTR amyloid fibrils particularly in the peripheral nervous system (PNS). Physiologically, TTR has access to the brain and nerve through the blood and CSF. To investigate TTR function in nervous system homeostasis, differential gene expression in wild-type (WT) and TTR knockout (KO) mice was assessed. We show that peptidylglycine α -amidating monooxygenase (PAM), the rate-limiting enzyme in neuropeptide maturation, is overexpressed in the PNS and central nervous system (CNS) of TTR KOs which, consequently, display increased levels of neuropeptide Y (NPY) in the PNS and CNS. NPY acts on energy homeostasis by increasing white adipose tissue (WAT) lipoprotein lipase (LPL) and by decreasing brown adipose tissue thermogenesis. Accordingly, we show increased LPL expression and activity in WAT, PNS and CNS as well as decreased body temperature in TTR KOs. Furthermore, in PC12 cells and primary cortical neurons, absence of TTR is related to increased PAM expression, NPY levels and LPL synthesis. Associated to increased NPY levels, TTR KOs display increased carbohydrate consumption and preference. Regarding NPY neuroprotective and neuroproliferative functions, we show that TTR KO mice hippocampal slice cultures are protected against AMPA-induced neurodegeneration but, contrary to an expected neuroproliferative role of NPY, TTR KO mice display fewer neural precursor cells in the subventricular zone. In the bone, we demonstrate for the first time NPY expression in the osteoblastic lineage. Furthermore, increased NPY levels were observed in TTR KO mice bone tissue, which might be responsible for the observed trend for increased bone mineral density and trabecular volume detected in TTR KO mice. These results contribute to a better characterization of TTR KO mice phenotype, as a model of increased NPY. Moreover, our data suggest that TTR internalization is a critical step for the regulation of PAM expression and, consequently, amidated NPY levels. In summary, our findings demonstrate that TTR plays a role in nervous system physiology, namely by regulating neuropeptide maturation through downregulation of PAM expression.

Sumário

A transtirretina (TTR) é uma proteína plasmática que actua fisiologicamente como transportadora de tiroxina e retinol. Os dois principais locais de síntese de TTR são o fígado e o plexus coróide do cérebro, que são, respectivamente, as fontes para a TTR encontrada no plasma e no líquido cefalorraquídeo (LCR). Várias mutações pontuais na TTR estão associadas com a polineuropatia amiloidótica familiar (PAF), uma doença autossómica dominante e letal caracterizada pela deposição sistémica e extracelular da TTR particularmente no sistema nervoso periférico (SNP). Fisiologicamente, a TTR tem acesso ao cérebro e ao nervo através do sangue e do LCR. Para investigar a função da TTR na fisiologia do sistema nervoso, a expressão diferencial de genes foi avaliada em murganhos controlo e em murganhos sem TTR, aqui referidos como ratinhos WT e ratinhos TTR KO, respectivamente. Observou-se que os ratinhos TTR KO apresentam tanto no SNP como no sistema nervoso central (SNC) um aumento de expressão da enzima limitante no processo de maturação dos neuropéptidos, de nome monooxigenase amidadora de glicinapéptidos (PAM). Consequentemente, os ratinhos TTR KO apresentam níveis aumentados do neuropéptido Y (NPY) tanto no SNP como no SNC. O NPY regula a homeostase energética através do aumento da lipoproteína lipase (LPL) no tecido adiposo branco e da diminuição da termogénese no tecido adiposo castanho. Consistentemente, observou-se nos ratinhos TTR KO um aumento da expressão e actividade da LPL no tecido adiposo branco, SNP e SNC, assim como uma diminuição da temperatura corporal. Em culturas de células PC12 e culturas primárias de neurónios corticais, verificou-se que a ausência de TTR está relacionada com o aumento da expressão de PAM, dos níveis de NPY e da síntese de LPL. Associado ao aumento de NPY, os ratinhos TTR KO apresentam um elevado consumo e preferência por carboidratos. Em relação às funções neuroprotectora e neuroproliferativa do NPY, observou-se que culturas organotípicas do hipocampo de ratinhos TTR KO estão protegidas contra a neurodegeneração induzida por AMPA mas, contrariamente ao papel neuroproliferativo do NPY, os ratinhos TTR KO apresentam menos células precursoras na zona subventricular. No osso, demonstrou-se pela primeira vez a expressão de NPY em células da linhagem osteoblástica e colocou-se a hipótese de os níveis elevados de NPY observados no tecido ósseo dos ratinhos TTR KO serem os responsáveis pela tendência para um aumento da densidade mineral óssea e do volume trabecular nos ratinhos TTR KO. Estes resultados contribuem para uma melhor caracterização do fenótipo dos ratinhos TTR KO como modelo de elevados níveis de NPY. Adicionalmente, os resultados obtidos sugerem que a internalização da TTR é um passo crítico na regulação da expressão da PAM e, consequentemente, dos níveis de NPY amidado. Em resumo, estes resultados demonstram que a TTR participa na fisiologia do sistema nervoso, nomeadamente por regular a maturação dos neuropéptidos através da diminuição da expressão da PAM.

Sommaire

La transthyrétine (TTR) est la protéine plasmatique qui transporte la thyroxine et le retinol. La TTR est synthétisée par le foie et le plexus choroïde du cerveau, qui sont respectivement les sources de TTR trouvées dans le plasma et dans le fluide cérébro-spinal (FCS). Plusieurs mutations dans la TTR sont associées à la polyneuropathie amyloïdote familiale (PAF), une maladie caractérisée par le dépôt extracellulaire systémique des fibrilles amyloïdes de TTR en particulier au niveau du système nerveux périphérique (SNP). La TTR a d'accès au nerf dans les conditions physiologiques par le sang et par le FCS. Pour étudier la fonction de la TTR dans l'homéostasie du système nerveux, l'expression différentielle de gène de souris WT et TTR knockout (KO) a été évaluée. Nous prouvons que la peptidylglycine α amidating monooxygénase (PAM), l'enzyme limitante dans la maturation de neuropeptide, est surexprimée dans le SNP et dans le système nerveux central (SNC) des TTR KO qui, par conséquent, ont des niveaux plus hauts de neuropeptide Y (NPY) dans le SNP et dans le SNC. Le NPY agit sur l'homéostasie d'énergie en augmentant la lipoprotéine lipase (LPL) du tissu adipeux blanc (TAB) et en décroissant la thermogenèse du tissu adipeux brun. En conséquence, nous montrons l'expression et l'activité accrues de la LPL dans le TAB, SPN et SNC aussi bien que la température de corps diminuée dans les TTR KO. En outre, dans des cellules PC12 et des neurones corticaux primaires, l'absence de la TTR est liée à l'expression accrue de PAM, à l'augmentation des niveaux de NPY et de la synthèse de LPL. Associé aux niveaux accrus de NPY, les TTR KO ont la consommation et la préférence d'hydrates de carbone augmentée. Concernant les fonctions neuroprotectives et neuroproliferatives du NPY, nous prouvons que des cultures organotypiques de hippocampus de souris de TTR KO sont protégées contre la neurodégénération induite par l'AMPA et que, contrairement à notre hypothèse, les souris TTR KO montrent moins de cellules neurales précurseurs dans la zone subventriculaire. Dans l'os, nous démontrons pour la première fois l'expression de NPY dans la lignée ostéoblastique et présumons que les niveaux accrus de NPY observés dans l'os des souris TTR KO sont responsables par la tendance observée pour une densité minérale de l'os et un volume trabéculaire accrus chez les souris TTR KO. Ces résultats contribuent à la meilleure caractérisation du phénotype des souris TTR KO comme modèle de NPY accru. D'ailleurs, nos données suggèrent que l'internalisation de la TTR soit une étape critique pour le règlement de l'expression de PAM et, par conséquent, des niveaux de NPY amidé. Nos résultats démontrent que la TTR joue un rôle à la physiologie du système nerveux, à savoir en réglant la maturation de neuropeptides en diminuant l'expression de la PAM.

PART I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Transthyretin (TTR) mutations are associated with familial amyloid polyneuropathy (FAP), a neurodegenerative disorder characterized by deposition of TTR aggregates and amyloid fibrils, particularly in the peripheral nervous system (PNS). The origin of TTR deposited in the PNS of FAP patients is unknown. Under physiological conditions, TTR has access to the nerve through the blood and the CSF. A function for TTR in nerve biology could explain its preferential deposition in the PNS. The aim of this research project was to unravel TTR involvement in the physiology of the nervous system. Several concepts are introduced in the following sections that constitute the basis for the development of this research project.

1 TRANSTHYRETIN

Transthyretin, shortly named TTR, was first discovered in 1942 in human cerebrospinal fluid (CSF) (Kabat et al., 1942). As it was the only protein that migrated ahead of albumin during electrophoresis, it was called prealbumin (Ingbar, 1958). At this time, prealbumin was found to bind thyroid hormones and its name was changed to thyroxine-binding prealbumin (Ingbar, 1958). A decade later, thyroxine-binding prealbumin was found to bind retinol-binding protein (RBP) (Raz and Goodman, 1969). Only in 1981, the name thyroxine-binding prealbumin was changed to transthyretin, which reflects its dual physiological role as the *transporter* of *thyroid* hormones and *retinol*-binding protein (Nomenclature Committee of the International Union of Biochemistry, 1981).

1.1 TTR gene structure

The gene encoding human TTR is a single copy gene (Tsuzuki et al., 1985) located on chromosome 18 (Whitehead et al., 1984) and assigned to the region 18q11.2-q12.1 (Sparkes et al., 1987). It spans about 7.0 kilobases and is composed of four exons of 95, 131, 136 and 253 base pairs, respectively, and three introns of 934, 2090 and 3308 base pairs (Sasaki et al., 1985; Tsuzuki et al., 1985). While the first exon encodes a signal peptide of 20 aminoacids, which is removed post-translationally, and 3 aminoacids of the mature protein, exons 2, 3 and 4 encode the aminoacid residues 4-47, 48-92 and 93-127,

respectively, of the mature TTR. One of the characteristic features of this gene is the presence of two independent open reading frames (ORF), in the first and third introns, with the same transcription direction as *ttr* and putative consensus regulatory sequences for transcription (Tsuzuki et al., 1985). Later, it was shown that these intronic ORFs corresponded to unspliced or partially spliced TTR that were not independently expressed *in vivo* nor part of functional transcripts (Soares et al., 2003). In the 5'-flanking region, upstream the transcription initiation site, several consensus sequences were found: two overlapping sequences with extensive homology to the glucocorticoid-responsive element at positions -224 and -212 base pairs, a CAAT box from -101 to -96 base pairs, a TATA box from -30 to -24 base pairs and a GC-rich region of about 20 base pairs (Sasaki et al., 1985). In the 3'-untranslated region, downstream the coding sequence, a polyadenylation signal (AATAAA) was identified at position 123 (Sasaki et al., 1985).

The *ttr* gene is highly conserved during evolution. The DNA sequence of the coding region of the mouse *ttr* gene shows 82% and 90% homology with the human and rat gene, respectively (the aminoacid sequence homology is higher, with 91% homology to the human and 96% to the rat aminoacid sequences) (Costa et al., 1986). Both mouse and rat genes are also composed of four exons (Costa et al., 1986; Fung et al., 1988). By comparing the mouse and human *ttr* promoters, a region of strong homology was observed that began about 290 base pairs upstream and extended to the mRNA cap site (Costa et al., 1986). The greatest degree of homology in this region occurs from -190 base pairs to the cap site, where homology value is similar to the one from the coding region (Costa et al., 1986). It was then proposed that the 190 base pairs immediately upstream of the cap site were good candidates to function in the regulation of the gene (Costa et al., 1986).

1.2 TTR gene regulation

The study of regulatory sequences performed using the mouse *ttr* gene allowed the identification of two major regulatory regions: a promoter sequence at -50 to -150 base pairs, and an enhancer sequence at -1.86 to -1.96 kilobases (Costa et al., 1986; Costa et al., 1988). The same regulatory sequences were found in the human *ttr* gene by comparative analysis (Costa et al., 1986). Several possible regulatory sites in both the promoter and enhancer regions of the mouse *ttr* gene were also identified, including DNA binding sites for liver-specific nuclear factors, such as the hepatocyte nuclear factors 1, 3, and 4 (HNF-1, 3, 4) (Costa et al., 1989; Costa and Grayson, 1991) and the CCAAT/enhancer binding protein (C/EBP) (Costa et al., 1988; Costa and Grayson, 1991).

The regulation of the human gene is less known, but homology search revealed that several regulatory signals exist in the 5' flanking region of the gene, including binding sites for the nuclear factors HNF-1, 3, 4 and C/EBP (Sakaki et al., 1989). Studies performed with transgenic mice carrying incomplete sequences of the human *ttr* gene, suggested that the liver and choroid plexus *ttr* genes are differentially regulated (Yan et al., 1990; Nagata et al., 1995). While a shorter sequence (< 1 kilobase) upstream the mRNA cap site is sufficient to drive TTR expression in the liver, choroid plexus expression requires the presence of a further upstream sequence (over 3 kilobases) (Yan et al., 1990; Nagata et al., 1995).

1.3 TTR expression

Transcription of the *ttr* gene results in approximately 0.7 kilobases mRNA containing a 5'-untranslated region, the coding region, and a 3'-untranslated region, preceding the poly(A) tail (Mita et al., 1984; Sasaki et al., 1984). As predicted by the nucleotide sequence of Mita *et al* (Mita et al., 1984) and Sasaki *et al* (Sasaki et al., 1984), TTR, like many other secreted proteins, is synthesized as a larger molecular weight precursor, pre-transthyretin, which includes a signal peptide at the N-terminal region that is cleaved upon TTR translocation to the endoplasmic reticulum (Soprano et al., 1985).

TTR is synthesized by the liver and secreted into the blood, where its concentration ranges from about 20 to 40 mg/dL (Vatassery et al., 1991). The liver is responsible for more than 90% of TTR present in the bloodstream. TTR plasma concentration varies with age: in healthy newborns it is about half of that in adults (Stabilini et al., 1968; Vahlquist et al., 1975), and begins to decline after age 50 (Ingenbleek and De Visscher, 1979). TTR is also synthesized by the choroid plexus epithelial cells and secreted into the CSF (Aleshire et al., 1983). It was reported that one gram of choroid plexus contained about 25 times larger amounts of TTR mRNA than 1 g of liver (Dickson et al., 1985), reflecting the very active synthesis of TTR in this tissue. TTR concentration in the CSF ranges from about 2 to 4 mg/dL, and reflects both a minor TTR fraction that enters the CSF via the blood-cerebrospinal fluid barrier (like albumin), and a major fraction that is produced and secreted by the choroid plexus (Weisner and Roethig, 1983). In the CSF, TTR represents 20% of the total proteins (Weisner and Roethig, 1983). Besides the liver and the choroid plexus of the brain, the retinal pigment epithelium in the eye is one more site of active TTR synthesis (Cavallaro et al., 1990). Both epitheliums, in the choroid plexus and retina, display structural and functional similarities, and TTR synthesis is one more factor in common. TTR synthesized by the retinal pigment epithelium is secreted across the apical

membrane into the extracellular matrix, together with RBP that is also produced in this tissue (Ong et al., 1994). More recently, TTR synthesis by the ciliary pigment epithelium in the eye was identified in the rabbit, at about one-third the levels found in the retinal pigment epithelium (Kawaji et al., 2005). Synthesis of small amounts of TTR in several other tissues was also demonstrated, as is the case of the pancreas (islets of Langerhans) (Kato et al., 1985), stomach, heart, muscle, spleen (Soprano et al., 1985) and meninges (Blay et al., 1993).

During human embryonic development, TTR is synthesized and detected in the fetal blood as early as the eighth week of gestation (Andreoli and Robbins, 1962; Jacobsson, 1989). First the protein is expressed in the tela choroidea, the precursor of the choroid plexus, followed by the expression in the liver (Harms et al., 1991; Richardson et al., 1994), and later in the pancreas (Jacobsson, 1989). TTR synthesis was also demonstrated in the human placenta (McKinnon et al., 2005). In mice, TTR mRNA was detected at the 10th day of gestation in endodermal cells of the visceral yolk sac, tela choroidea, and hepatocytes (Murakami et al., 1987).

In evolutionary terms, TTR synthesis occurs in fish (Santos and Power, 1999), reptiles, birds and mammalian ancestors (Richardson et al., 1994). In fish, TTR is produced mainly by the liver whereas in reptiles TTR is produced by the choroid plexus but not by the liver (Achen et al., 1993), while birds and mammals produce TTR in both tissues (Harms et al., 1991). These findings suggest two hypothesis: i) TTR expression occurred first in the choroid plexus and only a few million years later in the liver (Schreiber et al., 1993), and ii) TTR evolved in a common fish ancestor (Santos and Power, 1999).

1.4 TTR structure

The first X-ray crystal structure of human TTR was elucidated by Blake and coworkers in 1971 (Blake et al., 1971). TTR was found to be a tetrameric protein composed of four identical subunits with over-all molecular weight of 54,980 Daltons (Da). Each TTR subunit has 13,745 Da, is composed of 127 aminoacids (Kanda et al., 1974), and consists of 8 antiparallel β -strands designated A through H (**Figure 1**) (Blake et al., 1974). Each subunit contains two β -sheets formed by strands DAGH and CBEF. A single α -helix segment in each subunit is located at the end of β -strand E. Two TTR subunits associate forming one dimer by interaction between β -strands F and H of each subunit. The strand arrangement within the dimer is DAGHH'G'A'D' and CBEFF'E'B'C', with A' to H' being the β -strands from the second TTR subunit. The 2 β -sheets of 4 β -strands in the monomer are extended to 2 β -sheets of 8 β -strands in the dimer. Two dimers assemble to

form the tetramer by interactions involving the residues at the loops that join β -strands G to H and A to B (**Figure 2**). The quaternary structure of TTR has the shape of a globular protein whose overall size is 70 Å x 55 Å x 50 Å. The arrangement of the four subunits forms a central hydrophobic channel.

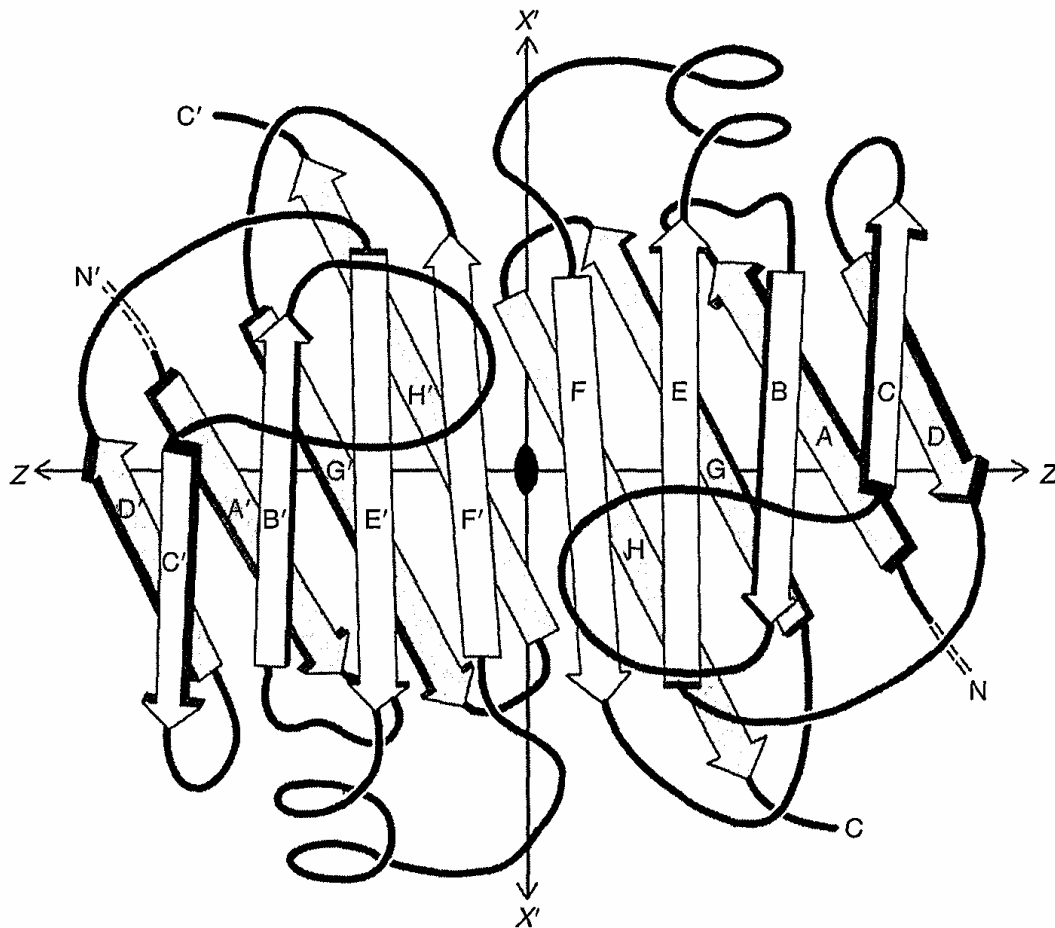


Figure 1. Structure of human TTR dimer showing eight antiparallel β -strands (labeled A-H or A'-H') and one α -helix in each subunit (Blake et al., 1974).

1.5 TTR physiological functions

Human TTR carries in blood all of the retinol-binding protein (RBP) and approximately one fifth of the thyroxine (T_4) hormone. Moreover, all circulating retinol (vitamin A) is bound to its specific plasma transport protein, RBP, leading to the formation of a complex TTR-RBP-retinol (Kanai et al., 1968). This makes the transport of T_4 and retinol the two major known TTR functions.

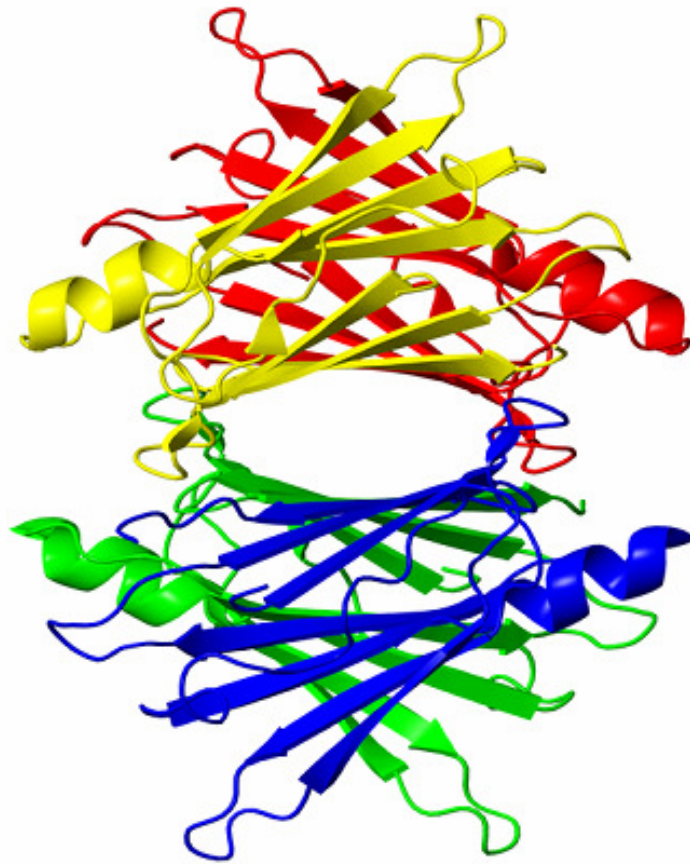


Figure 2. Representation of the quaternary structure of TTR (available at en.wikipedia.org/wiki/Transthyretin).

1.5.1 Transport of T_4

Thyroid hormones (THs) are essential for normal growth and development and for regulation of the basal metabolic rate. The two thyroid hormones are thyroxine (tetraiodothyronine, T_4) and triiodothyronine (T_3). A schematic representation of TH metabolism is presented in **Figure 3** and will be discussed throughout this subsection. THs are synthesized by the thyroid gland and secreted into the bloodstream. In mammals, most of the TH produced by the thyroid gland and in circulation in the plasma is T_4 , which has higher affinity for the distributor proteins in the blood than T_3 does. However, T_3 has higher affinity for the thyroid hormone receptors in the nucleus than T_4 , making T_3 the biological active form of THs. THs can be activated by deiodination of T_4 to T_3 in the thyroid gland or in the periphery. Cytosolic TH binding proteins are involved in their intracellular trafficking (Yamauchi and Tata, 1997). In the nucleus, T_3 can directly modulate expression of TH responsive genes through binding to its receptors, together with coactivator or corepressor proteins. More than 99% of THs in the blood are bound to

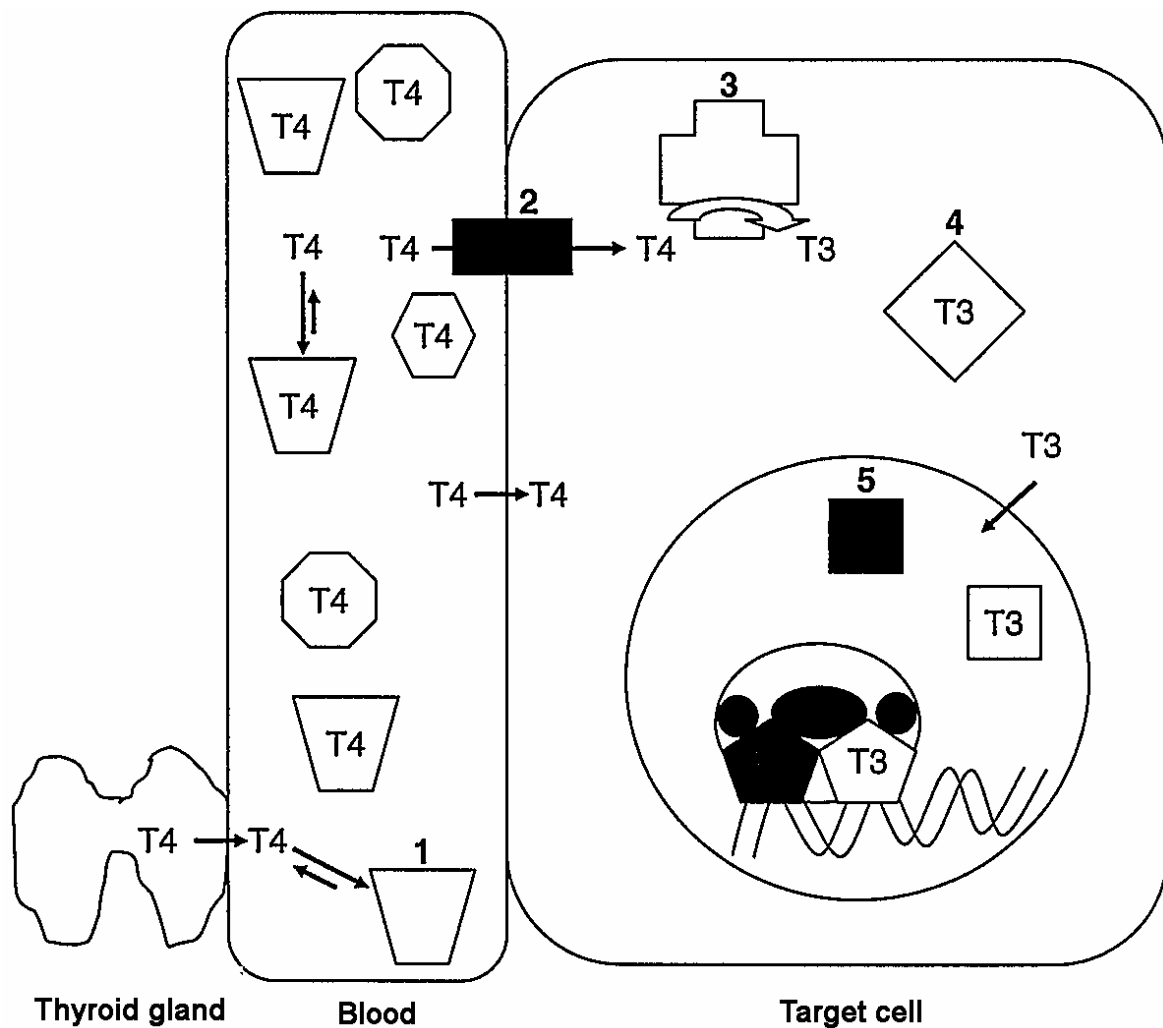


Figure 3. Thyroid hormones metabolism. 1: THDPs (TBG, TTR and albumin are represented by trapezoids, octagons and hexagons). 2: Thyroid hormone transporters. 3: Deiodinases. 4: Cytosolic thyroid hormone-binding proteins. 5: Thyroid hormone nuclear receptors. (Blake et al., 1974).

TH distributor proteins (THDP) (Mendel, 1989). In humans, the THDPs in blood are thyroxine-binding globulin (TBG), TTR and albumin (Bartalena, 1990). These three proteins are synthesized and secreted by the liver and are involved in the distribution of THs from their site of synthesis, via the bloodstream, to their sites of action: the cells and tissues throughout the body and brain. Together, they form a buffering network system for THs in blood, ensuring a sufficient amount of hormones in circulation by preventing the avid nonspecific partitioning of lipophilic THs into cell membranes. TTR has intermediate affinity for T_4 between those for albumin (lower affinity) and TBG (higher affinity) (Loun and Hage, 1992). These differences in affinity may justify the fact that, despite TTR concentration in serum is 20-fold higher than that of TBG, the former is responsible for the transport of about 15% of plasma T_4 , while the later transports 70% of the hormone. Albumin is thought to transport about 10%. In rodents, TTR is the major carrier of T_4 , transporting approximately 50% of the total hormone (Davis et al., 1970). TBG, the

principal T_4 carrier in humans, is present in neonatal but almost absent in the adult rodent serum (Vranckx et al., 1990). Other proteins are also able to bind THs though to a much lesser extent, namely immunoglobulins and apolipoproteins A and B (Benvenista et al., 2001). Only one THDP is produced in the brain: TTR, synthesized by the epithelial cells of the choroid plexus and secreted exclusively into the CSF. TTR is the major T_4 carrier in the CSF of both rodents and humans, transporting ~ 80% of T_4 in the human CSF.

TTR tetramer has a central hydrophobic channel with two structurally identical binding sites for T_4 , each located between two of the 4 subunits (Blake et al., 1974). These binding sites display different T_4 affinities (Andrea et al., 1980) and because of negative cooperativity only one site is occupied under physiological conditions (Wojtczak et al., 2001) (**Figure 4**).

The role of TTR in the delivery of thyroid hormones to target tissues has been controversial. Several hypothesis were raised throughout the years. Currently, the most widely accepted one is the free hormone hypothesis, which states that the concentration of free hormone in the blood is crucial for biological activity, rather than the protein-bound hormone concentration. This hypothesis does not exclude the possibility that in some cases hormone transfer into the tissues is made through the protein-bound fraction (Mendel, 1989). Free THs, dissociated from THDPs, can enter cells via TH transporters at the plasma membrane or by passive diffusion. Membrane-bound TH transporters have been identified (Sugiyama et al., 2003), which may assist in THs uptake into specific tissues. Studies performed with TTR null mice (Episkopou et al., 1993) further support the free hormone hypothesis for T_4 tissue uptake. The concentration of total T_4 in the blood of these mice was found reduced to 35% (Episkopou et al., 1993) or 50% (Palha et al., 1994) of that in wild-type mice, besides normal free hormone levels (Palha et al., 1994). Increased T_4 binding to TBG was observed in TTR null mice serum, without increased TBG expression, suggesting that TTR and TBG compete for T_4 binding (Palha et al., 1994). Furthermore, measurement of several parameters of thyroid hormone function indicated that these mice are euthyroid (Episkopou et al., 1993; Palha et al., 1994), despite the strongly reduced total T_4 plasma levels. The explanation for the euthyroid status in the absence of the major plasma T_4 carrier was the normal free hormone levels (Palha et al., 1994). These results suggest that TTR is not essential for thyroid hormone metabolism, even in conditions of increased hormone demand, as is the case of exposure to cold or thyroidectomy (Sousa et al., 2005). TTR is most likely a redundant protein in thyroid hormone homeostasis. A more active role for TTR was also suggested (Divino and Schussler, 1990), in which TTR bound to T_4 is recognized by specific cellular receptors and directly participates in the uptake of T_4 . The clarification of TTR role as the transporter of thyroid hormones entails further work and continues to be a matter of debate.

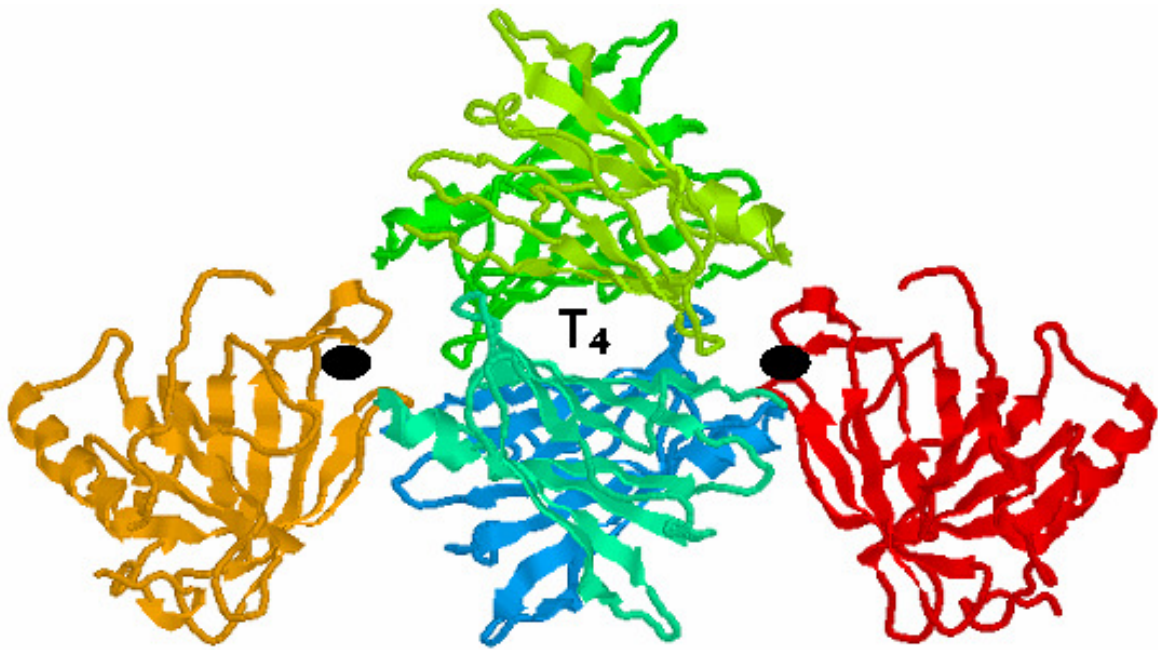


Figure 4. Representation of the complex thyroxine-TTR-RBP-retinol. TTR tetramer is shown in green/blue, thyroxine (T_4) is shown in the central channel, two RBP molecules are shown in orange/red at the sides (under physiological conditions only one RBP molecule is bound to TTR), and retinol is shown in black (adapted from Monaco et al., 1995).

1.5.2 Transport of retinol

TTR acts physiologically in the transport of retinol in the circulation, through the formation of a TTR-RBP-retinol complex (Kanai et al., 1968; Raz and Goodman, 1969) (**Figure 4**). RBP, which is synthesized and secreted primarily by hepatocytes, is the sole specific transport protein for retinol in circulation (van Bennekum et al., 2001). Retinyl esters obtained from the diet are delivered to the liver where they are either stored or secreted as retinol bound to RBP into the plasma. The secretion of RBP is strongly stimulated by its association with retinol, which alters the conformation of the protein (van Bennekum et al., 2001). Despite the presence of four binding sites for RBP in the TTR tetramer (van Jaarsveld et al. 1973), only one molecule is bound to TTR under physiological conditions, due to the limiting RBP physiological concentration in the plasma. In fact, serum TTR concentrations are usually in 2-3-fold molar excess of those of circulating RBP (van Bennekum et al., 2001). Association with TTR is proposed both to facilitate RBP release from its site of synthesis in the endoplasmic reticulum and to prevent renal filtration of the small (21,000 Da) RBP molecule (Raz et al., 1970). On the other hand, RBP is important to mobilize retinol from its sources in the liver and to deliver it to cells and tissues throughout the body via transport in the bloodstream. This pathway

constitutes the major mechanism through which cells acquire retinol, which is oxidized to the retinoic acid they need for regulating gene expression. Retinoic acid is essential for several physiological functions, namely for vision, reproduction, growth and development. TTR has also been found to bind retinoic acid but with less efficiency than RBP-retinol (Smith et al., 1994). Studies performed with TTR null mice (Episkopou et al., 1993) revealed a significantly decrease in plasma RBP and retinol levels in these animals as a consequence of increased renal filtration of the retinol-RBP complex (van Bennekum et al., 2001). This finding supports an important role for TTR in preventing the renal clearance of circulating RBP and retinol. Nevertheless, total retinol levels in the tissues of TTR null mice were similar to those observed in wild type mice, with mutant mice lacking symptoms of vitamin A deficiency (Episkopou et al., 1993; van Bennekum et al., 2001), suggesting that alternative mechanisms not yet identified are able to compensate the loss of RBP-mediated retinol delivery to tissues.

Regarding the mechanism of retinol uptake by cells, several hypothesis were raised over the years. Currently, the most accepted one involves the existence of a RBP receptor in the plasma membrane of target cells. The divergence regarding this mechanism is whether RBP is internalized during the retinol transference, by endocytosis, or remains outside the cell. TTR role during this process is not clarified yet as no consensus was reached regarding whether TTR is also recognized by the RBP receptor or not. Very recently, Kawaguchi *et al* identified in bovine retinal pigment epithelium cells STRA6, a multitransmembrane domain protein, as a specific membrane receptor for RBP (Kawaguchi et al., 2007). STRA6 was found to bind RBP with high affinity and to have robust retinol uptake activity from the retinol-RBP complex. The RBP-STRA6 retinol delivery mechanism does not depend on endocytosis (Kawaguchi et al., 2007). STRA6 is widely expressed during embryonic development and in adult brain, spleen, thymus, kidney, female genital tract, testis, placenta (and at lower quantities in heart, lung and liver) (Bouillet et al., 1997; Kawaguchi et al., 2007). A recent human genetic study found that mutations in STRA6 gene are associated with widespread birth defects in multiple organ systems (Pasutto et al., 2007), consistent with the expression of STRA6 and the diverse functions of retinol in embryonic development.

1.5.3 TTR proteolytic activity

In addition to the transport of T_4 and retinol in plasma, a fraction of TTR is carried in high-density lipoproteins (HDL) through binding to apolipoprotein A-I (apoA-I) (Sousa et al., 2000a). Characterization of the nature of TTR-apoA-I interaction revealed that TTR is a non-canonical serine protease with ability to cleave the C-terminus of apoA-I (Liz et al.,

2004; Liz and Sousa, 2005). When complexed with RBP, TTR activity was lost, whereas when complexed with T₄, only a slight decrease was observed (Liz et al., 2004). Although the exact mechanism of TTR proteolytic activity is not yet fully understood, the authors demonstrated that TTR cleaves not only lipid-free but also lipidated apoA-I (Liz et al., 2007). Furthermore, the relevance of apoA-I cleavage by TTR in lipoprotein metabolism was demonstrated by the reduced ability of HDL to promote cholesterol efflux and to bind their hepatic receptor upon TTR-mediated apoA-I cleavage (Liz et al., 2007). Additionally, the authors determined that TTR-cleaved apoA-I has a high propensity to form aggregated particles and fibrils than the full-length apoA-I (Liz et al., 2007). The results presented suggest that apoA-I cleavage by TTR may affect the development of atherosclerosis by reducing cholesterol efflux and increasing apoA-I amyloidogenic potential.

1.6 TTR metabolism

Not much is known regarding TTR physiological metabolism. It is known that the total body TTR turnover in humans is 250-300 mg/m²/day (Vahlquist et al., 1973). Furthermore, the biological half-life of TTR is about 2-3 days in humans (Socolow et al., 1965; Vahlquist et al., 1973), 22-23 hours in monkeys (Vahlquist and Peterson, 1972) and 29 hours in rats (Dickson et al., 1982). By intravenous injection of labeled TTR, Makover *et al* demonstrated that several tissues participate in rat TTR turnover and catabolism (Makover et al., 1988). TTR was found mainly in the liver (36-38% of total TTR, almost all in hepatocytes), muscle (12-15%), and skin (8-10%). Tissues as kidneys, adipose tissue, testes, and the gastrointestinal tract accounted each for 1-8% of total TTR. As no functional involvement for TTR has been ascribed for in these tissues, the presence of the protein in each of them was perceived as a possible site of TTR degradation. The mechanism for TTR cellular uptake is however poorly understood. Only one receptor, megalin, was identified as an important TTR receptor involved in renal uptake, thus preventing its filtration through the glomerulus (Sousa et al., 2000b). Megalin is a member of the low density lipoprotein (LDL) receptor family and is involved in the receptor-mediated endocytosis of a wide range of ligands. In the liver, a yet unidentified receptor-associated protein (RAP)-sensitive receptor was shown to mediate TTR internalization (Sousa and Saraiva, 2001). The authors showed that RAP, a ligand for all members of the LDL receptor family, was able to inhibit TTR internalization. Furthermore, as TTR uptake was inhibited by lipoproteins, it was suggested that a common pathway might exist between TTR and lipoprotein metabolism (Sousa and Saraiva, 2001). This RAP-sensitive receptor was

shown not to be megalin, as this protein is not expressed in the liver. Further studies are needed in order to clarify receptor-mediated TTR internalization.

2 TTR KO MICE

In order to further investigate the physiological role of TTR, namely its involvement in the development of the embryo, Episkopou *et al* reported, in 1993, the generation of a TTR null mutant mice (Episkopou *et al.*, 1993). Since then, several studies were performed using this strain, which contributed, on one hand, to the better understanding of the already known TTR physiological functions and, on the other hand, to the appearance of novel TTR functions.

2.1 Generation of the TTR KO mice

TTR knockout (KO) mice were generated by disruption of the *ttr* gene using gene targeting techniques in embryonic stem (ES) cells (Episkopou *et al.*, 1993). The targeting vector contained the bacterial neomycin-resistant gene (*neo*) sandwiched between the second exon of a 5.9 kilobases genomic mouse *ttr* gene fragment carrying exons 1 to 3. After transfection of the vector to the ES cells, the junction DNA fragment generated by homologous recombination was detected by PCR amplification and confirmed by hybridization to a *ttr-neo* probe and restriction analysis of the genomic DNA. Candidate ES cell clones yielding the *ttr* mutation were injected into host blastocysts and two germ-line chimeras were obtained. These animals were bred with females, transmitting the disrupted *ttr* allele to 50% of their progeny. After heterozygous animals were intercrossed, live-born homozygous TTR KO mice were obtained and underwent thorough confirmation of the null mutation at the *ttr* locus. By Western blot analysis, no TTR, nor TTR truncated forms were detected in the peripheral blood. Furthermore, no TTR protein, or altered forms of the protein were found by metabolic labeling experiments in the choroid plexus of homozygous mice. Hence, TTR KO mice had been created.

2.2 Characterization of the TTR KO mice

It was obvious from the frequency at which homozygous littermates were recovered from each litter that absence of TTR did not compromise fetal development (Episkopou *et al.*, 1993). Furthermore, TTR KO mice display no phenotypic abnormalities postnatally and their longevity does not differ from their heterozygous or wild type littermates

(Episkopou et al., 1993). Breeding experiments have also demonstrated that both male and female TTR KO mice have normal fertility (Episkopou et al., 1993). In sum, TTR KO mice are phenotypically normal, viable and fertile. However, some incongruences were observed regarding TTR major ligands, as described below.

2.2.1 Thyroid hormone metabolism in TTR KO mice

In rodents, TTR is the major carrier of thyroid hormones (THs) in the blood and cerebrospinal fluid (CSF), as referred in Section 1. Most of the TH in circulation in plasma is thyroxine (T_4), which has higher affinity for the distributor proteins in the blood than triiodothyronine (T_3) does. TTR is responsible for the transport of approximately 50% of the total T_4 in plasma (Davis et al., 1970). After the generation of the TTR KO mice, Episkopou *et al* revealed that the total T_4 and T_3 levels in the blood of these animals were reduced to 35% and 65%, respectively, of that in wild type (WT) mice (Episkopou et al., 1993). The pituitary thyrotropin (TSH) plasma levels were not altered, no morphological abnormalities were detected in the thyroid gland and as TTR KO mice had only slightly reduced T_3 plasma levels they were considered euthyroid (Episkopou et al., 1993). The same was already reported for humans with thyroxine-binding globulin (TBG) deficiency (Burr et al., 1980). One year after TTR KO mice generation, Palha *et al* justified their euthyroid status with the normal free T_4 levels they presented, further supporting the free hormone hypothesis as the mechanism for T_4 cellular uptake (Palha et al., 1994). The authors showed that despite a 50% decrease in total T_4 serum levels, the delivery of the hormone to tissues was not significantly affected by the absence of TTR. In fact, total and free T_3 and TSH plasma levels were similar in inbred TTR KO and WT mice (Palha et al., 1994). Increased circulating TSH is known to be a marker of hypothyroidism (Stein et al., 1989). Likewise, elevated deiodination is also found in hypothyroidism (Silva and Leonard, 1985; Berry et al., 1990), and the fact that deiodinase activity was not statistically different between strains suggested that tissues were not suffering from T_4 deprivation (Palha et al., 1994). Furthermore, TTR KO mice presented increased T_4 binding to thyroxine-binding globulin (TBG), another T_4 carrier in the blood, which may explain their normal free T_4 levels (Palha et al., 1994). The higher binding is not due to an increase in TBG plasma levels, suggesting that TTR and TBG compete for T_4 binding (Palha et al., 1994). In WT mice this battle is won by TTR as TBG levels are almost absent in the adult mouse serum (Vranckx et al., 1990). Conjugation with glucuronides, the major metabolic route for degradation of THs, was excluded as the cause for the reduced total T_4 plasma levels in TTR KO mice (Palha et al., 1994). In 1997, the same authors evaluated T_4 content in several tissues. No major differences were observed between TTR KO and WT mice in

the liver and kidney, but in the brain, TTR KO mice showed a 30% decrease in T_4 levels and the mean residence time of T_4 in this tissue was reduced from 58 min in WTs to 34 min in TTR KOs (Palha et al., 1997). The authors suggested that the low T_4 brain content reflected the absence of T_4 -TTR complexes in the mutant choroid plexus and CSF. However, no major changes were observed in brain T_3 concentrations, suggesting that availability of this hormone is not markedly altered in TTR KO mice (Palha et al., 1997). In sum, this study suggested that TTR is not essential for T_4 tissue uptake and for T_4 to reach the brain across the choroid plexus-CSF and/or blood-brain barriers (Palha et al., 1997). Subsequently, Palha *et al* demonstrated that the choroid plexus was the brain region responsible for the decreased whole brain T_4 content, presenting only 14% of WT levels, while in the cortex, cerebellum and hippocampus T_4 levels were normal (Palha et al., 2000). Despite only 48% T_3 levels were detected in the choroid plexus of TTR KO mice when compared to WTs, levels in the brain parenchyma were similar between strains (Palha et al., 2000). Once again, the authors concluded that, despite no other T_4 -binding protein was found to replace TTR in the CSF of TTR KO animals, interference with the blood-choroid plexus-CSF-TTR-mediated route of T_4 entry into the brain does not produce measurable features of hypothyroidism. In 2002, Palha and collaborators definitely proved that TTR is not necessary for thyroid hormone access to or distribution within the mouse brain by comparing THs distribution in TTR KO and WT mice using film autoradiography, a technique that yields definitive morphological results (Palha et al., 2002). Even under conditions of increased thyroid hormone demand, such as exposure to cold or thyroidectomy, TTR absence was found innocuous to the normal TH metabolism (Sousa et al., 2005).

2.2.2 Retinol metabolism in TTR KO mice

As referred in Section 1, TTR functions in the transport of retinol in the blood through the formation of a complex with retinol-binding protein (RBP). Plasma levels of both retinol and RBP in TTR KO mice were determined soon after their generation (Episkopou et al., 1993). Consistent with the hypothesis that TTR prevents loss of RBP-retinol by renal filtration, plasma levels of RBP and retinol were drastically reduced in TTR KO mice when compared to WTs, with TTR KOs presenting approximately 5% of the normal values (Episkopou et al., 1993; Wei et al., 1995). However, no symptoms of retinol deficiency were observed in TTR KOs and it was suggested that these animals were able to mobilize liver stored retinol despite a defective plasma retinol transport system (Episkopou et al., 1993). While total retinol (retinol plus retinyl ester) in the liver, testis, kidney, spleen, and eye cups was found similar in both strains, RBP levels in TTR KO

liver, but not in the kidney, were 60% higher than those of WT mice, suggesting that RBP secretion might be impaired in the absence of TTR (Wei et al., 1995). Nevertheless, this hypothesis was ruled out a few years later and it was emphasized that the low circulating levels of retinol and RBP in TTR KO mice were due to increased renal filtration of the RBP-retinol complex (van Bennekum et al., 2001). At the same time, another report demonstrated that retinal structure and function are not affected by the low plasma retinol and RBP levels observed in the absence of TTR (Bui et al., 2001). In sum, the mechanism by which TTR KO mice maintain normal tissue total retinol concentrations regardless of the low circulating retinol-RBP levels remains to be elucidated.

2.2.3 Neural stem cell niche in the brain of TTR KO mice

A very recent study suggests that the subventricular zone (SVZ) neural stem cell niche is greatly affected by the absence of TTR in TTR KO mice (Richardson et al., 2007). The SVZ of the adult mammalian brain contains neural stem cells (NSCs) that give rise to neural progenitor cells (NPCs), which in turn differentiate into neurons and glia. It constitutes one of the few brain regions where neurogenesis occurs in the adult. The normal fate of the rapidly dividing NPCs is apoptosis (Morshead and van der Kooy, 1992). In TTR KO mice, a 50% reduction was found in the number of cells in the SVZ undergoing apoptosis when compared to WT mice (Richardson et al., 2007). This level of apoptosis is equivalent to that observed in hypothyroid WT mice (Richardson et al., 2007), and the authors suggested that a central nervous system-specific hypothyroidism occurs in TTR KO mice. THs regulate the cell cycle in the neural stem cell niche of the adult rodent brain by influencing both proliferation and apoptosis (Lemkine et al., 2005). Richardson *et al* proposed that in TTR KO animals NPCs have a reduced T_4 supply due to the absence of TTR and, as a consequence, these cells are not induced into the apoptotic pathway (Richardson et al., 2007). Within the CSF of TTR KO mice, albumin is the only TH distributor protein (THDP) and is present in low amounts (due to the leakiness of the blood-brain barrier). The authors suggested that albumin is not able to distribute T_4 to cells of the SVZ, via the interstitial fluid, as the TH gradient from the CSF to subsequent brain layers is attenuated in the absence of TTR (Richardson et al., 2007). NPCs are more susceptible to the reduced TH levels than NSCs as they are furthest away from the ventricle. The fate of these NPCs remains to be investigated.

2.2.4 Sensorimotor performance of TTR KO mice

The sensorimotor performance of TTR KO mice was evaluated by SHIRPA

(Fleming et al., 2007), a procedure for behavioral and functional analysis of mice (Rogers et al., 1997). TTR KO mice behaved poorly than the WT littermates in several SHIRPA parameters, suggesting a sensorimotor impairment. In this respect, TTR KO mice locomotor activity was found increased when compared to the WT littermates at 3- and 6-month of age, in accordance with previous observations (Sousa et al., 2004), but not at 12-months of age when the tendency was reversed, probably as a consequence of the motor discoordination of older TTR KO mice (Fleming et al., 2007). The sensory defect of TTR KO mice was further confirmed by the hot plate test, in which TTR KO animals took more time to react to the noxious thermal stimulus than the WTs (Fleming et al., 2007). No morphometric or electrophysiological abnormalities were detected in TTR KO sciatic nerves that could justify their poorer sensorimotor performance (Fleming et al., 2007). Furthermore, morphological analysis of the cerebellum, a center coordinating motor function, revealed no differences between both strains (Fleming et al., 2007). The consequences arising from these observations were then examined following sciatic nerve crush, a subject addressed in Section 3.

2.2.5 Lipid and glucose metabolism in TTR KO mice

The metabolism of lipids and glucose was recently addressed in TTR KO mice (Marques et al., 2007). Several evidences were found supporting the idea that absence of TTR does not interfere with the regulation of lipid and glucose homeostasis. First, TTR KO mice did not differ from WT in body weight and white adipose tissue morphology, nor in basal or fast-induced circulating levels of glucose, lipids, and leptin (Marques et al., 2007). Furthermore, glucose tolerance tests showed that TTR KO mice have normal capacity to remove and metabolize energy substrates. Also, expression of genes encoding lipid transporters and nuclear receptors were similar in TTR KO and WT mice.

3 TTR IN DISORDERS OF THE NERVOUS SYSTEM

The main neurodegenerative disorder associated with TTR is familial amyloid polyneuropathy (FAP). However, in the last few years, several lines of evidence have drawn attention to the importance of TTR in the pathophysiology of other neurological and psychiatric diseases. Altered levels of TTR have been found in a number of neuronal dysfunctions. Whether these alterations are the cause or the consequence of the disease in most cases is still unclear. Further studies are needed to clarify TTR role in the disorders of the nervous system.

3.1 **Familial amyloid polyneuropathy (FAP)**

Familial amyloid polyneuropathy (FAP) was described more than 50 years ago in a group of portuguese patients who had a fatal hereditary amyloidosis characterized by a sensorimotor peripheral polyneuropathy and autonomic dysfunction (Andrade, 1952). Later, TTR was identified as the major protein present in amyloid deposits of FAP patients (Costa et al., 1978) and the most common molecular abnormality causative of the disease, a substitution of methionine for valine at position 30 of TTR (TTR Val30Met) was also unraveled (Saraiva et al., 1984). This condition is inherited in an autosomal dominant pattern (Andrade, 1952; Andrade et al., 1969; Ando et al., 1993). FAP is related to the systemic extracellular deposition of mutated TTR aggregates and amyloid fibrils throughout the connective tissue, with the exception of the brain and liver parenchyma, and affecting particularly the peripheral nervous system (PNS) (Coimbra and Andrade, 1971a, b). As a consequence of TTR deposition, axonal degeneration arises, beginning in unmyelinated and low diameter myelinated fibers, and ending up in neuronal loss at ganglionic sites (Dyck and Lambert, 1969; Thomas and King, 1974; Said et al., 1984; Sobue et al., 1990). TTR amyloid deposits are diffusely distributed in the PNS, involving nerve trunks, plexuses, sensory and autonomic ganglia (Said et al., 1984; Hanyu et al., 1989; Sobue et al., 1990). While in the nerve, TTR is mainly deposited in the endoneurium, in ganglia amyloid deposition occurs in close contact with satellite cells, which also accounts for progressive loss of neurons (Hofer and Anderson, 1975; Ikeda et al., 1987; Hanyu et al., 1989; Sobue et al., 1990).

Clinical symptoms generally appear before age 40 and consist of progressive and severe sensory, motor and autonomic polyneuropathy, fatal in about 10 to 20 years. The

initial symptom is usually a sensory impairment in the lower limbs, with pain and temperature sensations being the most severely affected (Dyck and Lambert, 1969). The majority of FAP patients have early and severe autonomic nervous system involvement manifested by impotence, urinary bladder dysfunction, motility disturbances of the gastrointestinal tract and postural hypotension (Canijo and Andrade, 1969; Alves et al., 1997). Motor impairments also occur with disease progression, causing wasting and weakness. Outside the PNS, malabsorption, cardiac insufficiency and vitreous opacities frequently take place. Besides the V30M mutation, a large number of other amyloidogenic TTR variants have been identified, most of them also associated with PNS involvement (Saraiva, 2001).

The precise mechanisms underlying TTR amyloid fibril formation are unknown. However, several studies suggest that amyloidogenic mutations destabilize the native TTR structure, thereby inducing conformational changes leading to the dissociation of tetramers into non-native monomers (Bonifacio et al., 1996; Quintas et al., 1999). These partially unfolded species can subsequently self-assemble forming high-molecular-mass aggregates, protofilaments assembled in short fibrils (protofibrils) and ultimately amyloid fibrils (Sebastiao et al., 1998; Quintas et al., 2001; Cardoso et al., 2002) (**Figure 5**).

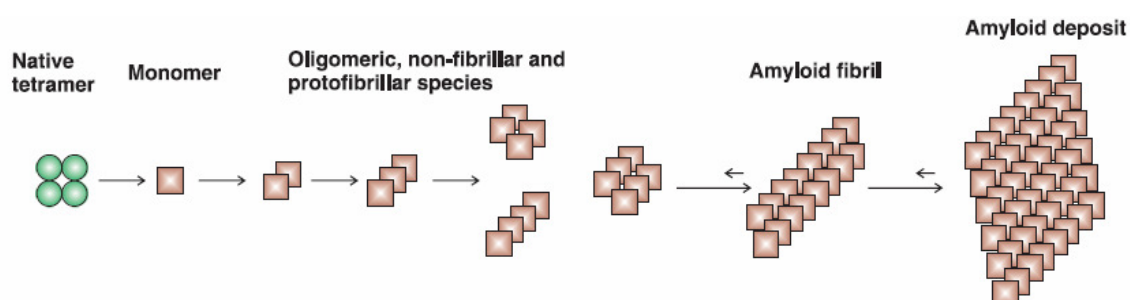


Figure 5. Proposed model for TTR amyloidogenesis (adapted from Hou et al., 2007).

Presence of non-fibrillar TTR aggregates was demonstrated in nerves from asymptomatic FAP patients, being also present in later stages of the FAP disease in association with mature fibrils (Sousa et al., 2001). At this time point, the hypothesis of toxicity of the non-fibrillar TTR aggregates was further investigated and, surprisingly, it was proved that mature TTR fibrils were essentially unable to cause cellular damage whereas TTR aggregates were toxic to cells therefore being potentially able to induce neurodegenerative effects (Sousa et al., 2001; Andersson et al., 2002).

Recently, a large number of studies provided important advances regarding the molecular mechanisms of TTR-mediated cellular toxicity. From the interaction of TTR low-molecular-mass aggregates with membranes, to the resulting intracellular events that will

ultimately lead to neuronal apoptosis, progresses have been made in order to better understand the pathophysiology of the disease (reviewed in (Hou et al., 2007). These developments are very welcome to the clinical field, leading to the expansion of strategies for FAP therapy, which so far rely on liver transplantation.

3.2 Nerve injury

TTR involvement in the process of regeneration following peripheral nerve injury was recently addressed in our lab (Fleming et al., 2007). As newly published, TTR KO mice presented a decreased regenerative capacity after sciatic nerve crush when compared to WT littermates (Fleming et al., 2007). At the functional level, TTR KO mice displayed a slower recovery of locomotor activity and a slower nerve conduction velocity, when compared to WT. The functional impairment throughout regeneration correlated with nerve morphometry, as 15 days after injury the number of myelinated axons was 20% decreased, and after 30 days of injury the density of unmyelinated axons was 40% decreased in TTR KOs relatively to WT. The decreased number of myelinated fibers 15 days after crush reached however WT levels 30 days post-injury and was proposed to be unrelated to a myelination impairment, as the g ratio determined in both WT and TTR KO mice was similar. Moreover, in transgenic mice expressing TTR in neurons, in a TTR KO background, this phenotype was rescued, reinforcing that TTR is the responsible factor for the enhancement of nerve regeneration. *In vitro*, neurite outgrowth was decreased in the absence of TTR, probably explaining the decreased regenerative capacity of TTR KO mice. These findings clearly demonstrate that TTR plays a role in nerve regeneration.

3.3 Alzheimer's disease

Many years have passed since the first observation that TTR levels in the CSF were decreased in Alzheimer's disease (AD) patients (Elovaara et al., 1986). Soon after, additional reports showed that TTR in the CSF was negatively correlated with the degree of dementia of Alzheimer type (Riisøen, 1988) and with senile plaque abundance (Merched et al., 1998). Subsequently, TTR was found to sequester amyloid β protein (A β), thereby preventing amyloid formation *in vitro* (Schwarzman et al., 1994). Schwarzman *et al* (Schwarzman et al., 1994) hypothesized that specific TTR variants could influence the development of this A β fibrils (Schwarzman et al., 1994). However, no TTR mutations have been found associated with AD (Palha et al., 1996). It was then

proposed that patients with late onset Alzheimer's disease might have increased risk of amyloid β fibril formation due to the lack of sufficient concentrations of TTR in the CSF to sequester Abeta (Serot et al., 1997). It is well known that AD may be caused by the abnormal processing of the amyloid precursor protein (APP) and the accumulation of Abeta (reviewed in (Hardy and Selkoe, 2002). However, APP can be proteolytically cleaved into multiple fragments, many of which have distinct biological actions. Although a high level of Abeta can be toxic, the alpha-secretase cleaved APP (sAPPalpha) is neuroprotective against a variety of insults, including Abeta toxicity (Stein and Johnson, 2003; Stein et al., 2004). In AD, Abeta levels increase while sAPPalpha levels decrease (Stein and Johnson, 2003). The mechanism of sAPPalpha protection is unknown, but some studies suggest that it induces TTR expression, thus protecting against the onset of AD neuropathology (Stein and Johnson, 2003; Stein et al., 2004). Furthermore, TTR neutralization both *in vitro* and *in vivo*, demonstrated that the sAPPalpha-stimulated expression of transthyretin is necessary for protection against Abeta-induced neuronal death (Stein et al., 2004). More recently, reduced levels of TTR in the CSF of AD patients were confirmed by the use of more powerful tools, such as comparative proteomics (Castano et al., 2006). Moreover, transgenic mouse models for AD revealed markedly increased TTR levels well before the onset of Abeta deposition (Wu et al., 2006), suggesting that TTR expression is induced in response to Abeta overproduction in an attempt to overcome its deleterious effects. Consistently, by crossing a transgenic mouse model for AD with TTR KO mice, Choi and co-workers reported that Abeta levels were increased and its deposition was accelerated in the brains of transgenic mice with the hemizygous deletion of TTR (Choi et al., 2007). The authors had already reported that transgenic mice exposed to an enriched environment exhibited reduced Abeta levels and deposition, in parallel with TTR upregulation (Lazarov et al., 2005). Besides these observations, the mechanism by which TTR protects against Abeta toxicity is poorly understood and requires further investigation. However, as recently highlighted, TTR involvement in AD neuropathology should take into account its unique site of synthesis in the brain, the choroid plexus, and its secretion to the CSF (Sousa et al., 2007a).

3.4 Memory impairment

Recently published data revealed that decreased levels of TTR may be critical to the development of memory impairments during aging (Brouillette and Quirion, 2007; Sousa et al., 2007b). A lower TTR expression was observed in aged memory-impaired (AI) rats when compared to aged memory-unimpaired (AU) animals following stimulation in a

spatial memory task (Brouillette and Quirion, 2007). Memory deficits were also found during aging in TTR KO mice (Brouillette and Quirion, 2007). The authors proposed that the mechanism underlying TTR role in the maintenance of normal cognitive abilities was related to its capacity to transport retinol since cognitive deficits in TTR KO mice and aged rats were reversed by treatment with retinoic acid, the active form of retinol (Brouillette and Quirion, 2007). It is known that retinoic acid can modulate a wide variety of biological processes, including synaptic plasticity and long-term potentiation, through binding to its specific nuclear receptors (Chiang et al., 1998; Etchamendy et al., 2001). In turn, Sousa and coworkers reported that young adult (5-month old) TTR KO mice display a spatial reference memory impairment when compared to age-matched WT animals (Sousa et al., 2007b). During aging, WT mice worsens its performance in spatial reference tasks, a fact that may be related to the 30% decline found in CSF TTR in old (18-month) compared to young adult mice (Sousa et al., 2007b). Interestingly, TTR KO mice show no longer memory impairment with increasing age, with no differences being found between both strains at 18-month old (Sousa et al., 2007b). These findings suggest that absence of TTR accelerates the poorer cognitive performance commonly associated with normal aging and accelerated in Alzheimer's disease. The authors consider the possibility that abnormalities in thyroxine and retinol brain distribution may occur during embryonic development, as a consequence of TTR absence, that may influence TTR KO mice cognitive performance in adult life.

3.5 Depression

A link between TTR and depression was first proposed by Jorgensen and coworkers, in 1988, with the observation that TTR levels in the CSF were 7.2% increased in depressed patients when compared to those found in other psychiatric patients (Jorgensen, 1988). Changes were partially normalized during recovery from depression and the authors hypothesized that a relationship exists between depression and increased choroid plexus activity and CSF production (Jorgensen, 1988). Since then, other studies (all from the same lab) addressed this subject although with opposing results (Hatterer et al., 1993; Sullivan et al., 1999; Sullivan et al., 2006). TTR was found decreased in the CSF of depressed patients when compared with control participants with neurological disorders (Hatterer et al., 1993) or healthy volunteers (Sullivan et al., 1999; Sullivan et al., 2006). In the latest study, the authors sought to replicate and extend their previous findings of lower CSF TTR in depression by using an improved TTR radioimmunoassay (Sullivan et al., 2006). It is not clear whether thyroid hormones (THs) play any role

regarding these observations. What is known is that reduced levels of THs can result in depression (Haggerty and Prange, 1995). Given this, it has been postulated that reduced levels of TTR in the CSF result in reduced levels of THs being distributed throughout the brain, resulting in TH-related depression. However, as already referred, studies performed with TTR KO mice suggest that TTR is not necessary for adequate delivery of THs to brain parenchyma (Palha et al., 1997; Palha et al., 2000; Palha et al., 2002). Furthermore, the major route of THs entry into the brain is likely to be the blood-brain barrier, not the choroid plexus-CSF barrier (as reviewed by (Palha, 2002)). While considering that differences may occur between mice and human, thyroid abnormalities are only rarely found among depressed patients (Fava et al., 1995), arguing against a central role for THs in the pathogenesis of depression. It is also noteworthy to refer that most patients included in these studies were under some kind of medication, which can alter TTR levels in the CSF in both ways. Thus, it was very important to address TTR role in depression by comparing TTR KO with WT mice. This study came out recently, and it was found that TTR KOs displayed reduced depressed-like behavior based on their increased activity in the forced swim test (Sousa et al., 2004). This test has been extensively used as a screening model for depression (Porsolt et al., 1977). Increased noradrenaline levels were found in the limbic forebrain of TTR KO mice and were suggested to cause TTR involvement in depression, as serotonin and dopamine concentrations were normal (Sousa et al., 2004). The increased levels of noradrenaline seem to be independent on metabolic activity, as both synthesis and degradation of the catecholamine were not affected in TTR KO mice (Sousa et al., 2004). The exact mechanism for TTR involvement in depression remains to be elucidated.

3.6 Psychosis

Psychosis is a severe mental condition that is characterized by a loss of contact with reality and is typically associated with hallucinations and delusional beliefs. There are numerous psychiatric conditions that present with psychotic symptoms, most importantly schizophrenia, bipolar affective disorder, and some forms of severe depression referred to as psychotic depression. The pathological mechanisms resulting in psychotic symptoms are not understood, nor is it understood whether the various psychotic illnesses are the result of similar biochemical disturbances.

3.6.1 Schizophrenia

Schizophrenia is the most devastating and enduring psychotic disorder affecting as many as 1% of the population worldwide, with a similar prevalence between the sexes and throughout diverse cultures and geographic areas (Susser and Wanderling, 1994; Thaker and Carpenter, 2001). It affects virtually all brain functions and, despite major research effort, its etiology is still unknown. The disorder is almost certainly the result of a complex interaction between numerous predisposing genes and environmental factors. Many studies have focused on the identification of schizophrenia genetic mutations and polymorphisms, and although such “risk genes” may play a role in a small number of schizophrenia cases, the great majority of patients do not exhibit any apparent genetic alteration (Sullivan, 2005). Several studies were performed in an attempt to identify disease biomarkers that could help to a better understanding of the pathogenesis of schizophrenia. TTR was consistently found downregulated in the CSF of first-onset, drug naïve schizophrenia patients when compared to healthy volunteers (Huang et al., 2006). To evaluate the contribution of blood TTR to the changes found in the CSF of schizophrenia patients (as it has been estimated that 3% and 10% of TTR in the ventricular and lumbar CSF, respectively, are derived from blood (Reiber, 2001)), serum TTR from the same individuals was also analyzed. A 15% reduction in plasma TTR levels was observed in schizophrenia patients compared to controls (Huang et al., 2006). However, no correlation was found between CSF and serum TTR from the same individuals, suggesting that TTR levels are independently regulated in the CSF and blood (Huang et al., 2006). In addition, an approximately 40% reduction in TTR protein was verified in post-mortem prefrontal cortex from patients with schizophrenia when compared to controls (Huang et al., 2006). Once again, the hypothesis was raised that decreased levels of TTR in the CSF might imply abnormalities in thyroid hormone transport from the bloodstream to the brains of schizophrenia patients (Huang et al., 2006) that could explain the diseased state. Decreased TTR levels in the CSF (Wan et al., 2006) and plasma (Yang et al., 2006) samples of schizophrenia patients was also reported by independent studies. Furthermore, Wan *et al* demonstrated plasma TTR upregulation in schizophrenia patients after antipsychotic valid medical treatment in relation to pretreatment levels (Wan et al., 2006). In nonrespondent patients, meaning the ones in which a reduction in psychiatric symptoms was not observed following medical treatment, TTR levels remained unchanged (Wan et al., 2006). This finding suggests that TTR is indeed correlated with the disease state. Given that ApoE was also found decreased in the CSF of schizophrenia patients, and since ApoE is a retinoid transporter as well, the authors hypothesized that a reduction of retinoid level may occur in the brain of patients with schizophrenia (Wan et

al., 2006). This conjecture is somehow bizarre since the authors state that no correlation was found between TTR and retinoid plasma levels before and after antipsychotic treatment (Wan et al., 2006). Moreover, a recent study reports that serum retinol-binding protein (RBP) levels did not differ between schizophrenia patients and healthy individuals (Ruano et al., 2007), suggesting that serum retinoid status is not altered in patients with schizophrenia, since retinol is the most common form of retinoids in circulation and RBP the only plasma carrier of retinol and a marker of retinol plasma levels (de Pee and Dary, 2002). Contrary to the above-mentioned studies, Ruano *et al* reported no difference in TTR serum levels between schizophrenia patients and healthy individuals (Ruano et al., 2007). The reason for these discrepancies is not understood. Clozapine is an effective antipsychotic drug in the treatment of schizophrenia. Long-term administration of clozapine was found associated with TTR upregulation in rat hippocampus, revealed by 2D-electrophoresis and confirmed by Western blot (Chen and Chen, 2007). Additionally, the authors reported that chronic treatment of rats with clozapine or olanzapine, but not haloperidol, induced an increase in TTR expression in the cerebral cortex, as addressed by RT-PCR (Chen and Chen, 2007). As well known from early TTR characterization and recently highlighted (Sousa et al., 2007a), the only site of TTR synthesis in the brain is the choroid plexus. The data obtained by Chen *et al* in the cerebral cortex (Chen and Chen, 2007) reflects a sample contamination with choroid plexus and, if confirmed, an increased TTR expression by the epithelial cells of the choroid plexus under those long-term treatments. These findings may support a clinical relevance of TTR in the pathophysiology of schizophrenia.

3.6.2 Bipolar disorder

Bipolar disorder, or manic depressive illness, is characterized by recurrent episodes of depression and mania. Evidence that TTR might be implicated in the pathophysiology of bipolar disorder came from a genetic study implicating *ttr* gene in a Danish pedigree of bipolar patients (Ewald et al., 1997). Later, TTR synthesis in the choroid plexus was found decreased following chronic administration of lithium chloride to rats (Pulford et al., 2006). Lithium has been used as a mood stabilizer agent in the treatment of bipolar disorder, but its therapeutic mechanism is still not understood. This report may have shed some light into this subject together with the knowledge that lithium modulates AP-1 binding to DNA (as reviewed by Ikononov and Manji, 1999) and that TTR promoter contains a functional AP-1 binding site (Qian et al., 1995). Whether TTR downregulation is lithium-specific or is also observed with other mood stabilizing agents is not known.

3.7 Other neurodegenerative disorders

3.7.1 Frontotemporal dementia

Frontotemporal dementia (FTD) is a neurodegenerative disorder that involves atrophy of the frontal and anterior temporal cortex regions, the anterior portion of the cingulate gyrus and with a relative sparing of postcentral cortex. The typical clinical picture is a slowly progressive dementia, often confounded with other neurodegenerative disorders, like Alzheimer's disease. TTR was found increased in the CSF of FTD patients compared to controls, using 2D-electrophoresis (Hansson et al., 2004). These results were further confirmed by the use of surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry (MS) (Ruetschi et al., 2005).

3.7.2 Parkinson's disease

The most distinctive feature of Parkinson's disease (PD) is the loss of dopaminergic neurons in the substantia nigra, with the subsequent decline of striatal dopamine levels, resulting in cardinal motor symptoms such as tremor at rest, bradikinesia, muscular rigidity, stooped posture, and instability (reviewed in (Moore et al., 2005). The estimated prevalence of PD is 100 to 200 per 100,000 people older than 40 years of age (Tanner and Goldman, 1996). A significant increase in TTR concentrations was reported in the CSF of a rat model of PD, as assessed by 2D-electrophoresis (Rite et al., 2007). Changes were further confirmed in the CSF and plasma by Western blotting (Rite et al., 2007).

3.7.3 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common adult motor neuron disease, affecting one in every 40,000 individuals (reviewed in (Jackson and Bryan, 1998). It typically affects individuals in their mid-50s and is characterized by rapidly progressive degeneration of motor neurons in the cerebral cortex, brainstem and spinal cord. The median survival in ALS is three to five years. TTR was found decreased in the CSF of ALS patients compared to controls, using surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry (MS) (Ranganathan et al., 2005).

4 NEUROPEPTIDES

What makes a peptide a neuropeptide? De Wied and his group (de Wied, 1984) and Kastin and his colleagues (Kastin et al., 1979) gave important contributions to the formulation of the neuropeptide concept: peptides that have a direct effect on neurons, via specific peptidergic receptors, and that have highly specific physiological properties. Today, the definition of a neuropeptide is even more encompassing. Besides neurons, neuropeptides affect non-neuronal tissues and organs, and it has become increasingly clear that one of the most important functions of neuropeptides is the integration of the functions of the brain and the systems of the body.

The long list of functions in which neuropeptides are involved include regulation of reproduction, growth, temperature control, food and water intake, cardiovascular, gastrointestinal, and respiratory control, behavior, memory, affective states, nerve development and regeneration. Multiple neuropeptides may regulate a specific physiological or behavioral function. Consequently, a complex coordination of the many neuropeptides has to occur *in vivo*. Neuropeptides are often co-localized with the classical neurotransmitters, such as acetylcholine, the monoamines and γ -aminobutyric acid (GABA). Depending on the tissue substrate, the stage of development, the metabolic state, the presence or absence of other hormones, growth factors, or even neurotransmitters, neuropeptide action may be highly specific, merely permissive, or perhaps even redundant. Neuropeptides may play a fail-safe role so that in the absence of other regulatory factors, neuropeptides provide the necessary critical control.

Neuropeptides (ranging in size from 3 to 40 aminoacid residues) are formed from large precursor pre-pro-neuropeptides and pro-neuropeptides, which are progressively split by specific proteolytic enzymes. The aminoacid sequence will influence the conformation of the active peptide chain, regulating its flexibility or rigidity. The neuropeptide system of communication requires its synthesis, processing, transport, storage, secretion, binding to receptor, and finally its metabolism. In most cases, synthesis of the neuropeptide is regulated by a feedback system. Neuropeptides can be classified into families on the basis of the similarity of their aminoacid sequences.

4.1 **Biosynthesis, processing and secretion of neuropeptides**

The initial precursor polypeptide chain containing the aminoacid sequence of the

neuropeptide plus a signal sequence at its N-terminus is known as the pre-pro-neuropeptide. The signal sequence guides pre-pro-neuropeptides into the lumen of the rough endoplasmic reticulum where it is rapidly cleaved by a signal endopeptidase. The peptide chain with the signal peptide removed constitutes the pro-neuropeptide. Almost all pro-neuropeptides are biologically inactive and attain potency only after they are processed. Folding occurs in the cisterna of endoplasmic reticulum. Pro-neuropeptides are then shuttled to the *cis*-face of the Golgi apparatus and targeted to the *trans*-Golgi network. Packaging of the precursors into secretory granules occurs in the *trans*-Golgi network, where they are actively sorted away from other proteins into the granules of the regulated secretory pathway. Neuropeptide precursors exit the *trans*-Golgi network in large dense-core vesicles (LDCVs), named for the opaque core seen in electron micrographs. Proteolysis, except for the removal of the signal peptide, begins at the *trans*-Golgi network and continues in the granules. Some precursors also undergo other modifications such as glycosylation, phosphorylation, sulfation, acetylation and amidation. The processing enzymes necessary to achieve all this modifications are co-packaged into the granules and sequentially attack the neuropeptide precursors as the granules move from the cell body down to the nerve terminals. Endoproteolysis of the pro-neuropeptide generally occurs at dibasic aminoacid sites through prohormone convertases. Then, exoproteolysis takes place by amino- and carboxypeptidases that trim the ends of the resulting peptide fragments. The processing events in secretory granules require a few to several hours. Neuropeptides are then stored in the mature LDCVs and secreted by exocytosis in response to secretagogues. Exocytosis entails the formation of fusion pores between membrane-bound vesicles and the presynaptic membrane, with the subsequent release of the vesicular content into the extracellular space. This process is Ca^{2+} -dependent. Recent studies suggest that neurons might release their vesicle cargo in places different than the classical synaptic cleft (like dendrites or soma) (reviewed in (Salio et al., 2006) and that they can control the amount of cargo released (reviewed in (Michael et al., 2006).

4.2 Amidation of neuropeptides

Peptidylglycine α -amidating monooxygenase (PAM) is the only enzyme that C-terminally α -amidates peptides. Each secretory granule is thought to contain only a few molecules of this processing enzyme (Mains et al., 1987; Prigge et al., 2000), making it rate-limiting in the process of neuropeptide maturation, as its substrates exist in excess. PAM is involved in the posttranslational processing of more than 50% of all known

neuropeptides (Eipper et al., 1992), which require α -amidation for biological activity. PAM catalyzes the formation of α -amidated peptides from precursor molecules with a COOH-terminal glycine (**Figure 6**).

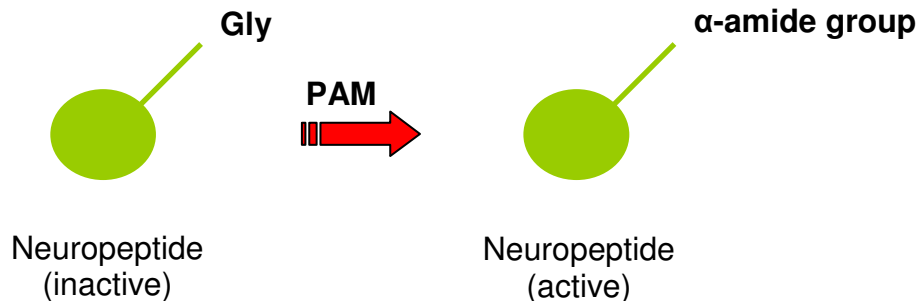


Figure 6. Schematic representation of PAM involvement in regulating neuropeptide amidation.

Generation of peptidylglycine substrates for PAM requires pro-neuropeptide cleavage by the appropriate prohormone convertases and carboxypeptidases. Conversion of a peptidylglycine substrate into an α -amidated product involves a two-step process resulting from the sequential actions of two enzyme activities (Tajima et al., 1990). Both enzymes are derived from the bifunctional PAM protein (Perkins et al., 1990). The first enzyme, peptidylglycine α -hydroxylating monooxygenase (PHM), produces an α -hydroxylated intermediate in the presence of copper, ascorbate, and molecular oxygen. The second enzyme, peptidyl- α -hydroxyglycine- α -amidating lyase (PAL), cleaves the peptidyl- α -hydroxyglycine intermediate to form the α -amidated peptide and glyoxylate.

PAM has a broad substrate specificity, is found in a variety of tissues, and is regulated in a tissue-specific fashion (Eipper et al., 1992). The highest levels of PAM are found in the pituitary, adrenal medulla, atrium of the heart, and central nervous system (Eipper et al., 1985), where the enzyme is detected in all major brain areas with the exception of the cerebellum (Bolkenius and Ganzhorn, 1998). In particular, the hypothalamus, hippocampus and olfactory cortex represent the major sources of the protein in the brain (Bolkenius and Ganzhorn, 1998). PAM activity was also detected in dorsal root ganglia and sciatic nerves (Takasugi et al., 1996). In both peripheral and central neurons PAM is localized to LDCVs in axons and nerve terminals (Oyarce and Eipper, 1993).

4.3 Neuropeptide Y

Neuropeptide Y (NPY), so named because it begins and ends in tyrosine (single-letter aminoacid code Y), is a member of the pancreatic polypeptide (PP) family, which also includes the peptide YY (PYY) and PP. Each of these peptides consists of 36 aminoacids and a C-terminal amide. While there is some overlap, in general NPY is found in abundance in the central and peripheral nervous system, PYY in the endocrine cells of the duodenum, and PP only in the pancreatic islets. NPY is one of the most evolutionary conserved peptides known. There is perfect sequence identity in mammalian NPY, except for cow, pig and sheep, the formers differing in one and the last in two aminoacids out of 36. Chicken NPY differs from human NPY by only one aminoacid.

4.3.1 NPY synthesis and processing

The NPY gene spans 7.2 kilobase pairs and is located on human chromosome 7, locus 7p15.1 (Cerdeira-Reverte and Larhammar, 2000). It is composed of four exons and three introns and results in the synthesis of a 97 aminoacid pre-pro-NPY, which undergoes at least four post-translational enzymatic events (Higuchi et al., 1988) (**Figure 7**). The prefix “pre-” indicates that there is an N-terminal sequence, the signal peptide, which is cleaved upon passage through the rough endoplasmic reticulum, yielding the 69-residue pro-NPY. Subsequently, pro-NPY undergoes cleavage by the prohormone convertases PC1/3 and/or PC2 at a single paired basic site (Lys³⁸-Arg³⁹), releasing a 30-aminoacid C-terminal peptide (C-flanking peptide of NPY, CPON) and NPY₁₋₃₉ (Minth et al., 1984). Kinetic studies demonstrated that PC1/3 was more efficient than PC2 in cleaving pro-NPY (Brakch et al., 1997). NPY₁₋₃₉ is further processed by a carboxypeptidase. The resulting NPY₁₋₃₇ is finally amidated at its C-terminal end by PAM, in a process that removes an additional aminoacid, glycine, and produces the active NPY₁₋₃₆. NPY amidation is therefore an essential step for its biological activity. Ultrastructural immunohistochemical studies have confirmed NPY localization within LDCVs in the cytoplasm of cell bodies as well as in terminals (Pickel et al., 1995; Pickel et al., 1998), namely in the rat hippocampal formation (Pickel et al., 1995), nucleus accumbens (Pickel et al., 1998) and hypothalamus (Iwai et al., 1989).

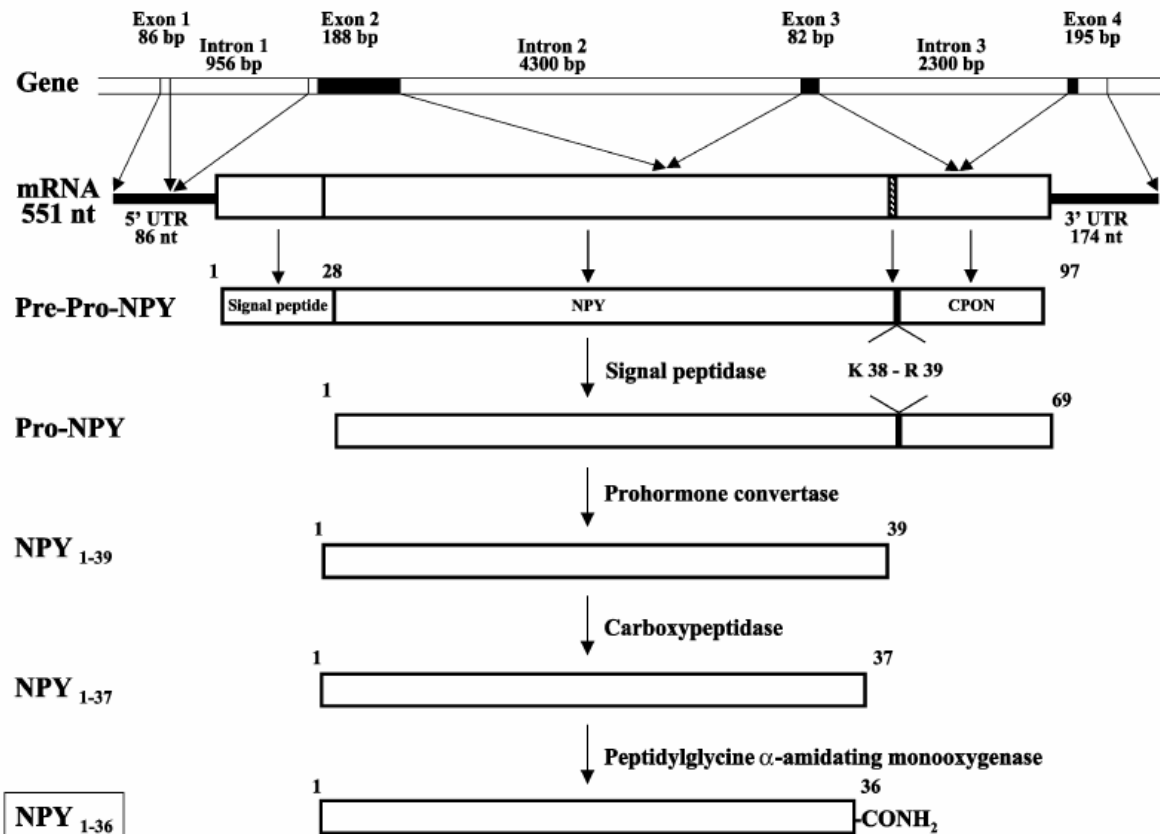


Figure 7. NPY synthesis and processing. bp: basepairs; nt: nucleotides; UTR: untranslated region. (Blake et al., 1974).

4.3.2 NPY localization

NPY is abundantly expressed in numerous brain regions including the hypothalamus, amygdala, hippocampus, nucleus of the solitary tract, locus coeruleus, nucleus accumbens and cerebral cortex (Chronwall et al., 1985). NPY is also abundantly expressed in the periphery. The adrenal medulla is the primary source of circulating NPY (Cavadas et al., 2002) though it is also expressed in pancreas, liver, heart, lung, spleen and in endothelial cells of blood vessels. NPY is abundant in the sympathetic nervous system where it is co-stored and co-released with norepinephrine. It is also expressed in a subpopulation of parasympathetic neurons. Brain NPY is found colocalized with norepinephrine, GABA and somatostatin in agouti-related protein containing neurons (reviewed in (Kask et al., 2002).

4.3.3 NPY functions

Since the discovery of NPY, in 1982, as an abundant peptide in brain tissue

(Tatemoto et al., 1982), a robust body of literature has developed around the potential functions of this peptide. NPY actions range from stress-related behaviors (such as anxiety and depression) to the regulation of energy homeostasis, cardiovascular and memory functions and to the control of seizure activity.

4.3.3.1 Energy homeostasis

NPY is the prototype of appetite-stimulating hormones, inducing particularly carbohydrate intake [216, 217]. The ability of NPY to induce food intake was reported very early after its first isolation. Intracerebroventricular injection of NPY elicits a strong feeding response [216, 219], even in satiated animals, eventually leading to obesity [220, 221]. Subsequently, a large body of evidence suggested that NPY was a naturally produced neurotransmitter that participates in the hypothalamic integration of energy homeostasis. For instance, NPY expression in the hypothalamus is largely increased in situations of poor metabolic conditions, such as fasting [222, 223]. High hypothalamic NPY levels are found also in obese Zucker rats [224, 225]. Intracerebroventricular NPY injection has two additional major effects on energy homeostasis: it decreases brown adipose tissue thermogenesis, thus reducing energy expenditure, and it increases white adipose tissue lipoprotein lipase, promoting storage of lipids in white adipose tissue and leading to a positive energy balance (Billington et al., 1994; Kotz et al., 1998).

4.3.3.2 Depression and anxiety

A growing body of evidence implicates NPY in depression: lower NPY-like immunoreactivity in the CSF of depressed patients (Widerlov et al., 1988), and in the brain of suicide victims (Widdowson et al., 1992) and depressed rats (Jimenez Vasquez et al., 2000) have been described. Furthermore, NPY central administration displays antidepressant-like activity in rodent swimming tests, reducing immobility (Stogner and Holmes, 2000; Redrobe et al., 2002). Experimental animals treated with clinically effective antidepressants were reported to have increased NPY levels in several brain areas (Heilig et al., 1988); electroconvulsive shock, another effective antidepressant treatment, also augments brain NPY levels both in depressed subjects (Stenfors et al., 1989) and rats (Jimenez-Vasquez et al., 2007). Lithium chloride, a mood-stabilizing agent also used in antidepressive treatments to augment its efficacy, is also known to increase hippocampal NPY synthesis (Zachrisson et al., 1995). In addition to its antidepressant activity, exogenous NPY reduces anxiety in several models. In the elevated plus maze test, a validated anxiety model, centrally injected NPY produces an anxiolytic-like effect (Heilig et

al., 1989). The anxiolytic properties of NPY appear to be mediated by Y1 receptors (Wahlestedt et al., 1993; Sorensen et al., 2004).

4.3.3.3 Memory

Several studies strongly suggest a physiological role for NPY in learning and memory. However the effects observed seem to depend on the area affected. For instance, NPY injection into the rostral hippocampus and septum enhanced memory retention, while administration into the amygdala or caudal hippocampus induced amnesia. No effects were observed after injection in the caudate/putamen, thalamus, or cerebral cortex (Flood and Morley, 1989). Moreover, the immunoneutralization of NPY into the same brain areas prevented the effects normally produced by NPY, suggesting that NPY has a physiological role in memory processing. The implication of endogenous NPY on memory was observed in transgenic rats with hippocampal NPY overexpression. These animals showed an impairment of spatial memory acquisition and retention in the Morris water maze (Thorsell et al., 2000). However, NPY KO mice performed normally in an inhibitory avoidance test, a model designed to measure memory retention (Bannon et al., 2000).

4.3.3.4 Epilepsy and glutamate excitotoxicity

Emerging evidence points toward an important role for NPY in the regulation of pathological conditions associated with excessive glutamate release, such as that occurring during seizures. Several *in vitro* and *in vivo* results agree with a powerful antiexcitatory, antiepileptogenic and neuroprotective actions of NPY. These effects seem to be mediated predominantly through presynaptic NPY Y2 receptors by depressing glutamate release through a mechanism involving the inhibition of the voltage-dependent Ca^{2+} influx in excitatory nerve terminals (Schwarzer et al., 1996; Weiser et al., 2000; Silva et al., 2001). At this respect, Silva and collaborators demonstrated in rat hippocampal synaptosomes the presynaptic modulation of intracellular Ca^{2+} concentration by activating Y1 and Y2 receptors (Silva et al., 2001). Y2 receptor activation displayed the highest inhibition of the total glutamate release in the whole hippocampus, sustaining a NPY inhibitory effect over evoked-glutamate release. Furthermore, NPY application to rat hippocampal slices potently and selectively reduced synaptic excitation mediated by glutamate release (Colmers et al., 1985; Haas et al., 1987). An anticonvulsive action of NPY in the epileptic human hippocampus was also shown to be mediated by NPY inhibition of glutamatergic excitation (Patrylo et al., 1999). Moreover, NPY overexpression

in the hippocampus has been reported in various seizure models (Gruber et al., 1994; Schwarzer et al., 1996; Takahashi et al., 2000; Silva et al., 2005) and its administration into the lateral ventricle was shown to lead to a powerful inhibition of kainic acid-induced seizures (Woldbye et al., 1997). NPY and NPY receptors were also shown to be neuroprotective against excitotoxic insult in organotypic hippocampal slice cultures (Silva et al., 2003; Xapelli et al., 2007). Additionally, NPY can regulate excitotoxicity in other brain areas such as amygdala and cortex (Cheung and Cechetto, 1995).

4.3.3.5 Neurogenesis

Recently, NPY has been shown to promote neurogenesis. Adult neurogenesis occurs in the human and rodent brain mainly in the dentate gyrus of the hippocampus and in the subventricular zone (SVZ) (Altman and Das, 1965; Doetsch et al., 1999; Gage, 2000). NPY was shown to promote adult neurogenesis in the dentate gyrus, as NPY Y1 receptor KO mice presented reduced proliferation of neuronal precursor cells and significantly fewer immature neurons when compared to controls (Howell et al., 2005). These results suggested that NPY modulates dentate neurogenesis through Y1 receptor. A previous report had already demonstrated that NPY induces the proliferation of hippocampal neuronal precursor cells *in vitro* via NPY Y1 receptor (Howell et al., 2003). Furthermore, NPY mediates neurogenesis in the olfactory epithelium, another, however less known, region of adult neurogenesis (Calof et al., 1998). NPY KO mice displayed significantly fewer dividing olfactory neuronal precursor cells when compared to the WT littermates, suggesting that NPY functions as a neuroproliferative factor (Hansel et al., 2001). Moreover, in SVZ cell cultures, NPY treatment increased neuronal proliferation and differentiation (Agasse F et al. 2007 2nd International Meeting of the Portuguese Society for Stem Cells and Cellular Therapy), demonstrating that NPY is also neurogenic in this brain region.

Evidence suggests that NPY may play a crucial role in injury-induced neurogenesis. In fact, following seizure activity, NPY was shown to be overexpressed in the hippocampus (Silva et al., 2005), and in parallel, neurogenesis was increased in the dentate gyrus (Cha et al., 2004; Jessberger et al., 2005; Ernst and Christie, 2006). Likewise, in depression disorders, in which neurogenesis is decreased in the dentate gyrus, antidepressant treatments like lithium and electroconvulsive therapy were reported to increase neurogenesis in this hippocampal subregion (Dranovsky and Hen, 2006). Decreased NPY mRNA levels were observed in rats from the strain Flinders sensitive line, characterized by depressive behavior, and electroconvulsive treatments increased NPY levels in the dentate gyrus of these rats (Jimenez-Vasquez et al., 2007). One might

speculate that there might be an association between increased neurogenesis and up-regulation of endogenous NPY upon antidepressive treatments.

4.3.3.6 Alzheimer's disease

Alterations in NPY levels have been reported in patients with Alzheimer's disease (AD), which lead to the speculation on the possible involvement between NPY and the development of the disease. NPY content in plasma (Koide et al., 1995) and CSF of AD patients was found significantly decreased when compared with that in healthy controls, a decrease that becomes more accentuated with increased severity and duration of the disease (Alom et al., 1990; Martignoni et al., 1992; Edvinsson et al., 1993). Furthermore, several studies have shown that NPY-immunoreactivity was decreased in temporal lobe, frontal lobe, occipital and frontal cortex of AD patients (Beal et al., 1986; Davies et al., 1990). Additionally, binding sites for NPY in the temporal cortex and hippocampus were reported to be decreased in AD (Martel et al., 1990). A significant negative correlation between NPY-immunoreactivity and duration of the disease was also observed by another study (Minthon et al., 1996). Whether these alterations are causal or just reflect collateral changes related to the development of the disease is still not understood. In transgenic mice models of AD, dystrophic and varicose fibers containing NPY were commonly seen in close proximity to amyloid β protein (A β) plaques (Diez et al., 2000; Diez et al., 2003). In addition, an increase in NPY-immunoreactivity was reported in several brain areas of these transgenic mice (Diez et al., 2000; Diez et al., 2003). This evidence suggested that the increase in NPY may reflect an attempt to counteract the degeneration by initiating protective mechanisms (Diez et al., 2000; Diez et al., 2003).

4.3.3.7 Parkinson's disease

Contradictory data have been reported regarding NPY in Parkinson's disease (PD). While some studies have shown no changes in NPY levels in PD hippocampus, cortex (Allen et al., 1985), and CSF (Yaksh et al., 1990), Martignoni *et al.* (Martignoni et al., 1992) reported a significant reduction in CSF NPY levels. Additionally, in PD striatum the number of NPY mRNA expressing interneurons was found markedly increased in the nucleus accumbens and caudate. Moreover, in the putamen, NPY was also increased but to a smaller extent when compared to control tissue (Cannizzaro et al., 2003). The explanations for the increased NPY levels in PD striatum were alterations of dopaminergic pathways and receptors caused either by the disease or the treatment (Cannizzaro et al., 2003).

4.3.4 NPY receptors

NPY receptors belong to the superfamily of G-protein coupled receptors, which represent more than 80% of neuropeptide receptors. G proteins are membrane-bound cytoplasmic -facing proteins that bind GTP and act as intermediaries between the receptor and several second-messenger systems. Six NPY receptors have been identified in mammals (Y1, Y2, Y3, Y4, Y5, and y6). The y6 receptor has been given the lower case designation (IUPAR nomenclature) since the receptor protein is truncated in most mammals including man and rat (Starback et al., 2000). Interestingly, y6 is functional in mice (and rabbits) and needs to be considered when interpreting results from receptor knockouts. Furthermore, while Y4 binds preferentially to PP, Y3 is the only receptor not yet cloned, but it has been characterized pharmacologically as the "NPY-preferring" receptor; i.e., not activated by PYY or PP (Berglund et al., 2003). Y1, Y2 and Y5 receptors are the best characterized ones and to which the majority of NPY functions are associated.

The Y1 receptor was the first NPY cloned receptor in the family. The highest levels of Y1 mRNA in the brain of several mammalian species are consistently seen in forebrain regions, including the cerebral cortex, the hippocampal formation, and several amygdaloid, thalamic, and hypothalamic nuclei (Parker and Herzog, 1999; Kishi et al., 2005). NPY Y1 receptors have been implicated namely in the regulation of food intake (Daniels et al., 2001) and anxiety (Sorensen et al., 2004). The lack of co-localization of Y1 mRNA with NPY mRNA observed in the human brain suggests that the Y1 receptor has a postsynaptic localization (terminal, dendritic, and/or somatic location) in the mammalian brain (Caberlotto et al., 2000). However, immunohistochemical studies identified the presence of Y1 receptors in NPY-immunoreactive terminals of the rat nucleus accumbens and rat hippocampal neurons in culture, which suggests that the Y1 receptor may also be a presynaptic receptor and may act as an autoreceptor to regulate NPY release (Pickel et al., 1998; St-Pierre et al., 2000).

In general, Y2 receptor brain mRNA levels are lower when compared to Y1 receptor (Parker and Herzog, 1999). Y2 receptor mRNA is discretely localized in the rodent brain, including the hippocampus, hypothalamus, thalamus, amygdala and brainstem (Naveilhan et al., 1998; Parker and Herzog, 1999). The presence of Y2 receptor in the central nervous system has attracted particular interest because of its possible implication in the control of food intake (Sainsbury et al., 2002), bone formation (Herzog, 2002), anxiety (Redrobe et al., 2003), neuronal excitability and epilepsy (reviewed in (Vezzani and Sperk, 2004), and learning and memory (Redrobe et al., 2004). The co-localization of Y2 receptor mRNA with NPY mRNA in some neuronal cells supports

pharmacological studies that indicate a presynaptic localization of the receptor, where it acts as an autoreceptor, inhibiting the release of NPY and other neurotransmitters (Martire et al., 1995; Caberlotto et al., 2000; King et al., 2000; Silva et al., 2001).

In the rat brain, the Y5 receptor mRNA is probably less abundant than either the Y1 or the Y2 receptors mRNA (Nichol et al., 1999; Parker and Herzog, 1999). In post-mortem human brain high levels of Y5 receptor mRNA is found in the amygdala, substantia nigra and hypothalamus (Jacques et al., 1996; Nichol et al., 1999) whereas in the hippocampus moderate levels are found (Nichol et al., 1999). A small, almost undetectable, Y5 mRNA level is found in the cerebral cortex (Jacques et al., 1996; Nichol et al., 1999). This receptor has been implicated in several physiological processes, namely food intake (Gerald et al., 1996), brain excitability and seizures (Woldbye et al., 1997; Benmaamar et al., 2005). Some histochemical studies have demonstrated the presence of Y5-immunoreactivity in fibers suggesting that activation of these receptors could modulate presynaptic neurotransmitter release as well (Campbell et al., 2001; Wolak et al., 2003). Co-localization of Y1- and Y5-immunoreactivity is observed in several brain regions, including the cerebral cortex, hippocampus, thalamus and caudate putamen (Wolak et al., 2003).

4.3.5 NPY metabolism

Active NPY can be degraded by dipeptidyl peptidase IV (DPPIV) and aminopeptidase P (AmP) to generate NPY₃₋₃₆ and NPY₂₋₃₆, respectively, which display some selectivity for particular NPY receptors. DPPIV is a membrane-bound protease which cleaves proline in penultimate position (Hopsu-Havu and Glenner, 1966). By removing the Tyr-Pro dipeptide from the NPY N-terminal, DPPIV generates NPY₃₋₃₆, a fragment with reduced affinity for the NPY Y1 receptor but that retains its capacity to bind to the NPY Y2 and Y5 receptors (Grandt et al., 1993). DPPIV is present in the kidneys, liver, ileum, and is abundant on endothelial and epithelial cells, astrocytes as well as activated T and B lymphocytes (Stange et al., 1996). A soluble form is found in the plasma (Shibuya-Saruta et al., 1996). Similarly, AmP can hydrolyze peptide bonds between the first and second amino acid residues at the N-terminal side of a protein provided that the second amino acid is a proline (Yaron and Mlynar, 1968). AmP removes the N-terminal tyrosine from NPY in human kidney and jejunum to generate NPY₂₋₃₆ (Medeiros and Turner, 1994), a potentially semi-selective Y2 and Y5 agonist. AmP is present in the lungs (Ryan et al., 1994), on astrocytes and neurons, as well as on smooth muscle and endothelial cells (Mentlein et al., 1990). A soluble form of the enzyme exists in circulation (Ryan et al., 1994). Nevertheless, the physiological importance of NPY processing to

regulate the activation of particular NPY receptors through the generation of selective ligands remains to be established.

NPY can be degraded in the central nervous system by proteases present, for instance, in the hippocampal synaptosomes. NPY is efficiently metabolized by a single cleavage between the residues Leu³⁰ and Ile³¹. Thus, catabolic processing of NPY results in formation of a C-terminal truncated fragment NPY_{1–30} and its counterpart, NPY_{31–36}. The enzyme involved in this process appears to possess properties of aspartyl proteases. Moreover, the neutral endopeptidase has a major NPY-hydrolyzing activity in striatal synaptic membrane and in the renal brush border (Medeiros Mdos and Turner, 1996).

4.3.6 NPY models

4.3.6.1 NPY overexpressing models

Two NPY overexpressing mice models (Inui et al., 1998; Thiele et al., 1998) and one NPY overexpressing rat model (Michalkiewicz and Michalkiewicz, 2000) have been generated and analyzed. Only a modest 1.15 increase in NPY protein was detected in the brain-restricted model (using the neuronal promoter Thy1) generated by Inui *et al* (Inui et al., 1998). The model produced by Thiele *et al* (using a construct containing 10 kb of the endogenous NPY promoter and 3 kb of the 3' flanking sequence) showed approximately 5-fold higher brain NPY mRNA levels and abundant NPY protein expression in the cortex, amygdala and hippocampus but interestingly not in the arcuate nucleus of the hypothalamus (Thiele et al., 1998). A 14.5 kilobase fragment containing the entire rat NPY gene including 5 kilobases of 5' and 1.5 kilobases of 3' flanking sequences was used to generate the transgenic NPY rat model (Michalkiewicz and Michalkiewicz, 2000; Thorsell et al., 2000). Significant increases of NPY levels in the central nervous system were only found in the CA1-2 region of the hippocampus, reaching a 32% increase. Considerable increased NPY levels were also found in peripheral tissues including kidney, lung, spleen and heart.

A summary of the major phenotypes found in NPY overexpressing models are shown in Table 1.

Species	Examined behavior	Effect	Reference
Mouse	Food intake	↔	Inui et al. (1998)
	Body weight	↔	
	Voluntary ethanol intake	↓	Thiele et al. (1998)
	Sensitivity to ethanol-induced sedation	↑	
Rat	Food intake	↔	Thorsell et al. (2000)
	Body weight	↔	
	Anxiety-related behaviors	↓	
	Spatial memory	↓	
	Locomotion	↔	
	Seizure susceptibility	↓	Vezzani et al. (2002)

Table 1. Major phenotypes observed in NPY overexpressing models. ↔: unaltered, ↑: increased, ↓: decreased.

NPY overexpressing mice generated by Inui *et al* did not show any change in food intake or body weight (Inui et al., 1998). However, an obese phenotype with transiently increased food intake could be induced once mice were put on a sucrose loaded diet (Kaga et al., 2001). At approximately 1 year of age these mice also develop hyperglycemia and hyperinsulinemia although without altered glucose excursion (Kaga et al., 2001). No information regarding feeding and body weight effects are available for the NPY overexpressing model generated by Thiele *et al* (Thiele et al., 1998). However, these NPY-overexpressing mice showed a significant reduction in voluntary ethanol consumption and a higher sensitivity to the effects of ethanol compared to control mice (Thiele et al., 1998).

No changes on body weight or food intake have been reported in rats overexpressing NPY. Locomotor activity and baseline performance on the elevated plus maze, a test for anxiety-like behavior, were also unaffected in these animals (Thorsell et al., 2000). However, the elevated NPY expression in this rat model seems to influence their sensitivity to the behavioral consequences of stress (Thorsell et al., 2000). In this respect, while restraint stress caused a significant reduction in exploratory behavior in the elevated plus maze in wild type (WT) rats, no such behavior was observed in the NPY overexpressing rats (Thorsell et al., 2000). A selective impairment of spatial memory acquisition was also found in the NPY overexpressing rats, as assessed in the Morris water maze (Thorsell et al., 2000). Likewise, under basal conditions, seizure susceptibility in NPY overexpressing rats is normal (Vezzani et al., 2002). However,

intracerebroventricular injection of kainic acid or stimulation by electrical kindling showed a significant reduction in the number and duration of electroencephalographic seizures in the NPY overexpressing rats when compared to WT animals. Moreover, under these conditions transgenic rats were less susceptible to epileptogenesis than WT littermates.

4.3.6.2 NPY knockout model

NPY knockout (KO) mice were generated by Erickson and colleagues in 1996 (Erickson et al., 1996). The targeting vector was designed to replace the coding sequence of the pre-pro-NPY peptide with the *lacZ* gene to verify the expression and functional activity of the *LacZ* gene in place of it.

A summary of the major phenotypes found in NPY KO mice are shown in Table 2.

Species	Examined behavior	Effect	Reference
Mouse	Food intake	↔	Erickson et al. (1996)
	Body weight	↔	
	Seizure susceptibility	↑	
	Fasting-induced refeeding	↓	Bannon et al. (2000)
	Anxiety	↑	
	Locomotion	↔	
	Memory	↔	
	Nociception	↓	
	Voluntary ethanol intake	↑	Thiele et al. (1998)
	Sensitivity to ethanol-induced sedation	↓	

Table 2. Major phenotypes observed in the NPY knockout model. ↔: unaltered, ↑: increased, ↓: decreased.

Similarly to NPY overexpressing models, NPY KO mice did not show any alteration in food intake or body weight under normal conditions (Erickson et al., 1996). However, a significant reduction of food intake after 24 or 48 h fasting was observed (Bannon et al., 2000). Alterations in body weight or food intake in the NPY KO mice were not even observed with a high fat diet loading (Hollopeter et al., 1998). Interestingly, although the high fat diet increased the weight of NPY KO mice fat pad depots, the actual body weight of these mice was not significantly different from the chow fed controls. Surprisingly, no difference in food intake, body weight or fat pad weight between NPY KO

and WT mice was detected after chemical lesions in the arcuate nucleus of the hypothalamus via mono-sodium-glutamate injection into neonates (Hollopeter et al., 1998). Similarly, intraperitoneal injections of gold-thio-glucose in adult mice, which destroys neurons selectively in the ventral medial hypothalamus, also known as the satiety center, did not reveal significant differences between NPY KO mice and the WT controls (Hollopeter et al., 1998).

One of the more obvious phenotypes observed in NPY KO mice was the approximately 30% increase in mild seizures when mice were exposed to an unfamiliar environment (Erickson et al., 1996). Furthermore, when challenged with a convulsing agent, hyperexcitability was strongly increased in NPY KO mice, with 80% developing motor convulsions compared to only 30% of control mice. Moreover, the severity of the seizures was increased and their onset decreased in NPY KO mice.

Investigations leading to the behavioral characterization of NPY KO mice suggested an anxiogenic-like phenotype for these mice (Bannon et al., 2000). In the open field test, NPY KO mice spent significantly less time in the center area of the apparatus when compared to control animals. However, the total distance traveled by NPY KO and WT mice was not different, suggesting no influence on locomotor activity. Experiments investigating the acoustic startle response showed that NPY KO mice have a significant increase in amplitude, indicating greater anxiety (Bannon et al., 2000). No differences were however observed between both genotypes in the elevated plus maze. NPY KO and WT mice were also tested in an inhibitory avoidance paradigm, a trial for memory/cognition. No differences were observed between both groups (Bannon et al., 2000). In the hot plate test, NPY KO mice displayed a bigger latency time when compared to WT animals, suggesting a role for NPY in nociception (Bannon et al., 2000).

Data regarding ethanol consumption are in agreement with the results from NPY overexpressing models, as NPY KO mice exhibited significant higher ethanol consumption and lower sensitivity to ethanol compared to control mice, while NPY overexpressing mice showed the opposite effects (Thiele et al., 1998).

In summary, besides the important role NPY is assumed to play on the regulation of food intake and body weight, there is little genetic evidence that overexpression or knockout of the NPY gene leads to altered body weight regulation. One possible explanation has to do with the complexity and the many factors involved in this function, where compensatory mechanisms may overcome NPY altered levels. Both overexpression and knockout NPY models were useful in consistently substantiate a role for NPY in anxiety, susceptibility to seizures and alcohol consumption.

5 CONCLUDING REMARKS

Transthyretin (TTR), a plasma protein that functions as the transporter of thyroxine and retinol, is mainly synthesized by the liver and choroid plexus of the brain, which are respectively the sources of TTR in the plasma and cerebrospinal fluid (CSF). Several TTR mutations are associated with familial amyloid polyneuropathy (FAP), an autosomal dominant lethal disorder characterized by extracellular deposition of TTR amyloid fibrils, particularly in the peripheral nervous system (PNS), leading to axonal and neuronal loss. The origin of TTR deposited in the PNS of FAP patients is unknown. Physiologically, two routes of access of TTR to the nerve are possible: i) through the blood-nerve barrier, which is effective in slowing but not in preventing the entry of proteins into the endoneurium, such that ganglion cells and their axons are probably constantly exposed to serum proteins; and ii) through the CSF-nerve barrier, as peripheral nerve roots contact with CSF, where TTR is present in high levels. A similar reasoning may be applied to the central nervous system (CNS): TTR may access the brain both through the blood-brain barrier and the CSF-brain barrier. In addition to FAP, several lines of evidence have drawn attention to the importance of TTR in the pathophysiology of a number of other neurological and psychiatric diseases. However, TTR involvement in the homeostasis of both the PNS and CNS is not fully understood. In particular, studies addressing a putative role for TTR in the PNS are lacking. The aim of the present work is to study TTR-mediated molecular signaling in the nervous system, with special attention to the PNS. TTR KO mice constitute a valuable tool to achieve this purpose.

PART II

RESEARCH PROJECT

Objectives

The aim of this research project was to study TTR-mediated molecular signaling in the nervous system, with special focusing to the peripheral nervous system.

We proposed to:

- Chapter 1: Study TTR-related differential gene expression in the nervous system – PAM overexpression in the absence of TTR.
- Chapter 2: Search for upstream and downstream targets involved in TTR-mediated regulation of PAM expression.
- Chapter 3: Characterize the TTR KO mice as a model for increased NPY levels.
- Chapter 4: Search for the relevance of LPL overexpression in the nervous system of TTR KO mice.

CHAPTER 1

TTR IS RELATED TO DIFFERENTIAL GENE EXPRESSION
IN THE NERVOUS SYSTEM: PAM UPREGULATION IN THE
ABSENCE OF TTR

TTR IS RELATED TO DIFFERENTIAL GENE EXPRESSION IN THE NERVOUS
SYSTEM: PAM UPREGULATION IN THE ABSENCE OF TTR

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Abstract

Transthyretin (TTR) has access to the brain and nerve through the blood and cerebrospinal fluid. To investigate TTR function in nervous system homeostasis, differential gene expression in wild-type (WT) and TTR knockout (KO) mice was assessed. We show that peptidylglycine α -amidating monooxygenase (PAM), the rate-limiting enzyme in neuropeptide maturation, is overexpressed in the peripheral (PNS) and central nervous system (CNS) of TTR KOs. Furthermore, in PC12 cells and primary cortical neurons, absence of TTR is related to increased PAM expression. Our findings demonstrate that TTR plays a role in nervous system physiology, namely by regulating neuropeptide maturation through downregulation of PAM expression. To determine if TTR role in the homeostasis of the nervous system is mediated by the action of its major ligands, thyroxine (T_4) and retinol, PAM expression was evaluated in PC12 cells grown in the presence of mutated forms of TTR presenting low affinity for both ligands. The results obtained suggested that TTR effect on PAM expression is facilitated through binding to its major ligands, particularly T_4 . However, a direct effect of TTR in the regulation of PAM expression was supported by the fact that TTR-mediated decrease of PAM expression was observed in cultures grown in a T_4 - and RBP-retinol-free medium. A possible TTR involvement in PAM mRNA stability was addressed in primary cortical neurons. We determined that PAM mRNA half-life was not decreased in the presence of TTR. As such, the mechanism underlying PAM regulation by TTR is still not understood and should be further addressed.

Introduction

Transthyretin (TTR), a plasma protein that functions as the transporter of thyroxine and retinol (Raz and Goodman, 1969), is mainly synthesized by the liver and choroid plexus of the brain, which are respectively the sources of TTR in the plasma and cerebrospinal fluid (CSF) (Soprano et al., 1985). Several TTR mutations are associated with familial amyloid polyneuropathy (FAP), an autosomal dominant lethal disorder characterized by extracellular deposition of TTR amyloid fibrils, particularly in the peripheral nervous system (PNS), leading to axonal and neuronal loss (Sousa and Saraiva, 2003).

The origin of TTR deposited in the PNS of FAP patients is unknown. Physiologically, two routes of access of TTR to the nerve are possible: i. through the blood-nerve barrier, which is effective in slowing but not in preventing the entry of proteins into the endoneurium, such that ganglion cells and their axons are probably constantly exposed to serum proteins, and ii. through the CSF-nerve barrier, as peripheral nerve roots contact with CSF, where TTR is present in high levels. A similar reasoning may be applied to the central nervous system (CNS): TTR may access the brain both through the blood-brain barrier and the CSF-brain barrier.

In agreement with the importance of TTR in the homeostasis of the PNS and CNS, a number of recent studies have revealed TTR involvement both in the PNS and CNS biology. Regarding the PNS, TTR KO mice were reported to present sensorimotor impairments and a role for TTR in enhancing nerve regeneration was demonstrated (Fleming et al., 2007). In agreement with a function for TTR in the CNS, recent studies have implicated TTR in behavior and CNS disorders. Altered TTR levels in the CSF have been linked to neuronal dysfunctions, such as Alzheimer's disease, in which TTR in the CSF is negatively correlated with the degree of dementia (Serot et al., 1997). Accordingly, genetic reduction of TTR was shown to accelerate amyloid deposition in a mouse model for Alzheimer's disease (Choi et al., 2007), reinforcing the neuroprotective function of TTR in the development of the disease. Additional evidence for a role of TTR in the physiology of the nervous system, namely in behavior, came from the observation that TTR knockout (KO) mice are less depressed than control mice (Sousa et al., 2004). Recently published data also revealed that decreased levels of TTR may be critical to the development of memory impairments during aging (Brouillette and Quirion, 2007; Sousa et al., 2007).

In the present work we elucidate TTR-mediated molecular signaling in the nervous system, by studying differential gene expression in wild-type (WT) and TTR KO mice. Our data show that TTR plays a role in the physiology of both the CNS and PNS.

Materials and Methods

Animals

Mice were handled according to European Union and National rules. WT and TTR KO mice (Episkopou et al., 1993), in the 129/Sv background, bred separately, were maintained at $24 \pm 1^\circ\text{C}$ under a 12 h light/dark cycle and fed regular chow and tap water ad libitum. Unless otherwise stated, comparisons comprised groups of 6 animals of each genotype, age and sex matched. All animals were accustomed to regular handling.

Microarray analysis

Dorsal root ganglia (DRG) and respective nerve roots from 5 TTR KO mice and 5 human TTR transgenic mice in an endogenous TTR KO background (Kohno et al., 1997), age- and gender-matched, were processed according to Affymetrix (New Orleans). Labelled cRNAs were hybridised to Test3 chips to assess the quality of cRNAs prior to hybridisation to Affymetrix U74A oligonucleotide arrays. Differential expression was analysed using Affymetrix MAS 5.0 with the arrays scaled to an average intensity of 150. After excluding genes absent in all samples and genes related to within group variance, TTR KO samples were compared to control samples. Genes scored as increased or decreased ($\geq \pm 3$ -fold change) in all pairwise comparisons were selected.

RT-PCR analysis

Total RNA was isolated with Trizol (Invitrogen) and subjected to RT-PCR with the Superscript II kit (Invitrogen). PCR was performed for the appropriate number of cycles for each cDNA (20-40 cycles) at 95°C for 1 min, 56°C for 2 min, and 72°C for 3 min. Primers were designed using PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the sequence from the National Centre for Biotechnology Information database. Sense and antisense primers are shown in **Table 1**. Ethidium bromide-stained gels were scanned using a Typhoon 8600 (Amersham) and amplified bands were quantified using the ImageQuant software (Amersham). Fluorescence density of each PCR-amplified band was normalized with the corresponding value of either β -actin or hypoxanthine-guanine phosphoribosyltransferase (HPRT).

Immunohistochemistry

Sections were incubated in blocking buffer (1% bovine serum albumin, BSA, and 4% horse serum in phosphate buffered saline, PBS) for 30 min at 37°C , and with the primary antibody rabbit polyclonal anti-PAM (Ab246, Dr. Mains, University of Connecticut

Health Centre) 1:500 in blocking buffer overnight at 4°C. Antigen visualization was performed with the biotin-extravidin-peroxidase kit (Sigma). On control sections, primary antibody was replaced by blocking buffer. Two observers performed the immunohistochemistry analysis independently.

PAM expression in PC12 cells and primary cortical neurons

Rat pheochromocytoma PC12 cells (American Type Tissue Culture Collection) were grown in 6 well plates in William's medium E (Invitrogen) supplemented with 2 mM L-glutamine (Sigma) and 10% fetal calf serum (FCS, Invitrogen). When cells reached 50% confluence, FCS was withdrawn. William's E medium was supplemented with 100 ng/mL 2.5S nerve growth factor (NGF) (Sigma) and 10% of either WT or TTR KO mouse serum, or 10% TTR KO mouse serum containing 300 µg/mL of either recombinant TTR, produced as described (Liz et al., 2004), or BSA (Sigma). For the analysis of the influence of TTR ligands on PAM expression, TTR KO mouse serum was supplemented with 300 µg/mL of either recombinant V30M TTR or recombinant I84S TTR, produced as described (Liz et al., 2004). After 72 h, cells were lysed with Trizol and total RNA was extracted from the aqueous phase and processed as described above. Cortical neurons were obtained from embryonic day 17 (E17) TTR KO mouse brains. After removing all meningeal shreds, the cortex was dissected and digested for 8 min at 37°C with 0.05% trypsin. The tissue was pelleted by centrifugation (600 g, 5 min), resuspended in 2 mL DMEM (Invitrogen) and triturated with decreasing diameter bore flame-polished glass pipettes. The cell suspension was filtered through a cell strainer (Becton Dickinson) and centrifuged 5 min at 600 g. Cells were resuspended in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen) and 0.5 mM L-glutamine (Sigma) and were plated at 1.6×10^6 cells/well onto six-well plates coated with poly-D-lysine/laminin (Sigma). Three days after, the culture medium was supplemented with 10% of either TTR KO mouse serum or TTR KO mouse serum containing 300 µg/mL recombinant TTR. After 72 h, total RNA was extracted as described for PC12 cells. For the analysis of PAM mRNA decay, 72 h after incubation with TTR, actinomycin D (5 µg/mL, Sigma) was added to the culture medium to block *de novo* transcription. Following different time points (0, 10, 15, and 24 h), total RNA was extracted as described above. The half-life of PAM mRNA was estimated by regression analysis of the decline in mRNA levels as a function of time.

Statistical analysis

Statistical analysis was performed using the Student's t-Test. Results were expressed as average $\pm$ SD. For all statistical analysis, $P < 0.05$ was accepted as being statistically significant.

	Sense primer (5'-3')	Antisense primer (5'-3')
Mouse β -actin	GTGGGCCGCTCTAGGCACCAA	CTCTTTGATGTCACGCACGATTTC
Rat β -actin	TGTCACCAACTGGGACGATA	TCTCAGCTGTGGTGGTGAAG
Mouse HPRT	GTAATGATCAGTCAACGGGGGAC	CCAGCAAGCTTGCAACCTTAACCA
Mouse PAM	CCTGGGGTCACACCTAAAGA	TGTAAGGACACACCGGAACA
Rat PAM	AAAAATGGAGCATCGGTCAG	TTCCCTTTCCTCGGAATCTT

Table 1. Sense and antisense primers used to amplify each cDNA of interest.

Results

The PNS of TTR KO and WT mice displays differential gene expression

To initiate the evaluation of TTR-mediated molecular signaling in the PNS, pilot cDNA microarray analysis of DRG from TTR KO mice and transgenic mice for human TTR (in a TTR KO background) was performed. Statistical analysis identified 11 genes differentially expressed with an average-fold change $\geq \pm 3$ (gene identity can be disclosed upon request). These included genes potentially involved in the physiology of the nervous system, namely peptidylglycine α -amidating monooxygenase (PAM) and lipoprotein lipase (LPL), both upregulated in DRGs from TTR KO mice (24-fold and 3.6-fold, respectively). Validation of microarray results was initiated by comparing PAM expression in WT and TTR KO mice (for validation of LPL results refer to Chapter 3 of this thesis).

Peptidylglycine α -amidating monooxygenase, a key neuropeptide processing enzyme, is increased in TTR KO mice

Neuropeptides are stored in secretory granules and are activated by processing enzymes; approximately 50% of all known neuropeptides are synthesized as biologically inactive glycine-extended precursors that require a carboxy-terminal posttranslational amidation for biological activity. PAM is the only enzyme that C-terminally α -amidates peptides, being therefore rate-limiting in amidated neuropeptide maturation (10). Microarrays revealed that PAM is upregulated in DRG from TTR KO mice when compared to human TTR transgenic mice. To address whether a similar upregulation would be observed in TTR KO and WT mice, we performed PAM immunohistochemistry in the PNS of both strains. In the DRG and nerve from TTR KOs (n=6), PAM immunolabeling was increased relatively to WT mice (n=7), presenting a perinuclear and axonal distribution, respectively (**Figure 1A, first and second rows, arrowheads**), showing that absence of TTR is correlated with increased PAM expression. To determine whether PAM overexpression in TTR KO mice is restricted to the PNS or is observed throughout the nervous system, we performed PAM immunohistochemical analysis in the CNS. In the brain of TTR KOs, intense PAM staining was seen in punctate vesicle-like structures throughout the perinuclear area of neurons of the cortex outer granular layer (n=5), whereas in WT mice (n=5) a comparably moderate staining was present (**Figure 1A, third row, arrowheads**); similar differential labeling was observed in the hippocampus (**Figure 1A, fourth row, arrowheads**). Likewise, increased PAM immunostaining was detected in the spinal cord of TTR KOs when compared to WT mice (not shown). To confirm PAM overexpression in the CNS of TTR KO mice, we performed semiquantitative RT-PCR;

both the brain (n=6) and spinal cord (n=4) of TTR KO mice presented increased PAM expression (1.4-fold and 2.5-fold, respectively) when compared to WT animals (n=5 and n=4, respectively) (**Figure 1B**). Our data suggest that TTR regulates neuropeptide maturation not only in the PNS but also in the CNS.

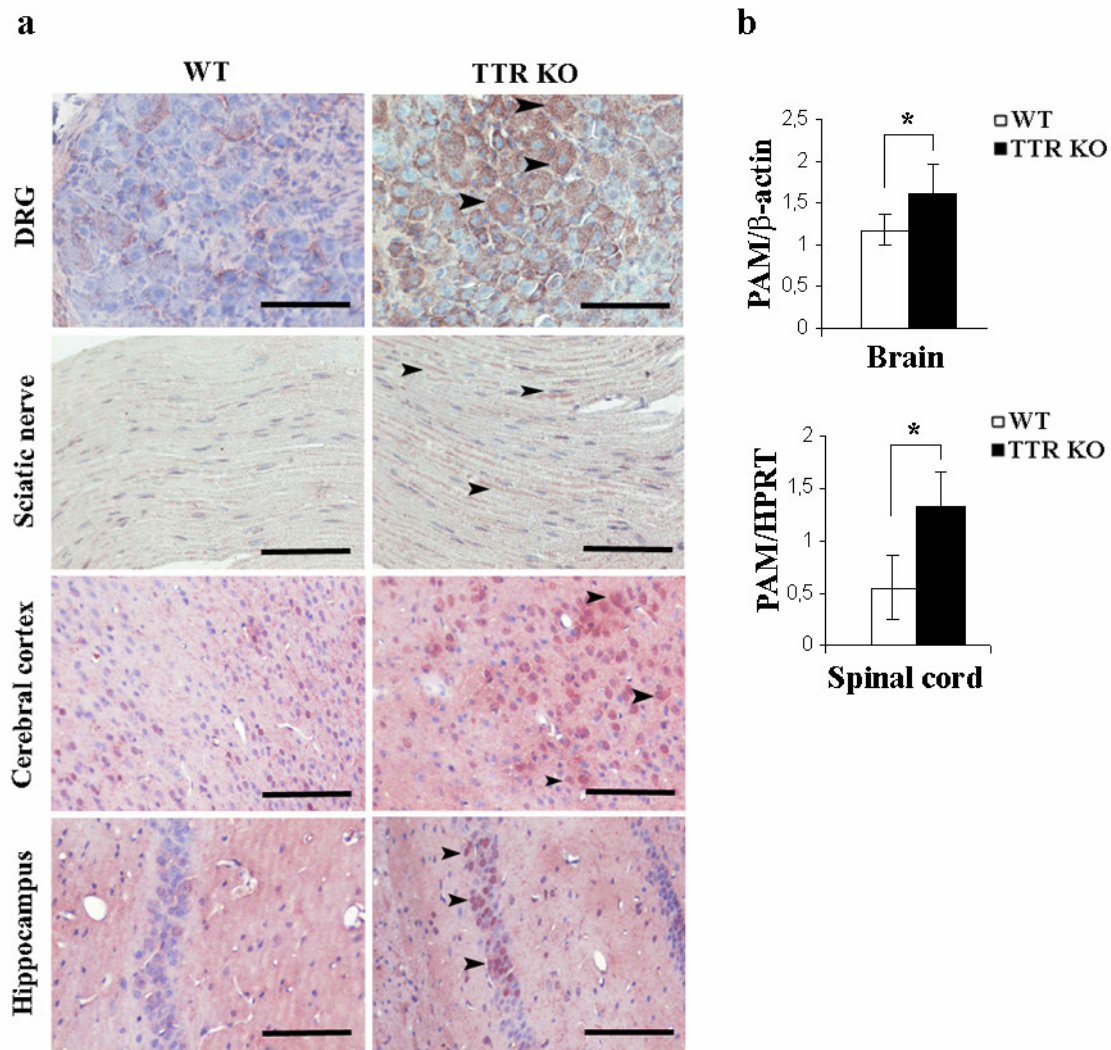


Figure 1. PAM expression in the PNS and CNS of TTR KO and WT mice. **A)** PAM immunohistochemistry in DRG, sciatic nerve, cerebral cortex, and hippocampus; arrowheads highlight structures with increased PAM staining; scale bar = 100 μ m. **B)** PAM semiquantitative RT-PCR analysis in brain and spinal cord samples; results are presented as average $\pm$ SD; * P < 0.05.

Absence of TTR leads to increased PAM expression in neuronal and neuronal-like cell cultures

To further confirm the hypothesis that TTR is responsible for decreased PAM expression, PC12 cells were used in cellular assays either in the presence or absence of

TTR. PC12 cells, which, undifferentiated, have an immature adrenal chromaffin cell phenotype once exposed to NGF differentiate and resemble sympathetic neurons. This cell line has been previously shown to be adequate to study PAM expression and activity (Ford and Mueller, 1993). As predicted, PC12 cells exposed to TTR KO serum displayed increased PAM expression when compared to cells grown with WT serum (**Figure 2, left and middle panels**). To establish whether TTR or another factor in WT serum was responsible for decreased PAM expression, PC12 cells grown with TTR KO serum were supplemented with either physiological concentration of TTR or albumin (negative control). Addition of TTR to KO serum was able to diminish PAM synthesis (**Figure 2, left and middle panels**), demonstrating that this protein is responsible for differential PAM expression. Similar results were obtained with primary cortical neurons grown either in the presence or absence of TTR (**Figure 2, right panel**).

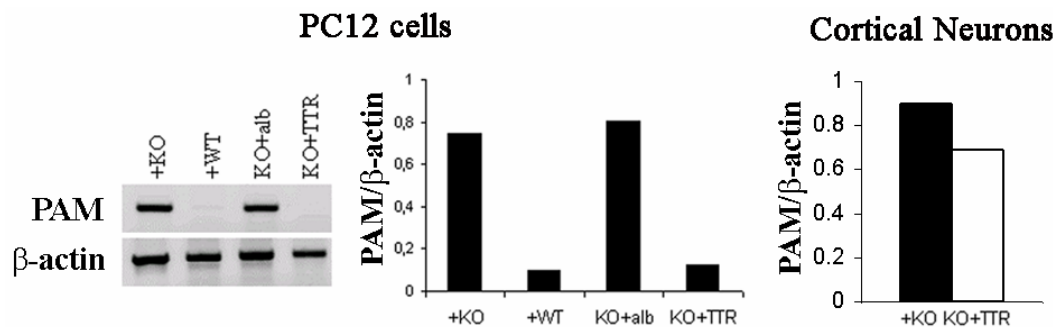


Figure 2. PAM expression in PC12 cells and primary cortical neurons. **Left and middle panels:** PAM semiquantitative RT-PCR analysis in PC12 cells grown in the presence of TTR KO mouse serum (+KO), WT mouse serum (+WT), or TTR KO mouse serum containing albumin (KO+alb) or TTR (KO+TTR). **Right panel:** PAM semiquantitative RT-PCR analysis in primary cortical neurons grown in the presence of TTR KO mouse serum with (KO+TTR) or without (+KO) added TTR.

TTR effect on PAM expression is facilitated through binding to its major ligands, T₄ and retinol

To address whether TTR-mediated decrease of PAM expression is a TTR-specific effect or is mediated by the action of its major ligands, thyroxine (T₄) and retinol, experiments were performed with PC12 cells in which TTR KO serum was supplemented with V30M TTR or I84S TTR. While V30M TTR presents considerable low affinity for T₄ (Rosen et al., 1993), I84S TTR displays a nearly negligible affinity for retinol binding protein (RBP) (Berni et al., 1994), the protein that binds retinol and is carried in the plasma through binding to TTR, and a 2-3-fold lower affinity for T₄ than wild type (WT) TTR (Murrell et al., 1992). As already shown in our previous experiments, and confirmed here, PC12 cells grown with TTR KO serum supplemented with WT TTR (KO+ WT TTR)

displayed decreased PAM expression when compared to cells grown with TTR KO serum alone (+KO) (**Figure 3**). The effect of WT TTR in decreasing PAM expression was not fully achieved by the mutants V30M TTR nor I84S TTR, in which TTR binding to its ligands is impaired (**Figure 3**). In fact, while incubation with V30M TTR produced an increase in PAM expression when compared to WT TTR (KO+ WT TTR), incubation with I84S TTR displayed a similar trend of increase, though a statistically significant difference was not reached, probably due to the increased standard deviation observed in this condition (**Figure 3**). These results suggest that binding to T_4 and RBP-retinol contributes to TTR-mediated effect in the regulation of PAM expression. If so, it seems that T_4 plays a more important role than retinol, as no V30M TTR additive effect on PAM expression was observed upon incubation with I84S TTR, in which binding to both ligands is defective.

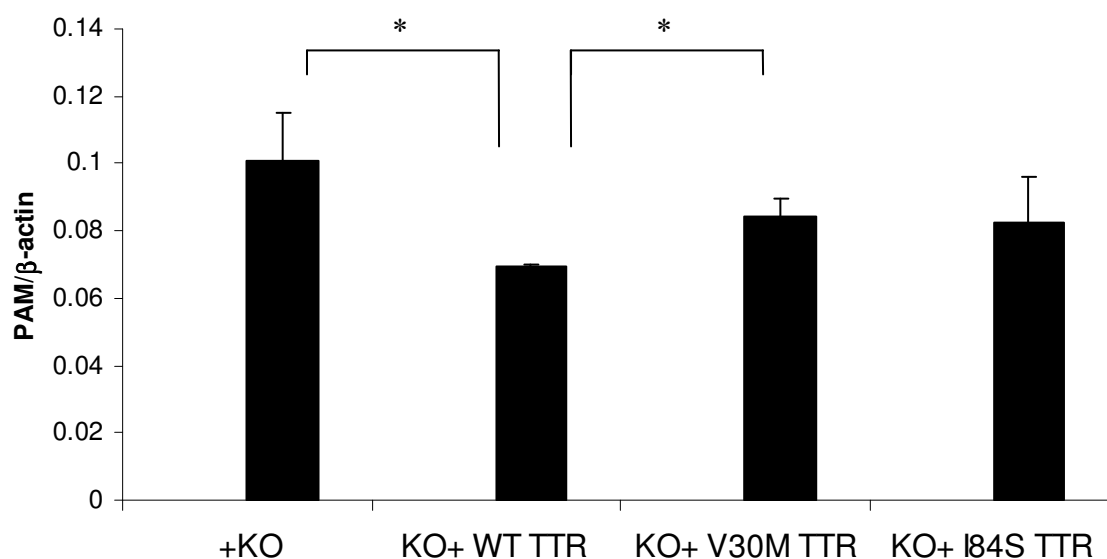


Figure 3. PAM semiquantitative RT-PCR analysis in PC12 cells grown in the presence of TTR KO mouse serum (+KO) or TTR KO mouse serum containing wild type TTR (KO+ WT TTR), V30M TTR (KO+ V30M TTR) or I84S TTR (KO+ I84S TTR); results are presented as average $\pm$ SD; * $P < 0.05$.

Increased PAM levels in the absence of TTR are not related to a TTR effect on PAM mRNA stability

The most commonly described mechanism for regulation of PAM expression involves alterations in PAM mRNA stability. Thyroid hormones, estrogens and α -adrenergic agonists are examples of molecules affecting PAM mRNA half-life (Fraboulet et al., 1996; El Meskini et al., 1997; Girard et al., 1999). Likewise, TTR could regulate

PAM levels by altering its mRNA stability in spite of influencing its transcriptional rate. To verify this hypothesis, PAM mRNA half-life was determined in primary cortical neurons grown either in the presence or absence of TTR. Actinomycin D was added to the culture medium to block *de novo* transcription and PAM mRNA decay was analyzed by RT-PCR 0, 10, 15, and 24 h after actinomycin D treatment. Despite the fact that the 10 h time point was deviated from our linear regression, for all time points a similar decay of PAM mRNA levels was observed with time in both conditions (**Figure 4**), however PAM mRNA half-life was not decreased in cells supplemented with TTR (KO+TTR) when compared to cells grown with TTR KO serum alone (KO). This result excludes a TTR involvement in PAM mRNA stability as the mechanism for TTR regulation of PAM expression. It also suggests that the increased PAM levels observed in the absence of TTR, both *in vivo* and *in vitro*, are the result of an increased transcriptional rate.

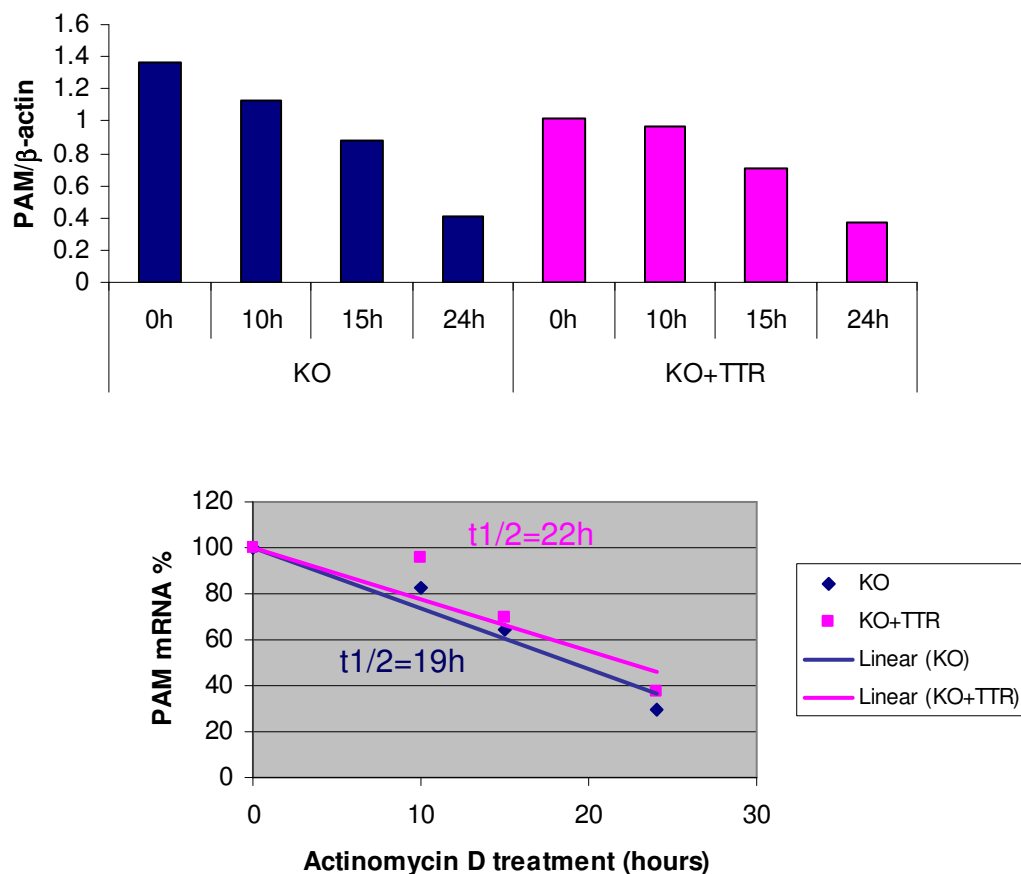


Figure 4. PAM mRNA decay assay in primary cortical neurons grown in the presence of TTR KO mouse serum with (KO+TTR) or without (KO) TTR addition to the culture medium. **Upper panel:** PAM semiquantitative RT-PCR analysis 0, 10, 15 and 24 h after actinomycin D treatment. **Lower panel:** estimation of PAM mRNA apparent half-life.

Discussion

We show that TTR is involved in the homeostasis of the nervous system, namely by regulating neuropeptide maturation through downregulation of PAM expression.

Active neuropeptides are synthesized from large precursor proteins by processing enzymes; PAM is the bifunctional protein that sequentially catalyzes the conversion of glycine-extended peptides into C-terminal amidated peptides. This integral membrane protein is expressed in a wide variety of cell types, including endocrine, glial and endothelial cells, and in many neurons (Prigge et al., 2000). It is of the utmost importance to unravel the mechanism underlying PAM regulation by TTR. TTR effect might be exerted by its major ligands, retinol (through binding to retinol-binding protein) and T_4 . Hypothyroidism induces PAM expression by increasing its stability in the cytoplasm (Fraboulet et al., 1996); however, although TTR is the major T_4 carrier in the CSF, TTR KO mice are euthyroid (Episkopou et al., 1993), suggesting that TTR effect on PAM expression is unlikely to result from impaired thyroid hormone homeostasis. In the case of retinol, the precursor of active retinoic acid has a wide influence on gene expression and could represent a mechanism for TTR regulation of PAM expression. However, despite low plasma retinol levels, TTR KO mice do not show any symptoms of vitamin A deficiency (Episkopou et al., 1993), suggesting that TTR effect on PAM expression does not result from impaired retinoic acid metabolism. TTR also binds pterins, that function as cofactors of catecholamine biosynthesis (Ernststrom et al., 1995); accordingly, TTR KO mice were shown to present increased limbic levels of noradrenaline (NE) (Sousa et al., 2004), which is a stimulator of PAM activity in *Xenopus* (Spindel et al., 1987); this NE increase could account for PAM overexpression in TTR KO mice.

From the analysis of the human PAM promoter, the triiodothyronine (T_3) receptor-alpha and T_3 receptor-beta were identified as being transcription factors involved in the regulation of PAM expression. By making use of the program AliBaba 2.1 (available at http://darwin.nmsu.edu/~molb470/fall2003/Projects/solorz/aliBaba_2_1.htm) for predicting transcription factor binding sites, these T_3 receptor elements were not however identified within the rat and mouse PAM promoter DNA sequences, obtained from the National Centre for Biotechnology Information database. T_3 receptors, also known as thyroid hormone receptors (TRs), belong to a family of ligand-activated transcription factors which regulate the expression of target genes by binding to specific DNA sequences. This family includes nuclear receptors for several hydrophobic ligands, such as glucocorticoids, estrogens, progesterone, vitamin D or retinoic acid. These receptors are composed of several domains important for hormone-binding, DNA-binding, dimerization and activation of transcription. The family of nuclear receptors has been classically divided in two

subfamilies on the basis of structural homologies. One subfamily includes the genes encoding steroid hormone receptors and the other comprises the receptors for the thyroid hormone (T_3) and retinoic acid. To date, two thyroid hormone receptors are known, alpha and beta, which are similar in overall structure. T_3 regulates gene transcription by binding to these receptors (Samuels et al., 1988). Protein-protein interactions are likely to determine the context in which TR binds to target genes and regulates the transcriptional response to thyroid hormone. In fact, T_3 receptors form heterodimers with either the 9-cis retinoic acid receptor (retinoid X receptor, RXR) or the all-trans retinoic acid receptor (RAR) (Tini et al., 1994). RXR was identified as a nuclear auxiliary protein for T_3 receptor as it enhances specific DNA binding (Zhang et al., 1992). The authors show that RXR can greatly enhance the transcriptional activity of T_3 receptors at low retinoic acid concentrations that do not significantly activate RXR itself. It is thus possible that, in addition to T_3 , retinoic acid can indirectly regulate PAM transcription in humans. Besides the fact that TTR KO mice are reported as being euthyroid and not showing any symptoms of vitamin A deficiency (Episkopou et al., 1993), and besides the fact that T_3 receptors were not identified as regulators of PAM transcription in mice, we thought it was worth to analyse this issue in order to assure what factors are involved in the regulation of PAM expression in the presence versus absence of TTR. By using TTR variants with low affinity for TTR major ligands, T_4 and retinol, a role for TTR ligands in mediating TTR effect on PAM expression was suggested. The results obtained seem to indicate that TTR binding particularly to T_4 contributes to the TTR-mediated decrease of PAM expression. This result is however difficult to explain when taking into account the fact that TTR-mediated decrease of PAM expression was observed in PC12 cells and primary cultures of cortical neurons grown in serum-free conditions, in which cells were cultivated in a T_4 - and RBP-retinol-free medium, as is the case of culture medium supplemented with B-27 (Chapter 2 and 3 of this thesis). It is possible that in the mutants used, structural changes occur unabling these variants to exert the normal TTR effect on a putative TTR-interacting molecule at the plasma membrane. This hypothesis would explain the results obtained with the TTR mutants and support a direct role for TTR as the major factor involved in the regulation of PAM expression.

TTR involvement in PAM mRNA stability as the mechanism for TTR regulation of PAM expression was excluded by the analysis of PAM mRNA decay in primary cortical neurons treated with actinomycin D, a blocker of *de novo* transcription. Alterations in PAM mRNA half-life constitute the most common mechanism for regulation of PAM expression reported in the literature. Several factors involved in the regulation of PAM expression share this mechanism, namely thyroid hormones, estrogens and α -adrenergic agonists (Fraboulet et al., 1996; El Meskini et al., 1997; Girard et al., 1999). This does not seem to

be the case for TTR, suggesting that the increased PAM levels observed in the absence of TTR, both *in vivo* and *in vitro*, are the result of an increased PAM gene transcriptional rate.

In summary, we show that TTR is involved in the homeostasis of the nervous system, namely by regulating neuropeptide maturation through downregulation of PAM expression. This finding constitutes an important achievement, as understanding the relevance of TTR presence in the PNS will probably have impact in understanding its physiology and the pathogenic events related to neurodegeneration/neuroprotection in PNS diseases. The mechanism underlying PAM regulation by TTR is still not understood and should be further addressed.

Acknowledgments

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CHAPTER 2

SEARCHING FOR UPSTREAM AND DOWNSTREAM
MOLECULES PARTICIPATING IN TTR REGULATION OF
PAM EXPRESSION

SEARCHING FOR UPSTREAM AND DOWNSTREAM MOLECULES
PARTICIPATING IN TTR REGULATION OF PAM EXPRESSION

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Abstract

Peptidylglycine α -amidating monooxygenase (PAM), the rate-limiting enzyme in neuropeptide maturation, is overexpressed in the peripheral and central nervous system of transthyretin (TTR) knockout (KO) mice. Furthermore, in PC12 cells and primary cortical neurons, absence of TTR is related to increased PAM expression. In the present work, primary cultures of cortical neurons were used as a model to further ascertain the mechanism by which TTR downregulates PAM expression. TTR KO primary cortical neurons grown either in the presence or absence of TTR in the culture medium were analyzed by Differential 2-Dimensional (2D) Fluorescence Gel Electrophoresis (DIGE) and GeneChip expression profile. Despite being highly valuable techniques, 2D-DIGE and cDNA microarrays did not fulfill our ambition, as several genes and proteins were found differentially regulated, but none that could be directly correlated with TTR regulation of PAM expression. Nevertheless, these approaches were useful in raising other questions and hypothesis, some of them already corroborated with work from our laboratory. Using 2D-DIGE we were able to identify 5 proteins as being consistently upregulated with a fold change higher than 1.5 in primary cortical neurons grown in the presence of TTR, namely heterogeneous nuclear ribonucleoprotein K, α - and β -tubulin, ornithine aminotransferase and histone H3. Furthermore, cDNA microarrays revealed that functions such as proliferation, neurogenesis, apoptosis, neurite outgrowth, retrograde axonal transport, synaptic transmission, integrin signaling, cholesterol/steroid biosynthesis and GABA biosynthesis might be influenced by TTR. Whereas we were unable to demonstrate TTR involvement in proliferation and apoptosis of cells of neuronal origin, our studies stress the need to further elucidate the role of TTR in neurite outgrowth and retrograde transport. Moreover, the putative involvement of TTR in synaptic transmission, integrin signaling, cholesterol/steroid biosynthesis as well as GABA biosynthesis arrests further studies.

Introduction

Animal and cell culture model systems are commonly used to understand mechanisms of neuronal signaling. These *in vivo* or *in vitro* systems have particular advantages and limitations. Although animal models can be used to reveal many aspects of neuronal functioning, in some cases the molecular, biochemical or structural observations are not simple to understand, due to the inherent complexity of the nervous system. With *in vitro* systems, although devoid of an intact tissue environment, homogeneous populations of cells that are undergoing synchronous and uniform changes can be studied. A major advance in neuronal cell culture occurred with the introduction of serum-free medium, namely a serum-free medium that effectively supported primary hippocampal and cortical neurons (Brewer, 1997). The use of cytotoxic antimetabolic drugs that can damage neurons could then be abolished, as proliferation of glial cells was inhibited. Primary neuronal culture is a powerful tool for isolating cellular and molecular mechanisms involved in several physiological and pathological conditions. This cell culture model has been used to study a vast diversity of neuronal features, including development, aging, death, neuroprotection, injury, survival, neurite outgrowth, signaling and RNA translocation (Knowles and Kosik, 1997; Barnea and Roberts, 2002; Lesuisse and Martin, 2002; Han et al., 2004; Ju et al., 2004; Lu et al., 2004; Wang et al., 2004; Chua and Tang, 2007; Madrigal et al., 2007).

In the present work, we used primary cultures of cortical neurons as a model to study transthyretin (TTR)-mediated downregulation of peptidylglycine α -amidating monooxygenase (PAM) expression. PAM is the only enzyme that C-terminally α -amidates peptides and over 50% of all known neuropeptides require this amidation for biological activity. PAM is therefore the rate-limiting enzyme in amidated neuropeptide maturation (Prigge et al., 2000). As previously reported (and discussed in Chapter 1 of this thesis), absence of TTR in the nervous system is related to increased PAM expression, both *in vivo* and *in vitro* (Nunes et al., 2006). In this respect, we demonstrated that PC12 cells and primary cortical neurons grown in the absence of TTR displayed increased PAM expression when compared to cells grown in the presence of TTR (Nunes et al., 2006). We chose primary cortical neurons and not PC12 cells as the model to ascertain the mechanism by which TTR downregulates PAM expression, as the first represents a more physiologically relevant system. Given that our first approaches in the search for this mechanism failed to give us the answers, as discussed in Chapter 1 of this thesis, the use of more refined tools was imposed. As such, to accomplish our aim, 2-dimensional (2D)

electrophoresis and cDNA microarrays were performed in TTR knockout (KO) primary cortical neurons grown either in the presence or absence of TTR.

Materials and Methods

Primary cultures of cortical neurons

Primary cultures of cortical neurons were performed as described in Chapter 1. Briefly, cortical neurons were obtained from the brains of embryonic day 17 (E17) transthyretin (TTR) knockout (KO) mice (Episkopou et al., 1993) in the 129/Sv background. Mice were handled according to European Union and National rules. After removing all meningeal shreds, the cortex was dissected and digested for 8 min at 37°C with 0.05% trypsin. The tissue was pelleted by centrifugation (600 *g*, 5 min), resuspended in 2 mL DMEM (Gibco) and triturated with decreasing diameter bore flame-polished glass pipettes. The cell suspension was filtered through a cell strainer (Becton Dickinson) and centrifuged 5 min at 600 *g*. Cells were resuspended in Neurobasal medium (Gibco) supplemented with 2% B27 (Gibco) and 0.5 mM L-glutamine (Sigma) and were plated at 7.2×10^6 cells/dish onto 100 mm culture dishes coated with poly-D-lysine/laminin (Sigma). Three days after, the culture medium of selected dishes was supplemented with 300 µg/mL recombinant TTR, produced as described (Liz et al., 2004). After 72 h, total RNA and protein were extracted.

Differential 2D Fluorescence Gel Electrophoresis (DIGE)

Total protein was extracted from primary cortical neurons grown either in the presence or absence of TTR according to the Ettan™ DIGE manual (Amersham) available at <http://keck.med.yale.edu/pdfs/Ettan%20DIGE%20User%20Manual.pdf>. Briefly, cells were pelleted by centrifugation (12,000 *g*, 4 min, 4°C) and washed four times in Standard Cell Wash Buffer (5 mM magnesium acetate in 10 mM tris, pH 8.0). The washed cell pellets were then resuspended in Standard Cell Lysis Buffer (8 M urea, 4% CHAPS, in 30 mM tris, pH 8.5) and left on ice for 10 min. After sonication, cell lysates were centrifuged (12,000 *g*, 10 min, 4°C) and the supernatants were collected. Protein concentration was determined using the PlusOne 2D Quant Kit (GE Healthcare), according to the manufacturer's instructions. The subsequent steps were performed as a service by the W.M. Keck Facility, Yale University, New Haven, USA, according to the above-mentioned Ettan™ DIGE manual. Briefly, 50 µg (gel 1) and 150 µg (gel 2) of protein extracts from primary cortical neurons grown either in the presence or absence of TTR were labeled with Cy-5 and Cy-3 N-hydroxysuccinimidyl ester dyes (GE Healthcare), respectively. 24 cm, pH 3-10 Immobiline (IPG) Drystrips (GE Healthcare) were rehydrated overnight with both samples and isoelectric focusing was carried out. 12.5% SDS polyacrylamide gel electrophoresis (second dimension) was run, and immediately after SDS PAGE, gels were

scanned at 2 wavelengths simultaneously on a GE Healthcare Typhoon 9410 Imager: 580 nm for Cy3, and 670 nm for Cy5. Gels were then fixed and stained with Sypro Ruby. GE Healthcare DeCyder software was used to quantify (this software includes an automatic background correction, spot volume normalization and volume ratio calculation), and to identify the differentially expressed protein spots with a fold change higher than 1.5. These spots were excised, high mass accuracy automated MALDI-MS/MS spectra was acquired on each target (using the Keck Laboratory's Applied Biosystems 4800 ToF/ToF instrument), and the resulting peptide masses were subjected to database searching using Mascot algorithms. As replicate samples are highly recommended to exclude biological variation, we performed the DIGE analysis in two independent sets of primary cortical neurons grown in the presence or absence of TTR and only spots altered in both experiments were identified.

RNA isolation, target synthesis and hybridization to Affymetrix GeneChips

Total RNA was extracted from primary cortical neurons grown either in the presence or absence of TTR using the RNeasy Mini Kit (Qiagen). The subsequent steps were performed as a service by the Genomics Unit, Instituto Gulbenkian de Ciência, Oeiras, Portugal. Briefly, RNA concentration and purity was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies). RNA was processed for use on Affymetrix (Santa Clara, CA, USA) GeneChip Mouse Genome 430A 2.0 Arrays, according to the manufacturer's One-Cycle Target Labeling Assay. Briefly, 4 µg of total RNA containing spiked in Poly-A RNA controls (GeneChip Expression GeneChip Eukaryotic Poly-A RNA Control Kit, Affymetrix) was used in a reverse transcription reaction (One-Cycle DNA synthesis kit, Affymetrix) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an *in vitro* transcription (IVT) reaction to generate cRNA (MEGAscript T7 kit, Ambion). The cRNA obtained was used for a second round of cDNA and cRNA synthesis, resulting in biotinylated cRNA (GeneChip Expression 3'-Amplification Reagents for IVT Labeling, Affymetrix). Labeled cRNA was fragmented in fragmentation buffer (40 mM Tris-acetate pH 8.0, 100 mM KOAc, 30 mM MgOAc) for 35 min at 95°C. Size distribution of the cRNA and fragmented cRNA, respectively, was assessed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay. 10 µg of fragmented cRNA was used in a 200-µL hybridization containing added hybridization controls. 130 µL of mixture was hybridized on arrays for 16 h at 45°C. Standard post hybridization wash and double-stain protocols (Midi_euk2v3_450) were used on an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned on an Affymetrix GeneChip scanner 3000 7G.

GeneChip data analysis

Scanned arrays were analyzed first with Affymetrix GCOS 1.4 software to obtain Absent/Present calls and for subsequent analysis with DNA-Chip Analyzer (dChip) Version Release 2006 (<http://www.dchip.org>, Wong Lab, Harvard). The arrays were normalized to a baseline array with median CEL intensity by applying an Invariant Set Normalization Method (Li and Wong, 2001). Normalized CEL intensities of the arrays were used to obtain model-based gene expression indices based on a PM (Perfect Match)-only model (Li and Hung Wong, 2001). Only genes called Present in at least one of the two arrays and within replicate arrays called Present were kept for downstream analysis (15299 genes). Thus, genes called Absent in both arrays were excluded. All genes compared were considered to be differentially expressed if the 90% lower confidence bound of the fold change between experiment and baseline was above 1.2. The lower confidence bound criterion means that one can be 90% confident that the fold change is a value between the lower confidence bound and a variable upper confidence bound. Li and Hung Wong (Li and Hung Wong, 2001) have shown that the lower confidence bound is a conservative estimate of the fold change and therefore more reliable as a ranking statistic for changes in gene expression. Annotations for the >22,000 mouse transcripts that are represented on the GeneChip Mouse Genome 430A 2.0 Array were obtained from the NetAffx database (www.affymetrix.com) as of December 2006 and imported into dChip using ChipInfo software (Zhong et al., 2003). The obtained genes underwent a meticulous analysis from which resulted the exclusion of genes with unknown function or known function unrelated to the nervous system physiology, as assessed by Pubmed search. Genes were then grouped according to their nervous system-related function.

TUNEL assay

For *in situ* determination of apoptosis by internucleosomal DNA fragmentation using terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL), primary cultures of cortical neurons were cultivated as described above, with the exception that cells were plated at a density of 2.4×10^5 cells/well onto 13 mm poly-D-lysine coated coverslips, placed in 24-well plates. 72 h after addition of TTR to the culture medium of selected wells, labeling of apoptotic cells was performed with the Apoptag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International), according to the manufacturer's instructions. After detection of nuclear staining in apoptotic cells using 3,3'-diaminobenzidine (DAB), neurons were labelled with the neuronal marker protein gene product 9.5 (PGP 9.5). Briefly, cells were incubated in blocking buffer (1% bovine serum albumin, BSA, and 4% horse serum in phosphate buffered saline, PBS) for 1 h at 37°C,

followed by incubation with the primary antibody rabbit anti-PGP 9.5 (Serotec) 1:500 in blocking buffer, overnight at 4°C. Antigen visualization was performed with the biotin-extravidin-alkaline phosphatase kit (Sigma), using fast red (Sigma) as substrate. Experiments were performed in duplicate.

Fluorescent immunocytochemistry

Primary cortical neurons grown on 13 mm poly-D-lysine coated coverslips in the presence or absence of recombinant TTR in the culture medium, were fixed with 4% neutral buffered formalin for 30 min and permeabilized by incubation with 0.2% Triton X-100 (Sigma) for 5 min. Fluorescence was quenched through incubation with fresh 0.1% sodium borohydride (Sigma) in PBS for 5 min, after which cells were incubated with MOM IgG blocking reagent (Vector) for 1 hour at room temperature. Incubation with the primary antibodies at the appropriate dilution in MOM diluent (Vector) was performed overnight at 4°C whilst secondary antibodies were diluted 1:1,000 and incubated for 1 h at room temperature. For staining of proliferative cells, monoclonal anti-alpha tubulin (Sigma) and rabbit anti-phosphohistone H3 antibodies (Upstate Biotechnology) (a kind gift from Paula Coelho, IBMC, Porto, Portugal) were used at 1:5,000 and 1:500, respectively. Donkey anti-mouse IgG-Alexa 488 and goat anti-rabbit IgG-Alexa 568 (Molecular Probes) were used as secondary antibodies. PGP 9.5 staining was performed as described above using donkey anti-rabbit IgG-Alexa 647 (Molecular Probes) as secondary antibody. Coverslips were mounted on glass slides using Vectashield Mounting Medium with DAPI (Vector) and visualized in the confocal laser scanning microscope Leica SP2 AOBS SE (Leica Microsystems).

For measurement of neurite outgrowth, PGP 9.5 staining was performed as described above using donkey anti-rabbit IgG-Alexa 488 (Molecular Probes) as secondary antibody. Neurite number was determined from 40x magnified fields. At least 150 neurons were analyzed for each condition.

Western blot

For western blot, primary cultures of cortical neurons were performed as described above, with the exception that cells were plated at a density of 3.6×10^6 cells/dish onto 60 mm poly-D-lysine/laminin coated dishes. 72 h after addition of TTR to the culture medium of selected dishes, cells were lysed with 300 μ L/dish lysis buffer (Kinexus). Protein concentration was determined using the PlusOne 2D Quant Kit (GE Healthcare), according to the manufacturer's instructions. 35 μ g of total protein per sample were run in 15% SDS polyacrylamide gels. After electrophoresis, samples were transferred to a nitrocellulose membrane (Amersham), blocked with blocking buffer (5% non-fat dried milk

in PBS), and incubated overnight at 4°C with the primary antibody rabbit anti-cyclin A (Abcam) diluted 1:100 in blocking buffer. Subsequently, incubation with horseradish peroxidase (HRP)-labeled secondary antibody anti-rabbit IgG-HRP (The Binding Site) diluted 1:10,000 in blocking buffer was performed for 1 hour at room temperature. Blots were developed using the ECL PlusTM Western blotting reagents (Amersham) and exposed to Hyperfilm ECL (Amersham).

Glutamate and GABA measurement

For measurement of glutamate and GABA intracellular levels, primary cultures of cortical neurons were performed as described above, with the exception that cells were plated at a density of 2.1×10^6 cells/dish onto 60 mm poly-D-lysine/laminin coated dishes. 72 h after addition of TTR to the culture medium of selected dishes, cells were lysed with 300 μ L/dish 0.1 M NaOH solution and cell lysate was collected and stored at -70°C until analysis. Intracellular glutamate and GABA levels were measured by reverse phase high-performance liquid chromatography (HPLC), as previously described (Rego et al., 1996). Briefly, samples were treated with 12% dithiotreitol (DTT) for 5 to 10 min and desproteinized with sulfosalicylic acid for 1 h at room temperature. After centrifugation at 15,800 *g*, for 2 min, to eliminate cell debris, supernatants were filtered and analyzed in a Gilson-ASTED HPLC system. Glutamate and GABA were detected as fluorescent derivatives after derivatization with o-phthaldialdehyde/2-mercaptoethanol, at 340 nm excitation and 410 nm emission. The concentrations of glutamate and GABA in the samples were determined by comparison with the peak areas of external standards.

Statistical analysis

Statistical analysis was performed using the Student's t-Test. Results were expressed as average $\pm$ SD. For all statistical analysis, $P < 0.05$ was accepted as being statistically significant.

Results

DIGE analysis revealed upregulation of five proteins in primary cortical neurons grown in the presence of TTR

To further ascertain the mechanism through which TTR downregulates PAM expression, DIGE analysis was performed in TTR KO primary cortical neurons grown either in the presence (termed KO+TTR condition) or absence (termed KO condition) of TTR in the culture medium. As replicate samples are highly recommended to exclude biological variation, we performed the DIGE analysis in two independent sets of primary cortical neurons grown in the presence or absence of TTR (**Figure 1**, gel 1 (50 μ g of protein extract) and gel 2 (150 μ g of protein extract)) and only spots altered in both experiments were identified.

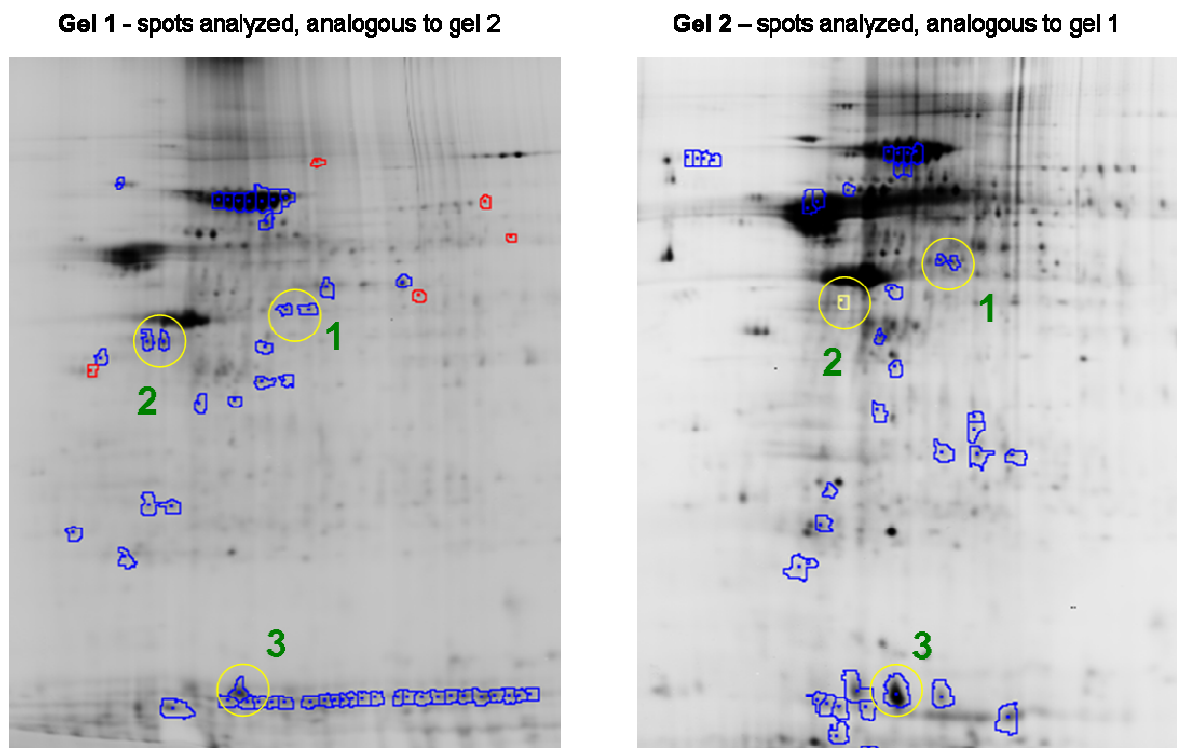


Figure 1. DIGE analysis in two independent experiments (gel 1 and gel 2) of primary cortical neurons grown in the presence or absence of TTR in the culture medium. Spots highlighted in blue correspond to proteins increased in the presence of TTR while spots in red correspond to proteins increased in TTR absence. Spots highlighted with a yellow circle (1, 2 and 3) correspond to spots altered in both experiments and the ones that were identified by MS.

Spots highlighted in blue correspond to proteins increased in the KO+TTR condition while spots in red correspond to proteins increased in the KO condition. **Table 1**

summarizes the results obtained. Heterogeneous nuclear ribonucleoprotein K (hnRNP K), alpha- and beta-tubulin, ornithine aminotransferase (OAT) and histone H3 were found consistently upregulated in primary cortical neurons grown in the presence of TTR, in the two independent experiments performed. While hnRNP K is involved in several aspects of the RNA life, alpha- and beta-tubulin are both markers of neuritic elongation, OAT is the enzyme that produces glutamate and histone H3 is involved in the regulation of cell proliferation. As shown in **Table 1**, these proteins are increased in the KO+TTR condition with a fold change higher than 1.5. These results were analyzed in association with data provided by the cDNA microarrays, as described below.

Protein Identified	KO+TTR/KO Ratio (CY5/CY3 Ratio)	Accession #
Pre-mRNA binding K protein, hnRNP K	2.08	gi 299029
Alpha-tubulin	1.58	gi 38328248
Beta-tubulin	2.68	gi 21746161
Ornithine aminotransferase, OAT	1.58	gi 8393866
Histone H3	2.32	gi 62642119

Table 1. DIGE results in TTR KO primary cortical neurons grown in the presence (KO+TTR) versus absence (KO) of TTR.

Differential gene expression in TTR KO primary cortical neurons grown in the presence versus absence of TTR

In parallel to DIGE analysis, cDNA microarrays were also performed in an attempt to further sort out the mechanism for TTR regulation of PAM expression. With these two approaches we cover a wide range of potential molecules involved in this process both at the protein and RNA levels. Only genes potentially involved in the physiology of the nervous system were selected and grouped according to their function. A large number of genes involved in cell proliferation was found upregulated in primary cortical neurons grown in the presence of TTR. For a matter of simplicity these genes are not enumerated in this thesis. It is noteworthy that histone H3 was one of the genes found differentially expressed in the microarrays, in accordance with the data from DIGE analysis in which this protein was also shown to be upregulated in the presence of TTR (as referred above). Additionally, 30 genes differentially expressed in primary cortical neurons grown in the presence versus absence of TTR were grouped in 8 major categories, according to their described function in the nervous system. These categories included neurogenesis, apoptosis, neurite outgrowth, retrograde axonal transport, synaptic transmission, integrin

signaling, cholesterol/steroid biosynthesis and GABA biosynthesis (**Table 2**). The lower bound of fold change of the differentially expressed genes was between 1.2 and 2.2 (**Table 2**). As the majority of categories were composed by several genes, some of which pointing towards opposing directions, our first approach was to address the functions in which genes were included and not the individual genes *per se*. We next describe the results obtained when attempting to evaluate the effect of TTR on cell proliferation, apoptosis, neurite outgrowth and GABA/Glutamate content.

GENE	Lower bound of fold change	Accession #
NEUROGENESIS		
G protein-coupled receptor 56 (secretin-like receptor)	1.29	NM_018882
Notch gene homolog 1/2	1.33	NM_008714
Bone morphogenetic protein 2 (BMP2)	1.42	AV239587
APOPTOSIS		
Baculoviral IAP repeat-containing 4 (BIRC4, XIAP)	-1.30	BF137345
Caspase 3	1.27	D86352
Fetal Alzheimer antigen	1.32	BB380312
Clusterin (apoJ)	1.34	NM_013492
Baculoviral IAP repeat-containing 5 (BIRC5, survivin)	1.48	BC004702
NEURITE OUTGROWTH		
Brain derived neurotrophic factor (BDNF)	-1.44	AY057913
Eph receptor A7	-1.35	BB075797
Eph receptor A3	-1.24	M68513
Microtubule-associated protein 2 (MAP2)	1.30	BM937510
Microtubule-associated protein 1B (MAP1B)	1.32	BB731480
Doublecortin	1.33	BB418548
Protein phosphatase 2, regulatory subunit B (B56), epsilon isoform	1.76	BB107628
RETROGRADE AXONAL TRANSPORT		
Myosin VI	1.24	BE133806
SYNAPTIC TRANSMISSION		
Synaptoporin	-1.21	BC026512
Synapsin II	1.25	NM_013681
Vesicle-associated membrane protein 2 (VAMP2, synaptobrevin 2)	1.31	BG871810
Piccolo	1.46	NM_011995
INTEGRIN SIGNALING		
Secreted phosphoprotein 1 (osteopontin)	-2.03	NM_009263
Poliovirus receptor-related 3 (nectin-3)	-1.37	NM_021495
Nephronectin	-1.33	AA223007
Vinculin	1.33	NM_009502
Reelin	1.47	NM_011261
Nischarin	2.23	BB025231

CHOLESTEROL/STEROID BIOSYNTHESIS		
NAD(P) dependent steroid dehydrogenase-like	1.32	BC019945
StAR-related lipid transfer (START) domain containing 4	1.41	AK014587
Lanosterol synthase	1.44	C77434
GABA BIOSYNTHESIS		
Glutamic acid decarboxylase 1 (GAD67)	1.41	AF326547

Table 2. Gene expression profiling in TTR KO primary cortical neurons grown in the presence (KO+TTR) versus absence (KO) of TTR. Genes displayed in black and with a positive value for the lower bound of fold change correspond to genes upregulated in the KO+TTR condition, while genes in red and with a negative value for the lower bound of fold change correspond to genes upregulated in the KO condition. In parenthesis the acronym or alternative name for the corresponding gene is indicated.

Proliferative cells in primary cultures of cortical neurons are not affected by the presence of TTR

Given the high number of genes involved in cell proliferation increased in primary cortical neurons cultured in the presence of TTR, we immunostained proliferative cells using antibodies for the phosphorylated form (at Serine 10) of histone H3 and for alpha-tubulin. The phosphorylation of Serine 10 occurs in cells that undergo mitosis starting at late G2 and this histone H3 modification was originally identified as a mitotic chromosome condensation marker. We have additionally used alpha-tubulin to easily identify the formation of mitotic spindle and subsequently to characterize the different mitotic stages. In both KO and KO+TTR conditions, few phospho-histone H3 (PH3)-positive cells were observed. Typically those cells exhibited several spots of PH3 staining colocalizing with brighter DAPI regions which are indicative of chromosome condensation. Nevertheless, we were not able to detect the presence of the mitotic spindle, as assessed by alpha-tubulin staining, in those PH3-positive cells, suggesting that these cells are in a pre-mitotic stage. A representative image of a proliferative, PH3-positive cell is shown in **Figure 2A**. Interestingly, nuclear chromosome condensation, observed by brighter DAPI staining regions and PH3 staining, was patent in cells of a non-neuronal origin, as determined by absence of staining with the neuronal cell marker PGP 9.5 (**Figure 2B**). Nevertheless, and in contrast with the results of the DIGE and microarrays which suggested increased cell division in the presence of TTR, no obvious differences in the number of proliferative cells were observed between KO and KO+TTR conditions.

TTR effect on proliferation was further investigated by western blot analysis of cyclin A, another marker of proliferation found upregulated in the presence of TTR by cDNA microarrays. Cyclin A is required for entry into mitosis, and for progress through mitosis until late prophase. No difference was observed in cyclin A levels from primary

cultures of cortical neurons grown either in the presence or absence of TTR in the culture medium (data not shown), confirming the lack of TTR effect on cell proliferation observed by immunofluorescent staining of proliferative cells in our culture system.

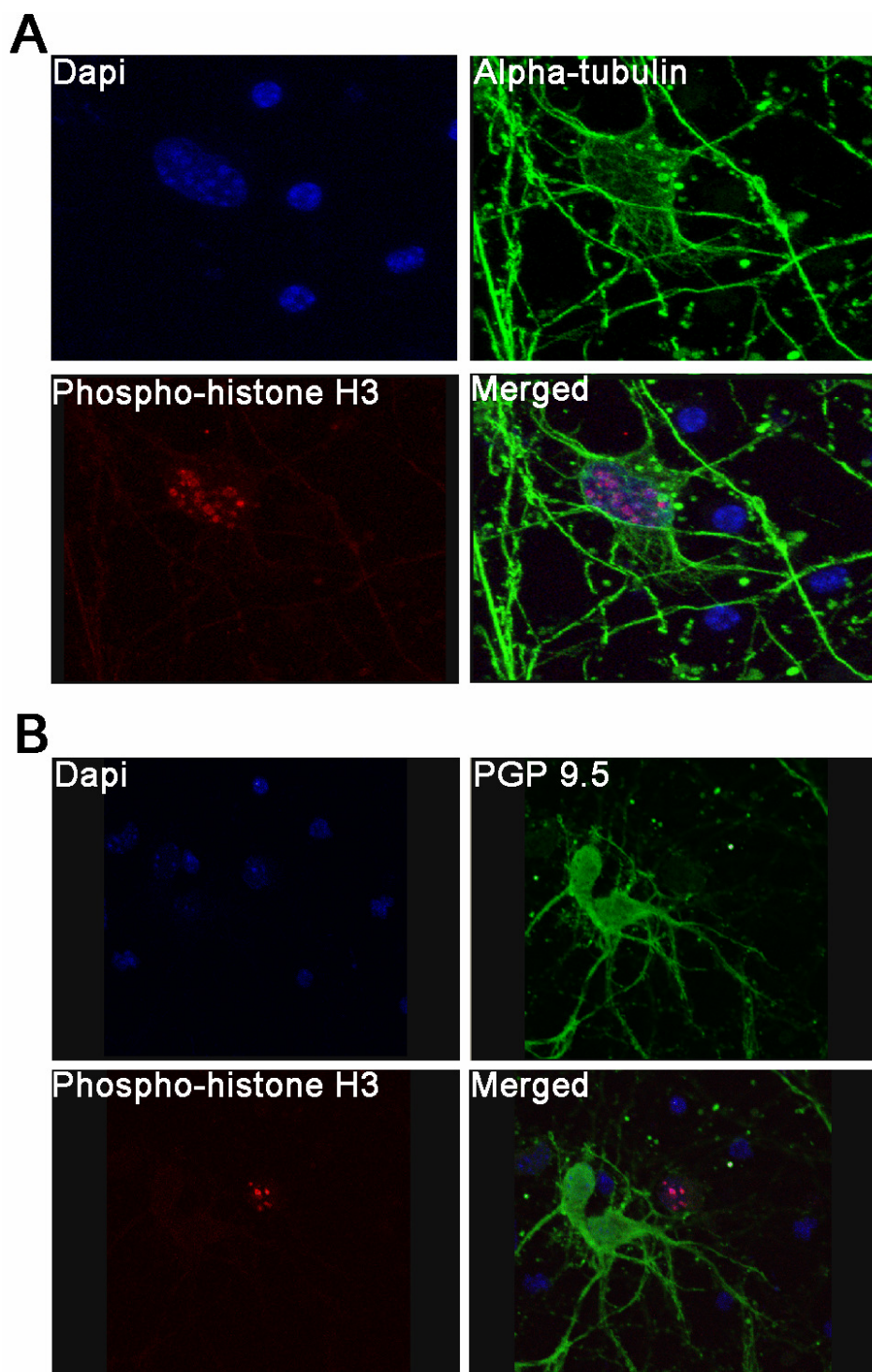


Figure 2. Representative images of proliferative cells in primary cultures of cortical neurons. **A)** DAPI staining of the cell nucleus is shown in blue, microtubules identified with alpha-tubulin staining are shown in green, phosphorilated-histone H3 is shown in red and the merged image of phosphorilated-histone H3 in the nucleus is shown in pink (63x magnification). **B)** DAPI staining of the cell nucleus is shown in blue, the neuronal marker PGP 9.5 is shown in green and phosphorilated-histone H3 is shown in red (40x magnification).

In primary cultures of cortical neurons, cells undergoing apoptosis are not neurons

To address apoptosis in primary cultures of TTR KO cortical neurons grown in the presence or absence of TTR, TUNEL assay was performed. As shown in **Figure 3**, DAB-positive, apoptotic cells (highlighted with arrows) were predominantly non-neuronal cells, as neurons were pink-labeled with the neuronal marker PGP 9.5. These contaminant cells were consistently present in our primary cultures of cortical neurons and had a morphology resembling blood-derived cells (erythrocytes, leucocytes) when observed under the microscope (data not shown). Given their great disparity in terms of morphology, function and overall metabolism when compared to neurons, the presence of blood cells in our preparation did not condition our study. The finding that these contaminant cells were undergoing apoptosis is not unexpected, as the culture media used in our assays is not appropriate for their survival. As no major apoptotic neurons were found, the putative influence of TTR on cell death was not further explored.

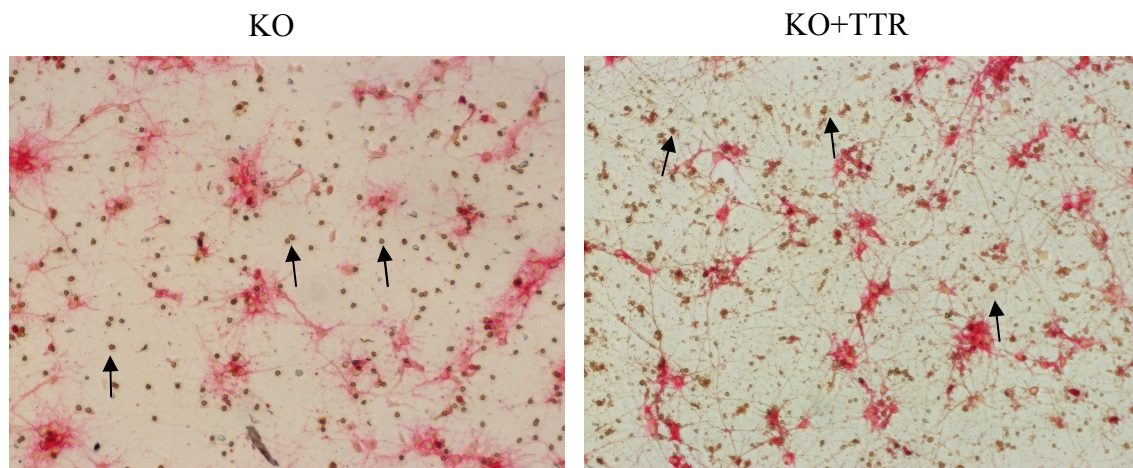


Figure 3. Representative images of TUNEL-positive cells in TTR KO primary cortical neurons grown in the absence (KO) or presence (KO+TTR) of TTR in the culture medium (20x magnification); arrows highlight DAB-labelled apoptotic cells.

In primary cultures of cortical neurons, TTR is not related to increased neurite number

Both DIGE and GeneChip analysis identified several genes and proteins involved in neurite outgrowth as being differentially regulated in the presence of TTR. To evaluate TTR effect in neurite outgrowth of primary cortical neurons, staining with the neuronal marker PGP 9.5 was performed for better visualization of neurites (**Figure 4**). We were however unable to determine neurite length due to the very intricate network of processes

in our primary cultures of cortical neurons. From the counting of neurite number per neuron, we observed that primary cortical neurons grown in the presence of TTR did not display increased neurite number when compared to neurons grown in its absence (**Figure 4**). This result was unexpected since a recently published study from our laboratory showed that addition of TTR to PC12 and DRG neuron cultures resulted in increased neurite number and length (Fleming et al., 2007). Moreover, the increased levels of alpha- and beta-tubulin in neurons grown in the presence of TTR as determined by DIGE, together with increased levels of MAP2 and MAP1B as determined by microarrays, further suggested that similarly to DRG neurons, cortical neurons grown in the presence of TTR displayed increased neurite outgrowth.

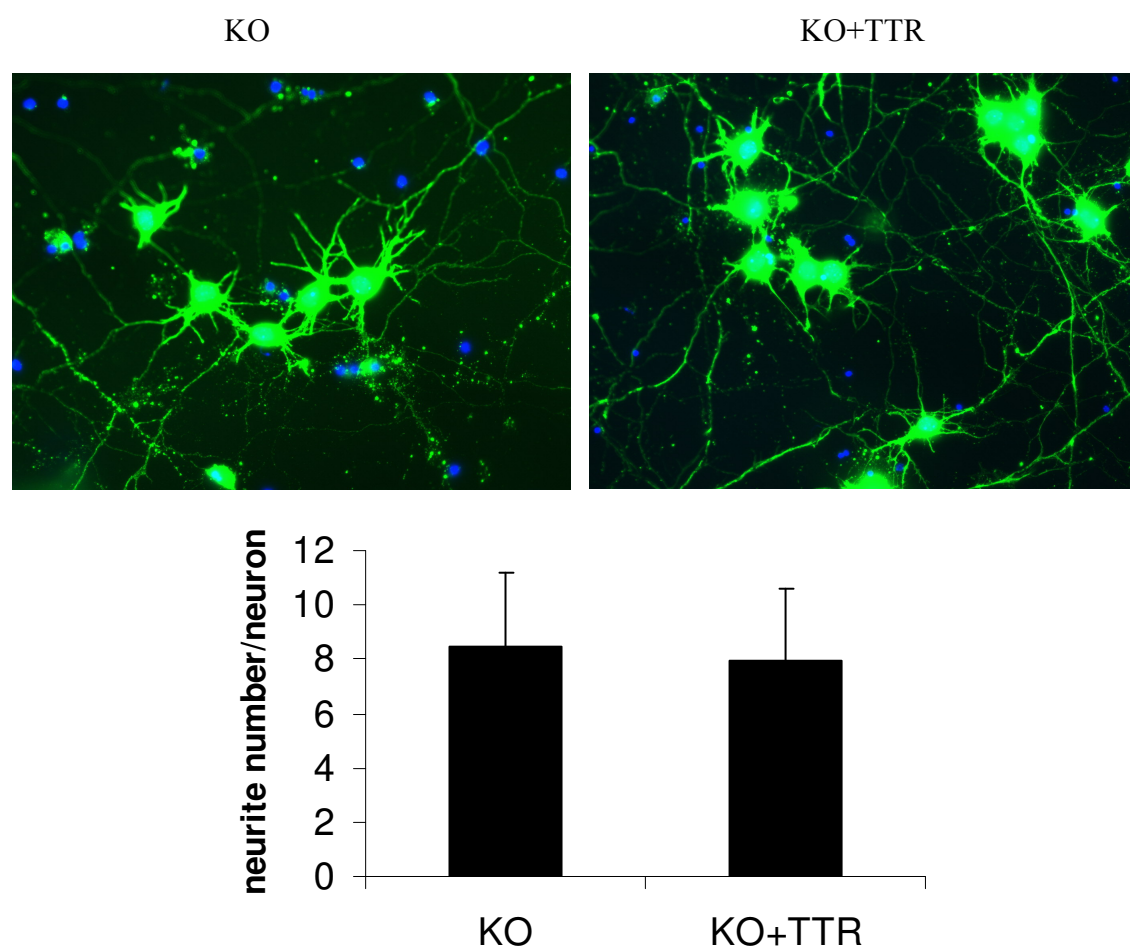


Figure 4. Neurite outgrowth in TTR KO primary cortical neurons grown either in the presence (KO+TTR) or absence (KO) of TTR in the culture medium. **Upper panel:** staining with the neuronal marker PGP 9.5 is shown in green while DAPI nuclear staining is shown in blue (40x magnification). **Lower panel:** quantification of neurite number per neuron. Results are presented as average $\pm$ SD.

Glutamate and GABA levels are decreased in primary cortical neurons incubated with TTR

To address the relevance of ornithine aminotransferase and glutamic acid decarboxylase 1 upregulation in primary cortical neurons grown in the presence of TTR, glutamate and GABA levels were determined, as the two enzymes are involved in the common biosynthetic pathway of both neurotransmitters. Surprisingly, glutamate and GABA intracellular levels were found strongly and consistently decreased in primary cortical neurons incubated with TTR when compared to cells grown in TTR absence (**Figure 5**). The relevance of this finding should be further addressed.

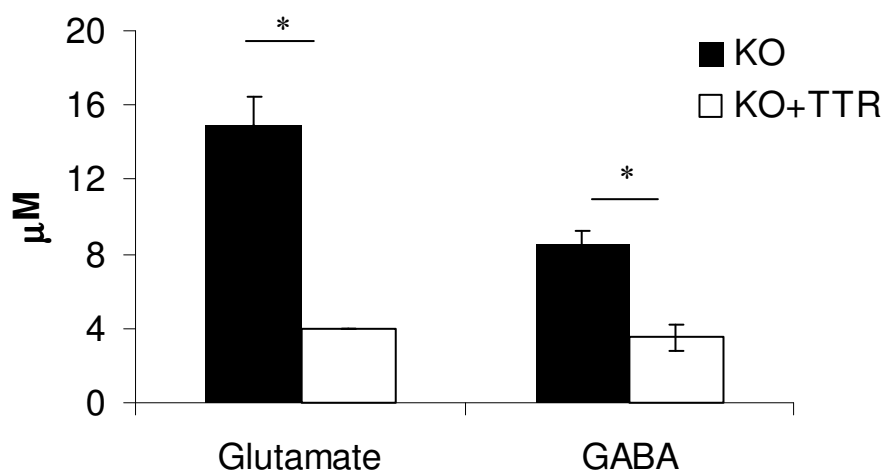


Figure 5. Glutamate and GABA levels in TTR KO primary cortical neurons grown either in the presence (KO+TTR) or absence (KO) of TTR. Results are presented as average $\pm$ SD; * $P < 0.05$.

Discussion

The high importance of establishing the mechanism through which TTR downregulates PAM expression (and as our first approaches failed to give us the answers, as discussed in Chapter 1), led us to the use of more powerful tools, such as comparative 2-dimensional (2D) electrophoresis and GeneChip analysis. By making use of these two highly valuable techniques we covered a wide range of molecules potentially involved in this process, both at the protein and RNA levels. Unfortunately, our major question remains to be answered as several genes and proteins were found differentially regulated by the presence of TTR, but none that could be directly associated with the regulation of PAM expression. On the other hand, many other questions were raised by the data obtained, some of which we tried to address.

It was very unexpected that PAM was not found differentially regulated by TTR neither in the protein nor in the gene arrays. However, it is known that RT-PCR is much more sensitive to detect gene variations than cDNA microarrays. In fact, by performing PAM semi-quantitative RT-PCR analysis using the same RNA samples used to perform the cDNA microarrays, we detected PAM overexpression in the absence of TTR (data not shown), further confirming our previous results. Similarly to PAM, and with the exception of histone H3, the proteins found by DIGE as being differentially regulated by TTR, did not appear differentially expressed in the GeneChip analysis, indicating that differences may exist when using distinct methodologies. By using complementary tools one may extend the range of molecules covered, though results obtained impose validation by other techniques, as false positives frequently arise.

With 2D-DIGE we were able to identify 5 proteins as being consistently upregulated with a fold change higher than 1.5 in primary cortical neurons grown in the presence of TTR, namely heterogeneous nuclear ribonucleoprotein K (hnRNP K), alpha- and beta-tubulin, ornithine aminotransferase and histone H3. Furthermore, GeneChip analysis revealed an extensive list of genes differentially regulated by TTR that were grouped according to their described function in the nervous system. These categories included neurogenesis, apoptosis, neurite outgrowth, retrograde axonal transport, synaptic transmission, integrin signaling, cholesterol/steroid biosynthesis and GABA biosynthesis. Some of the differentially regulated proteins found by DIGE were related with the data obtained from GeneChip analysis and as such, results were analyzed collectively.

The heterogeneous nuclear ribonucleoproteins comprise a large family of proteins that play important roles in telomere biogenesis, DNA repair, cellular signaling, and in the regulation of expression at both the transcriptional and translational levels. One of the

most extensively studied hnRNP family members, hnRNP K, has been implicated in a variety of processes, including chromatin remodeling, splicing, and translation events (reviewed in (Bomsztyk et al., 2004). Since its original identification in the nucleus as a component of the heterogeneous nuclear ribonucleoprotein complex, K protein has been also found in the cytoplasm and mitochondria. Through its multiple modules, K protein binds kinases while recruiting chromatin, splicing and translation factors, facilitating molecular cross-talk. Heterogeneous nuclear ribonucleoproteins are among the most abundant proteins in the eukaryotic cell nucleus and play a direct role in several aspects of the RNA life including splicing, export of the mature RNAs, stability and translation. With regard to a hnRNP K function in the regulation of PAM expression no reports exist in the literature. Moreover, a potential hnRNP K regulation of PAM mRNA stability was discarded, as no difference was observed in PAM mRNA decay in primary cortical neurons grown in the presence or absence of TTR (described in Chapter 1).

A large number of genes involved in the regulation of cell proliferation was found upregulated in primary cortical neurons grown in the presence of TTR by cDNA microarrays. One of these genes was histone H3 and its overexpression in the presence of TTR is in accordance with the data from DIGE analysis in which this protein was also shown to be upregulated by TTR. Histone H3 is one of the components of the nucleosome core particle, the basic unit of chromatin, which contains about 200 bp of DNA wrapping around an eight-histone complex. The histone octamer is composed of a central heterotetramer of histones H3 and H4, flanked by two heterodimers of histones H2A and H2B. Upregulation of histone H3 in the presence of TTR might indicate that DNA replication took place. Moreover, histone H3 phosphorylation has been observed during the initiation of chromosome condensation at G2 and entry into mitosis. Among the several genes involved in the regulation of cell proliferation found overexpressed in the presence of TTR was aurora B, originally identified as a mitotic protein and now known to be the major kinase responsible for the phosphorylation of histone H3 at Serine 10. For a matter of simplicity, and as genes related to proliferation constituted an extensive list, they were not enumerated in this thesis. However, the message they enclosed was that in the presence of TTR in our culture system, cell proliferation was increased. In fact, genes that were found differentially expressed regulate several cellular events that occur since DNA replication till mitotic progression, which are not commonly observed in cells that are differentiated. This result was unexpected as primary cultures of cortical neurons derived from embryonic day 17 (E17) are largely constituted by post-mitotic neurons. In fact, at this developmental stage, the emerging cerebral cortex beneath the pial surface is mainly composed of differentiated neurons whereas proliferating neuroblasts reside in the ventricular zone. In addition, the peak for astrocyte genesis spans a later period between

~ E18 and postnatal day one, suggesting that astrocytes, if present, are minimal in our culture system. By performing a staining with cell proliferation markers, we have indeed detected a few cells undergoing proliferation, although no difference was observed by the presence of TTR in the culture medium. These proliferative cells presented nuclear chromosome condensation, were probably in a pre-mitotic stage and, as expected, were non-neuronal. Their identity awaits to be unraveled. Absence of TTR involvement in cell proliferation was further confirmed by the analysis of cyclin A, another marker of proliferation found upregulated in the presence of TTR by cDNA microarrays. No difference was observed in the levels of this protein in primary cortical neurons grown either in the presence or absence of TTR in the culture medium, as assessed by western blot.

Microarray analysis revealed differential expression of five genes involved in apoptosis. Baculoviral IAP repeat-containing 4 (BIRC4, XIAP) and baculoviral IAP repeat-containing 5 (BIRC5, survivin) are both described as caspase inhibitors (reviewed in (Liston et al., 1997), and whilst the first was downregulated 1.3-fold in the presence of TTR the second was upregulated with a fold change of approximately 1.5 in the same culture condition. Furthermore, clusterin, caspase 3 and fetal Alzheimer antigen, all apoptotic factors (Han et al., 2001; Strachan et al., 2005; Kuribayashi et al., 2006), had their expression 1.3-fold increased by addition of TTR to the culture medium. Knowing that probably blood-derived cells in our primary cultures were the major contributors to the differentially regulated genes involved in apoptosis found by microarrays, led us to abandon the hypothesis that TTR might regulate cell death of brain-derived cells.

Interestingly, several genes involved in neurite outgrowth and microtubule maintenance were found differentially regulated in the presence of TTR. As discussed bellow, this finding was apparently in accordance with the recently described role of TTR as an enhancer of neurite outgrowth (Fleming et al., 2007). Eph receptors A3 and A7, whose expression was decreased around 1.3-fold in the KO+TTR condition, belong to the largest known family of tyrosine kinase signaling proteins, consisting of 8 different ligands and 14 well-characterized receptors (reviewed in (Zhou, 1998). Eph receptor ligands, the ephrins, are membrane-bound molecules that play important roles in regulating cell migration, axon pathfinding, and topographic mapping. Based on their specificity of interaction with ephrins, the receptors are classified in Eph A (ephrins A1-A5) or Eph B (ephrins B1-B3) receptors. Ephrins are well known for repulsive or inhibitory effects on axonal growth (reviewed in (Flanagan and Vanderhaeghen, 1998). However, some studies have shown that ephrins may also exhibit stimulatory effects, increasing axonal branching of neurons in cerebral cortex and medial hippocampus, and promoting neurite outgrowth of sympathetic neurons (Castellani et al., 1998; Gao et al., 1999; Gao et al.,

2000) and early cortical neurons (Zhou et al., 2001). Thus, the Eph/ephrin system may play a multifunctional role in neurite outgrowth, in a stage of development- and population-specific fashion. Brain derived neurotrophic factor (BDNF) was also found downregulated in the presence of TTR, with a 1.4 fold change. This neurotrophin supports neuronal development and survival, and stimulates neurite outgrowth (reviewed in (Wozniak, 1993). As such, and knowing that TTR enhances neurite outgrowth (Fleming et al., 2007), the decreased BDNF expression upon TTR addition is hard to explain. On the other hand, cDNA microarrays revealed upregulation of a number of genes involved in neurite outgrowth and microtubule maintenance in the presence of TTR, as is the case of the microtubule-associated proteins 1B and 2 (MAP1B, MAP2), upregulated 1.3-fold in the KO+TTR condition. MAP1B and MAP2 are the major components of the neuronal cytoskeleton and are best known for their microtubule-stabilizing activity and for their proposed roles in regulating microtubule networks in the axons and dendrites of neurons (reviewed in (Dehmelt and Halpain, 2005; Riederer, 2007). Strong evidence for a role in neurite outgrowth came from MAP1B and MAP2 suppression in both *in vivo* and *in vitro* models that lead to defects particularly at the level of neurite outgrowth initiation (Dinsmore and Solomon, 1991; Brugg et al., 1993; Gonzalez-Billault et al., 2002). In fact, there is a direct correlation between the increase in neurite length and the rate of microtubule assembly (Richter-Landsberg et al., 1983). Like MAP1B and MAP2, doublecortin is a microtubule-associated protein found 1.3-fold upregulated in the presence of TTR. This microtubule-stabilizing protein is mainly known for its involvement in neuronal migration (Ocbina et al., 2006). Finally from this group of genes, protein phosphatase 2, regulatory subunit B (B56), epsilon isoform was found 1.8-fold upregulated in the KO+TTR condition. The ubiquitously expressed serine/threonine-specific protein phosphatase 2A (PP2A) associates with regulatory subunits of different families. PP2A is particularly abundant in the brain where the various regulatory subunits show a subunit-specific expression pattern both during development and in the mature brain (reviewed in (Janssens and Goris, 2001). Its major neuronal substrates are the neurofilament subunit chains (Veeranna et al., 1995), and by regulating the phosphorylation of neurofilaments in neurons, PP2A preserves their filamentous form and regulates the interaction of cytoskeletal proteins during axonal outgrowth (Chiou and Westhead, 1992; Sacher et al., 1992; Saito et al., 1995). Furthermore, a role for PP2A in nerve growth factor (NFG)-mediated neurite outgrowth and maintenance of neurites was demonstrated (Chiou and Westhead, 1992). Additionally, alpha- and beta-tubulin were found upregulated by DIGE in primary cortical neurons grown in the presence of TTR. Alpha- and beta-tubulin are the building blocks of microtubules, being both markers of neuritic elongation (reviewed in (Ujike et al., 2002). In summary, the upregulation of

MAP1B, MAP2, doublecortin, protein phosphatase 2 regulatory subunit B (B56) and alpha- and beta-tubulin was in clear agreement with the ability of TTR to increase neurite outgrowth. Nevertheless, by addressing neurite number in primary cortical neurons grown either in the presence or absence of TTR, no TTR stimulatory effect in neurite outgrowth was observed. This is in sharp contrast to the report of increased neurite outgrowth in DRG neuron cultures incubated with TTR (Fleming et al., 2007) and might reflect the differential expression of stimulatory and inhibitory genes associated with axonal growth found in the cDNA microarrays. A different TTR effect in the peripheral and central nervous system may also occur. However, despite no effect of TTR in neurite number was found, an effect in neurite extension can not be excluded, as neurite length could not be determined due to the very intricate network of processes in our primary cultures of cortical neurons. This issue clearly needs to be further addressed in the future as at this time point it is still plausible that TTR may increase neurite length in both PNS and CNS neurons.

Myosin VI was also found overexpressed in primary cortical neurons grown in the presence of TTR with a fold change of 1.24. This actin-dependent motor protein is involved in the retrograde axonal transport of vesicles, playing a key role in clathrin-mediated endocytosis (Osterweil et al., 2005). Upregulation of this gene in the presence of TTR in primary cortical neurons is in agreement with data from our laboratory demonstrating that WT DRG neurons display increased retrograde axonal transport when compared to TTR KO DRG neurons, as assessed by incubation with transferrin conjugated with Texas red (Fleming et al., in preparation).

In relation to glutamate and GABA synthesis, two enzymes involved in this pathway were upregulated in the presence of TTR: ornithine aminotransferase (OAT, found by DIGE) and glutamic acid decarboxylase 1 (GAD67, found by microarrays). OAT was 1.5-fold upregulated by DIGE in primary cortical neurons grown in the presence of TTR. This mitochondrial matrix enzyme catalyzes glutamate synthesis from ornithine as part of the urea cycle (reviewed in (Seiler, 2000). Ornithine is not supplied in the diet but can be produced from endogenous or dietary arginine through the action of arginase. The vertebrate brain has the machinery to transport arginine and ornithine, and to form glutamate from these amino acids within nerve endings. OAT is thus a key enzyme of the Arg-->Orn-->Glu pathway in the brain. Interestingly, expression of GAD67 was found 1.4-fold increased in primary cortical neurons grown in the presence of TTR by GeneChip analysis. GAD67 is the enzyme that promotes the synthesis of GABA, the main inhibitory neurotransmitter in the brain. It was demonstrated more than 20 years ago that glutamate is the substrate for GABA production through glutamic acid decarboxylase, in nerve terminals (Johnson and Roberts, 1984). Thus, our results pointed to the hypothesis that a

common pathway exists in primary cortical neurons grown in the presence of TTR leading to increased levels of glutamate and, ultimately GABA. However, contrary to our hypothesis, both glutamate and GABA were found strongly reduced in our cortical neurons incubated with TTR. The relevance of this finding should be addressed *in vivo*, by determining the levels of these neurotransmitters in the cerebrospinal fluid.

Although GeneChip is a powerful tool, the data obtained need further validation. In this respect, additional studies should be performed to corroborate the influence of TTR in genes and functions not addressed in this thesis (namely synaptic transmission, integrin signaling and cholesterol/steroid biosynthesis). In relation to neurogenesis, the influence of TTR in this process was investigated in Chapter 3 – Part II of this thesis.

In summary, despite being highly valuable techniques, 2D-DIGE and cDNA microarrays did not fulfill our ambition. Several genes and proteins were found differentially regulated by the presence versus absence of TTR in primary cortical neurons, but none that could be directly associated with TTR regulation of PAM expression. Nevertheless, these approaches were useful in raising other questions and hypothesis, some of them already corroborated with work from our laboratory.

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CHAPTER 3

PART I

TRANSTHYRETIN KNOCKOUTS ARE A NEW MOUSE
MODEL FOR INCREASED NEUROPEPTIDE Y

TRANSTHYRETIN KNOCKOUTS ARE A NEW MOUSE MODEL FOR
INCREASED NEUROPEPTIDE Y

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Abstract

We have previously shown that the key enzyme in neuropeptide amidation, peptidylglycine α -amidating monooxygenase (PAM), is overexpressed in the peripheral (PNS) and central nervous system (CNS) of transthyretin (TTR) knockout (KO) mice. Here we report that, as a consequence of PAM overexpression, TTR KOs display increased neuropeptide Y (NPY) levels in the CNS and PNS. NPY acts on energy homeostasis by increasing white adipose tissue lipoprotein lipase (LPL) and by decreasing brown adipose tissue thermogenesis; accordingly, we show increased LPL expression and activity in white adipose tissue, PNS, and CNS as well as decreased body temperature in TTR KOs. Associated to increased NPY levels, TTR KOs display increased carbohydrate consumption and preference. In PC12 cells and primary cortical neurons, absence of TTR is related to increased PAM activity, NPY levels and LPL expression, reinforcing that TTR is involved in neuropeptide maturation and that increased NPY correlates with LPL overexpression in the nervous system. Furthermore, we provide molecular insights to the reduced depressive-like behavior of TTR KOs, as NPY is anti-depressant. TTR internalization seems to be a critical step for the regulation of PAM expression and, consequently, amidated NPY levels. Our findings demonstrate that TTR KOs are a model for increased NPY and that TTR plays a role in nervous system physiology.

Introduction

Our previous findings demonstrate that TTR plays a role in nervous system physiology, namely by regulating neuropeptide maturation through downregulation of peptidylglycine α -amidating monooxygenase (PAM) expression. PAM is involved in the posttranslational processing of many neuropeptides (Eipper et al., 1992). It catalyzes the formation of α -amidated peptides from precursor molecules with a COOH-terminal glycine. Conversion of a peptidylglycine substrate into α -amidated product involves a two-step process resulting from the sequential actions of two enzyme activities (Tajima et al., 1990). Both enzymes are derived from the bifunctional PAM protein (Perkins et al., 1990). The first enzyme, peptidylglycine α -hydroxylating monooxygenase (PHM), produces an α -hydroxylated intermediate in the presence of copper, ascorbate, and molecular oxygen. The second enzyme, peptidyl- α -hydroxyglycine- α -amidating lyase (PAL), cleaves the peptidyl- α -hydroxyglycine intermediate to form the α -amidated peptide and glyoxylate. Over half of the known neuropeptides require α -amidation for biological activity (Eipper et al., 1992), and PAM is the only enzyme known to promote this reaction.

As PAM knockouts do not exist, the significance of peptide amidation in several physiological functions is poorly understood. Elimination of PHM activity in *Drosophila* is embryonic lethal (Kolhekar et al., 1997) and the same happens with PAM null mutant mice (Czyzyk et al., 2005). Because PAM requires copper, *in vivo* and *in vitro* models of altered copper availability are used to examine neuropeptide functions. Mottled (Brindled) mice, which lack a functional Menkes copper transporter (ATP7A), limiting copper transport into the secretory pathway (Suzuki and Gitlin, 1999), serve as a model for Menkes disease (Grimes et al., 1997) and provide an *in vivo* partial loss-of-function PAM knockout. Despite normal levels of PAM protein, Mottled (Brindled) mice presented reduced levels of several amidated peptides in pituitary and brain extracts, namely joining peptide, α -melanocyte stimulating hormone and cholecystokinin (Steveson et al., 2003). Furthermore, by limiting the availability of copper *in vitro* or *in vivo* by additional methods, the production of α -amidated peptides such as α -melanocyte stimulating hormone (Mains et al., 1986; Mueller et al., 1993), cholecystokinin (Mueller et al., 1993), luteinising-hormone releasing hormone and substance P (Steveson et al., 2003) was found decreased.

PAM has a broad substrate specificity, is found in a variety of tissues, and is regulated in a tissue-specific fashion (Eipper et al., 1992). The highest levels of PAM are found in the pituitary, adrenal medula, atrium of the heart, and central nervous system (Eipper et al., 1985), where the enzyme is detected in all major brain areas with the exception of the cerebellum (Bolkenius and Ganzhorn, 1998). In particular, the hypothalamus, hippocampus and olfactory cortex represent the major sources of the

protein in the brain (Bolkenius and Ganzhorn, 1998). PAM activity was also detected in dorsal root ganglia and sciatic nerves (Takasugi et al., 1996). In both peripheral and central neurons PAM is localized to large dense core vesicles in axons and nerve terminals (Oyarce and Eipper, 1993). Generation of peptidylglycine substrates for PHM requires prohormone cleavage by the appropriate prohormone convertases and carboxypeptidases.

In the present work, the biological implications of PAM overexpression in TTR KOs was assessed. Our data show that TTR KOs are a model for increased NPY levels, further reinforcing TTR role in the physiology of the nervous system.

Materials and Methods

Animals

Mice were handled according to European Union and National rules. WT and TTR KO mice (Episkopou et al., 1993), in the 129/Sv background, bred separately, and TTR KO and WT littermate offspring of heterozygote breeding pairs, were maintained at $24 \pm 1^\circ\text{C}$ under a 12 h light/dark cycle and fed regular chow and tap water ad libitum. Unless otherwise stated, comparisons comprised groups of 6 animals of each genotype, age and sex matched. All animals were accustomed to regular handling. Mouse core body temperature was measured in the tail base, using a thermofocus infrared thermometer (Tecnimed) (Saegusa and Tabata, 2003).

RT-PCR analysis

Total RNA was isolated with Trizol (Invitrogen) and subjected to RT-PCR with the Superscript II kit (Invitrogen). PCR was performed for the appropriate number of cycles for each cDNA (20-40 cycles) at 95°C for 1 min, 56°C for 2 min, and 72°C for 3 min. Primers were designed using PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the sequence from the National Centre for Biotechnology Information database. Sense and antisense primers are shown in **Table 1**. Ethidium bromide-stained gels were scanned using a Typhoon 8600 (Amersham) and amplified bands were quantified using the ImageQuant software (Amersham). Fluorescence density of each PCR-amplified band was normalized with the corresponding value of either β -actin or hypoxanthine-guanine phosphoribosyltransferase (HPRT). For sequencing of the RT-PCR products of prohormone convertase 1 (PC1) and 2 (PC2), DNA from gel bands was extracted using the QIAEX II kit (Qiagen), according to the manufacturer's instructions. Quantities (100 ng) of each amplification product were sequenced as a service (Seqlab) using 20 pmol of the corresponding sense primer.

Immunohistochemistry

Sections were incubated in blocking buffer (1% bovine serum albumin, BSA, and 4% horse serum in phosphate buffered saline, PBS) for 30 min at 37°C , and with the primary antibody rabbit polyclonal anti-LPL (Dr. Ramji, Cardiff University) 1:100 in blocking buffer overnight at 4°C . Antigen visualization was performed with the biotin-extravidin-peroxidase kit (Sigma). On control sections, primary antibody was replaced by blocking buffer. Two observers performed the immunohistochemistry analysis independently.

Neuropeptide Y and substance P quantification

NPY and substance P quantification was performed by radioimmunoassay (RIA) with Bachem kits. Bachem's anti-NPY antibody has been developed using as antigen amidated NPY₁₋₃₆; measurements using as antigen NPY₁₋₃₆ free acid, i.e., lacking the C-terminal α -amidation (Bachem), in the same serial dilutions as the ones used for the NPY₁₋₃₆ amidated standard showed a cross reactivity of 39%. CSF was collected with a 26-gauge needle through the fourth ventricle using a stereotaxic device; samples presenting more than 10 leukocytes/ μ L and 100 erythrocytes/ μ L were excluded. Peptides from CSF, plasma and cell culture conditioned media were extracted by using C18 Sep-columns (Bachem), which yielded ~90% recovery as previously reported (Theodorsson-Norheim et al., 1987), and were subsequently lyophilised. Hippocampus and spinal cord were sonicated in 400 μ L RIA buffer. After 5 min centrifugation at 15,000 *g*, 100 μ L of supernatant was used in the assay. For peptide extraction from DRGs and sciatic nerves, samples were boiled 10 min in 10 volumes of extraction buffer (2 M acetic acid, pH 3.4), homogenised, sonicated and centrifuged 15 min at 3,000 *g*. Supernatants were lyophilised and pellets were used for total protein quantification using the BioRad reagent (Bio-Rad). To determine the percentage of NPY recovery from tissue samples, spiking experiments were performed. Tissues were equally divided in two halves, and 50 pg of amidated NPY₁₋₃₆ (Bachem) was added to one-half. Extraction procedures and NPY quantification by RIA were carried out as usual. Percentage of recovery (~50%) was calculated from the difference in NPY content obtained between the spiked and non-spiked half. After peptide extraction, lyophilized pellets were reconstituted in 210 μ L RIA buffer, to perform the assay with duplicate 100 μ L samples. Amidated NPY₁₋₃₆ was used as a standard in serial dilutions that ranged from 1 to 128 pg/tube. In brief, test tubes were incubated sequentially with rabbit anti-NPY primary antibody (except total count and nonspecific binding tubes) and radiolabeled ¹²⁵I-NPY tracer, for 24 h at 4 °C. Samples were subsequently incubated with goat anti-rabbit IgG serum and non-immune rabbit serum for 90 min at room temperature. After centrifugation at 3000 rpm, supernatants were aspirated (except in total count tubes) and tubes were counted in a gamma counter (Wizard 1470). The standard curve was generated by plotting the specific binding percentage, i.e., % sample specific binding/total specific binding of the assay (which corresponds to the difference between total binding and nonspecific binding) vs. the log of concentrations of standards. "Best fit" curves were obtained using the GraphPad Prism software. Based on the standard curve and on the specific binding percentage of each sample, the concentration of NPY in each tube was calculated. The coefficients of intra-assay and inter-assay variation were 5 and 20%, respectively. The range of detection of the kit is 10–1280 pg/mL, the IC₅₀ is 310

pg/mL and the limit of quantification is 80 pg/mL. NPY and substance P contents were normalized either per tissue weight, total protein or, in the case of CSF and plasma, per sample volume.

Binding of TTR to amidated NPY₁₋₃₆

Recombinant TTR was produced as described (Liz et al., 2004) and iodinated as previously reported (Sousa et al., 2000a). Binding assays were performed as established (Sousa et al., 2000b). Briefly, 96-well plates (Maxisorp, Nunc) were coated overnight with 5 µg/well of either amidated NPY₁₋₃₆ (Bachem), or the TTR ligand RAGE, used as a positive control (Sousa et al., 2000b). ¹²⁵I-TTR (30 ng) was added to each well alone or in competition with 1, 10, 100, or 500X molar excess of unlabeled TTR in 0.1% skim milk for 2 h at 37°C. Binding was determined after washing in phosphate buffered saline with 0.05% Tween 20 and elution in 0.1 M NaCl containing 1% nonidet P40 for 5 min at 37°C. The contents of each well were counted in the gamma counter.

Lipoprotein lipase activity assays

LPL activity assays were performed using the Total Lipase Test kit (Progen Biotechnic). Mice were starved overnight, and the procedure was completed during the following morning, due to the enzyme's diurnal variation. Tissues were weighted, homogenized in Krebs'-Ringer's buffer, and heparin (Sigma) was added to a final concentration of 100 U/mL. After 45 min incubation at 37°C and 10 min centrifugation at 14,000 g, supernatants were used in the assay. LPL activity was normalized either per tissue weight or total protein.

High-carbohydrate diet preference test

TTR KO (n=9) and WT (n=6) male mice were individually housed and their intake of regular chow was followed for the period of 4 weeks. Mice were then habituated to eat two different diets over 3 days: standard diet (68% carbohydrate, 27% protein, 4% fat) and high-carbohydrate diet (77% carbohydrate, 20% protein, 3% fat), both from Mucedola. In the experimental test, mice had free access to both diets during 4 days. The body weight and intake of each diet were measured every day, and placement of food was changed daily to avoid place preference. The average food intake per day was calculated and corrected for individual differences by normalizing to body weight of individual mice.

NPY levels and LPL expression in PC12 cells and primary cultures of cortical neurons

Rat pheochromocytoma PC12 cells (American Type Tissue Culture Collection) were grown in 6 well plates in William's medium E (Invitrogen) supplemented with 2 mM L-glutamine (Sigma) and 10% fetal calf serum (FCS, Invitrogen). When cells reached 50% confluence, FCS was withdrawn. For experiments in the presence of mouse serum, the William's E medium was supplemented with 100 ng/mL 2.5S nerve growth factor (NGF) (Sigma) and 10% of either TTR KO mouse serum or TTR KO mouse serum containing 300 µg/mL recombinant TTR, produced as described (Liz et al., 2004). For experiments in serum-free conditions, supplement B-27 (Invitrogen) was used; in selected wells, the PAM inhibitor 4-phenyl-3-butenoic acid (Sigma) or the non-selective NPY antagonist (D-Tyr^{27, 36}, D-Thr³²)-NPY₂₇₋₃₆ (Bachem) were added to a final concentration of 0.1 mg/mL or 0.1 µM, respectively. After 72 h, cells were lysed with Trizol and total RNA was extracted from the aqueous phase and processed as described above. The protein in each sample was extracted from the organic phase obtained after Trizol lysis, according to the manufacturer's instructions. Protein content was subsequently determined using the Bradford method, as described above. Cortical neurons were obtained from embryonic day 17 (E17) TTR KO mouse brains. After removing all meningeal shreds, the cortex was dissected and digested for 8 min at 37°C with 0.05% trypsin. The tissue was pelleted by centrifugation (600 g, 5 min), resuspended in 2 mL DMEM (Invitrogen) and triturated with decreasing diameter bore flame-polished glass pipettes. The cell suspension was filtered through a cell strainer (Becton Dickinson) and centrifuged 5 min at 600 g. Cells were resuspended in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen) and 0.5 mM L-glutamine (Sigma) and were plated at 1.6×10^6 cells/well onto six-well plates coated with poly-D-lysine/laminin (Sigma). Three days after, the culture medium was supplemented with 10% of either TTR KO mouse serum or TTR KO mouse serum containing 300 µg/mL recombinant TTR. After 72 h, total RNA and protein were extracted as described for PC12 cells.

Measurement of NPY levels in primary cultures of cortical neurons cultured in the presence of TTR coupled to FluoSpheres

Recombinant TTR was covalently bound to 1 µm-diameter carboxylate-modified FluoSpheres (Molecular Probes), using a carbodiimide cross-linking method, according to the manufacturer's instructions. The TTR-FluoSpheres complex was washed 3 times with PBS and the efficacy of the method was evaluated by running the 3 washes in a 15% SDS polyacrylamide gel to detect free uncoupled TTR; >90% TTR was bound to the

FluoSpheres with no detectable free TTR found within the last wash. Primary cultures of cortical neurons were performed as described above and TTR KO mouse serum (KO condition) was supplemented with: i) 300 µg/mL of free recombinant TTR (KO+TTR), ii) 300 µg/mL of recombinant TTR coupled to 0.12% FluoSpheres (KO+TTR-Fluo), and iii) 300 µg/mL of free recombinant TTR and 0.12% free FluoSpheres (KO+TTR+Fluo).

Analysis of TTR endocytosis by primary cortical neurons

Primary cultures of cortical neurons were performed as described above, with the exception that cells were plated at a density of 2.4×10^5 cells/well onto 13 mm poly-D-lysine coated coverslips, placed in 24-well plates. On day 3 after plating, culture medium was supplemented with 10% TTR KO mouse serum containing 300 µg/mL of recombinant TTR conjugated with Alexa 488 (TTR-Alexa 488, produced as described below) for 3 hours at 4°C or 37°C. Cells were fixed in 4% neutral buffered formalin for 30 min, washed with PBS, and immunocytochemistry for the neuronal marker protein gene product 9.5 (PGP 9.5) was performed. Briefly, cells were permeabilized by incubation with 0.2% Triton X-100 (Sigma) for 5 min, fluorescence was quenched through incubation with fresh 0.1% sodium borohydride (Sigma) in PBS for 5 min, after which cells were incubated with MOM IgG blocking reagent (Vector) for 1 hour at room temperature. Incubation with the primary antibody rabbit anti-PGP 9.5 (Serotec) 1:500 in MOM diluent (Vector) was performed overnight at 4°C while the secondary antibody goat anti-rabbit IgG-Alexa 568 (Molecular Probes) was diluted 1:1,000 in MOM diluent and incubation was performed for 1 hour at room temperature. Coverslips were mounted on glass slides using Vectashield Mounting Medium with DAPI (Vector) and visualized in the confocal laser scanning microscope Leica SP2 AOBS SE (Leica Microsystems). TTR was produced as previously described (Liz et al., 2004) and conjugated with Alexa 488 using the Alexa Fluor 488 labeling kit (Molecular Probes), according to the manufacturer's instructions. TTR-Alexa 488 was separated from free Alexa 488 by fine size exclusion chromatography using purification columns containing Bio-Rad BioGel P-30 resin. Subsequently, 1 µg TTR-Alexa 488 was run in a 15% SDS polyacrylamide gel, and, after electrophoresis, TTR-Alexa 488 was visualized using a Typhoon 8600 (Amersham) to check labeling efficacy.

Statistical analysis

Statistical analysis was performed using the Student's t-Test. Results were expressed as average $\pm$ SD or average $\pm$ SEM. For all statistical analysis, $P < 0.05$ was accepted as being statistically significant.

	Sense primer (5'-3')	Antisense primer (5'-3')
Mouse β -actin	GTGGGCCGCTCTAGGCACCAA	CTCTTTGATGTCACGCACGATTTC
Rat β -actin	TGTCACCAACTGGGACGATA	TCTCAGCTGTGGTGGTGAAG
Mouse HPRT	GTAATGATCAGTCAACGGGGGAC	CCAGCAAGCTTGCAACCTTAACCA
Rat PAM	AAAAATGGAGCATCGGTCAG	TTCCCTTTCCTCGGAATCTT
Mouse NPY	TGGACT GACCCTCGCTCTAT	GATGAGGGTGGAACTTGGA
Rat NPY	TGGACTGACCCTCGCTCTAT	TGTCTCAGGGCTGGATCTCT
Mouse LPL	GCGTAGCAGGAAGTCTGACC	AGCCCTTTCCTCAAAGGCTTC
Mouse NPY receptor Y1	GTCCTTGCAGTGGCTTCTTC	TGATTCGCTTGGTCTCACTG
Mouse NPY receptor Y2	CCTTGATGGGAGAGTGGA	CCTTCGCTGATGGTAATGGT
Mouse NPY receptor Y5	TGTGGATTGTCCCACAAAGA	CATCCAGCTAACAGCGAACA
Rat PC1	CCTGTAGGCACCTGGACATT	GGCTTGTTGAGCTTTTCCAG
Rat PC2	CAGCTTCACGATGAGGTTCA	GGTCATGAAAGGCCACTTGT

Table 1. Sense and antisense primers used to amplify each cDNA of interest.

Results

Neuropeptide Y is increased in TTR KO mice

To evaluate whether PAM overexpression in TTR KO mice has biological consequences resulting from increased PAM enzymatic activity, we compared amidated NPY content in TTR KO and WT animals. NPY is the major neuropeptide present in the mammalian brain (Pedrazzini et al., 2003) and its activation requires C-terminal α -amidation by PAM. As the CSF acts as a drain for brain neurotransmitters, in a way that CSF neurotransmitter content reflects CNS synaptic events (Valenzuela et al., 2000), the NPY content in the CSF of TTR KO and WT mice was evaluated; TTR KO mice (n=6) presented approximately 2-fold increased CSF NPY than WT mice (n=7) (**Figure 1A**). To exclude the interference of genetic background, WT and TTR KO littermates were also compared for CSF NPY, and similar results were obtained (not shown). As the hippocampus represents one of the most abundant NPY sources, we assessed its NPY levels; hippocampus from TTR KO mice (n=5) presented 5-fold increased NPY than WT hippocampus (n=5) (**Figure 1A**). In the PNS, TTR KO mice displayed 2 and 3.2-fold increased NPY in the DRG and sciatic nerve, respectively (**Figure 1A**). To evaluate the possibility that NPY might bind to TTR resulting in a decrease of neuropeptide having free access to the antibody used in the RIA assay, or to NPY receptors leading to functional consequences, NPY-TTR binding assays were performed. Amidated NPY₁₋₃₆ did not display any specific TTR binding, as demonstrated by the lack of displacement by increasing molar excess of cold TTR (**Figure 1B**). To exclude variation of NPY as a consequence of differential expression, we performed semiquantitative NPY RT-PCR analysis in DRG (**Figure 1C**), brain, hippocampus and sciatic nerve (not shown) from TTR KO (n=7) and WT mice (n=8); in all tested tissues, no statistical differences were found in NPY mRNA, supporting the hypothesis that increased NPY in TTR KO mice results from increased processing and activation by PAM. Semiquantitative RT-PCR analysis for NPY receptors Y1, Y2, and Y5 was performed in the nerve, DRG, and hippocampus from WT and TTR KO mice. No statistical differences resulting from the increase in amidated NPY were observed in the expression of NPY receptors between both strains (data not shown).

As PAM is responsible for amidating over 50% of all known neuropeptides, it is plausible that other amidated neuropeptides might be increased in TTR KO mice. To evaluate this hypothesis, we compared WT and TTR KO mice for substance P levels, a neuropeptide known to regulate inflammation and noxious information in the PNS (Hiruma et al., 2000). Although in the spinal cord, the major site of substance P production in the CNS, no differences were found between both strains (not shown), in the PNS, DRGs

from TTR KO mice presented 1.5-fold increased substance P (n=6) when compared to WT (n=8) (**Figure 1D**).

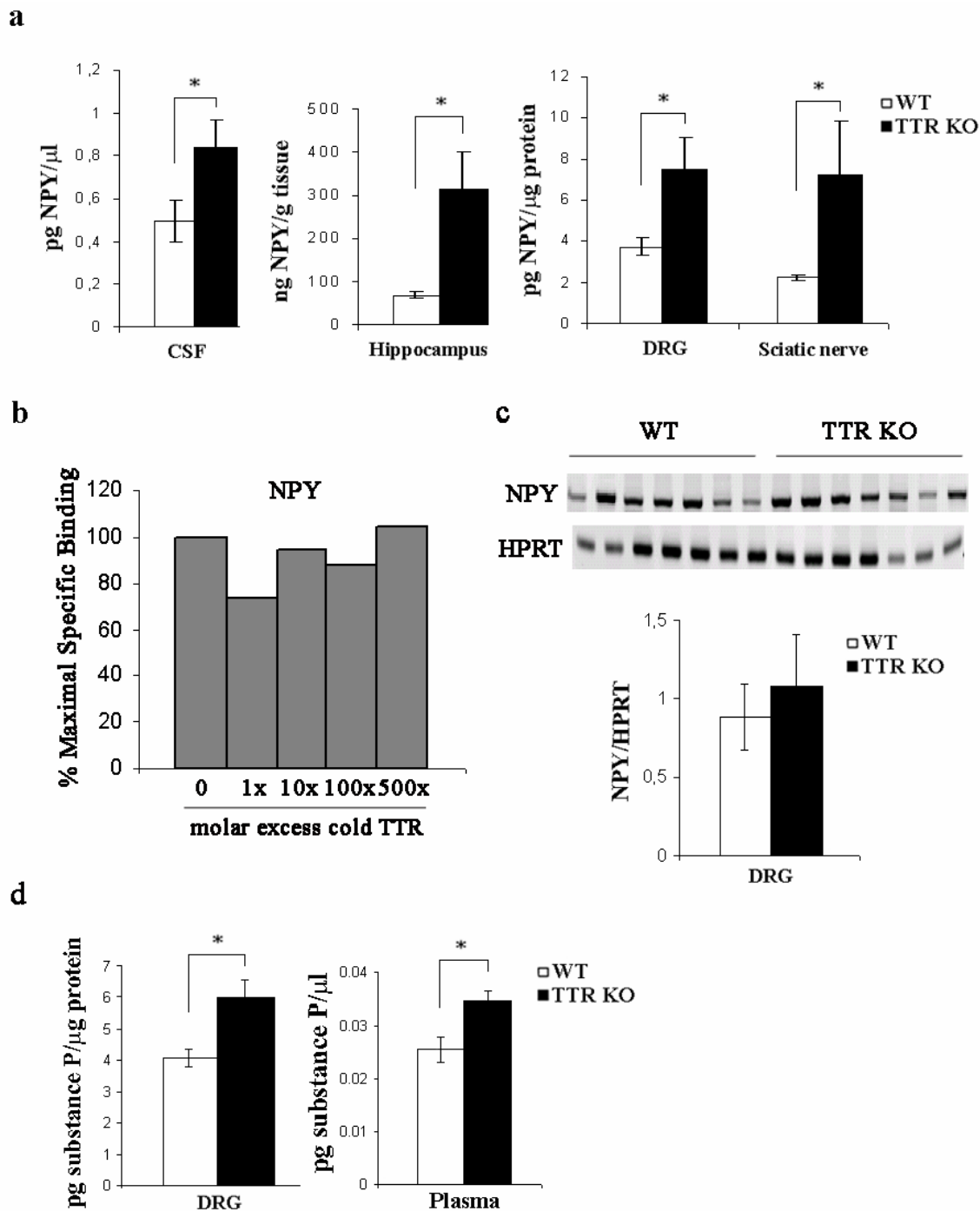


Figure 1. Neuropeptide content in TTR KO and WT mice. **A)** NPY content in the CSF, hippocampus, DRG, and sciatic nerve. **B)** NPY-¹²⁵I-TTR binding assay in the presence of excess molar concentration of cold TTR. **C)** Semi-quantitative RT-PCR analysis of NPY expression in DRGs. **D)** Substance P content in DRGs and plasma. **A, C, D)** Results are presented as average $\pm$ SEM; * $P < 0.05$.

As plasma substance P content reflects the levels of secretion of amidated substance P by the intestine, which is the non-neuronal peripheral tissue with higher substance P production, we compared the plasma levels of this neuropeptide in TTR KO and WT mice and found a 1.4-fold increase in the TTR KOs (**Figure 1D**), suggesting that PAM overexpression in TTR KO mice might also be responsible for increased levels of other amidated neuropeptides.

Lipoprotein lipase, a gene activated by NPY, is increased in TTR KO mice

One of the most prominent NPY effects is its action on energy balance by increasing energy intake and decreasing its expenditure. This effect is achieved by augmenting lipid storage in white adipose tissue (WAT) through increasing the expression and activity of LPL and by decreasing brown adipose tissue thermogenesis (Billington et al., 1994; Kotz et al., 1998). It is noteworthy that the microarray analysis referred in Chapter 1 revealed upregulation of LPL in TTR KOs, consistent with the increased PAM expression and NPY levels in these animals. The WAT is the only tissue where an NPY-induced increase in activity and expression of LPL has been described (Billington et al., 1994; Kotz et al., 1998), and is one of the tissues where LPL is most abundant. As expected, WAT from TTR KOs displayed a 2.6-fold increased LPL activity when compared to WT mice (**Figure 2A**). After demonstrating that increased NPY correlates with increased LPL activity in its key target tissue, we addressed whether a similar effect might be observed in the nervous system and analyzed the expression and activity of this enzyme in the CNS and PNS. Brain LPL immunostaining revealed augmented labeling in the cortex of TTR KO mice when compared to WT mice (**Figure 2B**). Furthermore, LPL mRNA levels were increased in the brain (1.6-fold), spinal cord (1.3-fold) and DRG (1.5-fold) from TTR KO mice (**Figure 2C**). In the activity assay, besides a positive trend, no statistical significant difference was seen between the brain of the two genotypes (not shown), but the cerebellum of TTR KOs presented higher LPL activity (1.2-fold) as compared to WT mice (**Figure 2D**). In addition, LPL overexpression observed in the spinal cord and DRG of TTR KO mice was correlated to a 1.3 and 2-fold increase, respectively, in LPL activity measured in the same WT tissues (**Figure 2D**). Apart from modulating energy storage through increasing WAT LPL expression and activity, NPY administration additionally decreases brown adipose tissue thermogenesis, thus reducing energy expenditure (Kotz et al., 1998). The balance between energy production and dissipation is a function of temperature regulation and any imbalance is manifested as changes in body temperature. Since NPY has been shown to induce hypothermia (Esteban et al., 1989; Balasko et al., 1999), we measured the core body temperature of WT (n=8) and TTR KO (n=8) mice. The basis of the tail, where the body temperature is lower than in other body parts (Saegusa

and Tabata, 2003), was chosen to perform the temperature measurements as, through the use of an infrared thermometer, animal handling is diminished, avoiding the resulting interference with body temperature. Although in young mice (1-3 months) no statistical difference was found, 12-month old TTR KO mice presented lower core body temperature than WT mice (**Figure 2E**), reflecting a decreased energy expenditure, possibly as a consequence of increased NPY levels.

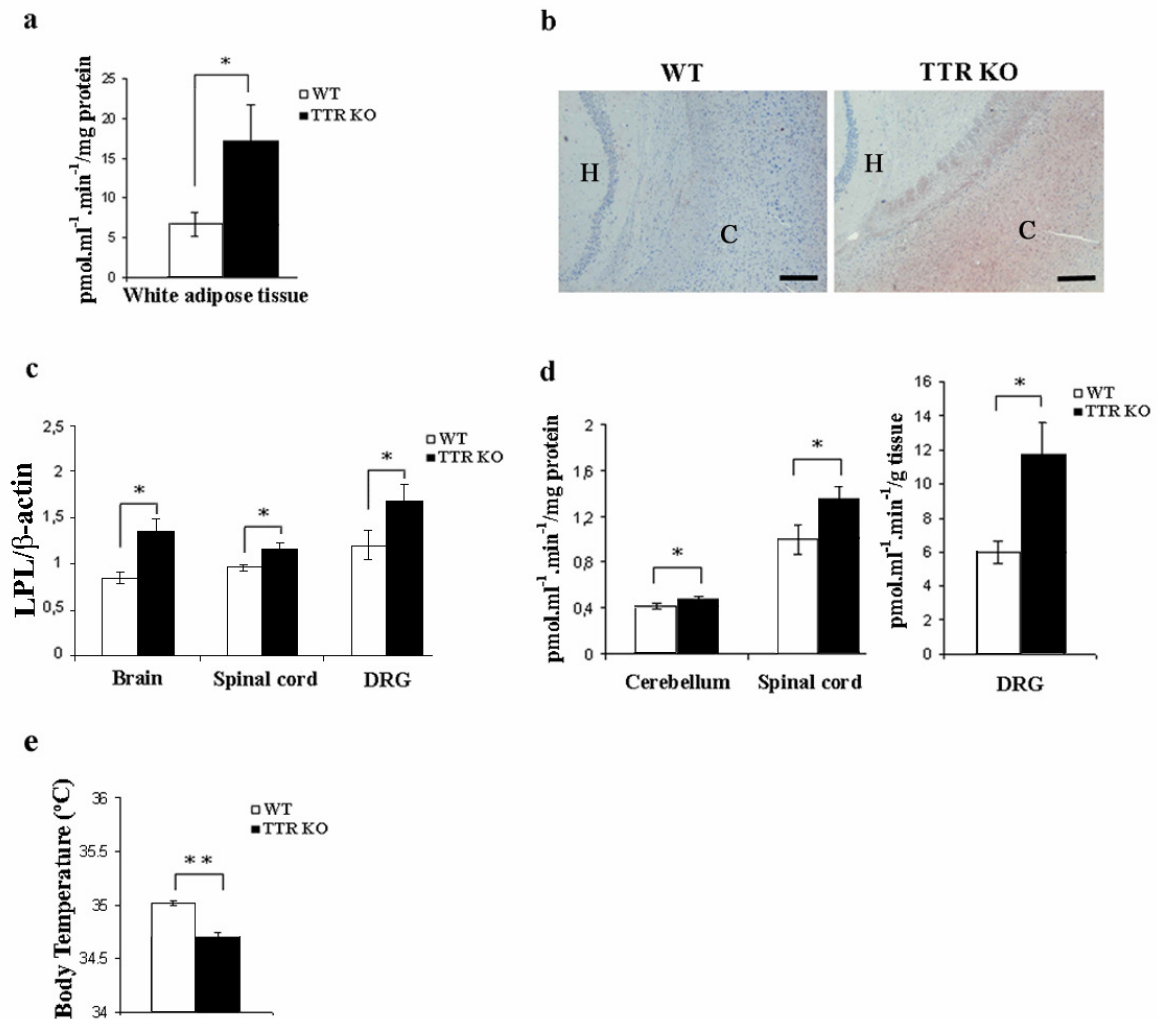


Figure 2. LPL activity and expression in TTR KO and WT mice. **A)** LPL activity in white adipose tissue. **B)** LPL immunohistochemistry in brain sections; C- cerebral cortex, H- hippocampus; scale bar = 200 μ m. **C)** LPL semiquantitative RT-PCR analysis in the CNS (brain, spinal cord) and PNS (DRG). **D)** LPL activity in the CNS (cerebellum, spinal cord) and PNS (DRG). **E)** Body temperature in TTR KO and WT mice. Results are presented as average $\pm$ SEM; * P < 0.05, ** P < 0.005.

TTR KO mice present increased carbohydrate consumption and preference

To evaluate whether increased NPY levels in TTR KO mice results in additional biological consequences, we compared TTR KO and WT mice for regular chow intake and

preference for a high-carbohydrate diet. The two strains did not differ in average body weight (WT, 28.23 ± 1.68 g; TTR KO, 28.24 ± 1.60 g) or average regular chow intake (WT, 3.59 ± 0.10 g per day; TTR KO, 3.54 ± 0.23 g per day) during the 4-week period. However, NPY has been reported to preferentially enhance carbohydrate ingestion (Pedrazzini et al., 2003). When we compared carbohydrate consumption, as predicted, TTR KO mice present increased intake and preference for the high-carbohydrate diet, when compared with WT littermates (**Figure 3A, B**, respectively). This result further stresses the existence of a NPY overexpressor phenotype in TTR KO mice.

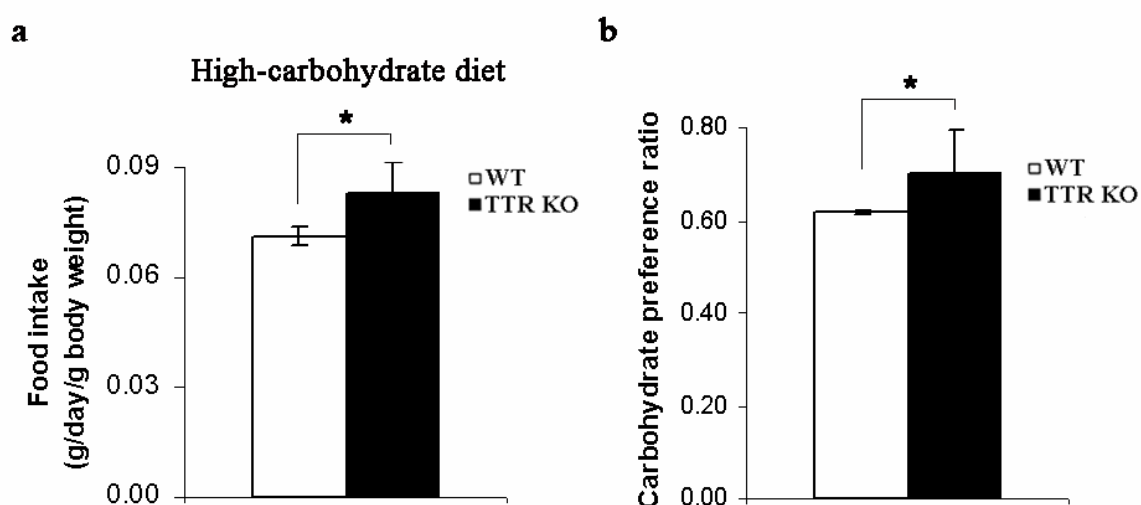


Figure 3. High-carbohydrate diet intake (**A**) and preference (**B**) in TTR KO and WT mice. Results are presented as average $\pm$ SD; * $P < 0.05$.

Absence of TTR leads to increased NPY levels and LPL expression in neuronal and neuronal-like cell cultures

PC12 cells, which, undifferentiated, have an immature adrenal chromaffin cell phenotype once exposed to NGF differentiate and resemble sympathetic neurons. This cell line has been previously shown to be adequate to study PAM expression and activity (Ford and Mueller, 1993), although there are conflicting reports in the literature (Rovere et al., 1996; Chen et al., 1997) as to their ability to express prohormone convertases and, therefore, be capable of processing the synthesized proNPY and secreting the amidated NPY form. The ability of the PC12 cells used in our studies to synthesize PC1 and PC2 was assessed by RT-PCR (**Figure 4A**) and confirmed by sequencing the RT-PCR products (not shown). We have previously reported (Chapter 1 of this thesis) that PC12

cells and primary cortical neurons grown in the absence of TTR displayed increased PAM expression.

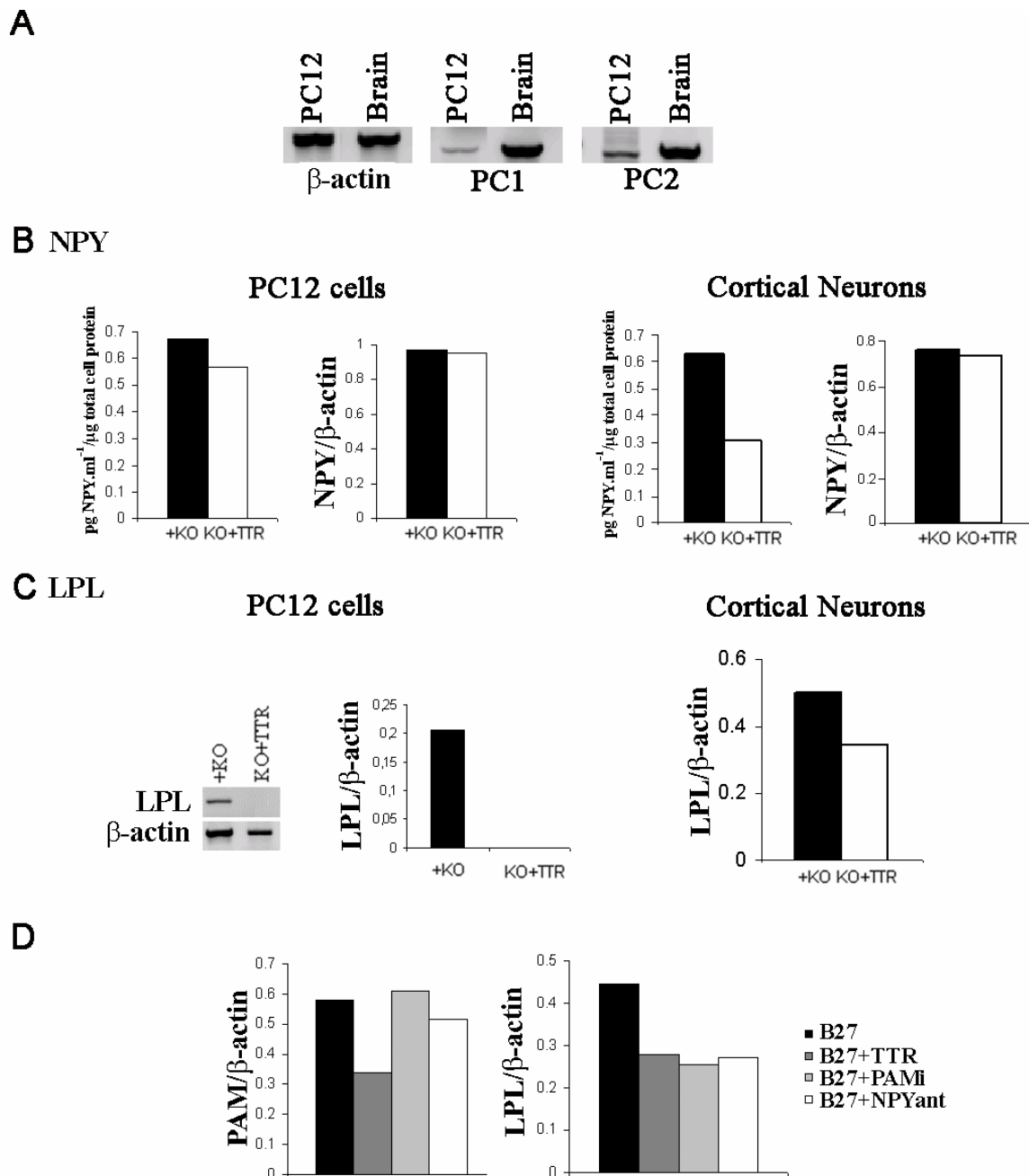


Figure 4. NPY levels and LPL expression in PC12 cells and primary cortical neurons. **A)** PC1 and PC2 expression in PC12 cells and brain (positive control). **B)** NPY levels in the conditioned media and NPY semiquantitative RT-PCR analysis in PC12 cells (left panels) and primary cortical neurons (right panels) grown in the presence of TTR KO mouse serum with (KO+TTR) or without (+KO) added TTR. **C)** LPL semiquantitative RT-PCR analysis in PC12 cells (left panels) or primary cortical neurons (right panel) grown in the presence of TTR KO mouse serum with (KO+TTR) or without (+KO) added TTR. **D)** PAM and LPL semiquantitative RT-PCR analysis in PC12 cells grown in the presence of B-27 (B27), B-27 supplemented with TTR (B27+TTR), with the PAM inhibitor (B27+PAMi) or with the NPY antagonist (B27+NPYant).

Here we show that conditioned media of both PC12 cells and cortical neurons grown with TTR KO serum, displayed increased NPY levels when compared to cells grown with TTR KO serum supplemented with TTR (**Figure 4B**). This further demonstrates that, not only absence of TTR leads to increased PAM expression, but also that this overexpression results in increased PAM activity, as reflected by increased NPY levels in the conditioned media, without NPY mRNA overexpression (**Figure 4B**). To verify that absence of TTR is related to increased LPL synthesis, cells grown with TTR KO serum, or with TTR KO serum supplemented with TTR were assessed for the expression of LPL. As predicted, in the absence of TTR, PC12 cells and primary cortical neurons displayed increased LPL synthesis (**Figure 4C**). To substantiate the hypothesis that LPL differential expression in the nervous system is related to NPY levels, experiments were performed with PC12 cells in serum-free conditions (in order to eliminate the putative interference of NPY present in mouse serum) in the presence of 4-phenyl-3-butenic acid, a PAM inhibitor, or (D-Tyr^{27, 36}, D-Thr³²)-NPY₂₇₋₃₆, a NPY antagonist. Both 4-phenyl-3-butenic acid, a potent irreversible inhibitor of PAM activity that covalently binds the enzyme, inhibiting neuropeptide amidation (Sunman et al., 2004), and a non-selective NPY antagonist, although having no effect on PAM synthesis, significantly reduced LPL expression (**Figure 4D**). This result further reinforces the hypothesis that increased NPY levels lead to LPL overexpression in other tissues than white adipose tissue.

TTR-mediated decrease of NPY levels requires TTR cell internalization

To understand if TTR internalization is needed for the downregulation of PAM expression and, consequently, for the decrease in amidated NPY levels, experiments were performed in primary cortical neurons in which TTR was coupled to 1- μ m-diameter polystyrene beads, FluoSpheres, which prevent TTR cell internalization due to their size. The biological activity of TTR-FluoSpheres complex was demonstrated to be maintained by its ability to efficiently bind T₄ (Fleming CE, in preparation). As already shown in our previous experiments, and confirmed here, primary cortical neurons grown with TTR KO serum supplemented with TTR (KO+TTR condition) displayed decreased amidated NPY content in the conditioned media when compared to cells grown with TTR KO serum alone (KO condition) (**Figure 5**). Interestingly, when TTR was coupled to FluoSpheres (KO+TTR-Fluo condition), its effect on decreasing NPY levels was absent (**Figure 5**). Incubation with both uncoupled TTR and free FluoSpheres (KO+TTR+Fluo condition) rescued the decreased NPY levels observed in the KO+TTR condition (**Figure 5**). The failure of TTR coupled to FluoSpheres to behave similarly to free TTR suggests that the mechanism by which TTR downregulates PAM expression and amidated NPY levels requires cell internalization.

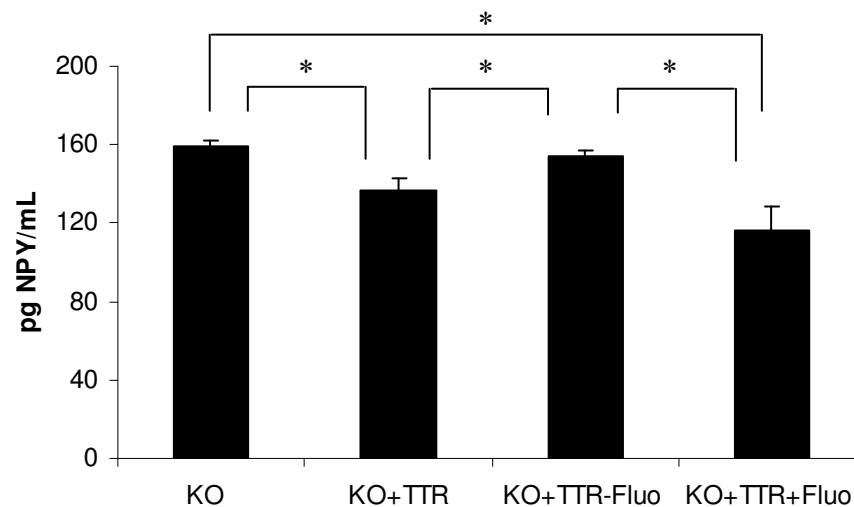


Figure 5. NPY levels in the conditioned media of primary cortical neurons grown with TTR KO mouse serum (KO), TTR KO mouse serum supplemented with TTR (KO+TTR), TTR KO mouse serum supplemented with TTR coupled to FluoSpheres (KO+TTR-Fluo) or TTR KO mouse serum supplemented with free recombinant TTR and free FluoSpheres (KO+TTR+Fluo). Results are presented as average $\pm$ SD; * $P < 0.05$.

TTR is internalized by primary cortical neurons

As TTR internalization seems to be required for its action in the nervous system physiology, namely for regulating neuropeptide maturation, our next step was to confirm whether TTR internalization was occurring in our culture system. To achieve this goal, primary cortical neurons were incubated with TTR-Alexa 488 for 3 hours at 37°C. Following immunocytochemical staining with the neuronal marker PGP 9.5 for better identification of neurons in the preparation, we could indeed visualize TTR labeling inside neurons (**Figure 6**). TTR staining was mainly seen in punctate vesicle-like structures throughout neuronal cell bodies (**Figure 6, arrows**); some non-neuronal cells were also labeled for TTR (**Figure 6, arrowheads**). To evaluate if TTR internalization was endocytosis-dependent, cultures were incubated with TTR-Alexa 488 for 3 hours at 4°C, as at this temperature receptor-mediated endocytosis is inhibited. No staining was observed at 4°C (data not shown), suggesting that TTR internalization in primary cortical neurons occurs via the endocytic pathway.

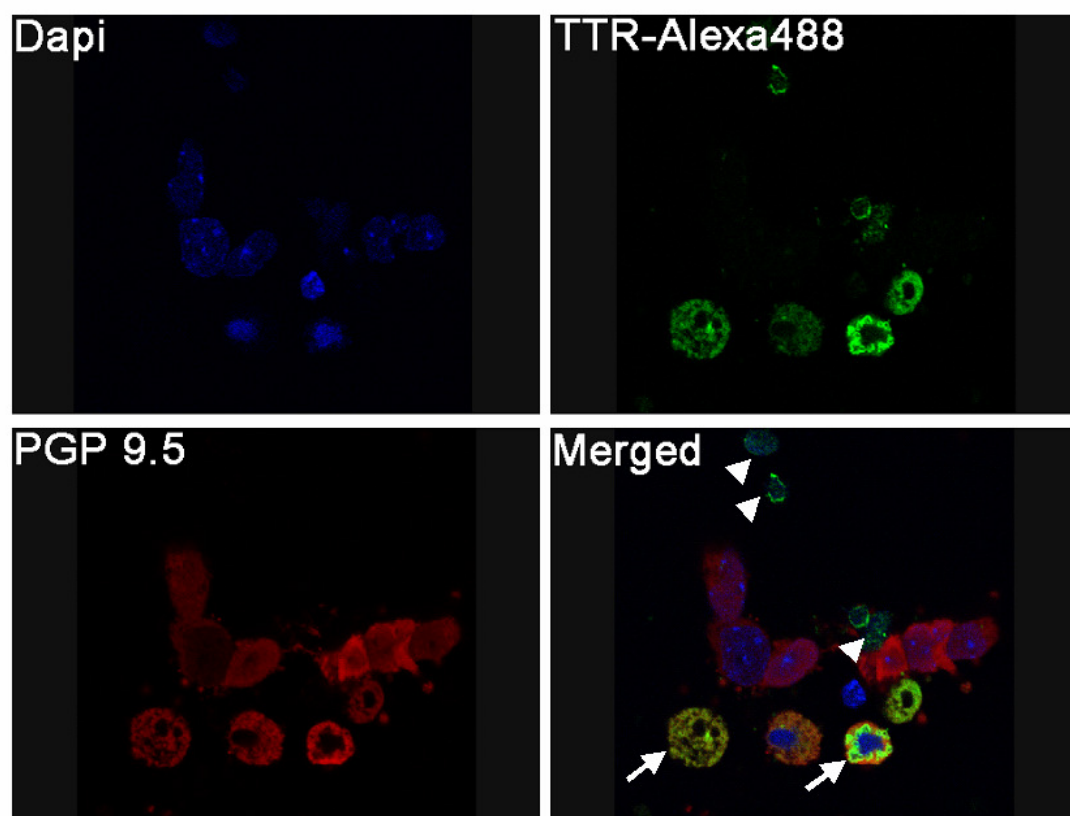


Figure 6. Representative image of TTR internalization in primary cortical neurons. DAPI staining of the cell nucleus is shown in blue, TTR-Alexa 488 is shown in green, the neuronal marker PGP 9.5 is shown in red and the merged image of TTR staining in neurons is shown in yellow (63x magnification).

Discussion

Agreeing with TTR role in regulating neuropeptide maturation through downregulation of PAM expression (Chapter 1), here we show that TTR KO mice display increased amidated NPY levels, without augmented NPY expression, and present a NPY overexpressor phenotype. The biological relevance of increased NPY in TTR KO mice is further demonstrated by its correlation with increased LPL expression and activity in the nervous system and white adipose tissue, as well as a lower core body temperature. Furthermore, our findings correlate with previous reports that TTR KO mice are less depressed than WT animals (Sousa et al., 2004), as NPY is an anti-depressant neurotransmitter (Heilig, 2004). TTR KO mice are, therefore, a new model for studying the effects of increased NPY.

Only two transgenic mouse models for NPY overexpression have been described: in the brain-restricted model (using the neuronal promoter Thy1), a modest 1.15 increase of NPY protein was found (Inui et al., 1998), whereas the second model (using a construct containing 10 kb of the endogenous NPY promoter and 3 kb of the 3' flanking sequence) refers 5-fold increased brain NPY mRNA levels (Thiele et al., 1998). None of these reports presents NPY levels in the PNS, nor in the CSF, not allowing comparisons with the TTR KO mice. It is, however, noteworthy that in a rat model of hippocampal NPY overexpression (Thorsell et al., 2000), NPY is increased 32% in the hippocampus, whereas in the TTR KO mice here reported, a higher increase is observed: 78% in the hippocampus, 41% in the CSF, 50% in the DRG and 69% in the sciatic nerve.

Neuropeptides are stored in secretory granules and are activated by processing enzymes. PAM is the only enzyme that C-terminally α -amidates peptides. Each secretory vesicle is thought to contain only a few molecules of the processing enzyme, PAM (Mains et al., 1987), making it rate-limiting in the process of neuropeptide maturation, as its substrates exist in excess. Therefore, its overexpression in TTR KO mice would result in increased levels of processed amidated NPY, without an increase in NPY expression, as shown by our data. The fact that both intracellular NPY levels (as measured in the hippocampus, DRG, and nerve) and secreted NPY levels (as measured in the cell culture conditioned media and CSF) are increased in the absence of TTR, strongly suggests that the differential levels of this neuropeptide are not related to its accumulation within the cells due to an impaired secretion, but are related to differential processing. It is plausible that changes in PAM expression lead to changes in levels of other amidated neuropeptides. In this respect we show that the PNS and plasma levels of another major amidated neuropeptide, substance P, are also increased in TTR KO mice. The physiological consequences of this observation should be further investigated as well as

the possible increase of other amidated neuropeptides. Although we do not discard this hypothesis that increased levels of other amidated neuropeptides may produce some complexity, TTR KO mice not only display increased NPY levels when compared with other NPY overexpressor models, but also present an accompanying NPY overexpressor phenotype, namely decreased energy expenditure, decreased depressive-like behavior and increased carbohydrate consumption and preference, most of which are not commonly observed in the NPY overexpressor models.

A growing body of data implicates NPY in depression (Heilig, 2004): central NPY is involved in the pathogenesis of human depression as NPY is decreased in the CSF of patients with major depression (Widerlov et al., 1988) and in the CNS of suicide victims (Widdowson et al., 1992). Experimental animals treated with clinically effective antidepressants were reported to have increased NPY levels in several brain areas (Heilig et al., 1988); electroconvulsive shock, another effective antidepressant treatment, also augments brain NPY levels both in depressed subjects (Stenfors et al., 1989) and rats (Jimenez Vasquez et al., 2000). Finally, administration of lithium (an affective stabilizer) leads to an up-regulation of hippocampal NPY synthesis (Zachrisson et al., 1995). In the case of NPY given intracerebroventricularly, it was shown to produce antidepressant-like effects in the forced swimming test (a test for depressive-like behavior), both in rats (Stogner and Holmes, 2000) and mice (Redrobe et al., 2002). TTR KO mice were reported to be resistant to depression based on their increased activity in this test (Sousa et al., 2004), which correlates with our finding of increased NPY levels in these animals. Increased norepinephrine levels in TTR KO mice were suggested to cause TTR involvement in depression (Sousa et al., 2004). In this report we show that TTR KOs present increased NPY levels, as a consequence of PAM up-regulation, and hypothesize that this neuropeptide might be responsible, at least in part, for the phenotype of TTR KOs. Lithium chloride is known to increase NPY (Zachrisson et al., 1995); again a negative correlation is found between NPY levels and TTR expression, as TTR synthesis is decreased in the choroid plexus following chronic administration of lithium chloride (Pulford et al., 2006). Besides the functions of NPY in the CNS, information is lacking regarding its effects in the PNS. NPY is upregulated following peripheral nerve injury (Shi et al., 1999) and has been involved in PNS regeneration (White and Mansfield, 1996) and axonal transport (Cougnon-Aptel et al., 1999). The effect of increased NPY levels in the PNS of TTR KOs, which are a novel model for studying peripheral NPY actions, remains, however, to be further elucidated.

NPY plays a pivotal role in coordinating energy homeostasis. Intracerebroventricular NPY injection has two major effects on energy homeostasis: it decreases brown adipose tissue thermogenesis, thus reducing energy expenditure, and it

increases white adipose tissue LPL, promoting storage of lipids in white adipose tissue and leading to a positive energy balance (Billington et al., 1994). Increased concentration of active amidated NPY in TTR KO mice, correlates with higher LPL expression and activity. Supporting this observation, we show that in the absence of TTR, PAM expression, as well as conditioned media NPY levels and LPL synthesis are increased in neuronal cells and that LPL overexpression is reversed by the presence of a PAM inhibitor as well as by a non-selective NPY antagonist. Therefore, our observations strongly suggest that, similarly to what has been reported in white adipose tissue, NPY might be responsible for LPL upregulation in the nervous system, where its function is largely unknown. LPL is expressed in Schwann cells, probably intervening in phospholipid and myelin biosynthesis (Huey et al., 1998); furthermore, LPL activity is increased following nerve crush, suggesting that it facilitates reutilization of degenerating myelin lipids (Huey et al., 2002). Given the differential expression of LPL between WT and TTR KO mice, putative differences in myelin lipid content in both sets of animals should be addressed.

Systematically observed in all mouse and rat models of NPY overexpression and NPY knockout is the lack of a major effect in energy homeostasis with unaltered body weight and food intake when compared with controls (Herzog, 2003). In this respect, TTR KO mice behave similarly, showing no difference in these parameters. However, an increase in carbohydrate consumption and preference, as expected from the increased NPY levels (Pedrazzini et al., 2003), is observed in the TTR KOs, whereas this has not been reported in other NPY models. Also, in TTR KO mice, not only we demonstrate increased LPL activity in white adipose tissue, but also decreased core body temperature, in agreement with the potent NPY central anti-thermogenic actions, decreasing whole-body energy expenditure (Esteban et al., 1989).

By using TTR coupled to 1- μ m-diameter polystyrene beads, FluoSpheres, which prevent TTR cell internalization due to their size, NPY levels in the conditioned media of primary cortical neurons were not decreased as happens following incubation with free TTR. This finding suggests that the mechanism by which TTR downregulates PAM expression and, consequently, amidated NPY levels requires cell internalization. A similar protocol was used to demonstrate nerve growth factor internalization in cultured rat sympathetic neurons (Riccio et al., 1997). However, despite its ability to efficiently bind T₄, we can not exclude the hypothesis that the coupling of TTR to FluoSpheres impairs the normal interaction between TTR and a potential receptor at the plasma membrane. Noteworthy, TTR-Alexa 488 staining was observed inside cortical neurons, and absence of labeling at 4°C suggests that TTR internalization occurs via the receptor-mediated endocytic pathway. It is possible that TTR internalization occurs through a clathrin-

dependent endocytic mechanism as recently observed in DRG neurons (Fleming CE, in preparation). Future studies should be performed to validate this hypothesis.

In summary, TTR KO mice offer a unique model to study both peripheral and central actions of NPY, as well as they provide a new animal model for increased NPY levels. Furthermore, understanding the relevance of TTR presence in the PNS will probably have impact in understanding its physiology and the pathogenic events related to neurodegeneration/neuroprotection in PNS diseases.

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CHAPTER 3

PART II

INCREASED NEUROPROTECTION AND DECREASED
NEUROPROLIFERATION IN TTR KO MICE

INCREASED NEUROPROTECTION AND DECREASED
NEUROPROLIFERATION IN TTR KO MICE

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Abstract

Accumulating evidence points to a key role for neuropeptide Y (NPY) in mediating neuroprotection and neuroproliferation. The aim of this study was to evaluate NPY-mediated neuroprotection and neuroproliferation in a new mouse model for increased NPY, the transthyretin (TTR) knockout (KO) mice. TTR KO mice display increased amidated NPY levels as a consequence of increased processing and amidation by peptidylglycine alpha-amidating monooxygenase (PAM), which is overexpressed in the absence of TTR. NPY amidated content is 2- and 4-fold increased in TTR KO mice cerebrospinal fluid and hippocampus, respectively. Here we show that TTR KO mice hippocampal slice cultures are protected against AMPA-induced neurodegeneration, despite the fact that TTR KO mice do not display decreased seizure susceptibility nor decreased neuronal death following kainate intraperitoneal injection. We also herein demonstrate that increased NPY levels in TTR KO mice are not related to an increase in the number of proliferating cells in the dentate gyrus of the hippocampus, but instead TTR KO mice display fewer neural precursor cells in the subventricular zone (SVZ), as assessed in SVZ neurosphere cultures. These results contribute to the better characterization of TTR KO mice phenotype, as a model of increased NPY.

Introduction

Neuropeptide Y (NPY) is the most abundant peptide in the mammalian central nervous system, being widely distributed throughout the brain. In the hippocampus, NPY is preferentially expressed by GABAergic hilar interneurons, although it is also present in long projecting neurons (de Quidt and Emson, 1986). NPY has been associated with numerous physiological processes in the brain, namely energy homeostasis, memory, learning, anxiety, depression and seizures (Hokfelt et al., 1998). Six G-protein-coupled NPY receptor subtypes, Y1-y6, have been identified, although only Y1, Y2 and Y5 have been shown to be most prominent (Blomqvist and Herzog, 1997). Whereas Y3 has never been cloned, Y4 has higher specificity for pancreatic polypeptide (PP), another member of the NPY family, and y6 is not functional in humans and rats (Silva et al., 2005a).

Emerging evidence points towards an important role for NPY in the regulation of pathological conditions associated with excessive glutamate release, such as that occurring during seizures. Spontaneous non-evoked seizure activity is the hallmark of the neurological disorder epilepsy (Morimoto et al., 2004). Glutamate is the major excitatory neurotransmitter in the brain and its effects can be mediated through interaction with metabotropic or ionotropic glutamate receptors (Ruzicka and Jhamandas, 1993). Both AMPA and kainate receptors are ionotropic receptors efficiently activated by either AMPA or kainate, in their respective preference order. Several studies have shown that overactivation of glutamatergic transmission leads to excitotoxic cell death (Choi, 1985; Garthwaite and Garthwaite, 1986). Glutamate excitotoxicity has been implicated in a number of neurodegenerative diseases, such as epilepsy, ischemia, Alzheimer's disease (Choi, 1994; Rothman and Olney, 1995; Alexander and Godwin, 2006), and the excitotoxicity involves excessive stimulation of ionotropic glutamate receptors, leading to increased intracellular concentration of Ca^{2+} and Na^{+} and ultimately to neuronal death (Choi, 1988, 1992). It is generally accepted that inhibition of glutamatergic hyperactivity may account for neuroprotection. In this respect, NPY application to rat hippocampal slices potently and selectively reduced synaptic excitation mediated by glutamate release (Colmers et al., 1985; Haas et al., 1987). An anticonvulsive action of NPY in the epileptic human hippocampus was also shown to be mediated by NPY inhibition of glutamatergic excitation (Patrylo et al., 1999). Furthermore, NPY overexpression in the hippocampus has been reported in various seizure models (Gruber et al., 1994; Schwarzer et al., 1996; Takahashi et al., 2000). Increased NPY levels are particularly evident in both inhibitory interneurons and in excitatory granule cells and mossy fibers that do not normally contain this peptide (Gruber et al., 1994; Schwarzer et al., 1996; Takahashi et al., 2000). Stimulation of glutamate receptors may be involved in the induction of NPY expression,

suggesting that excitatory glutamatergic neurotransmission regulates NPY mRNA levels (Schwarzer and Sperk, 1998). *In situ* hybridization demonstrated NPY mRNA presence in dentate granule cells from 12 hours to 90 days after seizures and the rapid confinement of the seizure inhibited NPY expression, further confirming that repeated seizures are directly responsible for NPY expression (Gruber et al., 1994). Transgenic rats overexpressing NPY were found less susceptible to epileptogenesis, as seizure activity, induced by kainate or electrical kindling, was less frequent and shorter (Vezzani et al., 2002). Moreover, long-lasting NPY overexpression in the rat hippocampus using a recombinant adeno-associated viral vector, decreased the generalization of seizures from their site of onset, delayed acquisition of seizures and afforded neuroprotection (Richichi et al., 2004). On the contrary, NPY knockout (KO) mice displayed mild, spontaneous seizures (Erickson et al., 1996) and presented increased susceptibility to pentylenetetrazol and kainic acid-induced seizures, as prolonged seizure activity, and most times death, were observed (Baraban et al., 1997). These effects were antagonized by intracerebral NPY administration (Baraban et al., 1997). Also, NPY administered into the lateral ventricle was shown to be a powerful inhibitor of kainic acid-induced seizures (Woldbye et al., 1997). These numerous *in vitro* and *in vivo* results obtained with different epilepsy models are in agreement with a powerful antiexcitatory, antiepileptogenic and neuroprotective action of NPY. These effects seem to be mediated predominantly through presynaptic NPY Y2 receptors by depressing glutamate release through a mechanism involving the inhibition of the voltage-dependent Ca^{2+} influx in excitatory nerve terminals (Schwarzer et al., 1996; Weiser et al., 2000; Silva et al., 2001).

More recently, NPY has also been shown to promote neurogenesis. Adult neurogenesis occurs in the human and rodent brain mainly in the dentate gyrus of the hippocampus and in the subventricular zone (SVZ) (Altman and Das, 1965; Doetsch et al., 1999; Gage, 2000). NPY was shown to promote adult neurogenesis in the dentate gyrus, as NPY Y1 receptor KO mice presented reduced proliferation of neuronal precursor cells and significantly fewer immature neurons when compared to controls (Howell et al., 2005). These results suggested that NPY modulates dentate neurogenesis through Y1 receptor. A previous report had already demonstrated that NPY induced the proliferation of hippocampal neuronal precursor cells *in vitro* via NPY Y1 receptor (Howell et al., 2003). Furthermore, NPY mediates neurogenesis in the olfactory epithelium, another, however less well-described, region of adult neurogenesis (Calof et al., 1998). NPY KO mice displayed significantly fewer dividing olfactory neuronal precursor cells when compared to the WT littermates, suggesting that NPY functions as a neuroproliferative factor (Hansel et al., 2001). Moreover, in SVZ cell cultures, NPY treatment increased neuronal proliferation and differentiation (Agasse F et al. 2007 2nd International Meeting of the Portuguese

Society for Stem Cells and Cellular Therapy), demonstrating that NPY is also neurogenic in this brain region.

Given all these asserted actions for NPY, the aim of this work was to study NPY-mediated neuroprotection and neuroproliferation in the transthyretin (TTR) KO mice, a new mouse model of increased NPY (Nunes et al., 2006). In addition to increased amidated NPY levels, TTR KO mice display a NPY-overexpressor phenotype, which includes increased carbohydrate consumption and preference, decreased body temperature and reduced depressive-like behavior (Sousa et al., 2004; Nunes et al., 2006). Our purpose was to evaluate whether neuroprotection and neuroproliferation could also take part in TTR KO mice NPY-overexpressor phenotype, thus reinforcing the pivotal role of this neuropeptide in both mechanisms.

Materials and Methods

Animals

Mice were handled according to European Union and National rules. Male wild type (WT) and transthyretin (TTR) knockout (KO) (Episkopou et al., 1993) littermate offspring of heterozygote breeding pairs, in the 129/Sv background, were maintained at $24 \pm 1^\circ\text{C}$ under a 12 h light/dark cycle and fed regular chow and tap water *ad libitum*. All animals were accustomed to regular handling.

Kainate treatment and seizure scoring

Kainate treatment was performed as previously reported on mice (Benkovic et al., 2004). Briefly, 3- and 8-month old male WT (n=5) and TTR KO (n=5) mice were injected intraperitoneally with 35 mg/kg kainic acid (KA, Sigma). KA was dissolved in sterile 0.9% NaCl at 3.5 mg/mL to keep the injection volume below 0.5 mL. A control group of animals was injected with the same volume of saline. Mice were observed for a period of 2 h post-injection and their behavior was scored according to the Racine scale: stage 1 – mouth and facial movements; stage 2 – head nodding; stage 3 – forelimb clonus; stage 4 – rearing; stage 5 – rearing and falling (Racine, 1972). 3- and 8-month old animals were sacrificed 30 and 6 days after KA injection, respectively, and brain tissue was examined for neurodegeneration.

Tissue preparation for histology

WT and TTR KO mice that underwent kainate injection were anesthetized with ketamine/medetomidine and perfused transcardially for 5 min with a wash solution (4% sucrose in 0.9% NaCl) followed by 20 min with the perfusion solution (4% paraformaldehyde in wash solution, pH 7.4). Brains were removed, fixed in the perfusion solution overnight at 4°C and left in 20% sucrose in phosphate buffer saline (PBS) at 4°C until frozen. Twenty micrometer sections were cut on a sliding microtome cryostat at -20°C and mounted onto gelatinized slides.

Nissl staining

Nissl staining was performed with a 0.2% thionin solution. Briefly, slides were incubated with 80% acetone in acetic acid for 5 min, rinsed briefly in deionized water and stained with a filtered solution of 0.2% thionin (Fluka and Riedel-de Haën) in 10% formol, for 5 min. Following a brief rinse in deionized water, sections were differentiated with 95%

isopropanol in acetic acid, rinsed in deionized water and dried at 37°C. Finally, slides were cleared and coverslipped.

Fluoro-Jade B staining

Slides were immersed in 100% ethanol for 3 min, followed by immersion in 70% ethanol and deionized water for 1 min each. Slides were then transferred to a filtered solution of 0.06% potassium permanganate for 15 min on a shaker to suppress background staining. Following a rinse in deionized water for 1 min, slides were incubated with the staining solution 0.001% Fluoro-Jade B (Histochem) for 30 min with gentle shaking. Staining solution was prepared by dissolving 2 mg Fluoro-Jade B in 20 mL deionized water and completing the final volume to 200 mL with 0.1% acetic acid. Slides were washed three times for 1 min each with deionized water and dried for 10 min at 50°C. Finally, slides were rinsed three times for 2 min each in xylene and coverslipped with DPX (Electron Microscopy Sciences).

Organotypic hippocampal slice cultures

7-8-days old WT and TTR KO mice were killed by quickly cutting the brain stem, brains were removed and the hippocampi isolated by dissection. Each hippocampus was then cut in 350 µm-thick transverse sections using a McIlwain tissue chopper. Slices were transferred to Gey's balanced salt solution (Sigma) supplemented with D-glucose (6.5 mg/mL) (Merck), trimmed for excess tissue under a microscope and placed on semiporous (0.4 µm pore size) insert membranes (30 mm in diameter) (Millipore Corp.) with 6 slices on each membrane. The inserts with the slices were transferred to six-well plates (Corning Costar) with 1 mL of culture medium in each well and placed in an incubator with 5% CO₂ and 95% atmospheric air at 33°C. The culturing medium was composed of 50% OPTI-MEM, 25% heat-inactivated horse serum, 25% Hank's balanced salt solution (all from Gibco), supplemented with D-glucose at a final concentration of 25 mM. On the seventh day of culture and thereafter, the medium was replaced by 1 mL of chemically defined, serum-free Neurobasal medium (Gibco) supplemented with 25 mM D-glucose, 1 mM L-glutamine (Sigma) and 2% B27 supplement (Gibco). No antimitotic drugs or antibiotics were used at any stages. At the same time medium was replaced to serum-free Neurobasal medium and until the end of the experiment, recombinant TTR, produced as described (Liz et al., 2004), was added to the culture medium in selected wells at a final concentration of 300 µg/mL (**Figure 1**). After this pre-incubation period (1 week), 8 µM AMPA (Sigma) was added to every well except for the control cultures, and inserts were transferred to an incubator at 36°C. This concentration was chosen based on previous studies on mice organotypic hippocampal slice cultures (Bernardino et al., 2005).

Cell death was determined using the fluorescence cell death indicator propidium iodide (PI) (Sigma). PI was added to the culture medium 24 h before AMPA treatment to yield a 2 μ M concentration. Recordings of cellular PI uptake by fluorescence microphotographs were performed immediately before the AMPA excitotoxic insult (day 0) and 24 h after it (day 1) using a tetramethyl rhodamine isothiocyanate filter (510-560/590 nm) and a Sensys KAF 1400 G2 (Photometrics, Tucson, AZ) digital camera (Noraberg et al., 1999). The PI uptake in the cell layers of the different hippocampal subfields (CA1, CA3, dentate gyrus and entire hippocampus) was quantified densitometrically, using NIH Image software (version 1.64), by delineating the different subfields. Basal death (day 0) was subtracted from values at day 1.

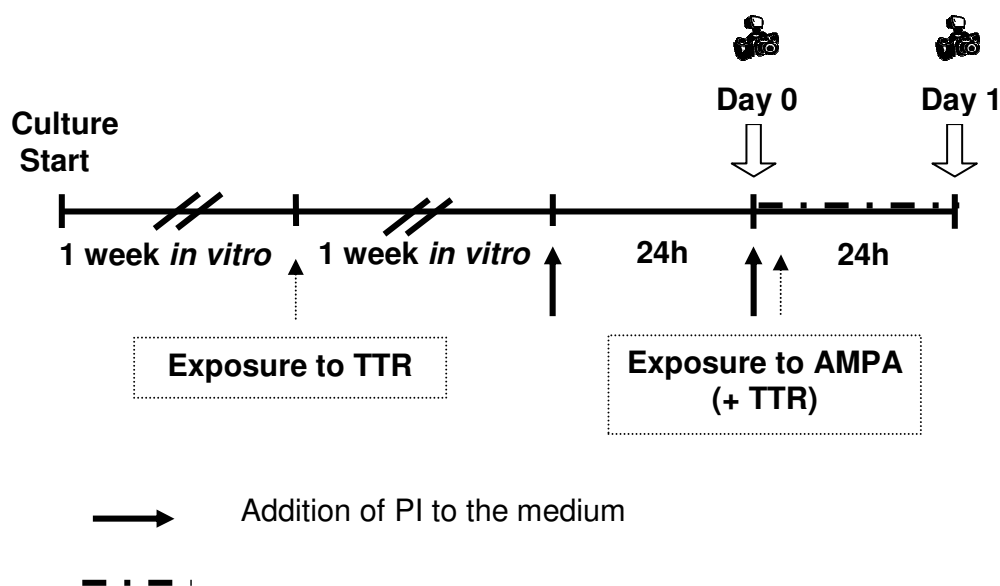


Figure 1. Schematic representation of the protocol used for studying the excitotoxic effect of AMPA in WT and TTR KO mice organotypic hippocampal slice cultures and the effect of TTR on AMPA-induced neuronal cell death (adapted from Silva et al., 2003).

BrdU treatment and immunohistochemistry

3-month old male WT (n=6) and TTR KO (n=6) mice were injected intraperitoneally with 50 mg/kg 5-bromo-2'-deoxyuridine (BrdU, Sigma) once a day for 3 consecutive days. BrdU was dissolved in sterile 0.9% NaCl at 10 mg/mL to keep the injection volume between 0.1-0.2 mL. 24 hours after the last injection, animals were anesthetized and perfused as referred above. Brain tissue was paraffin-embedded and serial 4- μ m-thick coronal sections were cut to yield 6 hippocampal slices per slide, each of them spaced 60 μ m apart. Sections were then deparaffinated, dehydrated in a modified alcohol series and blocked for the endogenous peroxidase activity. Antigen unmasking was performed with

2 N HCl for 20 min at 37°C, followed by 2 rinses in 0.1 M Borax ($\text{Na}_2\text{B}_4\text{O}_7$, Merck) for 2 min each, and finally incubation with 0.05% trypsin (Gibco) for 10 min was performed. BrdU immunohistochemistry was done with the MOM Kit (Vector), following the manufacturer's instructions. Briefly, sections were incubated in the MOM kit blocking reagent for 1 hour at room temperature, prior to the incubation with the monoclonal BrdU antibody (Sigma, 1:1,000 in MOM diluent) for 1 hour at 37°C. Antigen visualization was performed with the MOM Avidin:Biotinylated peroxidase Complex (ABC) reagent, using 3-amino-9-ethyl carbazole (Sigma) as substrate. On parallel control sections, the primary antibody was replaced by MOM diluent. For each animal, three consecutive coronal slides, corresponding to 300 μm depth each, were analyzed and all BrdU-immunopositive cells in the dentate gyrus were bilaterally counted.

Subventricular zone (SVZ) neurosphere culture and counting

The subventricular zone (SVZ) region was dissected from 3-month old male WT ($n=2$) and TTR KO mice ($n=4$) using standard microdissection techniques. Tissue from each animal in separate was collected in DMEM (Gibco) and enzymatically digested by incubation with a 1 mg/mL papain (Sigma) solution in DMEM/F12 1:1 (Gibco) for 30 min at 37°C, followed by enzymatic inhibition with ovomucoid (Sigma) 0.7 mg/mL in DMEM/F12 1:1. The cell suspension was filtered through a 40 μm cell strainer (Becton Dickinson) and centrifuged 5 min at 600 g . Cells were washed with DMEM and resuspended in DMEM/F12 1:1 supplemented with 2% B27 (Gibco), 20 ng/mL hEGF (Abcam), 10 ng/mL hFGF (Abcam) and 4 $\mu\text{g/mL}$ heparin (Sigma). Finally, 1 mg/mL cold collagen solution (Stem Cell Technologies) was added to the culture mixture to generate the collagen gel once inside the incubator. Each cell suspension, corresponding to 1 animal, was divided and dispensed in two 35 mm culture dishes (Stem Cell Technologies) with 1.5 mL culture medium each. Recombinant TTR was added to the culture medium of a single TTR KO culture dish from each animal at a final concentration of 50 $\mu\text{g/mL}$, corresponding approximately to the physiological concentration of TTR in the CSF. Dishes were placed in an incubator with 5% CO_2 and 95% humidity, at 37°C and cells were cultured for 21 days. Once every 7 days, cultures were replenished with 60 μL culture medium containing EGF, FGF, heparin and TTR (in the latter case, only for dishes cultured in the presence of TTR), in amounts guaranteeing the final concentrations detailed above. For neurosphere counting, 21-days old cultures were placed on a gridded scoring dish (Stem Cell Technologies) and all neurospheres from each dish were counted using a low power (4X) objective.

Statistical analysis

Statistical analysis was performed using the Student's t-Test. Results were expressed as average $\pm$ SEM. For all statistical analysis, $P < 0.05$ was accepted as being statistically significant.

Results

Increased NPY levels in TTR KO mice do not relate to decreased seizure susceptibility and neuronal death following kainate injection

Younger (3-month old) and older (8-month old) WT and TTR KO mice were compared regarding their susceptibility to kainate-induced seizures and neurodegeneration. A difference between both strains was expected as a consequence of their differential NPY levels. As NPY levels in TTR KO mice were found increased in the hippocampus as soon as 1-month of age but this increase became more generalized throughout the CNS with increasing age, two different ages were chosen. After kainate injection, 70% of animals in the 3-month old group, and all animals in the 8-month old group, experienced the ultimately stage of seizure (stage 5), according to the Racine scale (Racine, 1972), with no obvious differences observed between strains at both ages (data not shown). Moreover, no seizure activity was observed in the saline-injected control group. Despite no differences were seen regarding status epilepticus, we further addressed whether the increased NPY levels in TTR KO mice were protective against kainate-induced neuronal death. It was previously shown that NPY levels were increased in all rat hippocampal subregions 30 days after kainate injection (Silva et al., 2005b). Therefore, we chose 30 days as the time point to evaluate kainate-induced neurotoxicity in 3-month old WT and TTR KO mice, as at this time point a bigger difference between the two genotypes could probably be observed as a consequence of the increased NPY levels. Surprisingly, Nissl staining performed with thionin, revealed no major kainate-induced neuronal loss in the hippocampus from both WT and TTR KO mice compared to saline-injected control group, except for two TTR KO kainate-treated animals that presented loss of CA3 pyramidal neurons (**Figure 2A, arrow**). The hippocampal CA3 subregion is particularly sensitive to kainate-induced excitotoxicity due to its high levels of kainate receptors (Malva et al., 1998). By using Fluoro-Jade B as an additional marker of neuronal degeneration, no fluorescent staining was observed in both WT and TTR KO sections (data not shown), confirming the results obtained by Nissl staining. These results were unexpected as kainate-induced neuronal death should be evident. Given the general picture of absence of neuronal loss in 3-month old WT and TTR KO mice 30 days after kainate injection, the 8-month old mice group was sacrificed 6 days after kainate injection in order to assess if this earlier time point was more adequate for detection of kainate-induced neurodegeneration. No neuronal loss was likewise observed using the thionin staining in kainate-treated WT and TTR KO mice (data not shown), whereas Fluoro-Jade

B-labeled neurons were only observed in the hippocampal CA3 area of two animals, one from each genotype (**Figure 2B**).

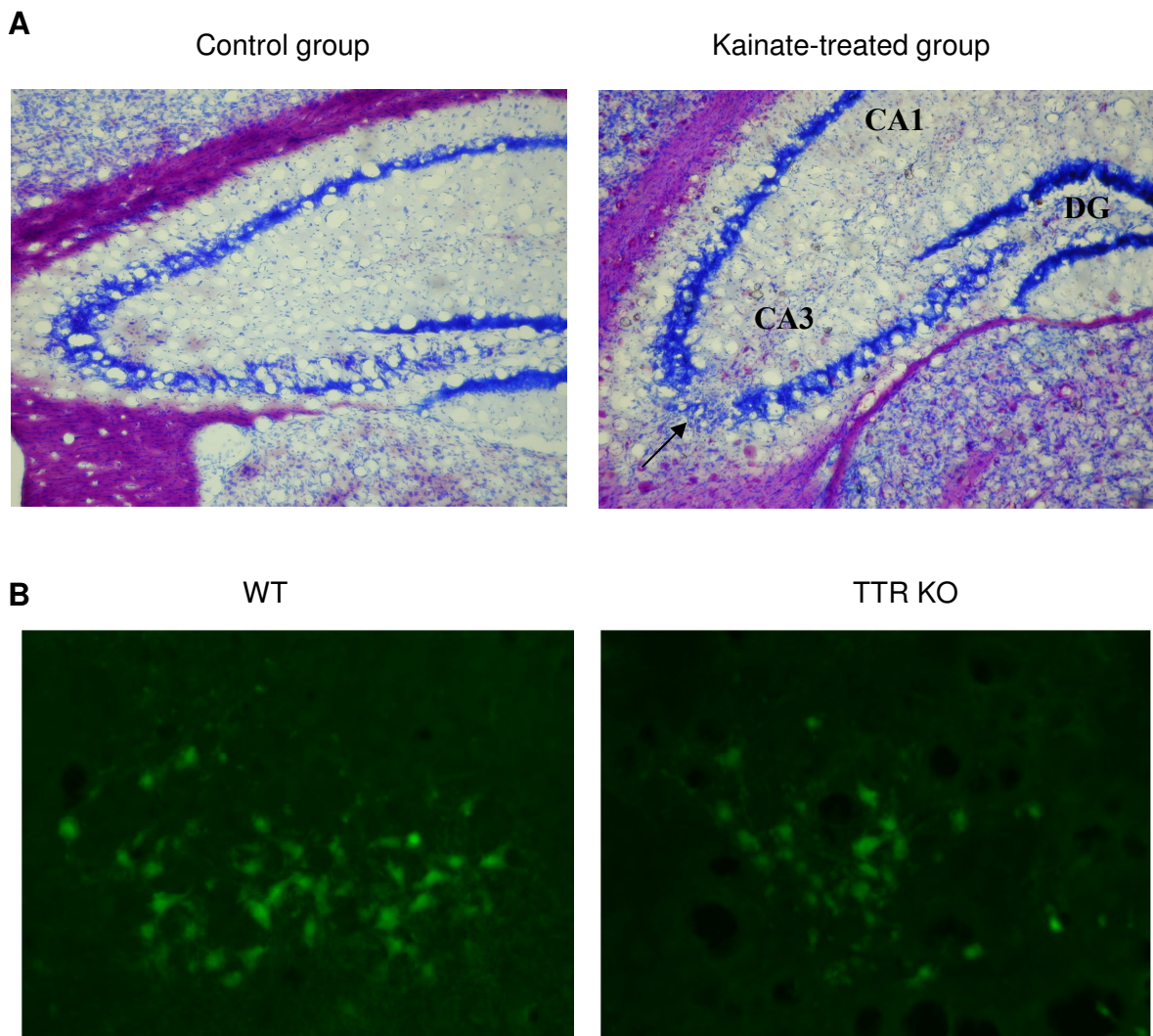


Figure 2. Histopathological analysis of the hippocampus following kainate treatment. **A)** Nissl staining in the hippocampus of both control and kainate-treated groups (10x magnification); arrow highlights CA3 region where pyramidal neurons were lost. **B)** Fluoro-Jade B stained neurons in the CA3 area of the hippocampus of both WT and TTR KO mice after kainate treatment (40x magnification).

TTR KO mice organotypic hippocampal slice cultures are protected against AMPA-induced neurodegeneration

Given that no major differences were found following kainate treatment *in vivo*, a different approach was used to address neuroprotection in WT and TTR KO mouse brains, which consisted in the use of organotypic hippocampal slice cultures. For detection of neuronal cell death in organotypic hippocampal slice cultures, propidium iodide (PI) has been extensively used. PI only enters cells with damaged cell membranes and once

inside the cells it binds to DNA, emitting fluorescence. Exposure of WT and TTR KO hippocampal slice cultures to 8 μ M AMPA for 24 hours induced an increase in cellular PI uptake in all hippocampal subfields (CA1, CA3 and dentate gyrus) when compared to control cultures (without AMPA treatment) (**Figure 3A, B**). Furthermore, in the AMPA-treated condition, TTR KO mice cultures displayed decreased PI uptake, in all hippocampal subfields, when compared to WT cultures, reflecting a decreased neurodegeneration in TTR KO hippocampal slice cultures following the AMPA excitotoxic insult (**Figure 3A, B**).

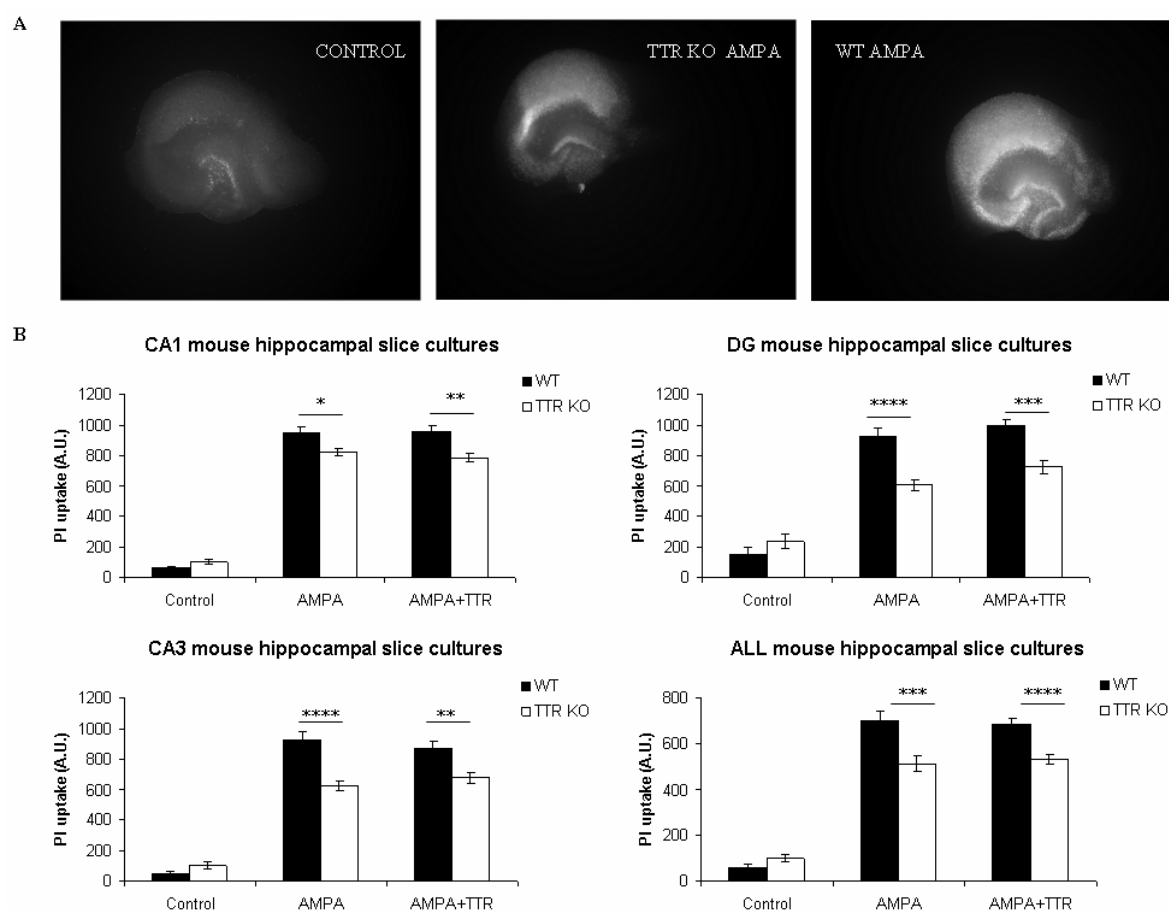


Figure 3. AMPA-induced excitotoxicity in WT and TTR KO mice organotypic hippocampal slice cultures and the effect of pre-incubation with TTR. **A)** Fluorescence photomicrographs of PI uptake at day 1 in representative slices from control and AMPA-treated WT and TTR KO mice organotypic hippocampal slice cultures. **B)** Densitometric measurements of PI uptake in control and AMPA-treated WT and TTR KO mice organotypic hippocampal slice cultures and the effect of TTR on AMPA-induced neurotoxicity. Densitometric measurements were performed in the CA1, CA3 and dentate gyrus (DG) regions, and also in whole hippocampus tissue (ALL), from hippocampal slice cultures. Results are presented as average $\pm$ SEM (n=12-30); * P < 0.05, ** P < 0.005, *** P < 0.0005, **** P < 0.00005.

Given that TTR is not expressed in the hippocampus (the only site of TTR synthesis in the brain is the choroid plexus) (Soprano et al., 1985), TTR was added to the culture media of selected wells in order to evaluate its direct effect on neuroprotection.

The one week pre-incubation period with TTR did not alter the PI uptake pattern obtained with AMPA treatment alone, in both WT and TTR KO cultures (Figure 3B). These results suggest that TTR KO cultures have an increased ability to resist against an excitotoxic insult when compared to WT cultures and that this difference is not abolished by addition of TTR to the TTR KO cultures.

Increased NPY levels in TTR KO mice are not related to an increase in the number of proliferating cells both in the DG and SVZ

The dentate gyrus of the hippocampus and the subventricular zone (SVZ) are the two main sites of adult neurogenesis (Gage, 2000). To address cell proliferation in dentate gyrus hippocampal subregion from adult WT and TTR KO mice, the thymidine analog BrdU was intraperitoneally injected and BrdU-positive cells (in S-phase of the cell cycle) were counted in this brain region. BrdU-positive cells were found in both WT and TTR KO mice, mainly in the granule cell layer but some also in the hilus, with the characteristic nuclear labeling (**Figure 4, arrows**).

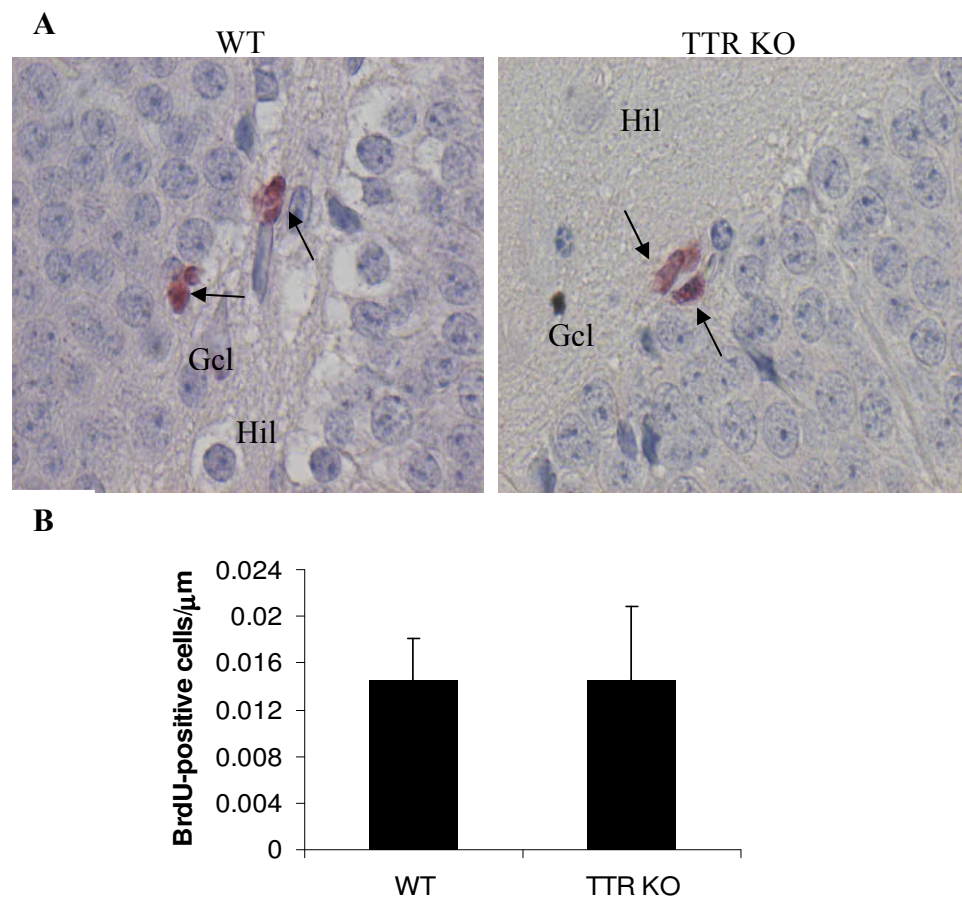


Figure 4. BrdU immunohistochemistry in WT and TTR KO mice hippocampus. **A)** Representative images of BrdU-positive cells in the dentate gyrus of the hippocampus from both WT and TTR KO mice (60x magnification); Gcl: granule cell layer, Hil: hilus. **B)** BrdU-positive cell counting in WT and TTR KO dentate gyrus. Results are presented as average $\pm$ SEM.

This pattern is in accordance with the literature: neural precursor cells in the dentate gyrus are mainly located in the subgranular zone and stem cell-derived progenitor cells migrate into the granule cell layer where they differentiate (Kempermann et al., 2004); a proliferative zone is also observed in the hilus (Gage et al., 1998). No difference in the number of proliferating cells between WT and TTR KO littermates was found (**Figure 4B**).

We further assessed if differences exist in the number of SVZ neural precursor cells between WT and TTR KO mice. Not only the SVZ is a site of active adult neurogenesis but also neural precursors in this region are close to CSF where NPY is 2-fold increased in TTR KO mice. To perform this analysis, SVZ neurosphere cultures from both strains were performed. A third experimental condition was analyzed by growing TTR KO cultures in the presence of TTR. SVZ cultures yielded neurospheres with less than 1.5 mm diameter in all conditions (**Figure 5A**), with no obvious differences in size between the different conditions analysed. Furthermore, TTR KO cultures presented decreased neurosphere number when compared to WT cultures (**Figure 5B**), suggesting that TTR KO mice display a decreased number of neural precursor cells in the SVZ. Addition of TTR to the culture media of TTR KO cultures had no significant effect on neurosphere number, being unable to recover the phenotype of TTR KO neurospheres (**Figure 5B**).

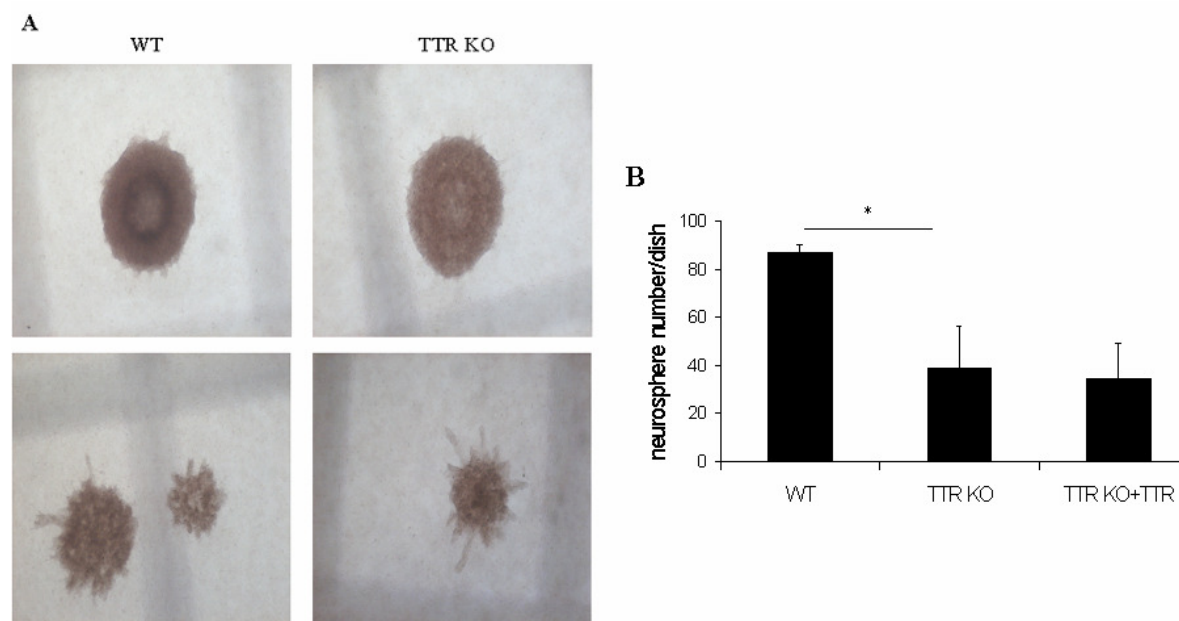


Figure 5. WT and TTR KO mice subventricular zone neurosphere cultures. **A)** Representative examples of different colony morphologies of individual neurospheres obtained (4x magnification). **B)** Neurosphere counting in WT, TTR KO and TTR KO + TTR subventricular zone cultures. Results are presented as average $\pm$ SEM ; * $P < 0.05$.

Discussion

In this study we show that TTR KO mice hippocampal slice cultures are protected against AMPA-induced neurodegeneration, despite the fact that TTR KO mice do not display decreased seizure susceptibility nor decreased neuronal death following kainate intraperitoneal injection. These results suggest that although *in vivo* in the chosen insult model, no neuroprotection was observed in TTR KO mice, *in vitro*, absence of TTR is related to decreased cell death. We also herein demonstrate that increased NPY levels in TTR KO mice are not related to an increase in the number of proliferating cells as would be expected given the neuroproliferative role of NPY.

Kainate was administered to both WT and TTR KO mice by intraperitoneal injection; this procedure has long been used to generate animal models of temporal lobe epilepsy (Ben-Ari, 1985), the most common adult form of treatment-resistant epilepsy. The model proposed for kainate excitotoxicity involves the activation of hippocampal presynaptic kainate receptors triggering the release of glutamate. This increase in glutamate levels accounts for an additional indirect effect of kainate neurotoxicity on postsynaptic glutamate receptors (Coyle, 1983). Kainate-induced epilepsy has been shown to be associated with increased NPY levels in the hippocampus (Gruber et al., 1994; Silva et al., 2005b) and increased NPY levels result in decreased susceptibility to seizures, as shown *in vitro* in animal (Haas et al., 1987; Klapstein and Colmers, 1997) and human samples (Patrylo et al., 1999) and also *in vivo* (Erickson et al., 1996; Baraban et al., 1997; Woldbye et al., 1997). This elevation in NPY levels may represent a compensatory mechanism in response to hyperexcitability, supporting the hypothesis that NPY can play a central role as an endogenous antiepileptic and neuroprotective agent.

In this study, we looked for these NPY properties in a new mouse model for increased NPY, the TTR KO mice (Nunes et al., 2006). TTR KO mice display increased NPY levels as a consequence of increased processing and amidation by peptidylglycine alpha-amidating monooxygenase (PAM), which is overexpressed in the absence of TTR (Nunes et al., 2006). As a consequence, NPY amidated content is 2- and 4-fold increased in the cerebrospinal fluid (CSF) and hippocampus, respectively, from TTR KO animals when compared to WT (Nunes et al., 2006). The increase in hippocampal NPY is evident in TTR KO mice as soon as 1-month of age (unpublished observation) and has never been determined before this age. Here we show that increased NPY levels in TTR KO mice do not relate to decreased seizure susceptibility and neuronal death following kainate injection. Nevertheless, TTR KO mice hippocampal slice cultures are protected against AMPA-induced neurodegeneration. This neuroprotection observed in TTR KO versus WT organotypic hippocampal cultures was not abolished by the exogenous

addition of TTR to the culture medium, suggesting that TTR KO and WT mice have intrinsic differences and that these differences probably derive from the environment in which the hippocampus was grown and developed *in vivo*. In this respect it is tempting to speculate, given the increased hippocampal NPY levels observed in TTR KO mice, that the exposure to increased NPY levels *in vivo* is responsible for the neuroprotection observed in TTR KO hippocampal cultures *in vitro*. However we can not exclude the hypothesis that TTR may regulate PAM expression, and consequently amidated NPY levels, through an indirect mechanism that may not be present in our culture conditions, and this could also justify the lack of neuroprotective effect in organotypic cultures grown in the absence of TTR. Consistent with a role for NPY in mediating neuroprotection in TTR KO hippocampal slice cultures, several reports showed that NPY and NPY receptors are involved in preventing neuronal cell death in organotypic hippocampal slice cultures exposed to an excitotoxic insult (Silva et al., 2003; Xapelli et al., 2007). When analyzing our results, we also have to consider that TTR-mediated changes in PAM expression may lead to changes in the levels of other amidated neuropeptides in addition to NPY, which may introduce some complexity to the *in vivo* system. This could also explain the absence of a NPY neuroprotective effect in TTR KO kainate-treated mice, as it is possible that other neuropeptides are counteracting NPY neuroprotective action *in vivo* but these may be absent in our *in vitro* model.

With regard to NPY-mediated cell proliferation, no differences were observed between WT and TTR KO mice in the number of BrdU-positive cells in the dentate gyrus of the hippocampus. We also addressed the number of SVZ-derived neurospheres in WT and TTR KO cultures and found that, contrary to our initial hypothesis, the number of neurospheres formed in TTR KO was lower than the one in WT cultures, suggesting that TTR KO mice display a decreased number of neural precursor cells in the SVZ. These results are in apparent disagreement with a recent report addressing neuroproliferation in the SVZ of WT and TTR KO mice (Richardson et al., 2007). According to that study, numbers of Ki67- and phosphorylated histone H3-positive cells in WT and TTR KO mice did not differ, but lack of TTR synthesis produced a significantly reduction in the number of neural precursor cells undergoing apoptosis (Richardson et al., 2007). Therefore, the two studies seem contradictory regarding the population of neural precursor cells in the SVZ. Addition of exogenous TTR to TTR KO SVZ cultures had no significant effect on neurosphere number, being unable to recover the phenotype of TTR KO neurospheres. Again, an indirect mechanism for TTR-mediated regulation of neural precursor cell number in the SVZ, absent in our culture conditions could justify the lack of TTR effect in cultures grown in its presence.

In summary, we herein report that TTR KO mice hippocampal slice cultures are protected against AMPA-induced neurodegeneration, an effect probably mediated by NPY. Furthermore, we also demonstrate that no increase in the number of proliferating cells in the dentate gyrus of the hippocampus arises from the increased NPY levels in TTR KO mice. Instead, TTR KO mice display fewer neural precursor cells in the SVZ. These results contribute to the better characterization of TTR KO mice phenotype, as a model of increased NPY, by addressing two well-known NPY functions, neuroprotection and neuroproliferation.

Acknowledgments

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CHAPTER 3

PART III

NPY EXPRESSION IN THE OSTEOLASTIC LINEAGE
REVEALS A POSSIBLE ROLE IN THE REGULATION OF
BONE FORMATION

NPY EXPRESSION IN THE OSTEOLASTIC LINEAGE REVEALS A POSSIBLE
ROLE IN THE REGULATION OF BONE FORMATION

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Abstract

The comprehension of NPY function in the regulation of bone mass is still very limited, with many opposing results found in the literature. The transthyretin (TTR) knockout (KO) mice display increased neuropeptide Y (NPY) levels and a NPY-overexpressor phenotype as a consequence of peptidylglycine α -amidating monooxygenase (PAM) overexpression, as PAM is the rate-limiting enzyme in neuropeptide maturation. In this work we aimed at better understand the role of NPY in bone biology by making use of this new mouse model of increased NPY. By immunohistochemistry, we demonstrate for the first time NPY synthesis in several bone cells, namely chondrocytes, osteoblasts and osteocytes. Additionally, TTR KO bone sections presented increased amidated NPY levels when compared to WT. We hypothesize that this increased NPY levels are responsible for the observed trend for increased bone mineral density and trabecular volume in TTR KO mice. RT-PCR analysis revealed the pioneer finding that NPY is expressed in MC3T3-E1 cells and bone marrow stromal cells (BMSCs) *in vitro*. This is also the first report showing that both WT and TTR KO BMSCs undergoing osteoblastic differentiation show PAM and NPY mRNA transcripts. While no difference was observed between WT and TTR KO BMSCs regarding NPY expression, PAM synthesis was elevated at day 14 of differentiation in TTR KO BMSCs. Consistent with the trend for increased bone mass, TTR KO BMSCs exhibited enhanced competence to undergo osteoblast differentiation. This work constitutes an important contribution to the better understanding of NPY role in the regulation of bone formation, by showing for the first time NPY expression in the osteoblastic lineage. Furthermore, we hypothesize that increased NPY levels in bone tissue are related to increased bone mass. Further studies are needed to unravel NPY role during fracture healing and, consequently, in the process of bone regeneration.

Introduction

The regulation of bone remodeling has been conventionally linked to local factors, hormones and mechanical loading (Pacifici, 1998; McDonald et al., 2003; You et al., 2007). However, in the last decade several reports provided evidences that bone homeostasis is also under the influence of central and peripheral neural control (Ducy et al., 2000; Baldock et al., 2002; Elefteriou et al., 2003; Chenu and Marenzana, 2005; Baldock et al., 2007). For this concept greatly contributed a number of histological studies revealing the existence of neuropeptide fibers in bone (reviewed in (Elefteriou, 2005). Neuropeptide-containing nerves richly innervate bone tissue and skeletal blood vessels, and these include neuropeptides of sensory, sympathetic, serotonergic and glutaminergic types. In addition, several neuropeptides and neuropeptide receptors are expressed by bone cells, namely calcitonin gene-related peptide (CGRP) and glutamate that are expressed by osteoblasts and osteoblast-like cell cultures (reviewed in (Elefteriou, 2005).

In bone, neuropeptide Y (NPY)-immunoreactive fibers were found mostly distributed in association to blood vessels and in the periosteum (Bjurholm et al., 1988; Hill and Elde, 1991; Tabarowski et al., 1996). Some fibers could be seen departing blood vessels, coursing only a short distance to be observed among cells of the lining layer and marrow cells, before terminating as free nerve endings (Hill and Elde, 1991; Tabarowski et al., 1996). NPY-immunoreactivity was dramatically reduced in sympathectomized animals, indicating the sympathetic origin of these nerves (Hill and Elde, 1991). NPY presence in the periosteum and bone marrow was also confirmed by radioimmunoassay (Ahmed et al., 1994). In addition, NPY expression was found in the rat bone marrow, namely in megakaryocytes (Ericsson et al., 1987). It was suggested that NPY could act in the control of the vascular tone in bone, due to its vasoconstrictive properties. Also, NPY could be involved in the process of immunological maturation, since a burst of NPY-immunoreactive fibers was observed with bone marrow maturation (Bjurholm, 1991). Recently, it was reported that the NPY receptor Y1, but not Y2, Y4, Y5 or y6, was expressed in cultured bone marrow stromal cells and osteoblasts (Lundberg et al., 2007). Despite the existence of NPY fibers and one of its receptors in the bone, NPY knockouts have normal bone mass, questioning NPY control in bone activity (Elefteriou et al., 2003). On the other hand, two different mouse models that have in common the fact they present increased NPY levels, the Y2-receptor and leptin- or leptin receptor-deficient mice, display high bone mass (Stephens et al., 1995; Ducy et al., 2000; Baldock et al., 2002), bringing NPY role in bone biology back into discussion. With regard to Y2 receptor deficient mice, both germline and conditional hypothalamic Y2 receptor KO mice share the same high

bone mass phenotype (Baldock et al., 2002), demonstrating that central hypothalamic Y2 receptors are crucial for this process. Interestingly, while germline Y1 receptor KO mice also display increased bone formation, conditional deletion of hypothalamic Y1 receptors did not alter bone homeostasis, suggesting a non-hypothalamic control of bone mass (Baldock et al., 2007). Furthermore, deletion of both Y1 and Y2 receptors did not produce additive effects on bone, suggesting a common pathway from hypothalamus to bone involving both Y2 and Y1 signaling (Baldock et al., 2007), with a probable central Y2 and peripheral Y1 effects on bone tissue.

NPY is an orexigenic peptide known to antagonize leptin's action on body weight and gonadal function (Elmquist et al., 1999; Inui, 1999). Genetically obese rodents that lack leptin (*ob/ob* mice) or its receptor (*db/db* mice) show increased NPY activity in the hypothalamus due to lack of leptin-induced inhibition of NPY expression (Stephens et al., 1995). Such increased hypothalamic NPY signaling contributes to the massive obesity and reproductive defects of these mice (Friedman and Halaas, 1998). Since NPY ablation in *ob/ob* mice attenuates all these malfunctions (Erickson et al., 1996) and leptin administration reverses the increased NPY hypothalamic expression in *ob/ob* mice, one would predict that intracerebroventricular (icv) infusion of NPY should have an opposite effect to that of leptin, and thus increase bone formation. Surprisingly, icv infusion of NPY decreases bone mass (Ducy et al., 2000), indicating that leptin and NPY do not antagonize each other's function in the control of bone formation as they appear to do in body weight control. Furthermore, this result is not in accordance with the above-mentioned increased bone mass in mouse models of elevated NPY levels. All these opposing results demand a closer look at NPY role in the regulation of bone mass.

In the present work we aimed at better understand the role of NPY in the control of bone formation, by making use of a new mouse model for increased NPY, the transthyretin knockout mice (Nunes et al., 2006). This new mouse model shows increased NPY levels due to peptidylglycine α -amidating monooxygenase (PAM) overexpression, as PAM is the key enzyme in NPY maturation. Here we show for the first time that NPY is expressed by several bone cells, namely osteoblasts. Furthermore, TTR KO mice display increased amidated NPY levels in bone tissue and an increased bone mass phenotype. We suggest that NPY is responsible for increasing bone formation in TTR KO mice.

Materials and Methods

Animals

Mice were handled according to European Union and National rules. Male wild type (WT) and transthyretin (TTR) knockout (KO) (Episkopou et al., 1993) littermate offspring of heterozygote breeding pairs, in the 129/Sv background, were maintained at $24 \pm 1^\circ\text{C}$ under a 12 h light/dark cycle and fed regular chow and tap water *ad libitum*. Prior to all experimental procedures, animals were anesthetized with ketamine/medetomidine. All animals were accustomed to regular handling.

Immunohistochemistry

For immunohistochemistry, femurs from 3 month old male WT (n=6) and TTR KO (n=5) littermates were fixed in 4% paraformaldehyde in PBS, decalcified in TBD-1 commercial solution (Thermo Electron Corporation), embedded in paraffin, and serial 4- μm -thick longitudinal sections were cut. Sections were then deparaffinated, dehydrated in a modified alcohol series and blocked for the endogenous peroxidase activity. NPY immunohistochemistry was performed with the MOM Kit (Vector), following the manufacturer's instructions. Briefly, sections from WT and TTR KO mice, as well as sections from NPY KO mice (a kind gift from Dr. Herbert Herzog, Garvan Institute, Australia) were incubated in the MOM kit blocking reagent for 1 hour at room temperature, prior to the incubation with the monoclonal NPY antibody, NPY05 (generously provided by Dr. Eric Grouzmann, University Hospital, Lausanne, Switzerland, 1:2,000 in MOM diluent) for 1 hour at room temperature. NPY05 is specific for the amidated form of NPY (Grouzmann et al., 1992). Antigen visualization was performed with the MOM Avidin:Biotinylated peroxidase Complex (ABC) reagent, using 3-amino-9-ethyl carbazole (Sigma) as substrate. On parallel control sections, the primary antibody was replaced by MOM diluent. Immunohistochemistry for neurofilament 200 (NF200) and protein gene product 9.5 (PGP 9.5), both markers of nerve fibers, for the FLAG epitope and mitochondrial trypanredoxin peroxidase (mTPx) from *Leishmania infantum* (a kind gift from Dr. Ana Tomás, IBMC, Porto, Portugal), were performed as negative controls. Immunohistochemistry for osteocalcin, a positive control for osteoblast staining, was also performed. Briefly, femur and brain sections (the latest being a positive control for anti-NF200 and anti-PGP staining) were incubated in blocking buffer (1% bovine serum albumin, BSA, and 4% horse serum in phosphate buffered saline, PBS) for 30 min at 37°C in a moist chamber, followed by the incubation with primary antibodies at the appropriate dilution in blocking buffer, overnight at 4°C . The dilutions used were 1:2,500 for rabbit anti-

NF200 (Sigma), 1:4,000 for rabbit anti-PGP 9.5 (Serotec), 1:1,000 for rabbit anti-FLAG (Sigma), 1:1,000 for rabbit anti-mTPx (Castro et al., 2002) and 1:500 for goat anti-osteocalcin (Biomedical Technologies). Antigen visualization was performed with the biotin-extravidin-peroxidase kit (Sigma), using 3-amino-9-ethyl carbazole (Sigma) as substrate. On parallel control sections, the primary antibody was replaced by blocking buffer. Immunohistochemistry analysis was carried out independently by two observers.

Bone Histology

Femurs were harvested from 3 month old male WT (n=6) and TTR KO (n=5) mice. After measuring their length, bones were fixed in 4% paraformaldehyde in PBS, decalcified as described above and embedded in paraffin. Serial 10- μ m-thick longitudinal sections were cut. Sections were then deparaffinated, dehydrated in a modified alcohol series and stained for hematoxylin/eosin.

Micro computed tomography analysis

Dissected hind limbs (femur plus tibia) were scanned with high resolution (5 μ m pixel size) micro computed tomography (microCT, Skyscan 1172, Belgium). The whole mouse femur and tibia were reconstructed, and the trabecular bone in the proximal metaphysis comprised in a region starting 0.25 mm from the growth plate and extending 1.5 mm (or 300 tomograms) distally was analysed. Histomorphometric analysis in 2- and 3- dimensions (2D, 3D) was performed by Skyscan software (CT-Analyser v.1.5.1.3). For the analysis of trabecular bone, cortical bone including the trabecular compartment was excluded by operator-drawn regions of interest and 3D algorithms were used to determine the relevant parameters, which included: bone volume percentage (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp).

Bone mineral density (BMD) measurement by microCT

Volumetric BMD (vBMD) values of the trabecular bone compartment within the femoral and tibial metaphysis were measured from the same regions of interest used to derive the microarchitectural parameters, using manufacturer's instructions. Briefly, two calibration phantoms (Skyscan) with a density of 0.25 and 0.75 g/cm³ and a sample of water were scanned and reconstructed using the same settings used for the femurs and tibiae. The grey scale density values were converted into Hounsfield units (HU) which were then used to compute the mean vBMD of each tibia.

Cell cultures

MC3T3-E1 Mouse osteoblastic cell line culture: MC3T3-E1 cells, established as an osteoblastic cell line from normal mouse calvaria, were grown in alpha-minimal essential medium (alpha-MEM, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen), 0.5% (v/v) gentamicin (Invitrogen), 1% (v/v) fungizone (Invitrogen), 50 µg/mL Vitamin C (Sigma), and 10 mM beta-glycerophosphate (Sigma) in a humidified 5% CO₂ incubator at 37°C. The medium was changed twice weekly. At confluence, the cells were trypsinized and seeded in 24-well plates at a cell seeding density of 4 x10⁴ cells/well.

Bone marrow stromal cells: Primary bone marrow stromal cells (BMSCs) were obtained according to the method developed by Maniopoulos et al (Maniopoulos et al., 1988). Briefly, femurs and tibias from 1 month old male WT and TTR KO littermates were aseptically excised from the hind limbs, the epiphyses cut off, and the marrow flushed with standard culture medium, which consisted of alpha-MEM supplemented with 10% FBS, 50 µg/mL gentamicin sulfate, and 2.5 µg/mL amphotericin B (all from Gibco). Cells were seeded in 75-cm² plastic culture flasks, and incubated in a humidified incubator (37°C and 5% CO₂). The medium was changed after the first 24 h to remove nonadherent cells. Subsequently, the adherent cells were cultured for 10 days, the medium being renewed every 3 days.

Differentiated bone marrow stromal cells: BMSCs were cultured as above, trypsinized and seeded in 24-well plates at a density of 4x10⁴ cells per well. Cells were then differentiated in osteoblasts by the addition of 50 µg/mL Vitamin C (Sigma) and 10 mM beta-glycerophosphate (Sigma) to the culture medium, and cultured for 3, 7, 14 and 21 days. Experiments were made in triplicate and a representative amplification is shown.

Reverse Transcriptase-PCR (RT-PCR) analysis

Total RNA from cell culture samples was isolated with the RNeasy Micro Kit (Qiagen) and subjected to RT-PCR with the Superscript II kit (Invitrogen, Carlsbad, CA). PCR was performed for the appropriate number of cycles for each cDNA (20-40 cycles) at 95°C for 1 min, 56°C for 2 min and 72°C for 3 min. Sense and antisense primers were: for β-actin, 5'-GTGGGCCGCTCTAGGCACCAA-3' and 5'-CTCTTTGATGTCACGCACGATTTC-3'; for NPY, 5'-TGGACT GACCCTCGCTCTAT-3' and 5'-GATGAGGGTGGAACTTGA-3'; for PAM, 5'-CCTGGGGTCACACCTA AAGA-3' and 5'-TGTAAGGACACACCGGAACA-3'; for osteocalcin, 5'-CTCTGTCTCTCTGACCTCACAG-3' and 5'-CAGGTCCTAAATAGTGATACCG-3' (Salingcarnboriboon et al., 2006); for Y1 receptor, 5'-CTCGCTGGTTCTCATCGCTGTGGAACGG-3' and 5'-GCGAATGTATATCTTGAAGTAG-

3' (Lundberg et al., 2007); for Y2 receptor, 5'-TCCTGGATTCTCATCTGAG-3' (Lundberg et al., 2007); for Y5 receptor, 5'-CGCTTCCATCTCAAGCAGA-3' and 5'-AAGTCGTCTACGCTGCCTCT-3'. Primers for β -actin, NPY, PAM and Y5 receptor were designed using PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the sequence from the National Centre for Biotechnology Information database. All primers used are located on two different exons to ensure that only properly spliced mRNA and not genomic DNA contaminants was amplified. Ethidium bromide-stained gels were scanned using a Typhoon 8600 (Amersham, Carnaxide, Portugal) and amplified bands were quantified using the ImageQuant software (Amersham). Fluorescence density of each PCR-amplified band was normalized with the corresponding value of β -actin.

Alkaline Phosphatase Histochemical Staining

Cytoenzymatic localization of alkaline phosphatase (ALP) was performed by histochemical staining using a standard procedure, according to the manufacturer's instructions (Sigma). BMSCs were washed twice with PBS, fixed in 4% v/v formaldehyde for 15 min, washed in water, and incubated for 30 min in Naphtol AS-MX phosphate/Fast Violet B salt at room temperature, in the dark. Finally, cells were washed in water, air dried, and observed under the microscope (6.3x magnification). ALP positive areas appear in red/pink.

Statistical analysis

Statistical analysis was performed using the Student's t-Test. Results were expressed as average $\pm$ SEM. For all statistical analysis, $P < 0.05$ was accepted as being statistically significant.

Results

Neuropeptide Y-immunoreactivity is detected in bone tissue

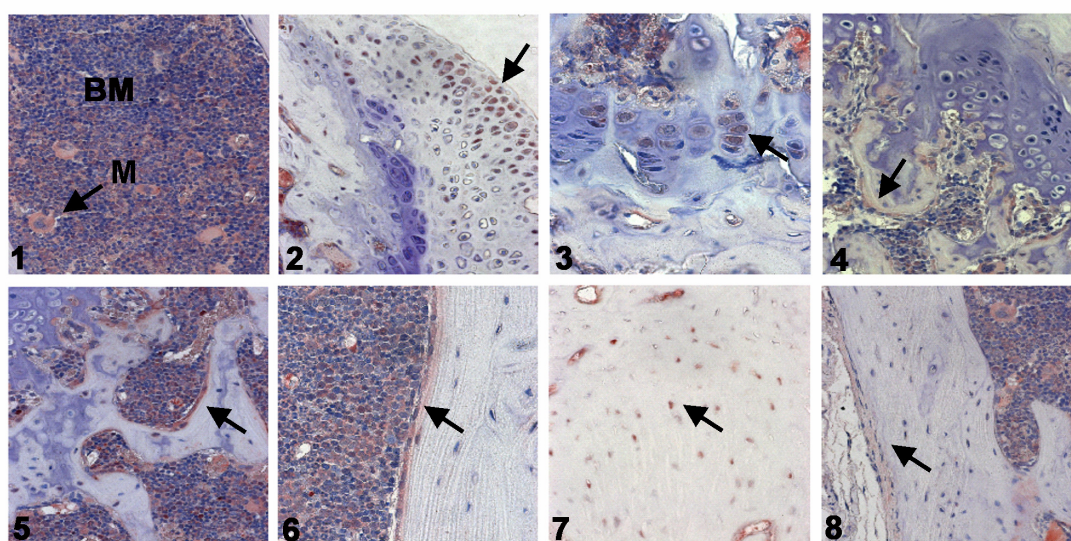
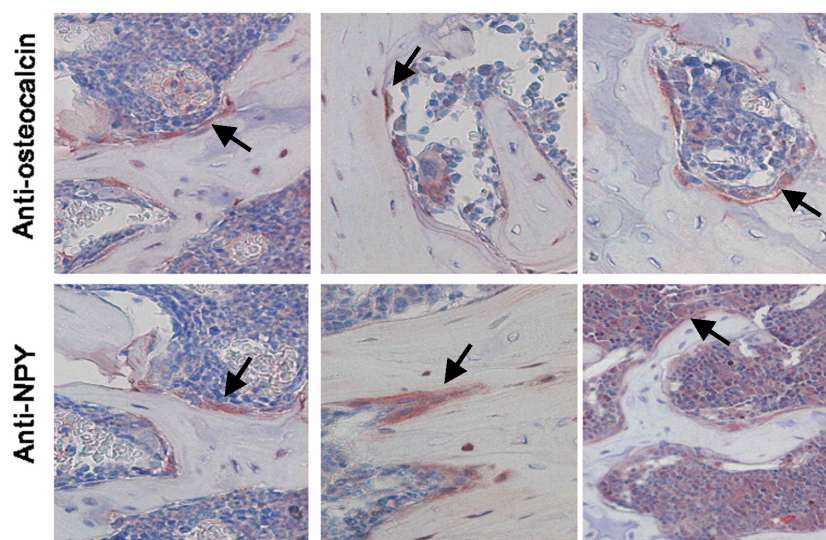
NPY expression was investigated in WT and TTR KO bone tissue by immunohistochemistry, using an antibody specific for the amidated form of NPY. NPY immunolabeling was observed in several bone cells (**Figure 1A, arrows**), namely bone marrow cells and megakaryocytes (**panel 1**), which was already described in the literature (Ericsson et al., 1987). Furthermore, we observed for the first time NPY immunostaining in articular cartilage chondrocytes (**panel 2**), hypertrophic chondrocytes (**panel 3**), osteoblasts lining trabecular bone (**panel 4, 5**), osteoblasts lining cortical bone (**panel 6**) and osteocytes (**panel 7**) from both WT and TTR KO mice. The periosteum (**panel 8**) also showed NPY-immunoreactivity, as already reported for WT mice and rats (Bjurholm et al., 1988; Hill and Elde, 1991; Tabarowski et al., 1996). To further demonstrate NPY synthesis in osteoblasts, osteoblast-specific staining was performed with the anti-osteocalcin antibody. The results obtained reveal that the pattern of staining is comparable to that obtained for NPY, thus confirming its expression in osteoblasts, as shown in the 3 panels of **Figure 1B (arrows)**.

To confirm that NPY is synthesized in these bone cells, a number of different negative controls were performed. Using anti-NF200 and anti-PGP antibodies, two nerve fiber markers, used in the same dilution as for brain labeling, no staining was observed in chondrocytes (C), bone marrow (BM) cells and osteoblasts (O) (**Figure 1C**), whereas in the periosteum a typical nerve fiber labeling was detected (**Figure 1C, lower panels, arrows**). No staining was observed in bone marrow (BM) cells, megakaryocytes (M), osteoblasts (O) and osteocytes (Os) with the anti-FLAG peptide nor with anti-mTPx antibodies (**Figure 1D**), both used to exclude non-specific labeling of the bone tissue by bone-unrelated antibodies. Furthermore, in NPY KO mice bone sections, none of the different bone cells showed NPY immunostaining (**Figure 1E**), suggesting that in fact the immunoreactivity observed in WT and TTR KO bone tissue is NPY-specific.

Increased amidated NPY levels in TTR KO bone tissue

From the comparison between WT and TTR KO NPY-immunoreactivity in bone sections came out the observation that TTR KO bone tissue display increased amidated NPY levels when compared to the WT (**Figure 2, arrows**). NPY immunostaining was increased in articular cartilage chondrocytes (**panel 1**), hypertrophic chondrocytes (**panel 2**), osteoblasts lining trabecular bone (**panel 3**), osteoblasts lining cortical bone (**panel 4**), osteocytes (**panel 5**), bone marrow cells and megakaryocytes (**panel 6**) from TTR KO

mice when compared to the same WT cells. This result is in accordance with the increased NPY levels reported in the nervous system of TTR KOs (Nunes et al., 2006), suggesting that this phenotype is not nervous system-restricted.

A**B**

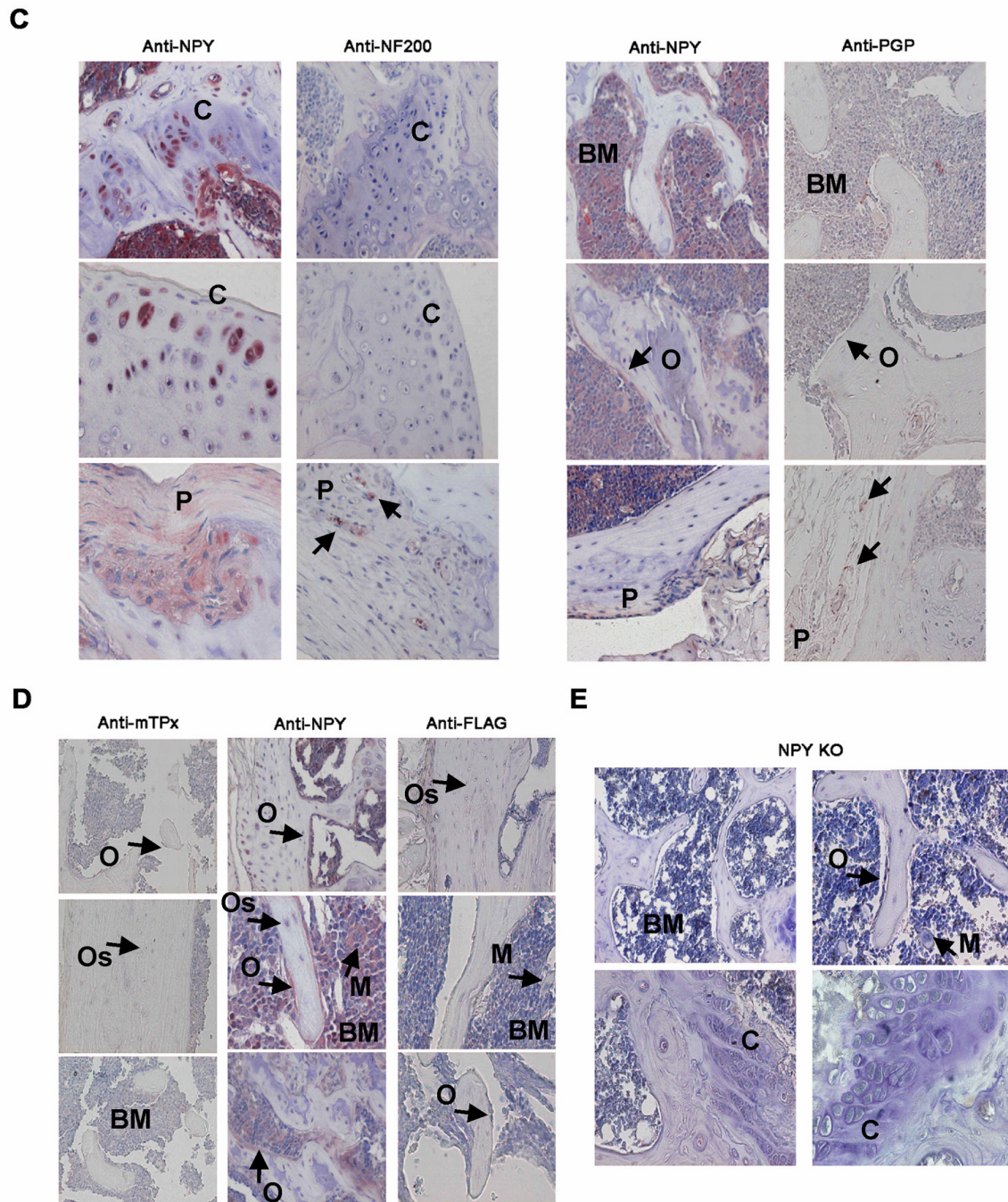


Figure 1. NPY immunohistochemistry in the bone tissue. **A)** NPY immunoreactivity in bone cells, namely bone marrow cells and megakaryocytes (**panel 1**), articular cartilage chondrocytes (**panel 2**), hypertrophic chondrocytes (**panel 3**), osteoblasts lining trabecular bone (**panel 4, 5**), osteoblasts lining cortical bone (**panel 6**), osteocytes (**panel 7**), and in the periosteum (**panel 8**). Arrows highlight labeled cells, and fibers in the case of the periosteum. **B)** Comparison between NPY and osteocalcin immunolabeling in the bone tissue. Arrows highlight labeled osteoblasts. **C)** NF200 and PGP immunohistochemistry, and **D)** mTPx and FLAG immunohistochemistry (as negative controls) in comparison to NPY immunoreactivity in bone tissue. Arrows in **C, lower panels**, highlight nerve fiber labeling in the periosteum. **E)** NPY immunohistochemistry in NPY KO bone sections. BM: bone marrow; C: chondrocytes; M: megakaryocytes; O: osteoblasts; Os: osteocytes; P: periosteum.

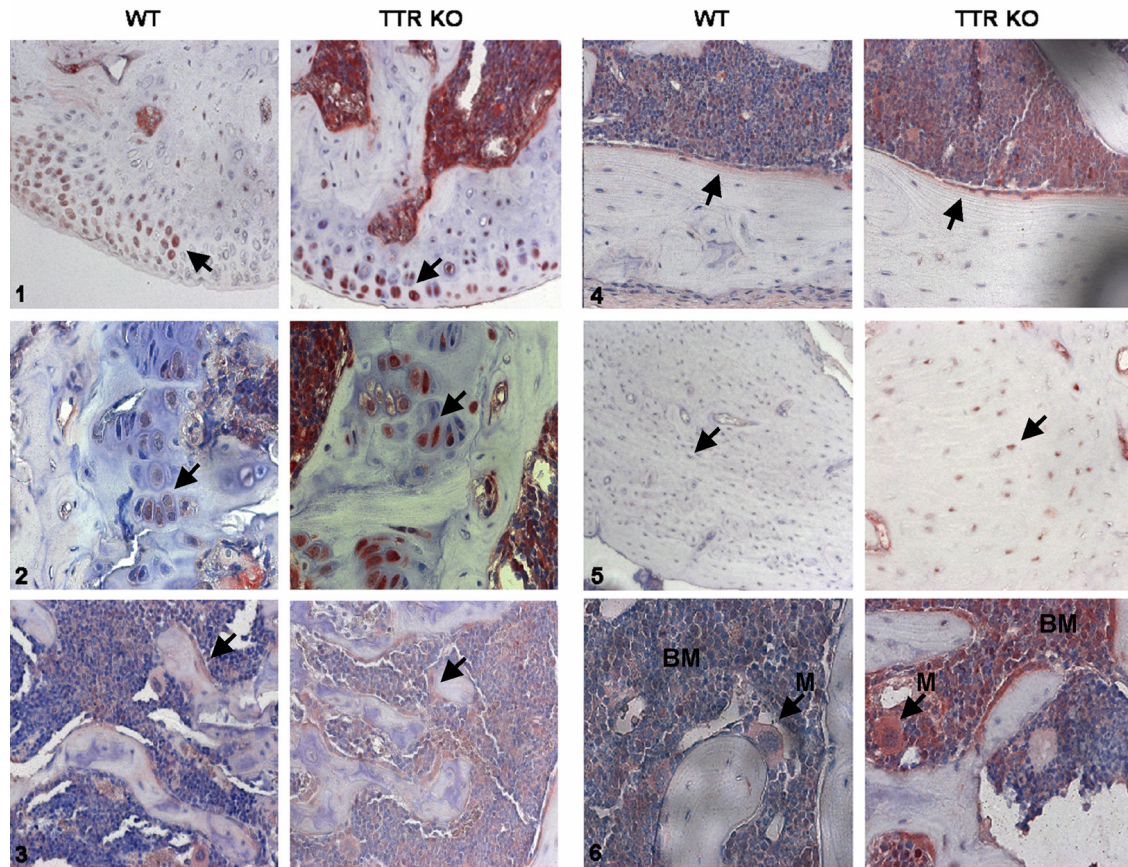


Figure 2. Comparison between NPY immunohistochemistry in WT and TTR KO bone sections. Arrows highlight the different cell types in evidence in each panel, namely articular cartilage chondrocytes (**panel 1**), hypertrophic chondrocytes (**panel 2**), osteoblasts lining trabecular bone (**panel 3**), osteoblasts lining cortical bone (**panel 4**), osteocytes (**panel 5**), bone marrow (BM) cells and megakaryocytes (M) (**panel 6**).

Trend for an increased bone mineral density and trabecular volume in TTR KO mice hind limbs

To address whether the increased NPY levels observed in TTR KO femurs have physiological consequences, we started by comparing bone histology in both WT and TTR KO mice. No obvious differences were found between femurs from the two strains (data not shown). Moreover, the femur length did not differ significantly in WT and TTR KO littermates (WT: 15.6 ± 1.4 mm; TTR KO: 15.9 ± 1.0 mm). To further characterize in detail the bone phenotype of TTR KO animals, microCT scanning analysis, including bone mineral density (BMD) measurement, was performed. As shown in **Figure 3A (left and middle panels)**, 2-dimensional trabecular number and thickness were apparently increased in TTR KO mice when compared to WT. Furthermore, 3-dimensional trabecular bone volume in the proximal metaphysis seemed also increased in TTR KO

animals (**Figure 3A, right panel**). From the statistical analysis of all WT (n=9) and TTR KO (n=10) samples, the results obtained demonstrate a clear trend for increased trabecular volume (BV/TV) and BMD in TTR KO mice when compared to the WT littermates (**Figure 3B**). No difference was however observed in trabecular thickness (WT: 0.053 ± 0.004 mm; TTR KO: 0.056 ± 0.007 mm), trabecular number (WT: 4.0 ± 0.6 ; TTR KO: 4.4 ± 0.7) and trabecular separation (WT: 0.17 ± 0.03 mm; TTR KO: 0.16 ± 0.02 mm). Despite no statistical differences were achieved, these findings suggest that increased NPY levels lead to increased bone density.

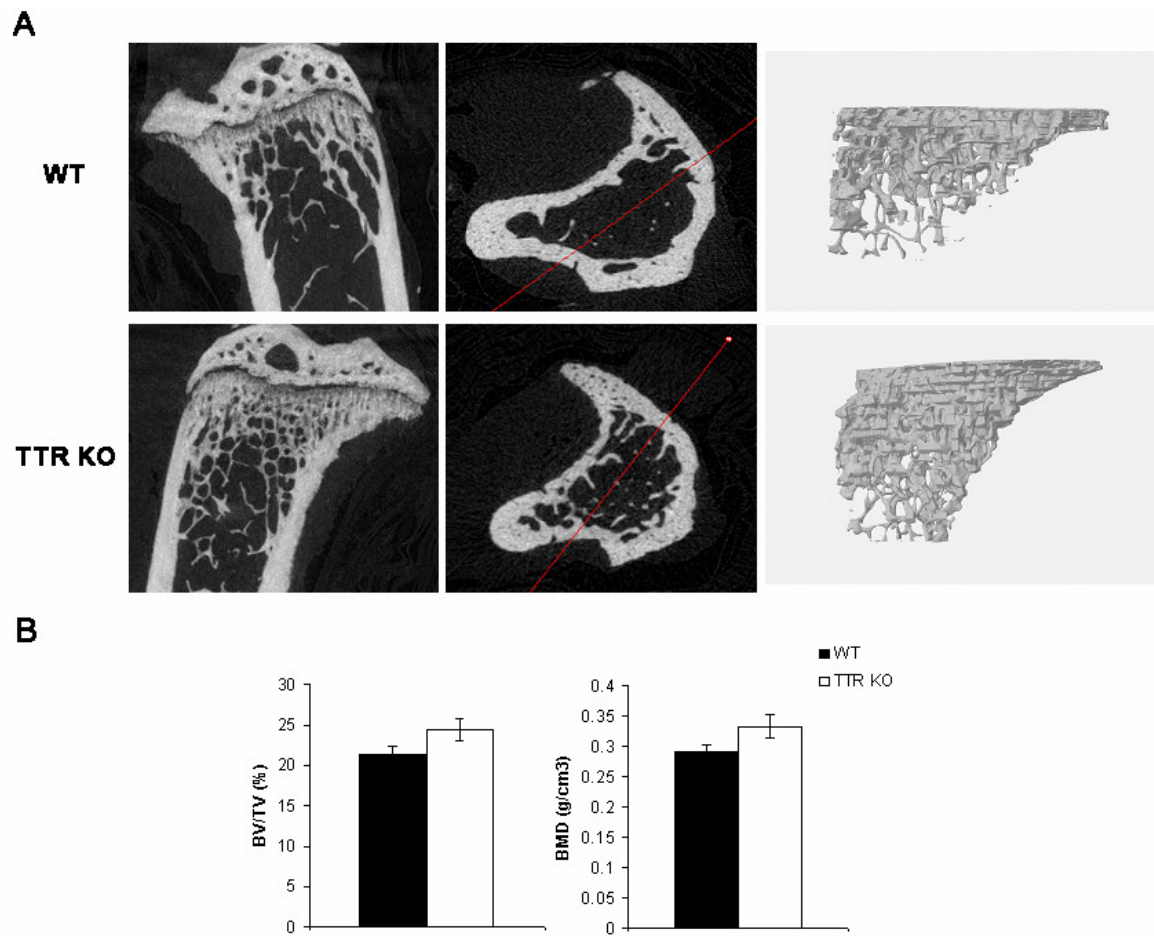


Figure 3. Micro computed tomography (microCT) in WT and TTR KO mice. **A)** Bone microarchitecture in WT and TTR KO mice. **Left and middle panels:** Two-dimensional microCT images of metaphyseal bone, showing reconstructed longitudinal sections (**left panel**) and transverse sections taken ~1 mm from the growth plate (**middle panel**). The red crossing line over the transversal sections indicates the orientation of the longitudinal sections. **Right panel:** Three-dimensional microCT images of metaphyseal trabecular bone in WT and TTR KO mice. **B)** Quantification of trabecular volume (BV/TV) and bone mineral density (BMD) in WT and TTR KO mice. Results are presented as average $\pm$ SEM.

Neuropeptide Y is expressed in bone cells

To further address NPY expression in bone cells, namely in MC3T3-E1 cells, an osteoblastic cell line, and in primary cultures of bone marrow stromal cells (BMSCs) and BMSCs throughout osteoblastic differentiation, we performed semi-quantitative NPY RT-PCR analysis. Using brain tissue as the positive control, we detected NPY expression in MC3T3-E1 cells and both in WT (data not shown) and TTR KO BMSCs (**Figure 4A**). NPY Y1 receptor expression, but not Y2 or Y5 (data not shown), was also detected in TTR KO BMSCs, in agreement with recent reports on WT mice BMSCs (Lundberg et al., 2007) (**Figure 4A**). Furthermore, both WT and TTR KO BMSCs on days 3, 7 and 14 of culture in osteogenic differentiation media presented NPY expression (**Figure 4B**). The NPY expression pattern was variable, with no statistical differences observed between WT and TTR KO BMSC cultures throughout the differentiation period (**Figure 4B**). Again, Y1 expression was revealed by RT-PCR in differentiating WT and TTR KO BMSCs (**Figure 4C**), with no Y2 or Y5 receptor amplification (data not shown), in accordance to the same recently published results (Lundberg et al., 2007). No statistical difference was observed between the two strains regarding Y1 expression (**Figure 4C**).

As peptidylglycine α -amidating monooxygenase (PAM) is the only enzyme that C-terminally α -amidates peptides (Prigge et al., 2000), and NPY requires this posttranslational amidation for biological activity, we assessed PAM expression in WT and TTR KO BMSCs on days 3, 7 and 14 of culture under osteoblastic differentiation conditions. As expected, given the fact that NPY is expressed in these cells, PAM expression was also detected at all time points in both WT and TTR KO cells (**Figure 4D**). Despite no difference in PAM expression was observed in cultures from both strains at day 3 and 7 of culture, at day 14 BMSCs from TTR KO mice displayed increased PAM expression when compared to BMSCs from WT mice (**Figure 4D**). As both WT and TTR KO BMSCs are grown with a similar TTR concentration (provided by the serum added to the culture medium), this finding suggests that TTR KO differentiated osteoblasts already display an intrinsic predisposition for augmented PAM expression in relation to the WT cells, as a consequence of their physiological TTR-free environment. This result is in accordance with our previous report of PAM overexpression in the nervous system of TTR KO animals (Nunes et al., 2006), suggesting that the TTR-mediated inhibition of PAM synthesis is not nervous system-restricted. Also in agreement with our previous report is the fact that neither NPY nor Y1 expression differ between strains.

An attempt was made to measure NPY levels in cell culture conditioned medias by radioimmunoassay (RIA), but it was technically not possible due to the fact that these cells have to be grown in serum-containing media and NPY present in serum interferes with the

assay as its levels are higher than the levels of cell-secreted NPY. For this reason we were unable to compare amidated NPY levels between WT and TTR KO cell cultures, which would be interesting particularly in BMSCs at day 14 of osteoblastic differentiation, a time when differences in PAM expression arise.

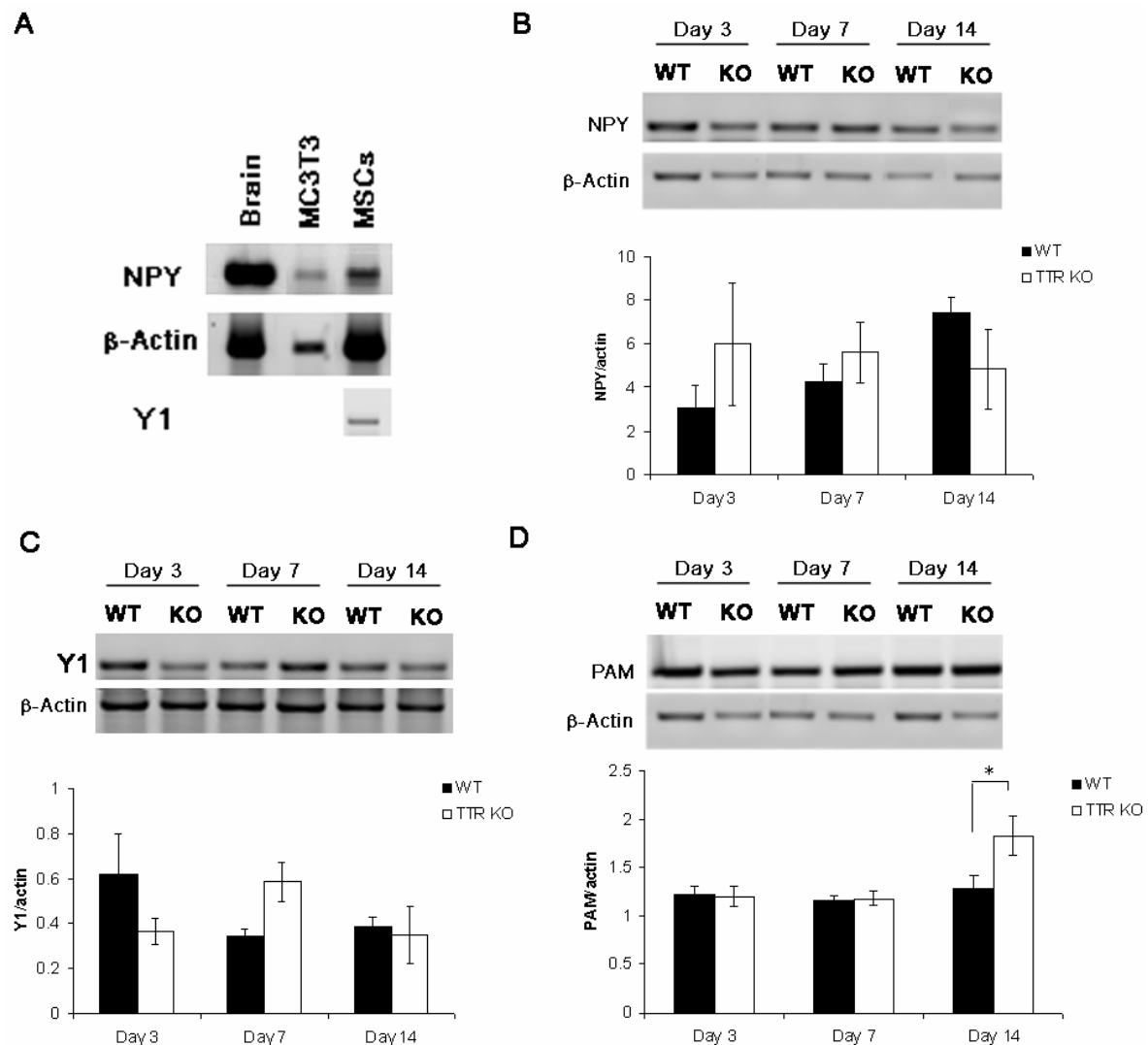


Figure 4. NPY, Y1 and PAM expression in bone cells from WT and TTR KO mice. **A)** NPY RT-PCR analysis in brain, MC3T3-E1 cells, and TTR KO BMSCs; Y1 RT-PCR analysis in TTR KO BMSCs. **B)** NPY, **C)** Y1, and **D)** PAM semiquantitative RT-PCR analysis in WT and TTR KO BMSCs at days 3, 7 and 14 of culture under osteoblastic conditions. Experiments were made in triplicate and a representative amplification is shown. Results are presented as average $\pm$ SEM; * P < 0.05.

Increased osteoblast differentiation of TTR KO bone marrow stromal cells

Given PAM overexpression in TTR KO BMSCs at day 14 of osteoblastic differentiation, it is plausible that amidated NPY levels are increased in these cells, despite the fact that it was technically not possible to demonstrate it by RIA. In fact, being PAM rate-limiting in the process of neuropeptide maturation, meaning that its substrates exist in excess, PAM overexpression in the nervous system of TTR KO mice results in increased levels of processed amidated NPY, without an increase in NPY expression (Nunes et al., 2006). TTR KO mice BMSCs undergoing osteoblastic differentiation seem to recapitulate our findings in the nervous system as PAM expression is increased on day 14, with no difference in NPY or Y1 expression throughout the differentiation process. To examine whether WT and TTR KO BMSCs differ in their capability to undergo osteoblast differentiation under appropriate culture conditions, as a possible consequence of their differential amidated NPY content, we assessed the levels of two osteoblast markers: alkaline phosphatase and osteocalcin. Surprisingly, despite PAM overexpression in TTR KO BMSCs was only evident at day 14 of osteoblastic differentiation, alkaline phosphatase staining was found increased in TTR KO osteogenic cultures as soon as day 3 and 7 and also at day 21 of differentiation when compared to WT cultures (**Figure 5A**). Furthermore, semi-quantitative RT-PCR analysis revealed increased osteocalcin expression in TTR KO BMSCs as soon as day 3 and also at day 14 of culture in osteogenic differentiation media (**Figure 5B**). While we do not have data supporting this hypothesis, we suggest that this increase in osteoblast markers in TTR KO cultures before day 14 of differentiation are, once again, the consequence of intrinsic differences between WT and TTR KO cells due to their different TTR and/or NPY physiological environment. If so, TTR KO BMSCs could already display intrinsic higher competence for osteoblastic differentiation. As expected, cultures from both strains displayed a time-dependent increase in alkaline phosphatase and osteocalcin levels, which is characteristic of the osteoblastic differentiation process. However, their pattern of increase is different: while alkaline phosphatase in WT cultures seems to increase from day 3 to day 14 of differentiation and then decrease at day 21, in TTR KO cultures alkaline phosphatase seems to increase from day 3 till day 21 of osteoblastic differentiation (**Figure 5A**). In summary, these data suggest that TTR KO BMSCs show an enhanced competence to undergo osteoblast differentiation *in vitro*.

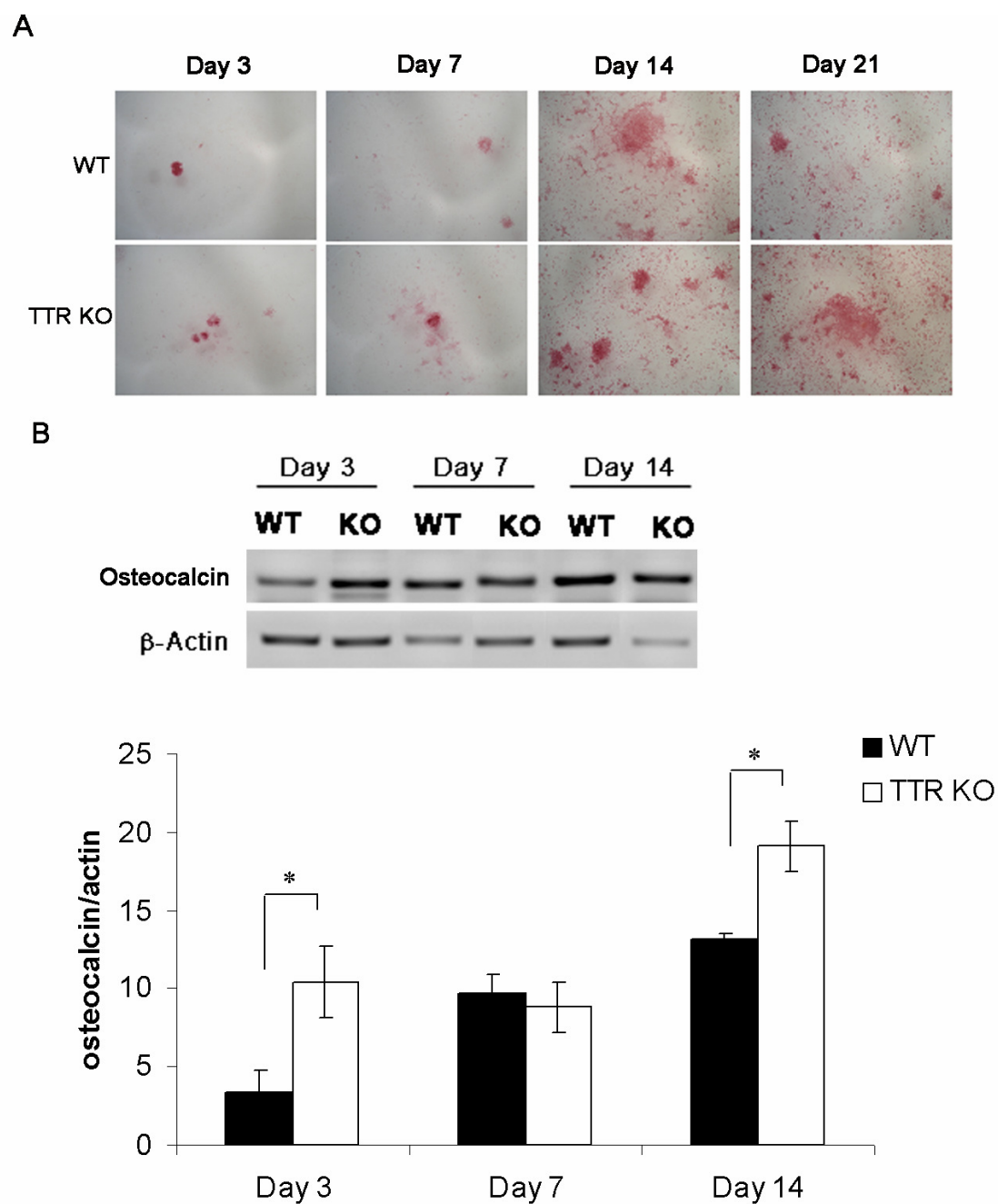


Figure 5. Osteoblast differentiation of WT and TTR KO BMSCs as assessed by alkaline phosphatase and osteocalcin levels. **A)** Alkaline phosphatase staining in WT and TTR KO BMSCs at days 3, 7, 14 and 21 of culture in osteogenic differentiation media. **B)** Osteocalcin semiquantitative RT-PCR analysis in WT and TTR KO BMSCs at days 3, 7 and 14 of culture under osteoblastic conditions. Results are presented as average $\pm$ SEM; * P < 0.05.

Discussion

The data presented in this study show that NPY is expressed in several bone cells, with evidence provided both *in vitro* and *in vivo*. Furthermore, amidated NPY is increased in TTR KO bone tissue, which can explain the slightly elevated bone density observed in TTR KO mice. Consistent with the trend for increased bone mass, TTR KO bone marrow stromal cells (BMSCs) display augmented competence for osteoblast differentiation under appropriate conditions.

Until now, NPY expression was only detected in bone marrow cells, including megakaryocytes (Ericsson et al., 1987). Here we show for the first time that the stromal cells also contribute to the observation of NPY mRNA in the bone marrow, as NPY is expressed in BMSCs and BMSCs undergoing osteoblastic differentiation. Moreover, this work is the first to report NPY expression in chondrocytes, osteoblasts, osteocytes and in the osteoblastic cell line MC3T3-E1. It is known that central NPY regulates bone mass, as conditional ablation of hypothalamic Y2 receptors results in increased bone formation (Baldock et al., 2002). This phenotype is very similar to the one observed in leptin- or leptin receptor-deficient mice, in which NPY is increased in the hypothalamus (Stephens et al., 1995; Ducy et al., 2000). Central NPY can also influence peripheral tissues by alterations in autonomic neuronal activity. This is probably mediated by NPY projections from the hypothalamus to the brain stem areas where sympathetic neuronal activity is modulated (Saper et al., 1976). Thus, to achieve its functions NPY may act centrally on hypothalamic receptors and/or peripherally on its osteoblastic receptor Y1 after being released from sympathetic nerve terminals supplying the skeletal tissue. With this work we have opened a new window in which NPY may also function as an autocrine or paracrine factor since it is expressed by osteoblasts as well. So far, the Y1 receptor is the only NPY receptor identified as being expressed in the bone (Lundberg et al., 2007). However, this is still an open question and future studies may provide more information.

In this work we further demonstrate that TTR KO bone tissue displays increased amidated NPY levels when compared to WT. TTR KO mice present elevated NPY levels in the nervous system as a consequence of increased processing and activation by peptidylglycine α -amidating monooxygenase (PAM) (Nunes et al., 2006). NPY and NPY receptors expression is not altered in this mouse model. Here we show that bone cells and tissue seem to recapitulate our findings in TTR KO nervous system. PAM expression is increased in TTR KO BMSCs on day 14 of osteoblastic differentiation, with no difference in NPY or Y1 expression throughout the differentiation process. Moreover, in the bone, increased amidated NPY levels are observed.

NPY control of bone mass is very controversial. On one side we have two different mouse models with increased NPY expression, displaying high bone mass (Stephens et al., 1995; Ducy et al., 2000; Baldock et al., 2002). This is the case of the NPY Y2 receptor KO mice, which display increased NPY expression (Baldock et al., 2002) due to the lack of Y2 receptors that are thought to act as autoreceptors inhibiting NPY secretion (King et al., 2000), and it is also the case of genetically obese mice that lack leptin (*ob/ob* mice) or its receptor (*db/db* mice) and show increased NPY activity in hypothalamus circuits due to the lack of leptin-induced inhibition of NPY expression and secretion (Stephens et al., 1995; Ducy et al., 2000). Both models share a similar high bone mass phenotype, with an increase in the parameters of osteoblast function and no impairment in the ones of bone resorption. On the other side, we have the report that NPY icv infusion decreases bone mass (Ducy et al., 2000). With regard to this last finding, however, it is not clear whether this central NPY inhibitory effect on bone is a direct consequence of NPY action or a secondary effect of the resulting increase in leptin levels (Sainsbury et al., 1996; Sainsbury and Herzog, 2001), as pointed out by Baldock *et al* (Baldock et al., 2002). The NPY KO mouse is not very helpful in this matter as its bone mass is normal (Elefteriou et al., 2003). The big advantage of the TTR KO mouse model is that, in addition to the increased NPY levels, leptin is not altered (Marques et al., 2007), excluding its interference in the bone phenotype observed. Thus, we claim the responsibility for the slightly increased bone mass in TTR KOs to the increased NPY levels observed in their bones. The bigger competence of TTR KO BMSCs to differentiate into osteoblasts supports the likelihood of our hypothesis, as PAM is overexpressed at day 14 of TTR KO osteogenic culture.

Several TTR mutations are associated with familial amyloid polyneuropathy (FAP), an autosomal dominant lethal disorder characterized by extracellular deposition of TTR amyloid fibrils, particularly in the peripheral nervous system, leading to axonal and neuronal loss (Sousa and Saraiva, 2003). There are few reports in the literature regarding bone involvement in neurological disorders, namely in FAP, in which a gain of TTR toxic function occurs. There is one particular FAP patient whose bone biopsy revealed amyloid deposition resulting in pathological fracture (Allard et al., 1991). However, bone necrosis has been described in 7% of FAP patients as a late clinical feature. More recently, a study showed decreased BMD in FAP patients when compared to other neuromuscular disorders, tending to decrease with disease duration (Conceicao et al., 2005). More than 50% of the FAP group was osteoporotic. Low BMD in FAP patients can be due to the autonomic nerve dysfunction, with diarrheas and malabsorption, which in turn can lead to the lower body mass index observed. It would be interesting to know the NPY levels of

these FAP patients. One can speculate that NPY levels are decreased as a result of the autonomic dysfunction, which in turn could explain the decreased BMD.

In summary, we provide evidence, for the first time, that NPY is expressed in bone cells, namely in osteoblasts. Furthermore, we suggest that the increased NPY levels observed in TTR KO bone tissue are responsible for the slightly increased bone mass phenotype they present. This work constitutes an important contribution to the better understanding of the role NPY may play in the regulation of bone formation, as we show that NPY is expressed in the osteoblastic lineage and is probably responsible for increasing bone mass. Finally, based on these findings, further work is needed to determine the localization of NPY and NPY receptors during fracture healing, and thereby elucidate the possible role of NPY in the bone regeneration process.

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CHAPTER 4

INCREASED LIPOPROTEIN LIPASE IS RELATED TO
INCREASED SPHINGOLIPID CONTENT FOLLOWING
NERVE CRUSH

INCREASED LIPOPROTEIN LIPASE IS RELATED TO INCREASED
SPHINGOLIPID CONTENT FOLLOWING NERVE CRUSH

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Abstract

Peptidylglycine α -amidating monooxygenase (PAM), the rate-limiting enzyme in neuropeptide maturation, is overexpressed in the peripheral (PNS) and central nervous system (CNS) of transthyretin (TTR) knockout (KO) mice. Consequently, TTR KOs display increased neuropeptide Y (NPY) levels and a NPY-overexpressor phenotype. The NPY-overexpressor phenotype of TTR KO mice includes increased expression and activity of lipoprotein lipase (LPL), an enzyme induced by NPY, both in the PNS and CNS. LPL is expressed in Schwann cells, probably intervening in myelin biosynthesis and in the reutilization of lipids from degenerating myelin. Here we show that under physiological conditions, no differences in myelin lipid content arise from increased LPL activity in the nervous system. Given the potential role of LPL in myelin lipids reutilization following nerve injury, together with the fact that TTR KO mice display an impairment in nerve regeneration, we compared the levels of PAM, NPY, LPL and myelin lipids in WT and TTR KO mice after sciatic nerve crush. Similarly to our observations under physiological conditions, the dorsal root ganglia (DRG) and spinal cord samples 15 days after crush revealed increased PAM immunostaining in TTR KO mice. Moreover, the amidated NPY content was also increased in the TTR KO DRG and nerve segment distal to the crush site. Accompanying the increased NPY levels, DRG and sciatic nerve revealed an increased LPL immunostaining in the TTR KOs. Moreover, LPL activity was increased in the distal nerve segment of TTR KO mice. As a probable consequence of LPL increase in the regenerating sciatic nerve of TTR KOs, the sphingolipids sphingomyelin and galactocerebroside were augmented in TTR KO mice distal nerve. Upregulation of these molecules in a system with decreased capacity for nerve regeneration, can only constitute a compensatory mechanism, given their reported ability to increase neurite outgrowth.

Introduction

Lipoprotein lipase (LPL) belongs to the triglyceride lipase gene family, together with the hepatic, pancreatic and endothelial lipases. LPL is the rate-limiting enzyme in the hydrolysis of triglycerides from triglyceride-rich lipoproteins in the blood, namely chylomicrons and very low-density lipoproteins (VLDL) (Eckel, 1989). Mature LPL is secreted to the vascular endothelium from the parenchymal cells of the adipose tissue and skeletal muscle, its major sites of synthesis. Its reaction products, fatty acids and mono- and diacylglycerol, are taken up by these tissues locally where they are processed in a tissue-specific manner: in adipose tissue the free fatty acids produced by LPL activity are re-esterified into neutral lipids (mainly triglycerides but also cholesteryl esters) and stored as fat; in the muscle the free fatty acids are oxidized and used for energy production (Zechner, 1997). After LPL interaction with lipoproteins in the vascular wall, the protein persists linked to the remnant lipoproteins; this catalytically inactive LPL can facilitate lipoprotein uptake by the tissues through binding to heparan sulfate proteoglycans (Eisenberg et al., 1992; Mulder et al., 1993) and lipoprotein receptors, such as members of the low-density lipoprotein (LDL) receptor family (Beisiegel et al., 1991; Medh et al., 1996). This dual action of LPL thus supplies the cells with the lipids they require, namely triglycerides, free fatty acids and cholesterol. Besides adipose tissue and skeletal muscle, LPL is also synthesized in several tissues, including the nervous system, where its function is still unclear. The presence of LPL mRNA in myelinating glial cells in the spinal cord implicated its involvement in the synthesis and maintenance of central nervous system myelin (Bessesen et al., 1993). In the brain, LPL was found to be widely expressed, having the highest levels in the pyramidal cells of the hippocampus (Paradis et al., 2004). Its function was once more associated with myelination as in the developing brain it is greatly expressed at a time point when extensive myelination of axonal fibers is required (Nunez et al., 1995). In the sciatic nerve, LPL immunostaining was reported throughout the endoneurium, with periaxonal and perivascular distribution (Huey et al., 2002). This pattern is in agreement with the finding that LPL is expressed by Schwann cells (Huey et al., 1998). Furthermore, when given an exogenous triglyceride source, Schwann cells efficiently incorporated the triglyceride-derived fatty acids into newly synthesized phospholipids, cholesterol and cholesteryl esters, an effect that was blocked upon incubation with a polyclonal anti-LPL antiserum (Huey et al., 1998). These results suggested that LPL functions in Schwann cells to provide free fatty acids for lipid biosynthesis, and thus that it may play a critical role in the maintenance of nerve myelin. Similar results were also reported in the central nervous system when cultured rat brain cells were shown to incorporate triglyceride-derived fatty acids into triglycerides,

phosphatidylcholine and sphingomyelin (Eckel and Robbins, 1984). After sciatic nerve crush, LPL protein and activity were increased in the nerve segment distal to the crush site (Huey et al., 2002). This suggested that LPL participates in the recycling of myelin lipids from degenerating fibers, perhaps by facilitating free fatty acids uptake and utilization by Schwann cells to remyelinate the regenerating nerve. In fact, following crush injury, the nerve undergoes a series of cellular responses leading to degeneration of the distal stump – Wallerian degeneration (Chen et al., 2007). The myelin sheath surrounding the axon degenerates and the released myelin lipids are scavenged by Schwann cells and macrophages that are recruited to the injury site, leading to the accumulation of intracellular lipid stores. Non-neuronal cells associated with the injury site, mainly macrophages and nonmyelinating Schwann cells, secrete large amounts of apolipoproteins (Boyles et al., 1990). The accumulation of myelin lipids and apolipoproteins leads to the local synthesis of lipoprotein particles that are uptaken by Schwann cells (Rothe and Muller, 1991), providing the lipids for remyelination of the regenerated axons. Besides the hydrolysis of triglycerides, LPL also possesses phospholipase activity, namely towards phosphatidylcholine and phosphatidylethanolamine (Huey et al., 1998). It is thus possible that after injury LPL hydrolyzes myelin phospholipids, the major myelin constituents (Garbay et al., 2000), further contributing to the recycling of degenerating myelin lipids.

The aim of this study was to evaluate the relevance of LPL overexpression in the nervous system of TTR KO mice as this new mouse model of increased NPY displays augmented LPL expression and activity as part of its NPY-overexpressor phenotype (Nunes et al., 2006).

Materials and Methods

Animals

Mice were handled according to European Union and National rules. 3- and 6-month old wild type (WT) and transthyretin (TTR) knockout (KO) (Episkopou et al., 1993) littermate offspring of heterozygote breeding pairs, in the 129/Sv background, were maintained at $24 \pm 1^\circ\text{C}$ under a 12 h light/dark cycle and fed regular chow and tap water *ad libitum*. Unless otherwise stated, comparisons comprised groups of 6 animals of each genotype, age and sex matched. All animals were accustomed to regular handling.

Sciatic nerve crush

Mice were anaesthetized with medetomidine/ketamine and an incision was made in the shaved thigh skin of both sides. The sciatic nerves were exposed and crush was performed bilaterally using Pean forceps, twice during 15 seconds. To standardize the procedure, the crush site was maintained constant for each animal at 35 mm from the tip of the third digit. A single skin suture, immediately above the crush site, served as an additional reference. Animals were allowed to recover for 15 days, after which the injury site was identified based on its position and on the presence of a subtle epineural scar. Mice were sacrificed and DRG L5, spinal cord corresponding to the L5 segment and sciatic nerves proximal and distal to the crush site were removed and frozen.

Immunohistochemistry

For immunohistochemistry (IHC), paraffin-embedded sections were deparaffinated, dehydrated in a modified alcohol series, blocked for the endogenous peroxidase activity and incubated in blocking buffer (1% bovine serum albumin, BSA, and 4% horse serum in phosphate buffered saline, PBS) for 30 min at 37°C in a moist chamber. Incubation with primary antibodies at the appropriate dilution in blocking buffer was performed overnight at 4°C . Primary antibodies used were: rabbit polyclonal anti-PAM (Ab246, a kind gift from Dr. Mains, University of Connecticut Health Center, 1:500) and rabbit polyclonal anti-LPL (Cell Applications, 1:50). LPL staining required boiling of sections for epitope exposure in 10 mM citrate buffer pH 6.0, for 20 min. Antigen visualization was performed with the biotin-extravidin-peroxidase kit (Sigma), using 3-amino-9-ethyl carbazole (Sigma) as substrate. On parallel control sections, the primary antibody was replaced by blocking buffer. Immunohistochemistry analysis was carried out independently by two observers and each tissue was scored from 0 (no labeling) to 3 (intense labeling).

Neuropeptide Y quantification

NPY quantification was performed by radioimmunoassay (RIA) using the Bachem NPY RIA kit. Bachem's anti-NPY antibody has been developed using as antigen amidated NPY₁₋₃₆, and showed a cross reactivity of 39% against NPY₁₋₃₆ free acid, i.e., lacking the C-terminal α -amidation, as previously described (Nunes et al., 2006). Spinal cord samples were sonicated in 400 μ L RIA buffer. After 5 min centrifugation at 15,000 *g*, 100 μ L of supernatant were used in the assay. For peptide extraction from DRGs and sciatic nerves (nerve segments proximal and distal to the crush site), samples were boiled 10 min in 10 volumes of extraction buffer (2 M acetic acid, pH 3.4), homogenized, sonicated and centrifuged 15 min at 3,000 *g*. Supernatants were lyophilized and pellets were used for total protein quantification using the BioRad reagent (Bio-Rad). Lyophilized samples were dissolved in RIA buffer and the assay was performed following the manufacturer's instructions. NPY content was normalized per total protein.

Lipoprotein lipase activity assays

LPL activity assays were performed using the Total Lipase Test kit (Progen Biotechnic). Mice were starved overnight and the procedure was completed during the following morning, due to the enzyme's diurnal variation. Briefly, nerve segments proximal and distal to the crush site were weighted, homogenized in Krebs'-Ringer's buffer, and heparin (Sigma) was added to a final concentration of 100 U/mL. After 45 min incubation at 37°C and 10 min centrifugation at 14,000 *g*, supernatants were used in the assay. LPL activity was normalized per tissue weight.

Myelin Lipid Content

For the analysis of myelin lipids in brain and sciatic nerve samples, tissues were homogenized and sonicated in 0.9% NaCl. After 3 min centrifugation at 13,000 rpm, the supernatant was collected and total lipids were extracted with chloroform:metanol (1:2). Lipids were fractionated according to their polarity in prepacked reversed-phase C18 columns (Macherey-Nagel) and both acidic and neutral lipids, with and without alkaline treatment, were separated by high performance thin layer chromatography in HPTLC silica gel plates (Merck). The lipids galactosyl- and lactosyl-sulfatide, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine and phosphatidylcholine were resolved with the solvent system chloroform:methanol:acetic acid:formic acid:water (35:15:6:2:1). To separate cholesteryl esters from triglycerides this system was also used plus a second dimension with n-hexan:diisopropyl ether:acetic acid (65:35:2). Galactosylcerebroside and sphingomyelin were separated by spraying the plates with a 1% sodium perborate

solution before sample application and resolution with chloroform:methanol:water (70:30:5). The sphingolipids gangliosides were resolved with the solvent system chloroform:methanol:15 mM calcium chloride (55:45:10). All solvents were analysis grade from Merck. A known amount of the corresponding lipid standards (Sigma) were also applied on the HPTLC plates and resolved accordingly. Lipid spots were visualized by spraying with a 1% anisaldehyde solution in sulfuric acid 80%, except for the ganglioside system in which the spraying was performed with a resorcinol solution (0.3% resorcinol, 80% hydrochloric acid, 0.25 mM copper sulfate). Quantitation was performed with the UN-SCAN-IT software (Silk Scientific, USA) and results were normalized per tissue weight. Whenever stated, semi-quantitative analysis was performed in cases where standard resolution did not allow quantitative measurements.

Statistical analysis

Statistical analysis was performed using the Student's t-Test. Results were expressed as average $\pm$ SEM. For all statistical analysis, $P < 0.05$ was accepted as being statistically significant.

Results

Under physiological conditions, no differences in myelin lipid content arise from increased LPL activity in the nervous system

To address whether LPL overexpression in the nervous system of TTR KO mice has biological implications, and as LPL is most likely intervening in myelin biosynthesis (Huey et al., 1998), we analyzed the myelin lipid content in the brain and sciatic nerves of WT (n=5) and TTR KO (n=5) mice. No difference was observed in the content of the examined lipids, namely in the glycerophospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the sphingolipids sphingomyelin (Sph) and galactocerebroside (Galc), and the neutral lipids triglyceride (TG) and cholesteryl ester (CE) (**Figure 1**). These results lead us to hypothesize that LPL effect might only be evident under exacerbating conditions, such as nerve injury/disease.

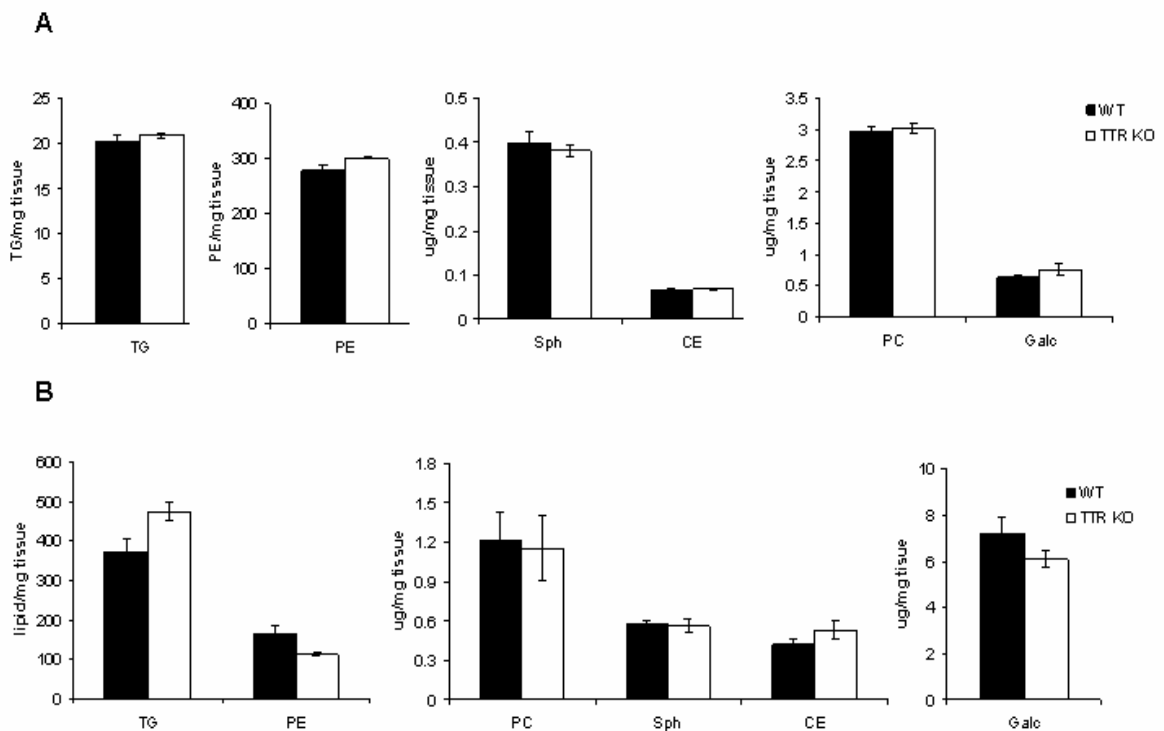


Figure 1. Myelin lipid content in the brain **A**) and sciatic nerve **B**) from WT and TTR KO mice under physiological conditions. TG: triglyceride (semi-quantification); PE: phosphatidylethanolamine (semi-quantification); Sph: sphingomyelin; CE: cholesterol ester; PC: phosphatidylcholine; Galc: galactocerebroside. Results are presented as average $\pm$ SEM.

Following nerve crush, PAM and NPY levels are increased in TTR KO mice

Given LPL potential role in the recycling of myelin lipids following nerve injury (Huey et al., 2002), together with the fact that TTR KO mice present an impairment in nerve regeneration (Fleming et al., 2007), we compared the levels of PAM, NPY, LPL and myelin lipids in the sciatic nerve of WT and TTR KO mice after crush. We choose to perform the analysis after 15 days of regeneration as at this time point TTR KO mice present a decreased number of myelinated fibers that is no longer observed at 30 days of regeneration (Fleming et al., 2007). Similarly to our observations under physiological conditions, the L5 dorsal root ganglia (DRG) and spinal cord samples of TTR KO mice 15 days after crush revealed a 2- and 1.25-fold increase, respectively, in PAM immunostaining when compared to WT animals (**Figure 2A**). In TTR KO mice DRG and spinal cord neurons, intense PAM staining was seen in punctate vesicle-like structures throughout the perinuclear area whereas in the spinal cord white matter, the typical punctate labelling was distributed along the axons (**Figure 2A, arrows**). No difference in PAM labeling was observed between WT and TTR KO nerve segments proximal or distal to the crush site (data not shown). These results suggest that PAM differential levels are more pronounced where neuronal cell bodies exist, that correspond to the site where PAM is being intensely produced, and are diluted all the way through nerve axons.

As a consequence of the increased PAM levels in TTR KOs after 15 days of regeneration, the amidated NPY content was 2-fold increased in 6-month old TTR KO mice DRG when compared to equivalent WT tissue (**Figure 2B**). No difference was however observed in the NPY content in the spinal cord (data not shown), probably reflecting the lower difference in PAM expression between strains in this tissue when compared to the DRG (1.25- and 2-fold, respectively, as stated above). Despite the absence of difference in the NPY levels in the nerve segment proximal to the crush site, the TTR KO mice distal nerve segment displayed a 2-fold increased NPY content when compared to WT littermates (**Figure 2B**). This increase in amidated NPY levels observed in the TTR KO mice distal nerve segment is probably DRG-derived, given the greater difference between strains in PAM expression in the DRG in relation to the spinal cord. Notably, the 2-fold increase in NPY levels in TTR KO mice DRG and distal nerve 15 days after crush is similar to the one reported previously under physiological conditions (Nunes et al., 2006). It is well documented that NPY expression and immunoreactivity are augmented in the DRG and spinal cord following peripheral nerve injury (Corness et al., 1996; Shi et al., 1998). Consistently, we found an approximate 3- and 4.5-fold increase in the amidated NPY levels in the DRG and sciatic nerve, respectively, 15 days after crush

when compared to the same tissues in the control situation (data on NPY levels under control conditions were reported previously by us (Nunes et al., 2006)).

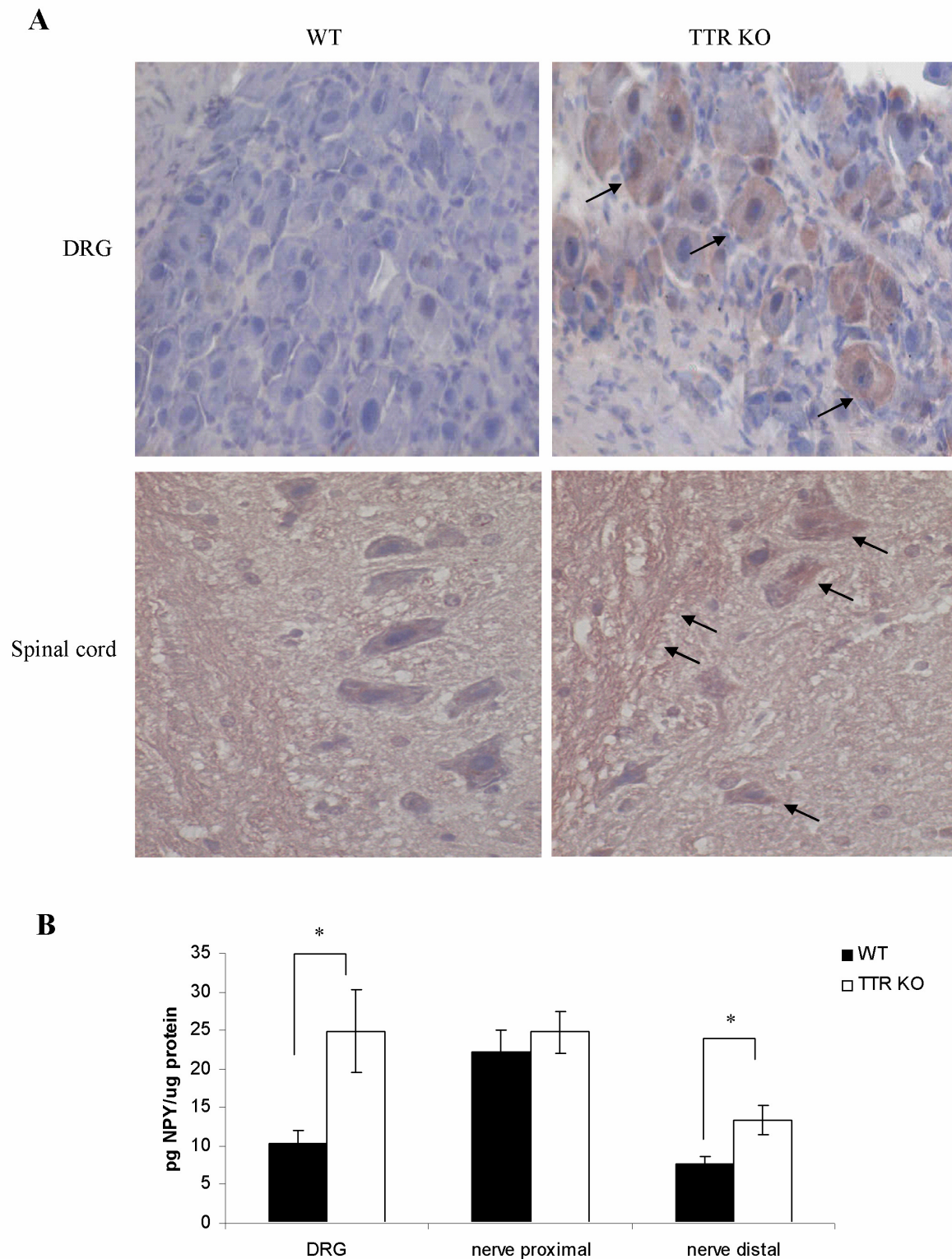


Figure 2. PAM and NPY levels in WT and TTR KO mice 15 days following sciatic nerve crush. **A)** PAM immunohistochemistry in the DRG and spinal cord (20x magnification); arrows highlight structures with increased PAM staining. **B)** NPY content in the DRG and sciatic nerve segments proximal and distal to the crush site. Results are presented as average $\pm$ SEM; * $P < 0.05$.

The NPY content in the DRG under physiological conditions was 4 ± 0.4 and 7 ± 1.6 pg/ μ g protein in WT and TTR KO mice, respectively, and after crush it increased to 10 ± 1.7 and 25 ± 5.4 pg/ μ g protein, respectively. Similarly, in the sciatic nerve, NPY levels under control situation were 2 ± 0.1 and 7 ± 2.6 pg/ μ g protein in WT and TTR KO mice, respectively, and after crush the values increased to 22 ± 3.0 and 25 ± 2.8 pg/ μ g protein, respectively, for proximal nerve, and 8 ± 0.8 and 13 ± 1.9 pg/ μ g protein, respectively, for the distal nerve segment.

LPL is expressed not only in Schwann cells but also in DRG neurons and its activity is increased in TTR KO mice following nerve crush

NPY is one of the key regulators of energy homeostasis, namely by increasing the expression and activity of LPL not only in the white adipose tissue (Billington et al., 1994; Kotz et al., 1998), but also in the nervous system, as previously reported (Nunes et al., 2006). Accordingly, associated to the increased NPY levels in TTR KO mice following crush, the L5 DRG and sciatic nerve distal to the crush site revealed an ~ 2-fold increased LPL immunostaining in TTR KOs when compared to WT (Figure 3A). In the DRG, LPL labeling was found in both large and small neurons whereas in the distal nerve LPL staining was mainly observed in non-axonal cells and in perivascular areas (Figure 3A, arrows). It is noteworthy that we here show for the first time LPL expression in DRG neurons. So far, LPL synthesis in neurons was only reported in the brain (Paradis et al., 2004) while in the peripheral nervous system only non-neuronal cells were shown to express the enzyme (Huey et al., 1998). Consistent with LPL expression in DRG neurons we have previously reported the presence of LPL mRNA and activity in this tissue (Nunes et al., 2006), not knowing which cells were responsible for it. To confirm that LPL is expressed by DRG neurons not only following injury but under physiological conditions, we performed LPL immunohistochemistry in normal DRG sections and found LPL staining in both large and small diameter neurons, similarly to our findings in the DRG following crush (Figure 3B, arrows). No LPL immunostaining was observed in control sections, in which incubation with the primary antibody was omitted (Figure 3B). In parallel with the increased LPL labeling in the distal nerve segment, LPL activity was increased in TTR KO distal nerve segments when compared to WT littermates, with no difference observed in proximal nerve segments (Figure 3C). In both proximal and distal nerve segments an approximate 2.5- and 2-fold increase, respectively, in LPL activity was found when compared to the activity of this enzyme in the sciatic nerve under control situation (Figure 3C). Moreover, following nerve crush a statistically significant difference is observed in LPL activity between WT and TTR KO nerve segments distal to the crush site while under physiological conditions only a positive trend was achieved.

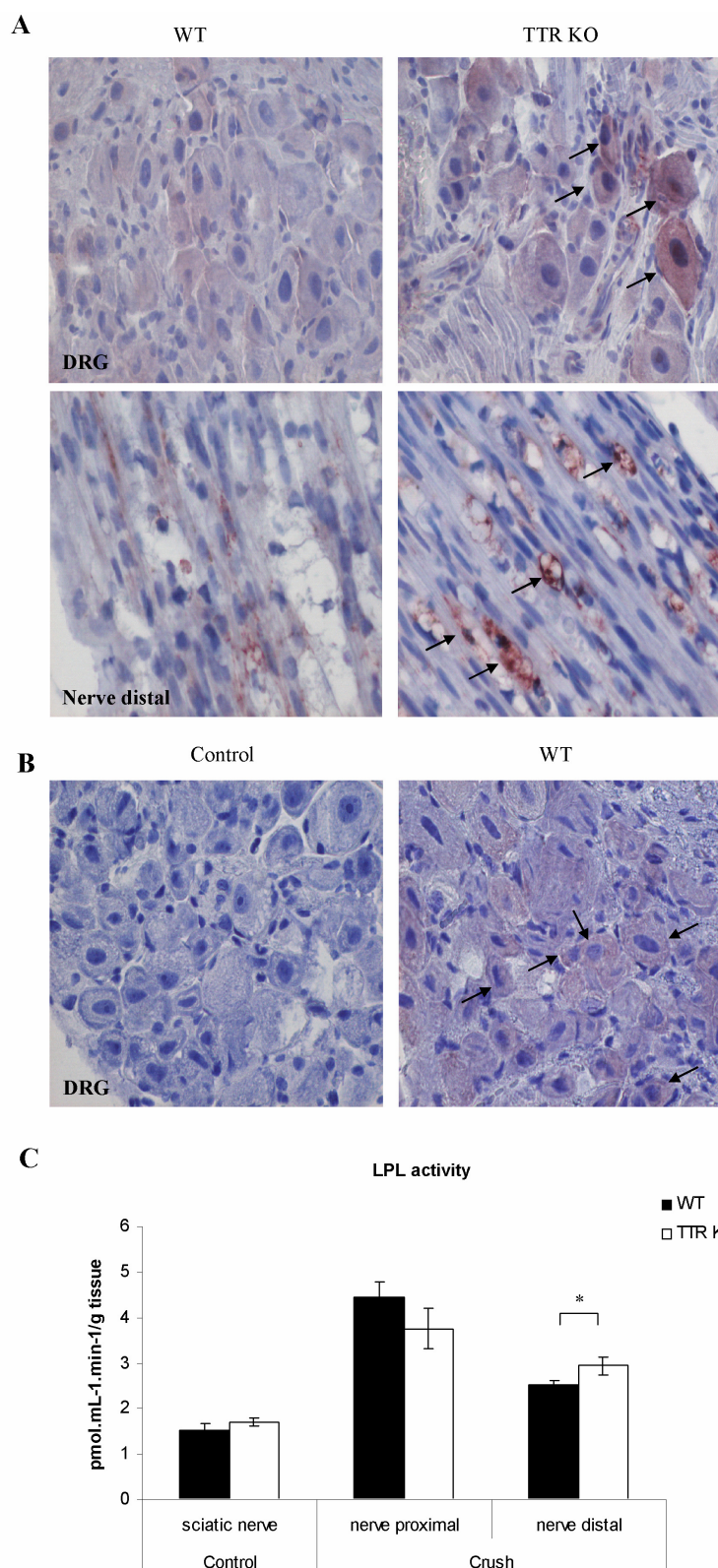


Figure 3. LPL immunohistochemistry and activity in WT and TTR KO littermates at 15 days of regeneration.

A) LPL immunohistochemistry in the DRG and sciatic nerve distal to the crush site (40x magnification); arrows highlight structures with increased LPL staining.

B) LPL immunohistochemistry in the DRG from WT mice under normal uninjured conditions (40x magnification); arrows highlight LPL staining. Negative control was performed by omitting the primary antibody.

C) LPL activity in the sciatic nerve under control and injured situations. Results are presented as average $\pm$ SEM; * $P < 0.05$.

Following nerve crush, increased sphingolipid content is observed in TTR KO mice distal nerve segments

The consequences of LPL increase in the distal nerve in the absence of TTR were assessed by determining the myelin lipid content in the regenerating nerve of TTR KO (n=5) and WT (n=5) mice subjected to nerve crush. From the quantitative analysis of myelin lipids in the sciatic nerve distal to the crush site we observed that the sphingolipids sphingomyelin (Sph) and galactocerebroside (Galc) were 2-fold increased in TTR KOs when compared to the WT littermates (**Figure 4**). No differences were observed in the neutral lipids triglyceride (TG) and cholesteryl ester (CE), and in the phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (**Figure 4**).

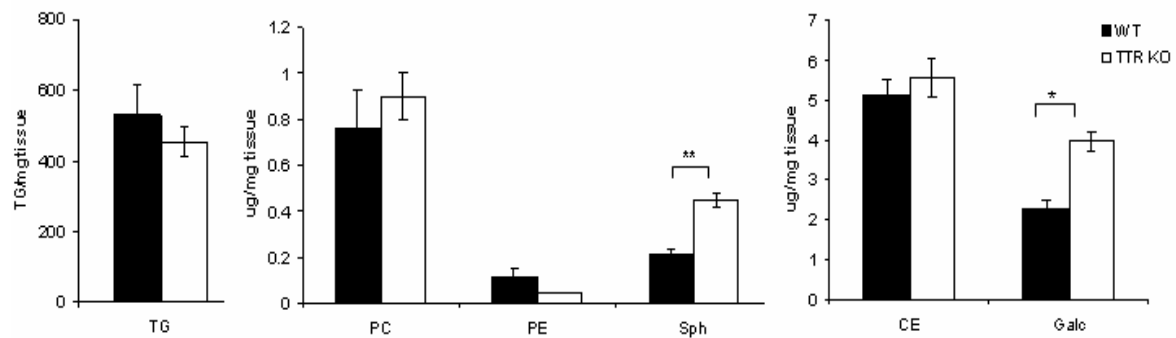


Figure 4. Myelin lipid content in WT and TTR KO mice sciatic nerve distal to the crush site 15 days after injury. TG: triglyceride (semi-quantification); PC: phosphatidylcholine; PE: phosphatidylethanolamine; Sph: sphingomyelin; CE: cholesterol ester; Galc: galactocerebroside. Results are presented as average $\pm$ SEM; * $P < 0.05$, ** $P < 0.005$.

Discussion

In this work we show that LPL is expressed not only by Schwann cells but also by DRG neurons and, that following nerve crush, increased LPL levels are related to increased sphingolipid content.

We have previously reported that TTR KO mice display increased expression of PAM, the rate-limiting enzyme in neuropeptide maturation, in the peripheral and central nervous system (Nunes et al., 2006). Consequently, TTR KOs display increased NPY levels and a NPY-overexpressor phenotype. The NPY-overexpressor phenotype of TTR KO mice includes increased expression and activity of LPL, an enzyme induced by NPY, both in the peripheral and central nervous system. Here we show that, similarly to our observations under physiological conditions, following sciatic nerve crush PAM immunolabeling is increased in the spinal cord and DRG from TTR KO mice when compared to WT. As a consequence of increased PAM levels, amidated NPY content is increased in TTR KO DRG and nerve segment distal to the crush site. The fact that a greater difference in PAM expression between WT and TTR KO mice is observed in the DRG in relation to the spinal cord, might imply a bigger contribution from the former to the increased amidated NPY levels observed in the TTR KO sciatic nerve. Consistent with this hypothesis, the DRG from TTR KO mice display increased amidated NPY content whereas no difference was observed in the spinal cord. Accompanying the increased NPY levels, DRG and sciatic nerve from TTR KOs revealed increased LPL immunostaining. LPL activity was also increased in the TTR KO sciatic nerve distal to the crush site, whereas under physiological conditions only a positive trend was observed. As a probable consequence of LPL increase in the regenerating sciatic nerve of TTR KOs, the sphingolipids sphingomyelin and galactocerebroside were augmented in TTR KO distal nerve when compared to WT animals. This elevation in sphingolipid levels might imply that the increased amount of free fatty acids available that are generated by LPL action are being used as a substrate for the sphingolipid biosynthetic pathway.

Little is known and reported in the literature regarding PAM response to peripheral nerve injury. To our knowledge, there is only one study comparing PAM levels in the contralateral and injured nerve. This report shows that PAM activity is decreased following sciatic nerve section (Rhodes et al., 1990). Given the markedly increase in NPY levels observed after injury, either transection or crush (Ohara et al., 1994), it is expected that PAM is also elevated, as PAM is the key enzyme in neuropeptide amidation. Each secretory vesicle is thought to contain only a few molecules of the processing enzyme PAM (Mains et al., 1987), making it rate-limiting in the process of neuropeptide maturation, as its substrates exist in excess. Therefore, an increase in the amidated active

NPY would only be achieved if PAM is also increased. Otherwise, the elevated NPY expression observed after crush would only contribute to increase the pool of immature NPY, the PAM substrate, and no overfunction would be accomplished. NPY is substantially upregulated in the DRG after sciatic nerve injury, mainly in large DRG neurons (Corness et al., 1996). We here report that in addition to DRG, NPY is also upregulated in both nerve segments proximal and distal to the crush site. This probably arises as a consequence of DRG neurons projection to the sciatic nerve and vesicular transport of NPY across the axons.

LPL was known to be upregulated following sciatic nerve crush in the distal stump (Huey et al., 2002). We here show increased LPL activity both in the proximal and distal nerve segments after crush. In accordance with the increased LPL activity in the nerve segment proximal to the crush site when compared to uninjured nerve, we show that amidated NPY is also increased in the proximal nerve segment after crush. Our data showing increased NPY on the proximal nerve segment also suggest that macrophages are not the sole responsible cell type for increased LPL activity after sciatic nerve crush, as macrophage infiltration only occurs in the distal stump.

The presence of LPL in the normal uninjured sciatic nerve supports the hypothesis that LPL has a function in the preservation of normal nerve structure and/or function, probably by providing free fatty acids to be uptaken and utilized by nerve cells. With regard to Schwann cells, they may depend upon LPL for lipid uptake and normal maintenance of the myelin sheath. In agreement with this hypothesis, phospholipids constitute the greatest portion of the myelin (Garbay et al., 2000) and LPL participates in phospholipid synthesis by Schwann cells (Huey et al., 1998). Furthermore, several models of LPL deficiency are associated with peripheral neuropathies. Rats rendered diabetic through streptozotocin injection display decreased LPL activity in the sciatic nerve, what could explain their decreased motor nerve conduction velocity (Ferreira et al., 2002). In the fatty liver dystrophy mouse model, deficiencies in LPL activity exist during the suckling period and a peripheral neuropathy with thinner myelin sheaths is evident (Langner et al., 1991). In addition, LPL deficient cats display nerve lesions and loss of myelinated fibers (Jones et al., 1986). Similar findings were observed in human patients with abetalipoproteinemia who have reduced LPL activity (Wichman et al., 1985). It is probable that LPL function in the peripheral nerve is similar to the well known function in the adipose tissue and other non-neuronal tissues, which is to supply the cells with triglyceride-derived fatty acids from circulating lipoproteins. Accordingly, lipoproteins and lipoprotein receptors are also present in the nervous system (Rothe and Muller, 1991; Ishiguro et al., 1995; Koch et al., 2001). Following injury, both the brain and sciatic nerve display increased LPL levels (Huey et al., 2002; Blain et al., 2004; Paradis et al., 2004). A

similar LPL involvement in the scavenging and recycling of lipids released by the degenerating nerve fibers was proposed. The recycled lipids are reassembled into locally produced lipoproteins to be endocytosed by surviving neurons and myelinating cells and lipids used in the regenerative processes. Other potential roles for LPL in the nervous system include the stimulation of neurite outgrowth and protection against oxidized lipoprotein-mediated toxicity (Postuma et al., 1998; Paradis et al., 2003). Taken together, these data suggest a beneficial role for LPL in the peripheral nervous system, which does not agree with our observations in the TTR KO mice. This new mouse model of increased NPY displays augmented expression and activity of LPL, both in the peripheral and central nervous system, as part of the observed NPY-overexpressor phenotype (Nunes et al., 2006). It was recently published that TTR KO mice present a sensorimotor impairment and delayed regeneration following sciatic nerve crush as a consequence of a decreased axonal outgrowth (Fleming et al., 2007). Moreover, in the process of regeneration, 15 days after crush, TTR KO mice have a decreased number of myelinated fibers that reaches however WT levels 30 days post-injury (Fleming et al., 2007). These findings would be expected in a situation of LPL deficiency, as referred above, not in a model with increased LPL. One possible explanation for these inconsistencies is that LPL is increased in TTR KO mice as part of a compensatory mechanism. Probably LPL is increased in TTR KOs to fight against the impairment caused by the absence of TTR in the process of regeneration. It is noteworthy that the decreased number of myelinated fibers in TTR KO mice 15 days post-crush is unrelated to a myelination impairment, as the g ratio determined in both WT and TTR KO mice was similar (Fleming et al., 2007). In agreement with that, we here report that no major differences were observed in WT and TTR KO mice phospholipids in the distal nerve following crush, with the exception of sphingomyelin, that was increased in TTR KOs. Phospholipids are the major myelin constituents in the peripheral nervous system, representing more than 50% of the total myelin lipids (Garbay et al., 2000). Sphingomyelin represents 11.5% of the total phospholipids in the mouse myelin, making it difficult to envisage that an increase in sphingomyelin alone would cause a notorious effect in the myelination process. Again, a compensatory mechanism can explain the increased sphingomyelin and galactocerebroside levels observed in TTR KO mice regenerating distal nerve segment, as they can be implicated on axonal growth. It was shown that some phospholipids, including sphingomyelin, and to a less extent also cerebroside, have neurite promoting activities on cultured neurons (Arakawa et al., 1991). Galactocerebroside constitutes 16% of the total peripheral nervous system myelin in the rat (Garbay et al., 2000) and, like sphingomyelin, is synthesized from ceramide. To our knowledge, no studies have been

performed regarding the effect of myelin lipids, in particular sphingolipids, in the process of remyelination following peripheral nerve injury.

NPY is known to contribute to the nerve injury-induced increase in neurite outgrowth of cultured DRG cells (White and Mansfield, 1996), thus promoting nerve regeneration. Nevertheless, TTR KO mice display increased NPY levels and delayed regeneration. These opposing observations may only be explained through the hypothesis that NPY is also a component of the compensatory mechanism established in TTR KO mice, together with the increased levels of LPL and sphingolipids.

In summary, we here report that following nerve crush TTR KO mice display increased LPL activity in the distal nerve segment and hypothesize that the increased amount of free fatty acids available generated by LPL action enter the sphingolipid biosynthetic pathway, as increased levels of sphingomyelin and galactocerebroside were found in TTR KO nerves. Furthermore, we provide for the first time evidence that LPL is expressed by DRG neurons.

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CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions and future perspectives

In this work we identified a novel role for transthyretin (TTR) in nervous system physiology: the regulation of neuropeptide maturation through downregulation of peptidylglycine α -amidating monooxygenase (PAM) expression. The biological relevance of this finding is that, as a consequence of PAM overexpression, TTR knockout (KO) mice display increased neuropeptide Y (NPY) levels and a NPY-overexpressor phenotype. NPY is the major neuropeptide present in the mammalian peripheral and central nervous system (PNS, CNS) and is involved in the regulation of several physiological functions, such as energy homeostasis, neuroprotection and neuroproliferation. We demonstrate that TTR KO mice display increased energy intake in parallel with decreased energy expenditure, reflected by the augmented lipoprotein lipase (LPL) expression and activity, carbohydrate consumption and preference, and diminished body temperature. Our findings suggest a role for NPY in mediating neuroprotection in TTR KO mice organotypic hippocampal slice cultures. In contrast to the neuroproliferative function of NPY, TTR KO mice display fewer neural precursor cells in the subventricular zone. In bone, where NPY function is still poorly understood, we demonstrate for the first time NPY expression in the osteoblastic lineage and hypothesize that the increased NPY levels observed in TTR KO mice bone tissue are responsible for the observed trend for an increased bone mass in these animals. These results contribute to the better characterization of TTR KO mice phenotype, as a model of increased NPY and highlight NPY role in diverse biological systems. Moreover, by demonstrating TTR regulation of PAM expression we have opened the doors to a new field of investigation, in which over half of the known neuropeptides might be involved. Similarly to NPY, it is probable that many other amidated neuropeptides are decreased by TTR action. Supporting this hypothesis, we here show increased levels of substance P, another major amidated neuropeptide, in the PNS and plasma of TTR KO mice. The physiological consequences of this observation, as well as the possible increase of other amidated neuropeptides, should be further investigated. Given the numerous physiological and pathological functions in which neuropeptides are involved, the modulation of PAM expression by TTR might impact several biological conditions.

While excluding TTR involvement in PAM mRNA stability, our data suggest that TTR internalization is a critical step for the regulation of PAM expression and, consequently, amidated NPY levels. The finding that TTR is most likely endocytosed by cortical neurons via receptor-mediated pathway underlies the need to unravel its mechanism of action. The mechanism underlying PAM regulation by TTR is still not

understood and should also be further explored. Using DIGE and GeneChip analysis, several other questions and hypothesis were raised, some of them remain unanswered and should be the subject of future investigation. While we were unable to demonstrate TTR involvement in proliferation and apoptosis of cells of neuronal origin, the physiological relevance of the decreased levels of glutamate and GABA in the presence of TTR should be addressed. Our results also stress the need to further elucidate TTR involvement in neurite outgrowth and retrograde transport. Moreover, validation of results from GeneChip analysis embraces the assessment of TTR role in synaptic transmission, integrin signaling and cholesterol/steroid biosynthesis.

LPL increase in TTR KO nervous system led us to evaluate putative differences in myelin lipid content, as LPL is probably intervening in myelin biosynthesis and in the reutilization of degenerating myelin lipids. No differences exist between WT and TTR KO mice under physiological conditions, though following nerve crush increased LPL is related to increased sphingolipid content. The relevance of this finding in the process of nerve regeneration should be further addressed.

In conclusion, our findings demonstrate that TTR plays a role in nervous system physiology, namely by regulating neuropeptide maturation through downregulation of PAM expression. This finding constitutes an important achievement, as understanding the relevance of TTR presence in the PNS and CNS will probably have impact in understanding its physiology and the pathogenic events related to neurodegeneration/neuroprotection in disorders of the nervous system.

PART III

APPENDIX

List of abbreviations

Abeta	Amyloid β protein
AD	Alzheimer's disease
ALP	Alkaline phosphatase
ALS	Amyotrophic lateral sclerosis
AmP	Aminopeptidase P
ApoA-I	Apolipoprotein A-I
APP	Amyloid precursor protein
BMD	Bone mineral density
BMSC	Bone marrow stromal cell
BrdU	5-bromo-2'-deoxyuridine
CNS	Central nervous system
CSF	Cerebrospinal fluid
DG	Dentate gyrus
DPPIV	Dipeptidyl peptidase IV
DRG	Dorsal root ganglia
FAP	Familial amyloid polyneuropathy
FTD	Frontotemporal dementia
GABA	Gamma-amino butyric acid
GAD67	Glutamic acid decarboxylase 1
HDL	High density lipoprotein
hnRNP K	Heterogeneous nuclear ribonucleoprotein K
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
KO	Knockout
LDCV	Large dense-core vesicle
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
mTPx	Mitochondrial tryparedoxin peroxidase
NE	Noradrenaline
NF200	Neurofilament 200
NGF	Nerve growth factor
NPC	Neural progenitor cell
NPY	Neuropeptide Y
NSC	Neural stem cell
OAT	Ornithine aminotransferase
PAM	Peptidylglycine alpha-amidating monooxygenase
PC	Prohormone convertase
PD	Parkinson's disease

PGP 9.5	Protein gene product 9.5
PI	Propidium iodide
PNS	Peripheral nervous system
PP	Pancreatic polypeptide
PYY	Peptide YY
RAR	Retinoic acid receptor
RBP	Retinol-binding protein
RXR	Retinoid X receptor
SVZ	Subventricular zone
T ₃	Triiodothyronine
T ₄	Thyroxine
TBG	Thyroxine-binding globulin
TH	Thyroid hormone
THDP	Thyroid hormone distributor protein
TR	Thyroid hormone receptor
TTR	Transthyretin
VLDL	Very low-density lipoprotein
WAT	White adipose tissue
WT	Wild type

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