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# TRANSTHYRETIN AS A SIGNALING MOLECULE: INFLUENCE ON IGF-IR PATHWAY AND 14-3-3ζ METABOLISM

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De acordo com o disposto no nº 2, alínea a, do artigo 31º do Decreto-Lei nº 230/2009, utilizaram-se neste trabalho resultados já publicados ou vias de publicação que a seguir se discriminam:

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No cumprimento do Decreto-Lei supra mencionado, o autor desta dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação e discussão dos resultados e na sua redação. Todo o trabalho experimental foi realizado pelo autor desta tese de doutoramento, Marta Virgínia Mota Vieira, exceto as experiências indicadas em contrário.

SUMÁRIO

À minha maravilhosa mãe,

a pessoa mais bonita que alguma vez conheci, a quem devo grande parte do que sou…

«Eras um pouco muito de mim…

Ficou o teu sorriso no que não esqueço, ficaste todo em mim.»

José Luís Peixoto

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# SUMMARY

Transthyretin (TTR) is the carrier protein of thyroxine ($T_4$) and retinol, through binding to retinol-binding protein (RBP), in plasma and cerebrospinal fluid (CSF). TTR is mainly synthetized by liver being secreted to blood. In brain, TTR synthesis occurs in choroid plexus followed by secretion to CSF. Beside these well known roles, TTR has been described as a neuroprotective molecule in the central nervous system (CNS): prevents Aβ toxicity; modulates Aβ levels in a gender dependent manner; improves nerve regeneration under nerve injury conditions and CSF TTR enhances survival of endangered neurons in cerebral ischemia. In an Alzheimer disease (AD) mouse model administration of insulin-like growth factor I (IGF-I) induced clearance of Aβ from brain, in part, through modulation of TTR levels. Activation of IGF-I receptor (IGF-IR) signaling cascade together with increased TTR expression levels were associated with reduced neurodegeneration in the same AD mouse model.

In the present work it was demonstrated the role of TTR as a signaling molecule through IGF-IR. It was shown that TTR binds to IGF-IR, increasing transcriptional receptor levels. It was also reported that TTR acts synergistically with IGF-I, the major ligand of IGF-IR, increasing activation of specific signaling pathways through phosphorylation of IGF-IR, Akt and FoxO, thus protecting HT22 hippocampal cell line from glutamate-induced cell death. *In vivo* studies demonstrated that young TTR null mice had decreased levels of pIGF-IR and pAkt when compared with age matched littermates. Total IGF-IR levels were also decreased in young TTR null mice. However, aging abolishes TTR effect on IGF-IR pathway. The synergistic effect of TTR and IGF-I can be explained by rearrangements in secondary structure and increased hydrophobic surface of TTR in the presence of IGF-I.

The present work also revealed neuritogenic effect of TTR in a dose response manner on hippocampal neurons, by increase in neurites number and length.

We proved that TTR regulates 14-3-3ζ levels in hippocampus of young and adult animals, being its effect abolished in old animals. The presence of TTR prevented 14-3-3ζ lysosomal degradation. TTR role was specific to the zeta isoform and did not occur at transcriptional level. A role of TTR in inhibiting autophagy was verified, probably by the regulation of 14-3-3ζ levels.

In summary, this work revealed important TTR functions on aspects that had never been described before: (i) increase of IGF-I receptor levels; (ii) synergistic effect in activation of the IGF-I receptor signaling cascade leading to excitotoxicity protection and (iii) modulation of autophagy by regulation of 14-3-3ζ levels.

# SUMÁRIO

A transtirretina (TTR) é uma proteína transportadora de tiroxina ($T_4$) e retinol através de ligação à proteína transportadora de retinol (RBP), no plasma e no líquido cefalorraquidiano (LCR). A TTR é sintetizada principalmente no fígado sendo secretada para o sangue. No cérebro, a síntese de TTR ocorre nos plexos coróideus seguida de secreção para o LCR. Para além destas funções muito bem conhecidas, a TTR tem sido descrita como uma molécula neuroprotetora no sistema nervoso: previne a toxicidade causada pelo péptido Aβ; dependendo do género modula os níveis do Aβ; melhora a regeneração nervosa em condições de lesão no nervo e promove a sobrevivência de neurónios lesionados em isquemia cerebral. Num modelo animal de murganho para a doença de Alzheimer (AD) a administração de fator de crescimento I semelhante à insulina (IGF-I) induziu a remoção do péptido Aβ do cérebro, em parte pela modulação dos níveis de TTR. A ativação da cascata de sinalização do recetor do IGF-I (IGF-IR) juntamente com o aumento dos níveis de expressão de TTR foram associados à reduzida perda neuronal no mesmo modelo animal.

Neste trabalho foi demonstrado o papel da TTR como molécula sinalizadora através do IGF-IR. Mostrou-se que a TTR se liga ao IGF-IR e ao seu ligando IGF-I e aumenta os níveis de transcrição do IGF-IR. Apresentaram-se evidências para a actuação sinergística da TTR com o IGF-I na ativação de vias de sinalização específicas, pela fosforilação do IGF-IR, Akt e FoxO. O efeito sinergístico da TTR e do IGF-I foi explicado por rearranjos de estrutura secundária assim como no aumento da superfície hidrofóbica da TTR na presença de IGF-I. Verificámos que tal sinergia se revela neuroprotetora para a linha celular de hipocampo, HT22, da morte celular provocada pelo glutamato.

Estudos *in vivo* demonstraram que murganhos jovens sem TTR apresentam níveis mais baixos de pIGF-IR e pAkt quando comparados com animais controlo da mesma idade. Os níveis totais de IGF-IR estão também diminuídos nos animais jovens sem TTR. Porém, com o avançar da idade o efeito da TTR na via de sinalização do IGF-IR é eliminado.

Este trabalho também revelou que a TTR induz o aumento do número e do comprimento das neurites em neurónios de hipocampo, numa forma dependente da concentração, ficando por determinar se tal facto se relaciona com a nova descrição do efeito da TTR no eixo IGF-I.

Estudos de proteómica em hipocampos de animais selvagens e animais sem TTR revelaram decréscimo nos níveis de 14-3-3ζ na ausência de TTR. Tal efeito é abolido em

animais idosos. A presença da TTR previne a degradação lisossomal da 14-3-3ζ. O efeito da TTR é específico para a isoforma zeta e não ocorre a nível transcricional.

Também foi verificado um efeito inibitório da TTR na autofagia, provavelmente pela regulação dos níveis da 14-3-3ζ.

# GENERAL INTRODUCTION

# TRANSTHYRETIN

In 1942, a protein migrating ahead of albumin during electrophoresis of plasma (Seibert and Nelson 1942) and cerebrospinal fluid (CSF) (Kabat, Moore et al. 1942) samples was identified and denominated prealbumin. The finding that prealbumin could bind thyroid hormones (THs), led to a change of its name to thyroxine-binding prealbumin (Ingbar 1958). In 1969, it was found that thyroxine-binding prealbumin could also bind retinol-binding protein (RBP) (Raz and Goodman 1969). In 1981 the International Union of Biochemists adopted the name 'transthyretin' (TTR) (Nomenclature 1981), due to its well-known role in the transport of thyroid hormone thyroxine ($T_4$) and retinol (vitamin A) through binding to retinol-binding protein (RBP).

TTR is found in many vertebrate species including mammals, marsupials, birds, reptiles, amphibians and teleost fish, indicating that it is an evolutionary conserved protein (Schreiber and Richardson 1997; Power, Elias et al. 2000).

Until now, TTR had only been characterized in vertebrates. However, recently, sequences homologous to TTR, known as transthyretin-like proteins (TLPs), have been found in bacteria, nematods and plants. In *Escherichia coli* and *Caenorhabditis elegans* TLPs form homotetramers, like TTR, although without the ability to bind $T_4$ (Eneqvist, Lundberg et al. 2003). TLP from *E.coli* was shown to be amyloidogenic and toxic in cellular studies (Santos, Costa et al. 2008).

## Gene structure and regulation

TTR is the product of a single copy gene (Tsuzuki, Mita et al. 1985) localized on the long arm of chromosome 18 (Whitehead, Skinner et al. 1984) ant it is allocated to the 18q11.2-q12.1 region (Sparkes, Sasaki et al. 1987). The gene has a size of about 7.0 kilobases (kb) and is constituted of four exons and 3 introns (Sasaki, Yoshioka et al. 1985). Exons 1-4 are composed of 95, 131, 136 and 253 base pairs (bp), respectively, and introns of 934, 2090 and 3308 bp. Exon 1 encodes a single peptide of 20 amino acids (which is removed post-translationaly) and 3 amino acids from the mature protein. Exons 2, 3 and 4 encode amino acids 4-47, 48-92 and 93-123 of the mature protein, respectively (Sasaki, Yoshioka et al. 1985; Tsuzuki, Mita et al. 1985). Interestingly, the *TTR* gene presents two independent open reading frames (ORF), localized in the first and third introns (Tsuzuki, Mita et al. 1985). Soares *et al*. described that both ORF are neither

productively expressed as part of a larger transcript nor as an independent polypeptide (Soares, Centola et al. 2003).

The mouse *Ttr* gene is highly conserved in evolution sharing 82% and 90% homology (in the DNA sequence of the coding region) with human and rat genes, respectively (Costa, Lai et al. 1986). Two major regulatory regions were identified in the mouse *Ttr* gene: a proximal promoter sequence between -108 and -151 nucleotides and an enhancer sequence within a 100-bp region between 1.96 and 1.86 kb 5' to the mRNA cap site (Costa, Lai et al. 1986; Costa, Lai et al. 1988). Regulatory sites were found in the promoter as well as in the enhancer regions. DNA-binding factors, namely hepatocyte nuclear factors (HNF) 1, 3 and 4 (Costa, Grayson et al. 1989) and an hepatocyte-enriched DNA-binding protein- CAAAT/enhancer binding protein (C/EBP) (Costa, Grayson et al. 1988; Costa and Grayson 1991) are important regulatory factors for TTR expression. Binding sites for HNF-1, 3, 4 and C/EBP in the 5' flanking region were also found in human *TTR* gene (Sakaki, Yoshioka et al. 1989).

## Expression

TTR is mainly synthetized by the liver (Felding and Fex 1982) and the choroid plexus (Aleshire, Bradley et al. 1983), which are the sources of TTR in plasma and CSF, respectively. 90% of plasma TTR is secreted from liver and its concentration ranges from 20 to 40 mg/dL (Smith and Goodman 1971). TTR levels in plasma change with age: they are decreased in healthy newborns when compared with adults (Stabilini, Vergani et al. 1968; Vahlquist, Rask et al. 1975) and after the age of 50 years start to decline (Ingenbleek and De Visscher 1979).

Synthesis of TTR by epithelial cells of the choroid plexus is the main source of CSF TTR (Aleshire, Bradley et al. 1983). TTR concentration in CSF ranges from 5-20mg/L (Vatassery, Quach et al. 1991) representing aproximately 25% of the total CSF protein content (Aldred, Brack et al. 1995). The choroid plexus has eleven times more mRNA than the liver, normalized for tissue weight, and synthetizes TTR thirteen times faster than the liver (Schreiber, Aldred et al. 1990).

TTR synthesis in brain areas other than choroid plexus has been a controversial subject. The presence of TTR mRNA in murine or human brains has been detected in brain areas such as cortex, hippocampus or cerebellum (Carro, Trejo et al. 2002; Stein and Johnson 2002; Buxbaum, Ye et al. 2008; Li, Masliah et al. 2011). Some authors claimed that the presence of TTR may be due to neuronal synthesis of the protein in these

tissues, while others proved, by laser microdissection technology, that TTR is not produced in brain parenchyma, suggesting that TTR contamination by choroid plexus may induce false positive results concerning TTR synthesis (Sousa, Cardoso et al. 2007).

Besides the liver and the choroid plexus, TTR synthesis has been described in several other tissues. TTR is highly transcribed and translated in the retinal pigment epithelium (RPE), a monolayer of cells that acts as blood barrier for the retina (Pfeffer, Becerra et al. 2004). RPE cells are the only cells in the eye where mRNA for TTR and RBP were found (Cavallaro, Martone et al. 1990), being secreted across the apical side of the cell into the extracellular matrix. It has been suggested that the TTR-RBP complex in this layer may act as a retinol transporter to other cells. TTR is also produced in the pancreatic islet of Langerhans (Kato, Kato et al. 1985; Jacobsson, Collins et al. 1989), and to a small extent in stomach, heart, skeletal muscle, spleen (Soprano, Herbert et al. 1985), visceral yolk sac endoderm (Soprano, Soprano et al. 1986), pineal gland (Martone, Mizuno et al. 1993) (Martone, Mizuno et al. 1993) and human placenta (McKinnon, Li et al. 2005).

## Metabolism

Although TTR production has been extensively studied, its catabolism is not fully understood. The biological half-life of TTR is about 2-3 days in humans (Socolow, Woeber et al. 1965), 23 hours in monkeys (Vahlquist 1972) and 29 hours in Buffalo rats (Dickson, Howlett et al. 1982). In 1988, Makover and colleagues demonstrated that the major sites of TTR degradation were the liver, muscle and skin. In their studies, 36-38% of total body TTR degradation occurred in liver, 12-15% in muscle and 8-10% in skin. In kidneys, adipose tissue, testes and gastrointestinal tract (GI) the degradation rate of body TTR was 1-8%, whereas less than 1% was degraded by the other tissues examined (Makover, Moriwaki et al. 1988). No evidence was found of TTR degradation in tissues of the nervous system. Liver and kidney were the most active organs of TTR catabolism, per gram of wet weight. TTR internalization in liver and kidney is receptor-mediated. Renal uptake of TTR was shown to be megalin-[(also known as low density lipoprotein-related protein 2(LRP2)] mediated (Sousa, Norden et al. 2000). Megalin is an endocytic multi-ligand receptor of the low-density lipoprotein (LDL) receptor family that is expressed in the epithelium of renal proximal tubes, among other epithelia. Sousa *et al*. demonstrated that TTR uptake in liver was mediated by a receptor member of LDL family sensitive to receptor-associated protein (RAP) (Sousa and Saraiva 2001). Inhibition of TTR uptake by

RAP suggested a common pathway between TTR and lipoproteins metabolism (Figure 1). Further studies need to be performed in order to clarify TTR internalization, since megalin is not expressed in liver.

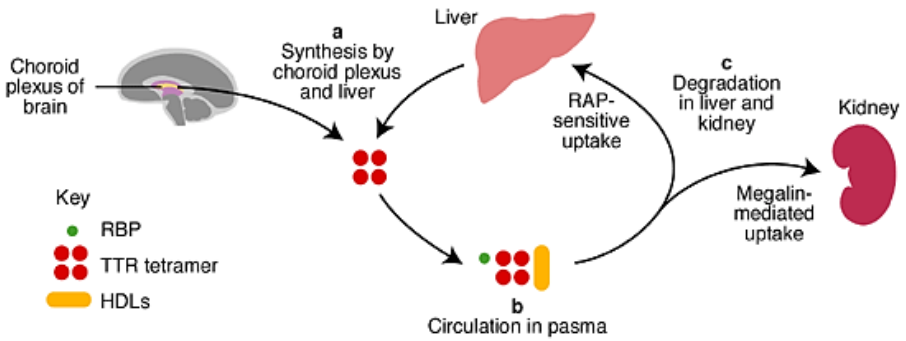


**Figure1.** TTR metabolism. a) TTR (red circles) is mainly synthetized by liver and choroid plexus. b) In circulation, TTR can bind RBP (green circle) and HDLs (yellow bar). c) Liver and kidneys are the main organs of TTR degradation. In liver, TTR uptake occurs through a receptor that binds RAP and in kidney TTR uptake is megalin-mediated (Saraiva 2002).

## Structure

In 1971 the first X-ray crystal structure of TTR was reported (Blake, Swan et al. 1971). TTR is a 54,980 Daltons (Da) homotetrameric protein, each subunit has 13, 745 Da and is composed of 127 amino acids (Kanda, Goodman et al. 1974). Each monomer consists of 8 antiparallel β-strands (A through H) which are organized into two four-stranded β-sheets (DAGH and CBEF) and only a short α-helix located on β-strand E (Blake, Geisow et al. 1978). A dimer is formed when β-strands F and H of each subunit interact by hydrogen bonds. Tetramer formation, results from interaction of the residues of the loops that join β-strands G to H and A to B. Native TTR has a globular shape with an overall size of 70 Å x 55 Å x 50 Å and a central hydrophobic channel (Figure 2).

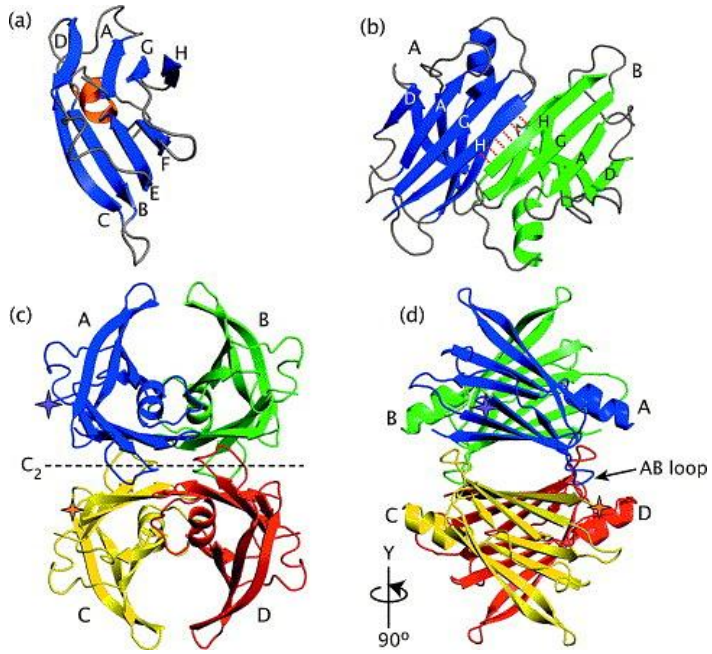


**Figure 2.** Schematic representation of TTR. a) TTR monomer with the 8 β-strands (A-H) in blue. b) A-B subunit interface showing the β-sheet formed by lateral association of H strands. Red dashes represent the hydrogen bonds that bridge the two subunits. c) TTR homotetramer showing each monomer in a different colour: subunit A is blue, subunit B is green, subunit C is yellow and subunit D is red. d) Same structure of C but rotated 90º around the y-axis (Foss, Kelker et al. 2005).

## Physiological functions

TTR has been mainly recognized by its role as a carrier protein of thyroid hormones and retinol in plasma and CSF. However, during the last years, a role in proteolysis, behavior, cognition, neupeptide amidation, neurogenesis, nerve regeneration and axonal growth has also been proposed.

### Transport of $T_4$

Thyroid hormones (THs) are iodinated compounds essential for development, tissue differentiation and regulation of metabolic balance in mammals. Thet thyroid gland synthetizes three THs: tetraiodothyronine ($T_4$), triiodothyronine ($T_3$) and a biologically inactive reverse $T_3$ ($rT_3$). $T_4$ has higher affinity to thyroid hormone-binding proteins (THBPs) in circulation than $T_3$, however $T_3$ has higher affinity to thyroid hormone receptors

in the nucleus, having the capacity to modulate expression of TH responsive genes together with co-activator or co-repressor proteins.

$T_4$ is the most abundant TH secreted by the thyroid gland. After synthesis, this hormone is secreted into the bloodstream, where it circulates bound to THBP, such as thyroxin-binding globulin (TBG), TTR and albumin. In humans, 65% of plasma $T_4$ is bound to TBG, 20% to albumin and 15% to TTR. TBG is the THBP with the highest affinity to $T_4$ (Loun and Hage 1992). Only 0.03-0.05% of $T_4$ circulates unbound or in a free form (Bartalena 1990). In rodents, 50% of total $T_4$ is carried by TTR (Hagen and Solberg 1974). In CSF, of both rodents and humans, TTR is the main carrier of $T_4$, transporting 80% of the hormone (Chanoine and Braverman 1992).

The homotetrameric structure of native TTR forms a central hydrophobic channel with two binding sites for $T_4$ (Blake, Geisow et al. 1974). As these binding sites exhibit negative cooperativity, just one molecule of $T_4$ is transported by TTR (Andrea, Cavalieri et al. 1980).

THBPs are synthetized by the liver and secreted into the bloodstream, being involved in the distribution of THs from the site of synthesis to the site of action. Their action prevents non-specific partitioning of lipophilic THs into cell membranes. The delivery of $T_4$ in cells is not a consensual subject; while some defend that uptake of $T_4$ occurs bound to the carrier proteins (Mendel 1989), others claim that $T_4$ enters the cell after dissociation of the complex formed with the carrier protein [for a revision see (Palha 2002)]. When THs dissociate from THBPs they can enter cells by passive diffusion or by TH transporters localized at plasma membrane. Studies on TTR null mice (Episkopou, Maeda et al. 1993) support the free $T_4$ tissue uptake hypothesis. TTR null mice exhibited a 50% reduction of total $T_4$ in the blood when compared with wild type animals, whereas the levels of free $T_4$ and total circulating $T_3$ were unaltered. In these mice, increased $T_4$ binding to TBG was observed, whereas the levels of TBG were the same, suggesting that TBG and TTR compete for $T_4$ binding (Palha, Episkopou et al. 1994). Several parameters were measured in TTR null mice to assess thyroid hormone function and indicated these animals to be euthyroid (Palha, Episkopou et al. 1994). This finding supported the ´free hormone hypothesis'; although the mice presented decreased levels of $T_4$, the free form levels were normal. Taken together, these results suggest that TTR is not pivotal to thyroid hormone metabolism, even in conditions of increased hormone demand as cold exposition or thyroidectomy (Sousa, de Escobar et al. 2005). Other studies showed that CSF of TTR null mice had 30% lower levels of $T_4$ when compared with wild type animals, but no differences were found in $T_4$ content in cortex, cerebellum or hippocampus (Palha,

Hays et al. 1997). Data revealed that TTR is not essential for the delivery of thyroid hormones to the brain or to other tissues.

The redundant role of TTR was also described for other thyroid THBPs such as albumin in rats (Mendel, Cavalieri et al. 1989) and TBG in humans (Refetoff 1989; Bartalena and Robbins 1993).

A recent study reported that $T_4$ transport from the CSF into the brain was dependent on TTR and mediated by receptor endocytosis (Kassem, Deane et al. 2006). Moreover, a critical role for TTR on $T_4$ transport across the placenta and delivery to the fetus was recently described (Landers, McKinnon et al. 2009; Patel, Landers et al. 2011). Further studies need to be performed to clarify the role of TTR on $T_4$ delivery into tissues.

## Transport of retinol

Retinol (vitamin A) and related metabolites are obtained from the diet. Oxidation of retinol originates retinoic acid which is very important in several functions including vision, reproduction, growth and development (Gudas 2012). Vitamin A is transported in the circulation by RBP, a 21 kDa protein (Kanai, Raz et al. 1968). RBP is mainly synthetized in liver and its secretion is stimulated by retinol binding. TTR associates to the RBP-retinol complex before secretion into the plasma (Raz and Goodman 1969). The TTR-RBP complex is a very stable form of retinol transport, allowing its delivery to cells. The association of TTR with RBP is important to prevent RBP from being filtered and degraded in kidney (Goodman 1984; Noy, Slosberg et al. 1992).

TTR tetramer has four RBP-binding sites, two in each dimer at the protein's surface, although, because of streric hindrance, just two RBP are transported by each molecule of TTR (Figure 3). Under physiological conditions, due to low levels of RBP when compared with TTR, just one molecule of RBP is transported by the TTR tetramer (Monaco, Rizzi et al. 1995; van Bennekum, Wei et al. 2001). $T_4$ binding to TTR is not influenced by RBP binding (Raz and Goodman 1969).

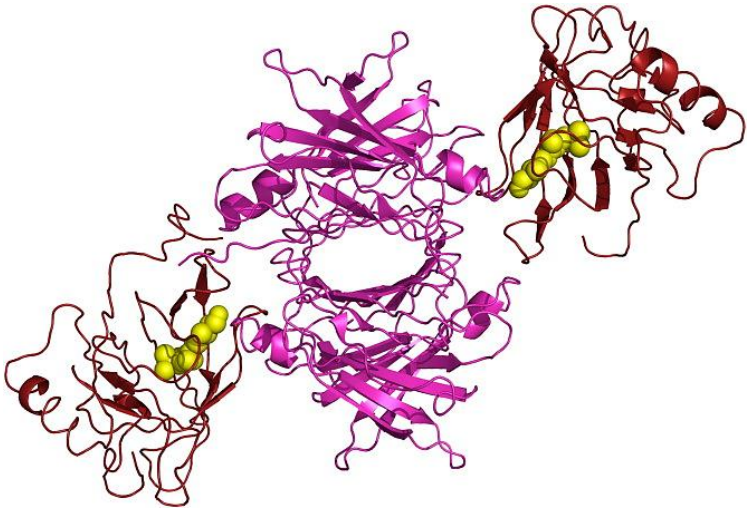

**Figure 3**. The three dimensional crystal structure of the retinol–RBP–TTR complex. TTR tetramer (pink) is linked with two RBP molecules (red) each one with a retinol molecule (yellow) inside. RBP-binding sites are independent of TH-binding sites (located in the central of TTR channel) (Berry and Noy 2012).

Studies in TTR null mice showed a dramatic reduction of retinol and RBP plasma levels (around 95%) when compared with wild type littermates. This finding could be explained by increased renal filtration of the retinol-RBP complex (van Bennekum, Wei et al. 2001). Increased hepatic RBP levels were found in TTR null mice (Wei, Episkopou et al. 1995). However, *in vitro* and *in vivo* studies demonstrated that RBP liver secretion from plasma was unchanged (van Bennekum, Wei et al. 2001), which indicates that diminished levels of RBP and retinol in plasma are not due to a failure in secretion.

Symptoms of vitamin A deficiency, such as loss of weight, infections and eye abnormalities were not observed in TTR mutant mice (van Bennekum, Wei et al. 2001). These findings suggest that TTR role on transport of RBP-retinol, does not have a critical role on retinol metabolism.

Interestingly, in kidney, testis, spleen and liver retinol and retinyl esters levels were unaltered in TTR null mice when compared with wild type animals (Episkopou, Maeda et al. 1993). Retinol uptake was suggested to be mediated by a TTR independent membrane receptor (Sundaram, Sivaprasadarao et al. 1998). Recently, Stra6 (a multi-transmembrane domain protein) was reported as a mediator of RPB4 binding to cell membranes and being crucial for cellular uptake of retinol (Kawaguchi, Yu et al. 2007). A new retinol transporter was identified: RBPR2, which is expressed in liver and intestine, suggesting a role in retinol absorption (Alapatt, Guo et al. 2013).

## Proteolytic activity

Another important function of TTR besides its role in transport of $T_4$ and retinol is its proteolytic activity on several substrates. A small fraction of plasma TTR (1-2%) is carried by high-density lipoproteins (HDL) through binding to apolipoprotein (apo) A-I (Sousa, Berglund et al. 2000). The interaction of TTR-apoA-I was further investigated and TTR was described as a non-canonical serine protease capable to cleave apoA-I carboxyl terminal domain (Liz, Faro et al. 2004). Cleaved apoA-I reduced cholesterol efflux and had an amyloidogenic potential (Liz, Gomes et al. 2007). Besides apoA-I, TTR has also the ability to cleave neuropepetide Y (NPY) (Liz, Fleming et al. 2009) and Aβ peptide (Costa, Ferreira-da-Silva et al. 2008). Cleavage of Aβ can occur at several different sites, and the resulting peptides were shown to have decreased amyloidogenic potential when compared with the complete peptide. TTR was also able to degrade aggregated forms of Aβ; inhibition of TTR activity resulted in increased Aβ fibril formation (Costa, Ferreira-da-Silva et al. 2008). TTR proteolytic role on NPY (Heilig 2004) and Aβ peptide represents additional important features of this protein in the nervous system.

# TTR IN THE NERVOUS SYSTEM

## Findings from TTR knockout mice

To better understand the physiological role of TTR, a mouse model with disruption in the *Ttr* gene was developed- TTR null mice. These animals are viable, phenotypically similar to wild type and heterozygous littermates, and fertile (Episkopou, Maeda et al. 1993). TTR null mice present reduced signs of depressive-like behavior, increased exploratory activity and anxiety (Sousa, Grandela et al. 2004). The authors suggested that increased levels of norepinephrine in the limbic forebrain observed in these mice could be a possible explanation for the observed phenotype. A few years later, Nunes *et al.* demonstrated that TTR null mice presented increased levels of NPY in dorsal root ganglia (DRG), sciatic nerve, spinal cord, hippocampus, cortex and CSF. Elevated levels of this amidated neuropeptide were shown to be a consequence of up-regulation of peptidylglycine α-amidating monooxygenase (PAM) – the only enzyme that amidates

neuropeptides, being crucial for the maturation process of NPY (Prigge, Mains et al. 2000; Nunes, Saraiva et al. 2006). These findings corroborate the importance of TTR in modulating depressive behavior.

Cognitive performance analysis of young/adult TTR null mice showed memory impairment when compared with wild type littermates (Sousa, Marques et al. 2007; Brouillette and Quirion 2008; Buxbaum, Ye et al. 2008). With aging, TTR wild type animals presented worsened cognitive performance, attributable to reduced levels of CSF TTR. This fact enhances the important role of TTR in cognition (Sousa, Marques et al. 2007).

Increased locomotor activity in young/adult TTR null animals was confirmed by Fleming *et al*; in older mice, a sensorimotor impairment was observed (Fleming, Saraiva et al. 2007). No morphological differences in sciatic nerves and cerebellum were found in TTR null animals that could explain the absence of sensorimotor impairment at young ages. However, under nerve crush conditions, absence of TTR slowed nerve regeneration (Fleming, Saraiva et al. 2007). TTR null mice have slower recovery of locomotor activity and slower nerve conduction velocity. Neuropathological parameters such as decreased levels of myelinated and unmyelinated axons were also observed in TTR null animals when compared with wild type littermates. TTR properties as a nerve regeneration enhancer were further demonstrated when TTR delivery to crushed sciatic nerves rescued the regeneration phenotype of TTR null animals (Fleming, Mar et al. 2009).

TTR has also the capacity of inducing neurite outgrowth in DGR and PC12 cells (Fleming, Saraiva et al. 2007). Neuritogenic activity of TTR seems to be independent of its major ligands, $T_4$ and retinol: TTR induced neurite outgrowth in TTR null DRG neurons cultured in a $T_4$ and retinol- free medium; I84S TTR, a TTR mutant with very low affinity for $T_4$ and RBP, was able to rescue the phenotype of PC12 cells grown in the presence of TTR null serum as wild type TTR does (Fleming, Saraiva et al. 2007). Retrograde transport was also impaired in TTR null mice (Fleming, Mar et al. 2009). Neuritogenic activity of TTR in DRG neurons depends on its internalization, a process that is clathrin-dependent and megalin-mediated. *In vivo* studies in a mouse model with reduced levels of megalin demonstrated that these animals had decreased nerve regeneration capacity. Together, these findings suggest that reducted megalin levels impair TTR action as an enhancer of regeneration (Fleming, Mar et al. 2009).

## TTR and neurodegenerative disorders

When the word 'transthyretin' is mentioned, the first neurodegenerative disorder associated with it is familial amyloidotic polyneuropathy (FAP). In the last years TTR has been associated with Alzheimer's disease and ischemia. Moreover, decreased TTR levels and/or increased oxidation have been described in several other neuropathologies such as syndrome Guillain-Barré, Huntington disease, frontotemporal dementia, amyotrophic lateral sclerosis among others [for a review see (Fleming, Nunes et al. 2009)].

TTR role on FAP, ischemia and AD will be discussed in the next sections.

### Familial amyloidotic polyneuropathy

FAP is an autosomal dominant disorder described for the first time in 1952 in a group of Portuguese patients (Andrade 1952). It is characterized by the presence of extracellular deposits of mutated TTR, in several organs, affecting particularly the peripheral nervous system (PNS) (Coimbra and Andrade 1971; Costa, Figueira et al. 1978). These deposits induce cell damage and organ dysfunction, ultimately leading to death [ for a review see (Sousa and Saraiva 2003)]. Clinical symptoms appear between the ages of 20 and 35 years being fatal within 10-15 years. In the early stages, symptoms include impairment of temperature and pain sensations in the lower limbs (Dyck and Lambert 1969). With progression of the disease, motor impairments, weakness, malabsorption, cardiac insufficiency, impotence, urinary bladder dysfunction, among other symptoms, will take place (Sousa and Saraiva 2003). Besides PNS, gastointestinal tract, vitreous and heart are other affected organs. More than 100 TTR mutations were identified and related with amyloid deposition, most of them in the PNS (Saraiva 2001). The most common TTR mutation is the substitution of valine for a methionine at position 30 (TTR V30M) (Saraiva, Birken et al. 1984).

It was demonstrated that increase in amyloidogenicity was associated with decreased tetramer stability (Bonifacio, Sakaki et al. 1996). The monomers resulting from tetramers dissociation could aggregate in oligomeric, non-fibrillar, protofibrills and in amyloid fibrils (Quintas, Vaz et al. 2001; Cardoso, Goldsbury et al. 2002).

Until now, liver transplantation is the only therapeutic option to FAP patients. Although this approach has proven to be very successful in some cases, it has some disadvantages such as limited availability of livers to transplant as well as persistence of some neurological lesions after transplant (Furtado, Oliveira et al. 1999). In order to overcome these problems new drugs are being developed: i) to stabilize the TTR tetramer; ii) inhibit fibril elongation and iii) disrupt of preformed amyloid fibrils (Oliveira, Cardoso et al. 2012). At this moment, two clinical trials are ongoing for FAP using either a tetramer stabilizer (Fx-1006A) (Sekijima, Kelly et al. 2008): phase II/III (ClinicalTrials 2009); or a mixture of doxycycline/TUDCA able to disrupt amyloid deposits (Macedo, Batista et al. 2008; Cardoso, Martins et al. 2010): phase II of clinical trial (ClinicalTrials 2010).

## Cerebral ischemia

Ischemia is a significant cause of brain injury worldwide, leading to high mortality, physical and cognitive incapacities. Some reports have been published in order to clarify the TTR role in brain ischemia. In young rats subjected to focal cerebral ischemia TTR was one of the differentially expressed proteins identified in plasma (Chen, Vendrell et al. 2011). In a rat model of transient middle cerebral ischemia artery occlusion, monomeric form of TTR was found to be increased in CSF (Suzuyama, Shiraishi et al. 2004). Furthermore, up-regulation of brain TTR was found by RT-PCR in a mouse model of oligemia (blood flow reduction without acute tissue damage) (Liverman, Cui et al. 2004). TTR excretion in urine was observed in stroke-prone spontaneously hypertensive rats before stroke (Sironi, Tremoli et al. 2001). More recently, using permanent middle cerebral artery occlusion (pMCAO) as the induction model to cerebral ischemia, TTR was revealed as a neuroprotective molecule. TTR null mice did not display significant differences in the cortical infarct area 24 hours after pMCAO when compared with wild type animals. However, TTR null mice heterozygous for the heatshock transcription factor 1 ($TTR^{-/-}$/$HSF1/^{+/-}$) presented increased infarct area, cerebral edema and microglial-leukocyte response when compared with $TTR^{+/+}$/$HSF^{+/-}$. Interestingly, TTR was localized in disintegrated β-tubulin III-positive neurons and cell debris. Elimination of TTR secreted by liver by treatment with RNAi had no effect on the distribution of TTR in endangered neurons, indicating that TTR mobilization to neurons was due to CSF and not to serum TTR. These results demonstrated that in a compromised heat-shock response, CSF TTR

is neuroprotective, influencing the survival of endangered neurons (Santos, Lambertsen et al. 2010).

## Alzheimer's disease

Alzheimer's Disease (AD) is the most common cause of dementia, affecting millions of people worldwide. It is an incurable neurodegenerative disorder characterized by a slow progressive decline in cognitive functions leading to death. The two main histopathological marks of AD are neurofibrillary tangles (aggregates of hyperphosphorylated tau protein and senile plaques (aggregates of Aβ peptide) (Goedert and Spillantini 2006). Aβ peptide results from the proteolytic cleavage of the amyloid precursor protein (APP). Briefly, APP can be cleaved by α-secretase or β-secretase, producing soluble N-terminal fragments, sαAPP or sβAPP, and two membrane-bound carboxyl-terminal fragments (CTF), αCTF and βCTF, respectively. Further, the CTFs are cleaved by γ-secretase originating the p3 peptide or the Aβ40 and Aβ42 peptides.(LaFerla, Green et al. 2007).

A role for TTR in AD has been suggested by several groups. The first description of decreased TTR levels in CSF of AD patients dates back to 1986 (Elovaara, Maury et al. 1986). Two years later, TTR in CSF was identified as being negatively correlated with the degree of dementia in AD (Riisoen 1988) and with abundance of senile plaques (Merched, Serot et al. 1998). More recently, with the use of powerful tools, a 2-fold decrease in TTR levels in CSF of AD patients was demonstrated (Castano, Roher et al. 2006; Gloeckner, Meyne et al. 2008). As observed in CSF, TTR plasma levels are decreased in AD patients when compared with non-demented controls (Han, Jung et al. 2011). TTR was also suggested as an early biomarker of AD, since it is decreased in serum of mild-cognitive impairment (MCI) and of AD patients in comparison with non-demented controls (Ribeiro, Santana et al. 2012).

TTR is able to bind Aβ peptides, preventing the formation of amyloid fibrils (Schwarzman, Gregori et al. 1994). Analysis of Aβ aggregation kinetics, showed that in the presence of TTR, the aggregation rate of this peptide was decreased (Liu and Murphy 2006). TTR is able to bind soluble, oligomeric and fibrillar forms of Aβ, having also the ability to inhibit fibril formation and disrupt already formed fibrils (Costa, Goncalves et al. 2008).

Transgenic mouse models have been very useful in providing information about TTR role in AD. Induction of TTR expression was the suggested mechanism to explain sAPPα protection. Furthermore, neutralization of TTR induced increased Aβ levels, tau phosphorylation, neuronal loss and apoptosis within hippocampus (Stein and Johnson 2003); however the antibody used in these studies was also reactive to proteins than TTR. *$APP_{swe}$/PS1ΔE9* animals, when exposed to an enriched environment, exhibited reduced levels of Aβ and amyloid deposits and TTR up-regulation (Lazarov, Robinson et al. 2005). Further, Aβ levels and deposition were increased in the hippocampus and cortex of *$APP_{swe}$/PS1ΔE9/$TTR^{+/-}$* animals when compared with *$APP_{swe}$/PS1ΔE9/$TTR^{+/+}$* mice (Choi, Leight et al. 2007). Furthermore, overexpression of human TTR in *APP23* mice decreased Aβ levels and deposition and also improved cognition (Buxbaum, Ye et al. 2008). More recently, it was demonstrated that TTR could modulate Aβ brain levels in a gender dependent manner, since *$APP_{swe}$/PS1A246E/$TTR^{+/-}$* female mice presented higher levels of $A\beta_{42}$ when compared with *$APP_{swe}$/PS1A246E/$TTR^{+/+}$* littermates, whereas no differences were found in males of the different genotypes (Oliveira, Ribeiro et al. 2011).

The neuroprotective role of TTR in AD, however, has not been a consensual subject. In a mouse model lacking TTR, Tg2576/$TTR^{-/-}$, vascular Aβ burden was decreased when compared to Tg2576/$TTR^{+/-}$ (Wati, Kawarabayashi et al. 2009). Another report demonstrated that Aβ plaque burden was reduced in the hippocampus of 4-month-old TgCRND8/$TTR^{+/-}$ when compared to TgCRND8/$TTR^{+/+}$ (Doggui, Brouillette et al. 2010). These results suggest that TTR effect depends on the mouse strain used. In this strain, TgCRND8, plaques appear very precociously; possibly, TTR protective role in AD is exerted since young ages.

Despite some controversy, TTR is considered a neuroprotective molecule in AD. More studies are needed to clarify the mechanism of TTR action.

## INSULIN-LIKE GROWTH FACTOR SYSTEM

The insulin-like growth factor (IGF) system is complex, being composed of small peptides (IGF-I, IGF-II and insulin), cell surface receptors [IGF-I receptor (IGF-IR), mannose-6-phosphate/IGF-II receptor (M6P/IGF-IIR), insulin receptor (IR), hybrid receptors IR/IGF-IR) and six binding proteins (IGFBP1-6) and their proteases. Their action

includes several biological processes such as proliferation, cell survival and growth, development, metabolism, plasticity, among others (Annunziata, Granata et al. 2011; Benarroch 2012).

## Insulin-like growth factor I

IGF-I, or somatomedine C, was first described as humoral mediator of growth hormone (GH) action (Daughaday, Hall et al. 1972; Klapper, Svoboda et al. 1983). The name of this peptide derives from its close homology with insulin and its roles on growth.

IGF-I can be found in biological fluids (blood and CSF) or in tissues. Within circulation, IGF-I is bound to high affinity binding proteins (IGFBPs) which act as its carrier proteins, extending IGF-I half-life and modulating its bioavailability. The majority of IGF-I in circulation is produced by the liver, but it can also be synthetized in many other organs exerting autocrine and paracrine functions (Bach, Headey et al. 2005). This peptide can also have an endocrine role, circulating in plasma and acting at distant sites.

The human *IGF-I* gene is located on the long arm of chromosome 12 (Brissenden, Ullrich et al. 1984; Tricoli, Rall et al. 1984), being composed of 6 exons. Four of these are subjected to alternative splicing originating different precursors; nevertheless the structure of the mature precursor is the same (Rotwein, Pollock et al. 1986; Smith, Spurrell et al. 2002). This precursor is subjected to proteolysis at both ends, originating a single peptide with 7.5 kDa. The structure of IGF-I reveals 3 $\alpha$-helices as the main secondary structural elements. IGF-I is composed of 70 amino acids in one single chain, classified into four domains (B, C, A and D in order from the N to the C terminus). Helix one is in the B domain, helix two and three are in the A domain. Domains B and C appear very flexible by nuclear magnetic resonance (NMR). The three-dimensional fold of this peptide is sustained by three disulphide bridges in residues Cys6-Cys48, Cys18-Cys61 and Cys47-Cys52 (Denley, Cosgrove et al. 2005) (Figure 4).

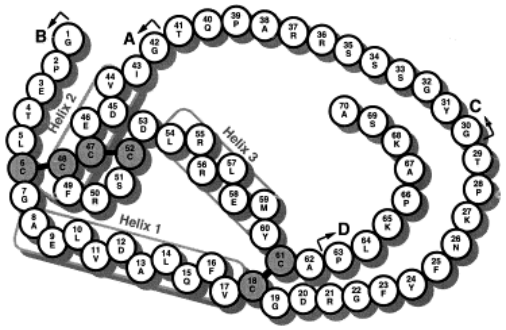


**Figure 4**. A two dimensional representation of IGF-I where amino acids (circles), disulphide bridges between cysteine residue (dark circles), helixes (1-3) and the four domains (A-D) can be seen (Denley, Cosgrove et al. 2005).

## Comparison with IGF-II and Insulin

IGF-I is structurally very similar to other peptides of the IGF-I axis: IGF-II and insulin.

IGF-II is a 67 amino acid polypeptide, three less than IGF-I, but it is also divided in four domains (A-D); it has the same tertiary structure of IGF-I, with 3 $\alpha$-helices and also three disulphide bridges contributing to its three dimensional form.

Despite the structural similarity between IGF-I and IGF-II, each ligand has unique single effects. IGF-II cannot compensate the loss of activity in patients with IGF-I deficiency, leading to severe growth and mental retardation (Woods, Camacho-Hubner et al. 1997; Denley, Wang et al. 2005; Walenkamp, Karperien et al. 2005). Mice with disruption in IGF-I or IGF-II are born with 60% reduced birth weight when compared with wild type littermates (DeChiara, Efstratiadis et al. 1990; Liu, Baker et al. 1993).

Insulin has 48% amino acid identity with IGF-I, despite having two disulphide-bonded peptide chains (B and A chains) of 30 and 21 residues, respectively. Like IGFs, insulin has a tertiary structure containing three $\alpha$-helices held together by two inter domain disulphide bridges and a single intra domain disulphide bond. Absence of C and D domains in mature insulin produces a major structural difference from IGFs (Figure 5) (Rinderknecht and Humbel 1978; Blundell, Bedarkar et al. 1983; Li, Yamashiro et al. 1983).

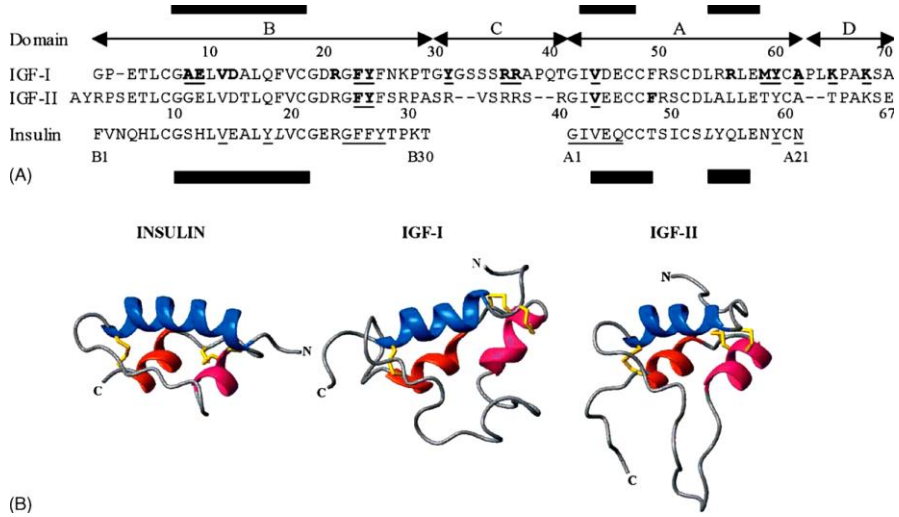


**Figure 5**. Comparison between IGF-I, IGF-II and insulin. A) Sequence alignment with domains and helices (black boxes). B) Ribbon structures of IGF-I, IGF-II and insulin where helix one is blue, helix two is pink and helix three is red. (Denley, Cosgrove et al. 2005).

IGF-I is involved in development, cell differentiation, plasticity, DNA repair, energy balance, protection from apoptosis (namely in the CNS), among other actions (Arnaldez and Helman 2012; Benarroch 2012). Moreover, IGF-I actions in the metabolism of lipids, proteins and glucose (Riedemann and Macaulay 2006), as well as its critical role in cancer, (Werner and Bruchim 2009) are well-known functions of this polypeptide. IGF-I effects are mainly mediated through the IGF-I receptor.

## Insulin-like growth factor binding proteins

A very important difference that distinguishes IGF-I from insulin is that it has specific amino acids (positions 3, 4, 15, 16) that are absent in insulin, conferring the ability to bind to specific IGFBPs. These proteins bind IGF-I and IGF-II with equal or even greater affinity than that of IGF receptors (Rapp, Deger et al. 1988; Le Roith, Bondy et al. 2001). IGFBPs modulate IGFs action, since they act as carrier proteins to IGFs within circulation. In serum, more than 75% of IGFs are found in a 150kDa ternary complex composed of IGF-I bound to IGFBP-3 (the most abundant IGFBP) and to acid labile subunit (ALS) (Boisclair, Rhoads et al. 2001). A 130 kDa complex formed by IGF:IGFBP-5:ALS can also be found in circulation (Twigg and Baxter 1998). IGFBPs affinity to ALS is increased in the presence of IGF-I or IGF-II, because IGF-I/IGF-II induce a conformational

change in the IGFBPs. Just IGFBP-3 and IGFBP-5 can bind ALS. Although IGFBP-5 has higher affinity to IGF-II, the binding to ALS is favored in the presence of IGF-I. Due to their size, these complexes cannot cross the vascular epithelial layer, prolonging the half-life of plasma IGF-I from 8 minutes to 15 hours, and acting as reservoirs within circulation (Guler, Zapf et al. 1989). The other IGFBPs can also bind IGFs, forming 50 kDa binary complexes that increase the half-life of IGF to 30 minutes but are able to cross the vascular epithelial layer and deliver IGFs in target tissues (Boes, Booth et al. 1992).

IGFBPs can modulate IGFs actions by inhibition of their binding to the receptors. The binding affinity of IGFs to IGFBPs can be changed by several processes, including phosphorylation, binding to extracellular matrix (ECM), or proteolysis (LeRoith and Roberts 2003). Serine proteases, cathepsins and matrix metalloproteinases have been described as IGFBP-3 proteases (Rechler and Clemmons 1998; Jogie-Brahim, Feldman et al. 2009). These processes decrease affinity to IGF-I increasing its bioavailability.

One of the possible manners of releasing IGFs from the ternary complex is proteolysis of IGFBP-3, resulting in a 30 kDa N-terminal fragment with 50 times lower affinity to IGF-I and a C-terminal fragment still attached to the complex (Figure 6).

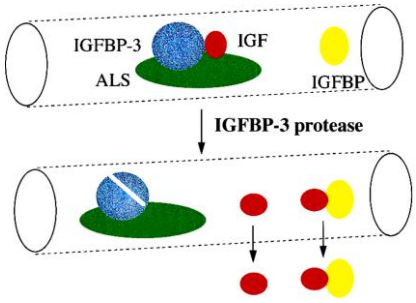


**Figure 6**. Mobilization of IGF-I from 150 kDa ternary complex by limited proteolysis. Proteolysis of IGFBP-3 (blue) associated with ALS (green) induced release of IGF-I (red) from the ternary complex, being able to cross the endothelial barrier alone or in a binary complex with other IGFBPs (Rechler and Clemmons 1998).

Besides their important role in IGF-dependent actions, IGFBPs also have IGF-independent functions. They have the ability to interact with ECM components and integrins promoting their entry into cells and migration to the nucleus (Perks, Newcomb et al. 1999; Granata, Trovato et al. 2004; Wheatcroft and Kearney 2009). Nuclear localization of IGFBP-3 is a well-described phenomenon. The major action of IGFBP-3 in the nucleus is to favor apoptosis. In the nucleus, IGFBP-3 can bind nuclear retinoid X receptor (RXR)-$\alpha$, and it was proposed that this binding modifies RXR$\alpha$/Nur77 (very

nuclear receptor transcription factor in the regulation of apoptosis) DNA binding complex, releasing DNA and targeting mitochondria, leading to apoptosis (Jogie-Brahim, Feldman et al. 2009). Phosphorylation of IGFBP-3 $Ser^{156}$, by DNA-dependent protein kinase (DNA-PK), induces its accumulation in the nucleus, a critical step to interaction with RXR$\alpha$ (Cobb, Liu et al. 2006). Although IGFBP-3 location in the nucleus is important to its proapoptoctic actions, this binding protein can induce apoptosis being present in the cytoplasm or without binding to RXR$\alpha$ (Bhattacharyya, Pechhold et al. 2006).

## Des-(1-3) IGF-I

IGF-I is widely expressed in the CNS; its highest rate of expression occurs before birth being decreased during adulthood. It is mainly expressed in neocortex, hippocampus, cerebellum, brainstem, hypothalamus and spinal cord (Benarroch 2012). In brain, one of the IGF-I forms is a glycine-proline-glutamate tripeptide, des-(1-3)IGF-I, generated by N-terminal cleavage of the mature peptide by an acidic protease (Sara, Carlsson-Skwirut et al. 1989; Yamamoto and Murphy 1994). This truncated form has reduced affinity for IGFBPs, being more available for receptor binding (Sara, Prisell et al. 1986), enhancing its neurotrophic actions *in vivo* and *in vitro* (Giacobini, Olson et al. 1990; Russo, Gluckman et al. 2005).

# Insulin-like growth factor receptor

## Gene regulation

The *IGF-IR* gene is located on chromosome 15q26 (Ullrich, Gray et al. 1986) and contains 21 exons (Abbott, Bueno et al. 1992). Exons 1-10 encode the 5'-untranslated region (UTR), the single peptide and the $\alpha$-subunit; exon 11 encodes the proteolytic cleavage site that generates $\alpha$ and $\beta$ subunits of the polypeptide precursor; exons 12-20 encode the $\beta$ subunit and exon 21 encode the 3'-UTR region (Werner, Hernandez-Sanchez et al. 1995). The promoter of IGF-IR is highly conserved between human and rodents (Werner, Stannard et al. 1990; Cooke, Bankert et al. 1991). Several transcription factors can regulate IGF-IR expression: Sp1, Wilm's tumor 1 (WT1) and p53 (Beitner-

Johnson, Werner et al. 1995; Werner, Shen-Orr et al. 1995; Sarfstein, Maor et al. 2006), among others. Recently it was described that IGF-IR can migrate to the nucleus, bind to IGF-IR promoter, and induce its transcription (Sarfstein, Pasmanik-Chor et al. 2012).

## Structure

IGF-IR is a transmembrane tyrosine kinase receptor, synthetized as a precursor that is proteolytic cleaved and glycolysed on extracellular regions. After proteolytic cleavage of the pro-receptor, mature IGF-IR is heterotetrameric composed of two extracellular $\alpha$-subunits and two $\beta$-subunits linked by disulphide bonds. The ligand binding site is located within $\alpha$-subunits (each with ~ 135kDa), and the intrinsic tyrosine kinase domain is located in $\beta$-subunits (each with ~95kDa) (Steele-Perkins, Turner et al. 1988).

IGF-IR and IR are structurally related having 84% homology in their tyrosine domain, 20-26% in the transmembrane domain and 45% in the C-terminal domain (Pedrini, Giorgino et al. 1994). Although the similarity between these receptors, ligand binding affinities are very different. IGF-IR binds to IGF-I with high affinity, being able to bind other ligands but with lower affinity: 6 fold lower for IGF-II and 100 fold lower for insulin (Annunziata, Granata et al. 2011). Due to the close homology between IGF-IR and IR, hybrid receptors can be formed, with $\alpha\beta$ hemireceptor of IGF-IR and $\alpha\beta$ hemireceptor of IR. These hybrid receptors were first described in human placenta (Soos, Nave et al. 1993) and bind IGF-I and IGF-II with affinities similar to IGF-IR, but have lower affinity to insulin (Soos, Field et al. 1993).

Two isoforms, formed by alternative splicing of exon 11 of the insulin receptor, are known: IR-A (lacks exon 11) and IR-B (contains exon 11). Insulin can bind both isoforms of IR with high affinity, whereas binding to IGF-IR and hybrid receptors has low affinity (Gallagher and LeRoith 2011).

Another receptor of the IGF system is IGF-IIR. This receptor binds IGF-II with high affinity, IGF-I with lower affinity and is not able to bind insulin. IGF-IIR, also known as cation-independent mannose-6-phosphate receptor (M6P/IGF-IIR), does not have the capacity of induce intracellular signaling transduction. Its main function is to sequester, internalize and degrade IGF-II. Therefore, IGF-II in not available to bind IGF-IR, and cannot activate its signaling pathway (Figure 7) (Scott and Firth 2004).

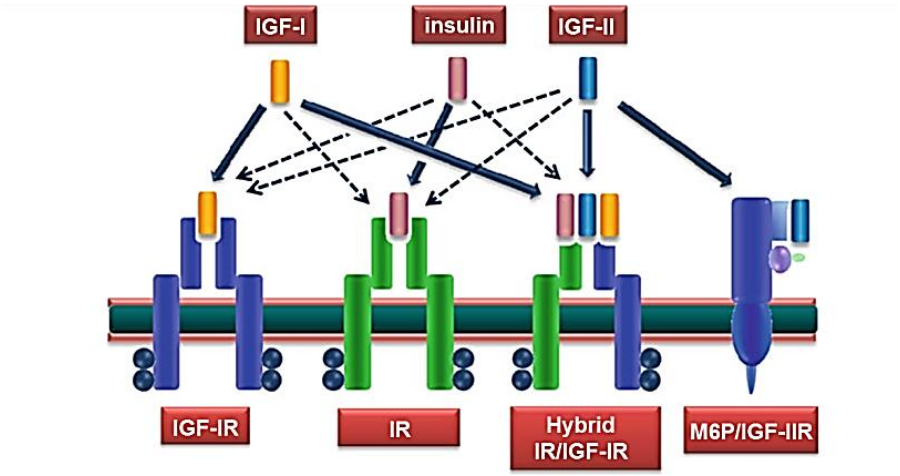


**Figure 7**. Representative scheme of ligands (IGF-I, IGF-II and insulin) and receptors (IGF-IR, IGF-IIR, IR and hybrid receptors) that comprises the IGF-axis. Full arrows and dotted arrows indicate high affinity and low ligand affinity, respectively (Annunziata, Granata et al. 2011).

## IGF-I/IGF-IR interaction

IGF-I binds IGF-IR with high affinity, having a $K_D$ of 1nM, much higher when compared with IGF-II, ($K_D$=15-20nM) and insulin ($K_D$=100nM). Binding of IGF-I causes a conformational change and subsequent autophosphorylation of tyrosine residues (Y1131, Y1135, Y1136) on the intracellular domain of IGF-IR, enhancing tyrosine kinase activity (Arnaldez and Helman 2012).

Activation of IGF-IR induces signaling mainly by two branches: Mitogen-activated protein kinase (MAPK)/Ras-Raf-Extracellular signal related kinase (Erk) and phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR).

Ligand binding induces recruitment of several signaling molecules such as insulin-receptor substrates (IRS-1 - IRS-4), Src homology domain C (Shc) (Gualco, Wang et al. 2009) and 14-3-3 proteins (Furlanetto, Dey et al. 1997; Spence, Dey et al. 2003) that connect IGF-IR with different pathways. Other molecules have the ability to bind directly IGF-IR including GRB10 adaptor (Morrione, Valentinis et al. 1996), Crk (Beitner-Johnson, Blakesley et al. 1996), PI3 kinase (Yamamoto, Altschuler et al. 1992), Syp phosphatase (Seely, Reichart et al. 1995) and C-terminal Src kinase (CSK) (Figure 8) (Arbet-Engels, Tartare-Deckert et al. 1999).

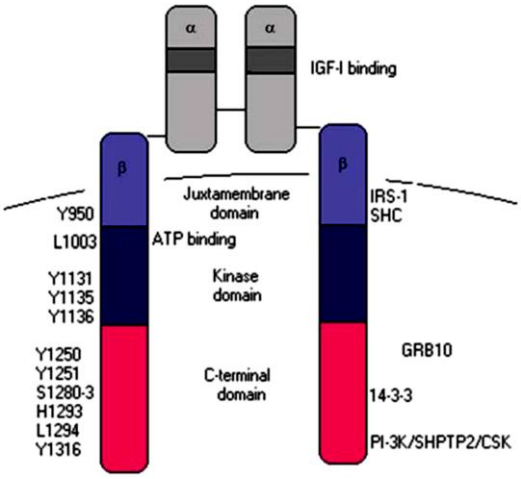


**Figure 8**. Illustration of Insulin-like growth factor receptor (IGF-IR). Heterotetrameric receptor with two extracellular α-subunits, with ligand binding region, and 2 β-subunits composed of a transmembrane domain and a cytoplasmatic region where tyrosine kinase and C-terminal domains are located. On the left side are residues involved in IGF-IR activation and on the right side are molecules that bind directly to IGF-IR after ligand binding (Gualco, Wang et al. 2009).

## MAPK/Ras-Raf-Erk

After ligand binding, $Tyr^{950}$ is phosphorylated and acts as a docking place to several substrates, IRS included. Phosphorylation of IRS-1 recruits Grb2/SOS or Shc, leading to activation of small G-protein Ras, that activates the protein serine kinase Raf and Erk. However, some isoforms of Shc have opposite roles on Erk signaling cascade (Laviola, Natalicchio et al. 2007).

IGF-IR activation can also activate other MAP kinases such as c-Jun $NH_2$-terminal kinase (JNK)-1, JNK-2 (Derijard, Hibi et al. 1994) and p38 MAP kinase (Rouse, Cohen et al. 1994). Ribosomal S6 kinase (Rsk 90), phospholipase A2 and several transcription factors are examples of downstream MAP kinase signaling molecules. Cell proliferation is one of main effects in this pathway (Grey, Chen et al. 2003).

## PI3K/Akt/mTOR

Once phosphorylated, IRS-1 recruits and activates class PI3-kinase through two Src-homology-2 (SH2) domains of the adaptor protein p85, leading to synthesis of

membrane-associated phosphorylated inositols. These molecules recruit and activate phosphoinositide-dependent kinases (PDKs) that will further activate other kinases such as Akt/protein kinase B (PKB), p70rsk and protein kinase Cζ (PKCζ).

PI3K can bind directly to pY1316 of IGF-IR and two additional molecules can bind pY608 and pY939 of activated IRS-1 (Reiss, Wang et al. 2001). Thus, a single activated IGF-IR can recruit at least 3 activated molecules of PI3K. Akt phosphorylation has several different functions, including: i) enhancement of protein synthesis by mTOR activation; ii) survival through regulation of Bad, GSK, FoxO, CREB phosphorylation and iii) blocking of caspase activation (Leinninger, Backus et al. 2004; Tzivion, Dobson et al. 2011).

Forkhead box O (FoxO) is a family of transcription factors, composed of four members (FoxO1, FoxO3, FoxO4 and FoxO6) that are very similar in structure, function and regulation. They have important roles in apoptosis, cell proliferation, metabolism and longevity, among others. Akt is one of the most important regulators of FoxO functions (Tzivion, Luo et al. 1998; Burgering 2008). Akt phosphorylates FoxO in three sites (T32, S253, S315) and this results in nuclear exclusion of FoxO, preventing the expression of genes that trigger apoptosis such as Fas ligand. An adaptor protein, 14-3-3ζ, facilitates FoxO nuclear/cytoplasmatic shuttling through the binding to FoxO by T32 and S253 phosphorylated residues (Brunet, Bonni et al. 1999).

## 14-3-3

14-3-3 proteins are a family of highly conserved acidic proteins, isolated from soluble extracts of cow brain (Moore and Perez 1967) and represent 1% of the total amount of brain protein (Boston, Jackson et al. 1982). The name is due to its fraction number on diethylaminoethyl-cellulose (DEAE) chromatography and migration position in starch gel electrophoresis (Moore, Perez et al. 1968). These proteins have approximately 30 kDa of molecular weight and are very conserved through evolution.

There are seven known isoforms of 14-3-3 proteins in mammalian cells (β,η, γ, τ, ζ, ε, σ) (Martin, Patel et al. 1993), each of them encoded by different genes; just σ and τ/θ are non-neuronally expressed (Hermeking 2003). 14-3-3 α and δ are the phosphorylated forms of β and ζ respectively (Tsuruta, Sunayama et al. 2004).

14-3-3 proteins can form homo- and heterodimers. Isoforms σ and γ prefer to homodimerize, while the other family members tend to heterodimerize (Tzivion, Luo et al. 1998; Kjarland, Keen et al. 2006). The dimers formed can interact with more than 400

molecules through phospho-serine/phospho-threonine residues (Bustos 2012). The main binding motifs are RSXpSXP and RXϕXpSXP, where pS represents phospho-serine, ϕ is an aromatic or aliphatic amino acid and X is any amino acid (Yaffe, Rittinger et al. 1997). The interaction of 14-3-3 can also occur with the C-terminal of the protein through the -pS/pT $X_{1-2}$-COOH sequence (Ganguly, Weller et al. 2005). It is important to note that not all interactions are phosphorylation dependent.

These proteins have important roles in several biological processes including cell-cycle regulation, cell survival, cellular trafficking, cytoskeletal organization, protein synthesis, redox-regulation and protein folding (Berg, Holzmann et al. 2003; Kjarland, Keen et al. 2006).

14-3-3 proteins are closely related with IGF-IR signaling. Several isoforms can physically interact with the intracellular domain of this receptor (Craparo, Freund et al. 1997; Furlanetto, Dey et al. 1997), and can also be involved in signaling pathways related to IGF-IR activation by interacting with phosphorylated Bcl-XL/Bcl-2- associated Death Promoter (Bad), stabilizing it in the cytoplasm, thus blocking the interaction with Bcl-2 and preventing apoptosis (Hsu, Kaipia et al. 1997). An intimate cooperation is observed between 14-3-3, Akt and FoxO molecules in preventing cell survival (Tzivion, Dobson et al. 2011). Phosphorylation of 14-3-3 by JNK releases proapoptoctic proteins such as Bad and FoxO from 14-3-3, antagonizing Akt survival effects (Tsuruta, Sunayama et al. 2004).

## IGF-I/IGF-IR role in cancer

The role of IGF-I in proliferation and protection from apoptosis is widely known. IGF-I levels have been identified as a risk factor for several malignancies such as breast (Hankinson, Willett et al. 1998; Ahlgren, Melbye et al. 2004), prostate (Chan, Stampfer et al. 1998; Stattin, Bylund et al. 2000), lung (Yu, Spitz et al. 1999), colorectal (Ma, Pollak et al. 1999) , endometrial (Petridou, Koukoulomatis et al. 2003) and bladder cancers (Zhao, Grossman et al. 2003). On the other hand, elevated IGFBP3 levels have been considered as a protective factor against cancer, due to the capacity to reduce IGF-I levels in circulation.

IGF-IR overexpression has been associated with progression of several tumors, since activation of IGF-IR pathway promotes several metastasis processes, such as cell adhesion, migration and invasion, among others (Werner and Bruchim 2009).

Upon activation, IGF-IR has anti-apoptotic and mitogenic roles that are crucial to tumor development. Activation of IGF-IR signaling pathway induce resistance against

cytotoxic therapies, such as radiation or chemotherapy, and targeted therapies (Casa, Dearth et al. 2008).

Epidermal growth factor receptor (EGFR) is a well-known receptor tyrosine kinase, and similarly to IGF-IR is involved in cell proliferation, inhibition of apoptosis and anchorage-independent growth, among other functions (van der Veeken, Oliveira et al. 2009). Interestingly, a crosstalk between EGFR and IGF-IR has been described by several groups (Morgillo, Woo et al. 2006; Saxena, Taliaferro-Smith et al. 2008; Ludovini, Bellezza et al. 2009). Upon ligand binding, both receptors undergo autophosphorylation, providing docking sites for proteins with SH2 and phosphotyrosine binding domain activating PI3K-Akt and Ras-Raf/MAPK pathway (Wells and Marti 2002).

Nowadays, several strategies are being applied to neutralize IGF-IR action on tumor growth, namely IGF-I antagonists, neutralizing antibodies against IGF-IR, and small molecules inhibitors targeting IGF-IR (Atzori, Traina et al. 2009; Arnaldez and Helman 2012). Blockade of both EGFR and IGF-IR has has been used as a therapeutic strategy against tumor growth (van der Veeken, Oliveira et al. 2009; Croasdale, Wartha et al. 2012).

Although these advances could be useful to tumor growth suppression, the prolonged exposure to IGF-IR blocking agents could be toxic of CNS, since IGF-IR has a neuroprotective role in brain. Further investigation is necessary to overcome these challenging issues.

## IGF-IR in the brain

IGF-I, IGF-II and IGF-IR are highly expressed in embryonic and early postnatal life, although their expression decreases with aging (Bondy and Lee 1993). IGF-IR is most expressed in choroid plexus, neocortex and thalamus, although it can be expressed in many other parts of the brain including hippocampus (Fernandez and Torres-Aleman 2012) (Figure 9).

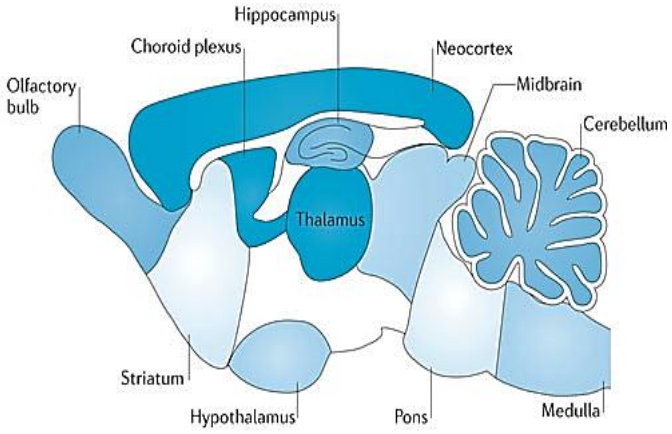


**Figure 9.** Distribution of insulin-like growth factor receptor in brain. Neocortex, thalamus and choroid plexus are the areas where IGF-IR is most abundant. Intensity of colours indicates increased levels of expression (Fernandez and Torres-Aleman 2012).

IGF-I has several known important functions in brain, namely: i) cell proliferation and survival; ii) differentiation of neural precursors; iii) regulation of cognition by modulation of synaptic plasticity, synapse density and neurotransmission; iv) stimulation of adult neurogenesis; v) regulation of metabolism (Benarroch 2012; Fernandez and Torres-Aleman 2012). It protects neurons against different toxic insults such as oxidative stress, high glucose, glutamate, N-methyl-D-aspartate (NMDA) and oxid nitric induced apoptosis. Neuronal apoptotic cell death is a marker of several neurodegenerative diseases. The protective effect of IGF-I against cellular death is becoming a very important candidate in neuroprotection. IGF-I has a protective role in AD (Carro, Trejo et al. 2002), Huntington disease (Humbert, Bryson et al. 2002), ischemic condition (Guan, Bennet et al. 2003), playing also an important role in cognitive functions and neuroplasticy (Aleman, Verhaar et al. 1999; Llorens-Martin, Torres-Aleman et al. 2009).

## Overview of IGF-I signaling in brain

Upon GH stimulation, IGF-I is produced by the liver and can act locally or can enter the circulation having an endocrine role. The majority of IGF-I in circulation is bound to IGFBPs, until delivery into the target tissue. Circulating IGF-I can enter into brain cells through binding to IGF-IR or by a megalin-mediated process. Furthermore, neurons, astrocytes and microglia can synthetize IGF-I. The biological actions of IGF-I are mainly mediated by IGF-IR, although it can also bind hybrid receptors, and, with less affinity, IR and IGF-IIR. Activation of IGF-IR triggers two different signaling pathways namely PI3K-

Akt and MAPK/Ras-Raf-Erk that will influence several biological processes such as protein synthesis, apoptosis, oxidative stress, proliferation and synaptic plasticity among others (Figure 10).

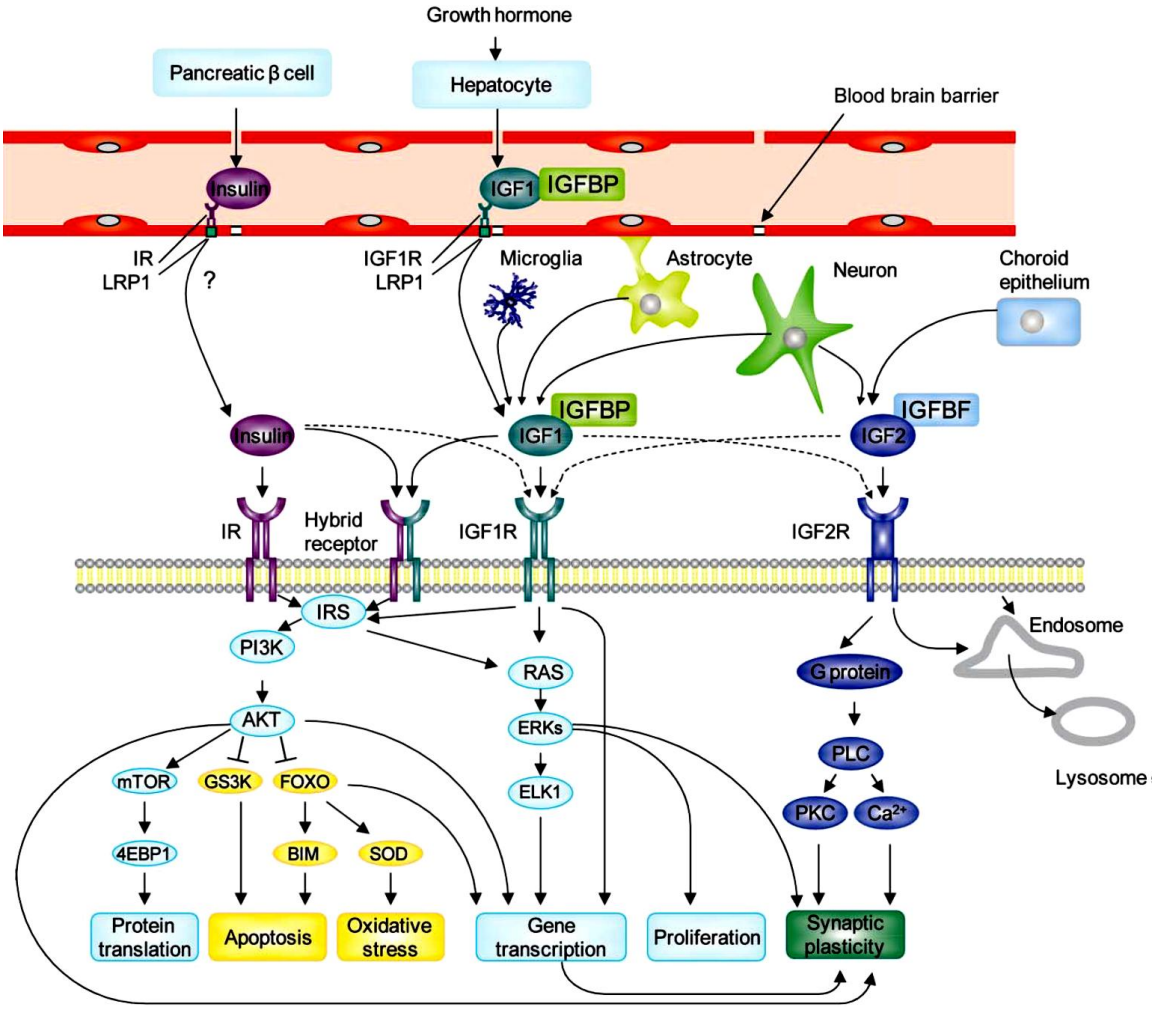


**Figure 10.** Insulin-like growth factor I signaling in brain. IGF-I can be produced by neurons, astrocytes and microglia, or it can cross BBB and enter the brain. Its bioavailability is regulated by IGFBPs and their actions are mediated by IGF-IR and hybrid receptors. Binding to these receptors activate two major signaling pathways: PI3K-Akt and Ras-Erk, leading to activation of several downstream molecules that influence many cellular processes (Benarroch 2012).

## CONCLUDING REMARKS

The neuroprotective roles of TTR and IGF-I as independent molecules have been described. TTR, besides its roles as a carrier protein of thyroid hormone and retinol, enhances nerve regeneration, promotes neuropeptide maturation and has a protective role in ischemia and AD. IGF-I has neuroprotective effects against several toxic insults, promoting cell survival under different pathological conditions namely, ischemia and AD. IGF-I actions occur mainly through binding to the IGF-I receptor. In 2002, microarray analysis of hippocampus of $APP_{swe}$ [characterized by Aβ deposition in cortical neurons and limbic regions without neuronal loss (Irizarry, McNamara et al. 1997)] demonstrated that TTR and IGF mRNA were increased when compared with nontransgenic littermates. Immunohistochemistry results corroborate this finding. Activation of the IGF-IR axis assessed by downstream activation of Akt, Erk and Bad, was also observed in this transgenic mouse strain, suggesting that reduction of neuronal death was due to activation of survival pathways (Stein and Johnson 2002). In the same year, Carro *et al*. described that IGF-I administration in Tg2576 mice increased Aβ clearance and up-regulation of TTR and albumin (Aβ carrier proteins) levels in CSF, cortex and choroid plexus (Carro, Trejo et al. 2002). Together, these findings suggest a close relationship between TTR and IGF-I. The aim of the work described in the next pages is to dissect the connection between these molecules, evaluating if TTR neuroprotective role could involve the IGF-IR signaling pathway. Proteomics and use of TTR null mice were the approaches employed for this purpose.

# RESEARCH PROJECT

# OBJECTIVES

The main objective of this work is to investigate TTR function as a signaling molecule through IGF-IR pathway in CNS. We propose to:

1) Search for TTR role in IGF-IR signaling - Chapter I
    - Analysis of TTR effect on IGF-IR signaling cascade;
    - Study TTR and IGF-I interaction and its cellular consequences;
    - Unravel IGF-IR as a new receptor for TTR action in brain.

2) Investigate TTR effect on IGF-IR levels – Chapter II
    - Evaluate TTR effects on IGF-IR levels *in vitro* and *in vivo*;
    - Understand if the TTR effects occurs at transcription or translational level;
    - Test if TTR neuritogenic effects occur in hippocampal neurons and if it is dependent on IGF-IR.

3) Assess brain protein expression in the absence of TTR - Chapter III
    - Validate results obtained by proteomics;
    - Perform molecular and cellular analyses of differences found in the absence of TTR.

# CHAPTER I

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# A new role of transthyretin as a signaling molecule: synergistic effect of transthyretin and insulin-like growth factor I, through IGF-I receptor, induces protection against HT22 glutamate-induced cell death

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## Abstract

Transthyretin (TTR) has a neuroprotective role in the central nervous system (CNS) as in Alzheimer disease (AD) and cerebral ischemia. Increased levels of TTR and insulin-like growth factor (IGF-I) receptor activation are associated with reduced neurodegeneration in anAD mouse model. In the present study, we found that TTR and IGF-I have a synergistic effect on activation of one of the IGF-I receptor (IGF-IR) signaling pathways. Hippocampus of TTR null mice present decreased levels of phosphorylated IGF-IR and Akt when compared with TTR wild type littermate animals. *In vitro* studies showed that TTR and IGF-I interact, being the interaction a possible explanation to the observed synergy. The data demonstrated that this synergistic effect protects HT22 cells from glutamate induced toxicity. In summary, our results point to a new neuroprotective TTR role through the IGF-I axis.


**Key words**: ischemia, glutamate, excitotoxicity, synergy

## Introduction

Transthyretin (TTR) is a secretory protein mainly synthetized by liver and the choroid plexuses of brain (Aleshire, Bradley et al. 1983; Soprano, Herbert et al. 1985), being these organs the source of TTR in plasma and cerebrospinal fluid (CSF), respectively. It is the carrier protein of thyroxine ($T_4$) (Woeber and Ingbar 1968) and retinol (vitamin A) through the retinol-binding protein (RBP) (Kanai, Raz et al. 1968). TTR is also associated with high density lipoproteins (Nakamura, Tanaka et al. 1996), through binding to apolipoprotein A-I (ApoA-I) (Sousa, Berglund et al. 2000). Cryptic protease activity of TTR was found for ApoA-1 (Liz, Faro et al. 2004) and Aβ peptide(Costa, Ferreira-da-Silva et al. 2008). TTR has been also described as a neuroprotective molecule in several contexts. Studies in TTR null mice revealed that absence of TTR reduces signs of depressive- like behavior (Sousa, Grandela et al. 2004), increases the levels of neuropeptide Y (Nunes, Saraiva et al. 2006) and delays nerve regeneration in nerve injury conditions (Fleming, Saraiva et al. 2007). TTR has also a protective role in Alzheimer's disease (Choi, Leight et al. 2007), prevents Aβ toxicity (Costa, Ferreira-da-Silva et al. 2008; Costa, Goncalves et al. 2008) and is able to modulate brain Aβ levels (Oliveira, Ribeiro et al. 2011). In cerebral ischemia, CSF TTR influences the survival of endangered neurons (Santos, Lambertsen et al. 2010). Absence of TTR decreases the susceptibility to neurodegeneration caused by excitotoxic insult AMPA (Nunes, Montero et al. 2009).

Insulin-like growth factor I (IGF-I) is a 70-amino-acid peptide that plays important roles in cell survival and differentiation. IGF-I has an antiapoptotic role against several insults such as reactive oxygen species (Heck, Lezoualc'h et al. 1999), serum deprivation (Zheng, Kar et al. 2002), TNF-α (Yadav, Kalita et al. 2005) or UV radiation (Kulik, Klippel et al. 1997). Binding of IGF-I to its receptor (IGF-IR) induces tyrosine autophosphorylation, triggering activation of Akt pathways (Zheng, Kar et al. 2000). Akt activation is a crucial event on the prosurvival effect of IGF-I (Dudek, Datta et al. 1997).

IGF-I has neuroprotective roles in neurological pathologies such as Huntington's disease (Humbert, Bryson et al. 2002) and AD (Carro, Trejo et al. 2002). In a study by Carro *et al.*, serum IGF-I levels increase Aβ clearance from brain, modulating levels of its carriers proteins, such as TTR (Carro, Trejo et al. 2002), which promotes Aβ transcytosis from CSF to blood (Carro, Spuch et al. 2005). In another study, reduced neurodegeneration in anAD mouse model was associated with increased level of TTR and increased activation of the IGF-IR signaling pathway (Stein and Johnson 2002). These findings suggest TTR and IGF-I may reciprocally influence their neuroprotective role. The

present study aims to dissect the relation of these two molecules, namely if the protective role of TTR could encompass the IGF-IR signaling pathway.

## Materials and Methods

### Animals

The number of mice handled for this research was approved by the Institutional and National General Veterinary Board Ethical Committees according to National and European Union rules. Three and nine-month-old TTR wild-type ($^{+/+}$) and TTR knock-out ($^{-/-}$) (Episkopou, Maeda et al. 1993), in a 129/svJ background were obtained from the littermate offspring of heterozygous breeding pairs. The animals were maintained under a 12h light/dark cycle and fed with regular rodent's chow and tap water *ad libitum*. Genotypes were determined from tail extracted genomic DNA, using primers for the detection of exon 2 of TTR (which is disrupted in $TTR^{-/-}$ by insertion of neomycin resistance gene) as previously described (Episkopou, Maeda et al. 1993). Mice were sacrificed with a lethal injection of a premixed solution containing ketamine (75mg/Kg) plus medetomidine (1mg/Kg). Hippocampus and plasma were frozen at -80ºC.

Efforts were made to minimize pain and distress; all animal experiments were carried out in accordance with the European Communities Council Directive.

### TTR production and purification

Recombinant TTR was produced in a bacterial expression system using *Escherichia coli* BL21 (Furuya, Saraiva et al. 1991) and purified as previously described (Almeida, Damas et al. 1997). Briefly, after growing the bacteria, the protein was isolated and purified by preparative gel electrophoresis after ion-exchange chromatography. Protein concentration was determined using the Lowry method (Lowry, Rosebrough et al. 1951).

### Endotoxin removal

To remove endotoxin, a polymixin B column (Thermo Scientific) was used. Briefly, the column was regenerated with 1% sodium deoxycholate (Sigma) and washed with pyrogen-free buffer to remove detergent. Recombinant TTR was applied to column and incubated during one hour at room temperature. Aliquots of pyrogen-free buffer were added and the flow-through was collected. Protein concentration was determined by the Bradford method (Hammond and Kruger 1988).

### Cell culture

NIH3T3 and R- (*IGF-IR deficient* murine fibroblasts, kindly given by Dr Renato Baserga, Philadelphia) cells were grown in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10% inactivated fetal bovine serum (FBS), 100μg/L streptomycin, 100U/mL penicillin, 300 μg/mL of L-glutamine and maintained at 37ºC in a humidified incubator 5% $CO_2$ /95% atmosphere. Once cells reached 80% confluency, were serum starved for two hours, rinsed with phosphate buffered saline (PBS) and then stimulated with either IGF-1(100ng/mL, Sigma), TTR (55μg/mL) or both for 30 min at 37 ºC.

HT22 (kindly given by Dr. Christian Behl, Mainz) cells were grown in high glucose DMEM with 10% inactivated FBS, 1mM sodium pyruvate, 100μg/L streptomycin and 100U/mL penicillin. Cell cultures were maintained at 37ºC in a humidified incubator until reaching 80% confluency. Cells were then serum starved during two hours, rinsed with PBS, stimulated either with IGF-I (100ng/mL) or TTR (55μg/mL) or both for 30 minutes before 5 mM glutamate exposure during 24 hours.

All culture media and supplements used were from GIBCO.

**Cell death assay**

Cell death assay was performed using Live/Dead Viability Cytotoxicity kit (Invitrogen). Briefly, HT22 live cells were stained for 30 minutes in the dark with 10μg/ml fluorescein diacetate and 20μg/ml propidium iodide diluted in PBS. Cells were then rinsed for 5 minutes in PBS, mounted in coverslips and counted. Live cells (green) are labeled by uptake and cleavage of fluorescein diacetate whereas death cells (red) are labeled by propidium iodide incorporation in DNA, indicating loss of membrane integrity.

**Western blot analysis**

Cultured cells and hippocampus were homogeneized in lysis buffer containing 20 mM MOPS, 2mM EGTA, 5mM EDTA, 30mM sodium fluoride, 60mM β-glycerophosphate, 20mM sodium pyrophosphate, 1mM sodium orthovanadate , 1mM phenylmethylsulphonyl fluoride, 1% Triton X-100 and 1x protease inhibitors mixture (GE Healthcare). After 20 minutes of centrifugation at 14000 rpm, supernatants were removed and total protein concentration was determined using the Bradford method. 50μg of protein were applied and separated by 10% SDS-PAGE and transferred to a nitrocellulose Hybond-C membrane (GE Healthcare), using a wet system. For IGF-I analysis, 1.5μL of plasma was applied and separated in 15% SDS-PAGE gel. Membranes were dried, blocked one hour at room temperature in blocking buffer, 5% BSA in phosphate-buffered saline Tween-20 (PBST), and then incubated overnight a 4ºC with primary antibodies diluted in blocking buffer, namely rabbit polyclonal phospho Akt (Ser473), total Akt and IGF-IR (1:1,000; Cell Signaling), IGF-I (1:200,R&D Systems) and TTR (1:1000, Dako). To phospho IGF-IR (1:500, Cell Signaling) analysis, membranes were boiled 10 minutes with PBS and then

blocked 1 hour at room temperature with 5% BSA/PBST and incubated with primary antibody over night at 4ºC. Membranes were then incubated with anti-rabbit IgG-HRP (1:10,000; Binding Site), during 1 hour at room temperature. The blots were developed using Immun-Star WesternC Chemiluminescent kit (BioRad) and exposed to Hyperfilm ECL (GE Healthcare). Blots were first incubated with phospho-specific antibodies, stripped with Re-Blot Plus Mild Solution (Chemicon) according to the manufacturer's instructions, reblocked, and then incubated with the respective anti-total protein antibody for total protein normalization. Quantitative analyses were performed using the ImageJ software.

**Immunoprecipitation**

NIH3T3 cells were incubated with TTR and TTR plus IGF-I for 30 minutes and whole cell lysates were performed as described above. For immunoprecipitation, protein extracts were pre-cleared 1hour at 4ºC with rabbit immunoglobulins (IgGs) coupled with protein G (GE Healthcare). After 10 minutes centrifugation at 13000 rpm, supernatants were removed and incubated with rabbit anti-IGF-IRβ (1:100; Cell Signaling) for 2 hours at 4ºC. After, the protein G slurry was added and incubated overnight at 4ºC. Immunoprecipitates were washed, samples were boiled for 10 minutes and separated in a 15% SDS-PAGE gel. Western blot to TTR was performed using as primary antibody TTR (1:1000; Dako).

**Immunofluorescence**

HT22 cells were grown on glass coverslips under appropriated conditions. Cells were washed with PBS, fixed with 4% paraformaldehyde during 20 minutes at room temperature (RT), permeabilised 15 minutes with 0.25% Triton X-100/PBS, blocked for 30 minutes at 37ºC with in PBS containing 4% of FBS and 1% bovine serum albumin (BSA) and then incubated overnight at 4ºC with rabbit polyclonal to anti-p-FKHR ($Ser^{256}$) (forkhead in rhabdomyosarcoma; 1:50; SantaCruz*).* After washing with PBS, cells were incubated with secondary antibody, anti-rabbit 488 (1:1,000; Molecular Probes) for 30 minutes at room temperature and protected from light. Coverslips were mounted in Vectashield with DAPI (Vector). Coverslips were visualized by a Widefield Fluorescence Microscope (Zeiss Axio Imager Z1) and images were analyzed using Fiji software.

**N-terminal sequencing**

An equimolar mixture of TTR and IGF-I diluted in 50 mM Tris pH 7.5, was incubated overnight at 37ºC. The sample was adsorbed in to PVDF membrane, and

submitted to N-terminal sequencing using the Edman reaction and a Procise 491 HT protein sequencer (Applied Biosystems).

**Circular Dichroism**

Measurements were recorded on a JASCO J-815 spectrometer possessing a Peltier-thermostated cell supports, set in all measurements at 20ºC. Each spectrum was obtained in a cell with a path length of 0.1cm (Hellma) and represents an average of ten scans of a 0.25mg/ml TTR sample in 10 mM Tris buffer pH= 7,5.

**Fluorescence emission spectra**

These spectra were acquired with a Cary Varian Eclipse instrument, equipped with Peltier temperature control set for all measurements at 20ºC. Intrinsic aromatic fluorescence emission spectra were acquired between 300 and 400 nm, after excitation at 280 nm ($slit_{ex}$: 5nm $slit_{em}$: 10 nm). ANS binding assays was used as an extrinsic fluorescence probe to detect the hydrophobic exposure of the domain (Stryer 1965; McClure and Edelman 1966). A 10-fold molar excess of 1-anilinonaphthalene-8-sulfonic acid (ANS) was added to the samples, and ANS fluorescence emission enhancement was evaluated upon 60 min incubation in the dark using a 370 nm excitation wavelength. All protein measurements were performed in the concentration range of 5 μM protein concentration in 10mM Tris buffer, pH=7,5.

**Statistical analysis**

Quantitative data are presented as Mean ± SEM. Statistical analysis was carried out using Graphpad Prism 5 software. Differences among groups were analyzed by one-way ANOVA (followed by Bonferroni's Multiple Comparison Test), comparisons between two groups were made by Student's *t* test. *P* values of lower than 0.05 were considered significant.

## Results

### TTR activates IGF-IR signaling in hippocampus

To search the action of TTR on IGF-IR signaling in the hippocampus, western blot of molecules downstream of the IGF-IR pathway were performed in hippocampus homogenates from TTR null mice and control littermates. Analysis of pIGF-IR (Figure 1A) and pAkt (Figure 1B) demonstrated being decreased in TTR null mice when compared with TTR wild type littermates.

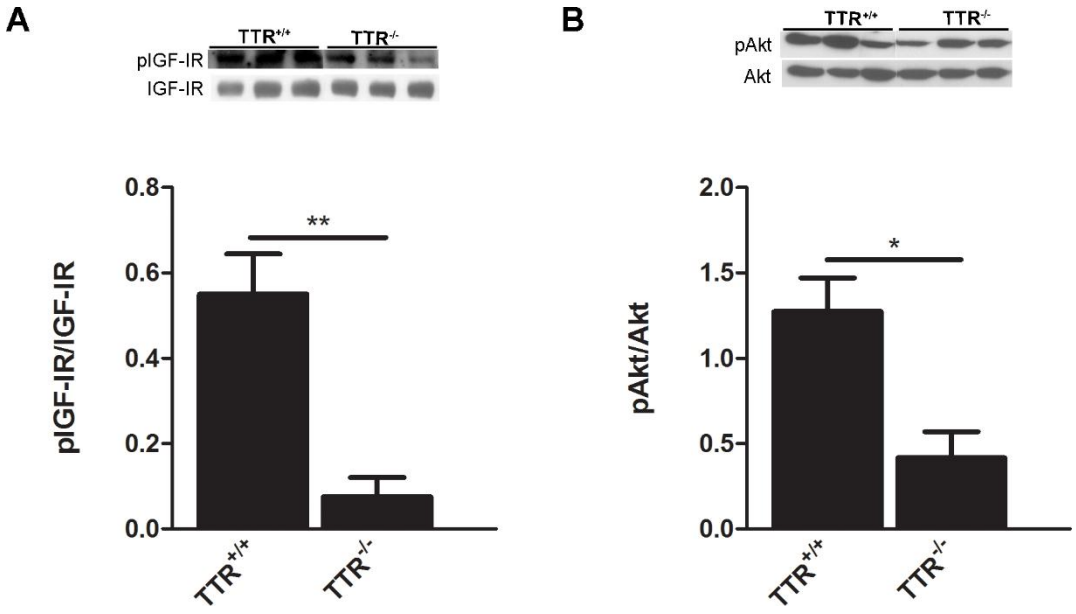


**Figure 1**. TTR activates IGF-IR signaling in young mice hippocampus. Representative images of western blot analysis and quantitative charts of pIGF-IR (A) and pAkt (B) levels of hippocampus samples at 3 months $TTR^{+/+}$ (n=5) and $TTR^{-/-}$ (n=5) mice. Error bars represent SEM. *$p < 0.05$; **$p < 0.01$ in a Student's t test.

IGF-I western blot analyses in plasma of the same animals revealed no differences between TTR wild type and TTR null animals (Figure 2). The observed results on IGF-IR signaling pathway in the hippocampus do not probably relate to IGF-I levels in plasma, being attributable to TTR. A tendency in reduced plasma IGF-I levels was observed in TTR null mice when compared with wid type littermates. This aspect will be clarified increasing animal number analysis.

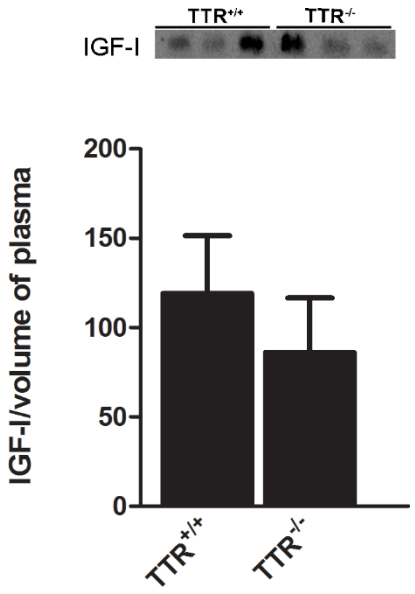


**Figure 2**. Plasma IGF-I levels in young animals. Representative images of western blot analysis and quantitative charts in plasma of 9 months $TTR^{+/+}$ (n=5) and $TTR^{-/-}$ (n=4) mice. Error bars represent SEM.

Hippocampus analysis of older animals revealed that in TTR null mice the levels of pIGF-IR were increased when compared with control littermates (Figure 3A).

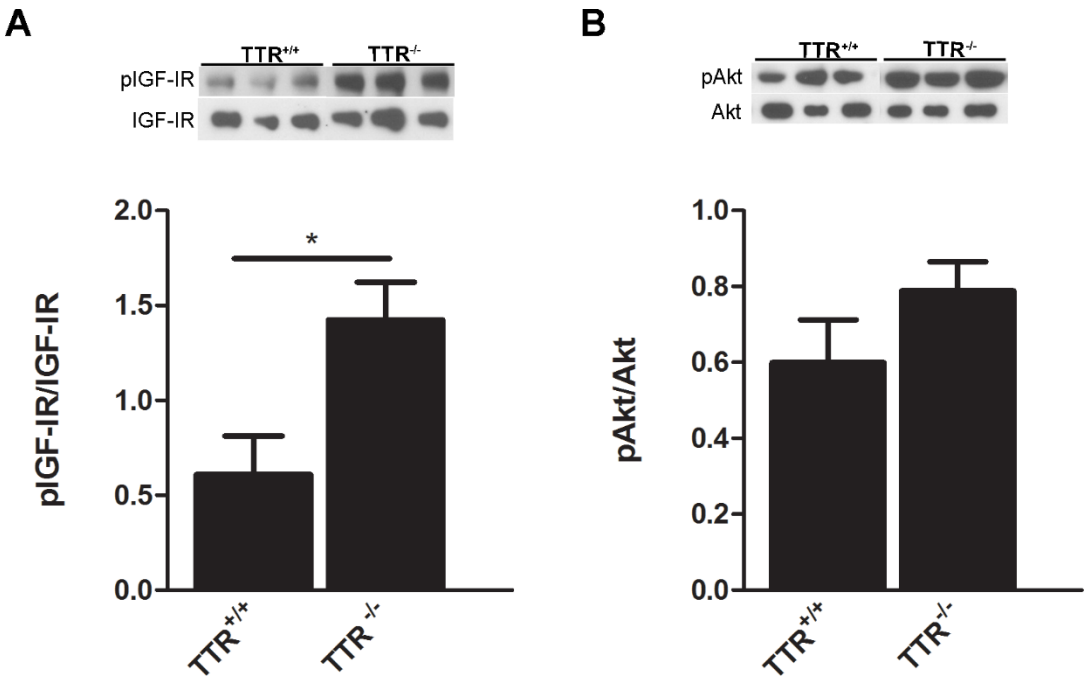


**Figure 3**. TTR effect on IGF-IR signaling in old mice hippocampus. Representative images of western blot analysis and quantitative charts of pIGF-IR (A) and pAkt (B) levels in hippocampus samples of $TTR^{+/+}$ (n=5) and $TTR^{-/-}$ (n=5) animals. Error bars represent SEM. *$p < 0.05$ in a Student's t test.

No differences were observed in Akt activation (Figure 3B) and in IGF-I plasma levels between TTR wild type and TTR null mice (Figure 4).

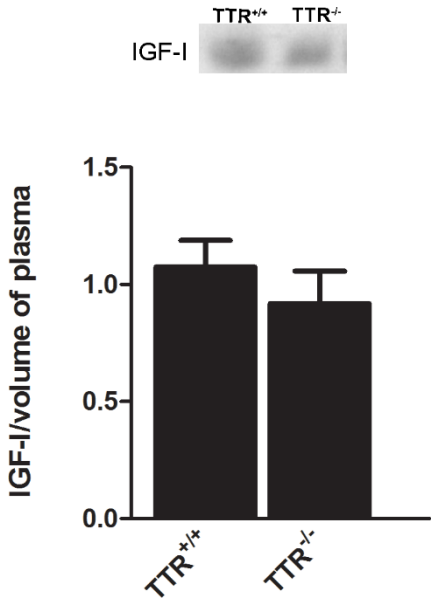


**Figure 4.** IGF-I plasma levels in 9 old animals. Representative images of western blot analysis and quantitative charts in plasma of 9 months $TTR^{+/+}$ (n=5) and $TTR^{-/-}$ (n=5) mice. Error bars represent SEM.

**TTR and IGF-I act synergistically to promote enhanced Akt activation through IGF-IR**

In order to dissect the molecular mechanism underlying the TTR effect on IGF-IR pathway, NIH3T3 fibroblasts were serum starved for two hours, followed by stimulation with either TTR, IGF and TTR plus IGF-I during 30 minutes. Analysis of Akt activation showed that TTR alone had no effect, but when TTR and IGF-I were simultaneously added to cells, up-regulation of Akt was observed when compared with activation caused by IGF-I alone (Figure 5, black bars).

IGF-I signaling occurs almost exclusively through IGF-IR, but IGF-I can also bind, with significantly lower affinity, to both isoforms of Insulin Receptor (IR) (Annunziata, Granata et al. 2011; Martin and Baxter 2011). In order to clarify if the up-regulation of Akt in NIH3T3 occurs through IGF-IR, the same experiment was performed in a mouse embryonic *fibroblasts cell line,* with a targeted disruption of the *IGF-IR* (R-). In this case, no significative activation of Akt occured in the presence of IGF-I or TTR plus IGF-I (Figure 5, white bars).

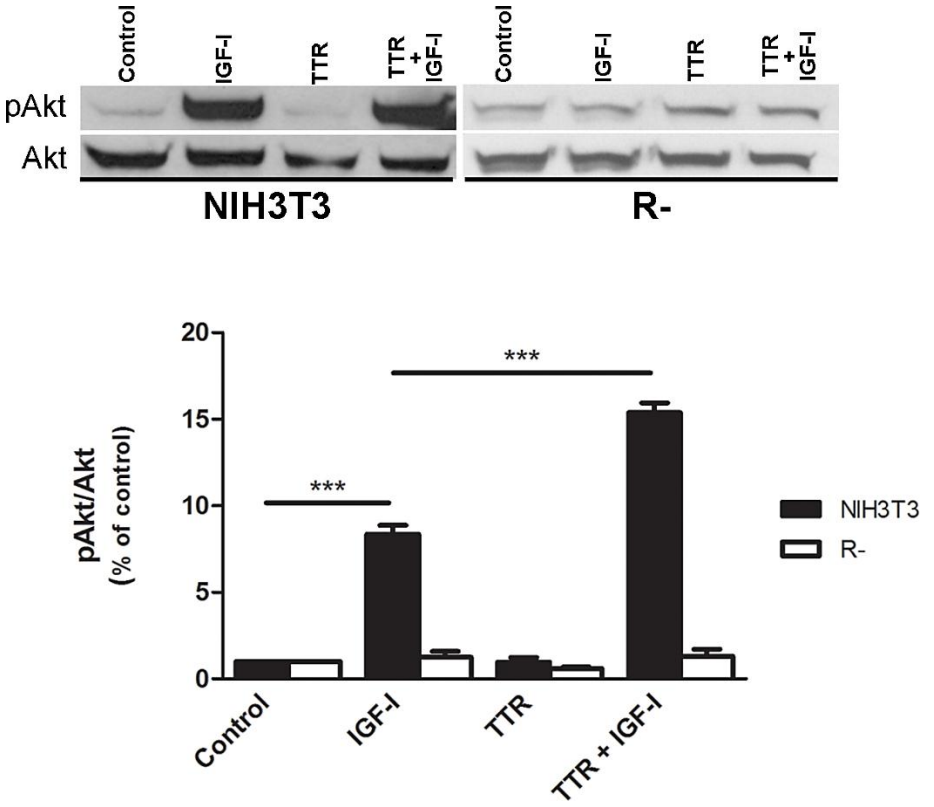


**Figure 5**. Synergistic effect of TTR and IGF-I on Akt activation. Western blot analysis of phosphorylated Akt (upper panel) and respective quantification (lower panel) in serum-starved NIH3T3 (black bars) and R- (white bars) stimulated with IGF-1, TTR, and TTR + IGF-I for 30 min. Data represent the means +- SEM of three independent experiments. Error bars represent SEM. ***$p < 0.001$ in one-way ANOVA, with Bonferroni's post test.

Taken together these results revealed that TTR and IGF-I act synergistically on Akt activation, and this effect occurs through IGF-IR. The synergistic effect of TTR and IGF-I became more interesting when we notice that TTR did not induce any up-regulation of Akt by itself. To clarify the mechanism responsible for the activation of IGF-IR by TTR and IGF-I, TTR was added to cells 10 minutes after IGF-I stimulation. pAkt levels were determined 30 minutes after TTR addition (Figure 6).

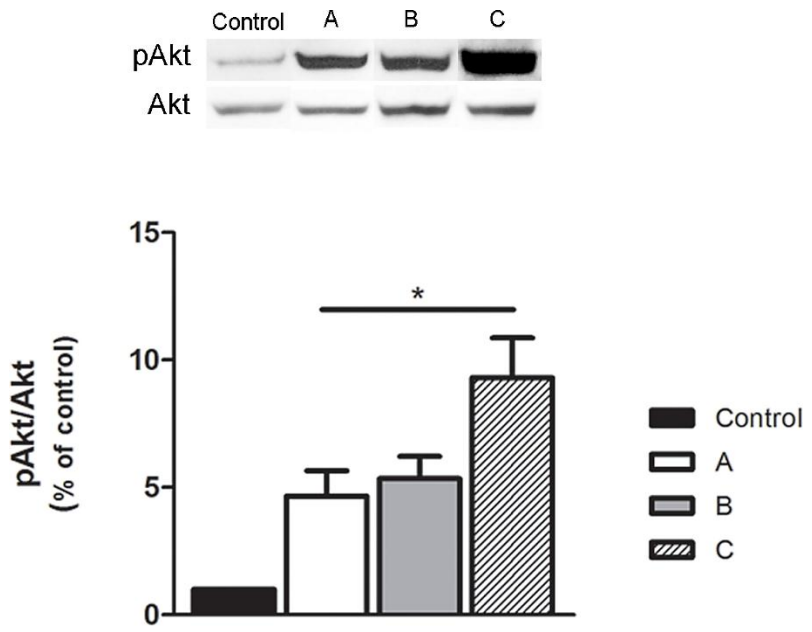


**Figure 6.** Synergistic effect of TTR and IGF-I only occurs when both molecules are added to cells at same time. NIH3T3 cells were exposed to IGF-I alone (white bar), IGF-I for 10 minutes followed by addition of TTR (grey bar) and TTR plus IGF-I (striped bar). Phosphorylation of Akt was assessed after 30 minutes stimulation. Data represents the means +- SEM of three independent experiments. Error bars represent SEM. $^{**}p < 0.05$ in one-way ANOVA, with Bonferroni's post test.

Previous activation of IGF-IR, abolished the TTR and IGF-I synergistic effect. Both molecules needed to be added to cells at same time to induce increase in pAkt levels. If IGF-I had already activated IGF-IR, TTR was not effective in IGF-IR and Akt activation.

IGF-I peptide can be cleaved, originating a truncated form of IGF-I called des-(1-3) IGF-I (Sara, Carlsson-Skwirut et al. 1989). This tripeptide, Gly-Pro-Glu, absent from the N-terminus, is much more potent because it has lower affinity to IGF-binding proteins, possibly due to the absence of glutamate on position 3 (Ballard, Francis et al. 1989) (Ballard, Wallace et al. 1996). Generation des-(1-3) peptide can be obtained by a serum protease (Yamamoto and Murphy 1994). As TTR has been described as a protease (Liz, Faro et al. 2004; Costa, Ferreira-da-Silva et al. 2008; Liz, Leite et al. 2012) it was reasonable to hypothesized that TTR could be a protease associated with IGF-I cleavage. To answer this question, TTR and IGF-I were incubated overnight at 37ºC and N-terminal sequence performed. The mixture of the two molecules did not result in a formation of a 3 amino acid peptide, excluding the possibility of IGF-I cleavage by TTR (data not shown).However, we cannot exclude if changes in des-(1-3) IGF-I levels occur *in vivo* in the absence of TTR.

**TTR binds to IGF-IR**

To determine if TTR action on IGF-IR activation involved binding to IGF-IR, NIH3T3 cells were incubated with TTR alone and TTR plus IGF-I during 30 minutes. Immunoprecipitation to IGF-IRβ followed by western blot to TTR were performed. In the control situation (unstimulated cells), no TTR was detected; however when cells were incubated with TTR alone or with TTR plus IGF-I, TTR was detected, indicating the presence of TTR/IGF-IR complexes (Figure 7). This result demonstrated that the action of TTR on IGF-IR signaling occurs through direct binding to the receptor.

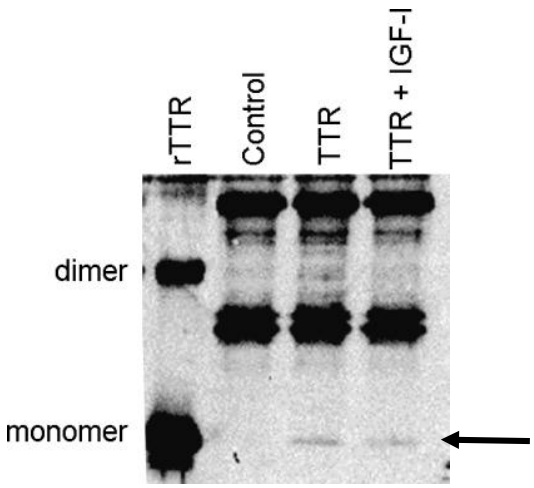


**Figure 7.** TTR binds IGF-IR. Western blot to TTR of whole NIH3T3 cell lysates stimulated with TTR alone or TTR in the presence of IGF-I for 30 minutes and immunoprecipitated with anti-IGF-IRβ. As a control was used unstimulated NIH3T3 cells. Recombinant TTR was used as a western blot marker to TTR. (→) highlightsTTR monomer.

**TTR interacts with IGF-I**

In order to establish if IGF-I, a major ligand of IGF-IR, also interacted with TTR, we probed TTR conformational changes through different biophysical and spectroscopic methods. Variations on secondary structure and tertiary contacts of TTR were investigated by far ultra violet- circular dichroism (UV-CD) and aromatic fluorescence emission, respectively. Enhancement of 1,8-ANS fluorescence provided a tool to investigate the exposure of hydrophobic regions. This panoply of techniques allowed a detailed evaluation of TTR conformational changes upon incubation with IGF-I, at distinct structural levels.

Secondary structure

The far-UV CD spectrum of TTR denotes a β-sheeted protein with a prominent negative band centered at 216 nm (Figure 8A). This far-UV CD signature is in agreement with the available structural data for this protein, which comprises nine strands and one α-helix in each domain in an extended β-sheet sandwich topology. Upon incubation with IGF-I, an increase in TTR secondary structure content was observed (Figure 8A). Moreover, this effect is concentration dependent up to an IGF-I/TTR ratio of one (Figure 8B). These results provide some evidence that IGF-I may interact with TTR, leading to secondary structure rearrangements.

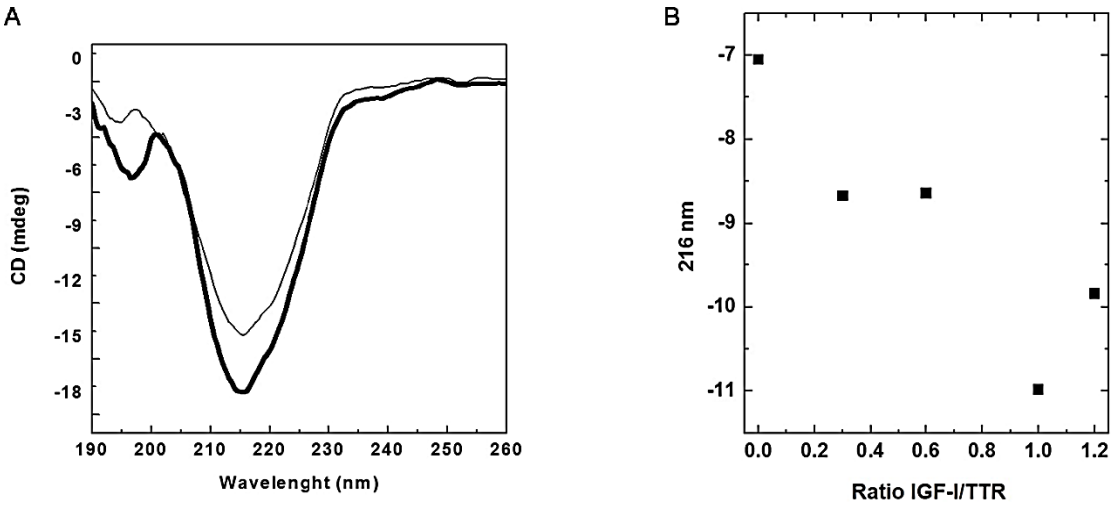


**Figure 8**. IGF-I impacts on TTR secondary structure. (A) Far-UV CD spectrum of TTR alone (light line) ~~or~~ and in the presence of IGF-I (thick line). (B) Concentration effect of IGF-I on TTR secondary structure monitored at 216 nm.

Tertiary Contacts

Comparison of the intrinsic fluorescence emission spectrum evidenced subtle effects on the tertiary structure. Protein fluorescence deriving from the naturally fluorescent aromatic residues is known to provide information on proteins conformational changes (Ladokhin 2000). In this respect, each TTR domain presents several aromatic residues ($Trp^{41,79}$, $Phe^{33,44,64,87}$, $Tyr^{69, 78, 105, 114, 116}$) spread all over the protein structure that represent an excellent probe to evaluate the effect of IGF-I on TTR tertiary structure. The fluorescence emission spectra of native TTR exhibited a broad emission band centered at 337 nm (Figure 9). Upon incubation with IGF-I, which lacks aromatic residues, only a very

slight increase in emission intensity is observed which could be an indication that the environment around TTR aromatic residues is not changed in the presence of IGF-I.

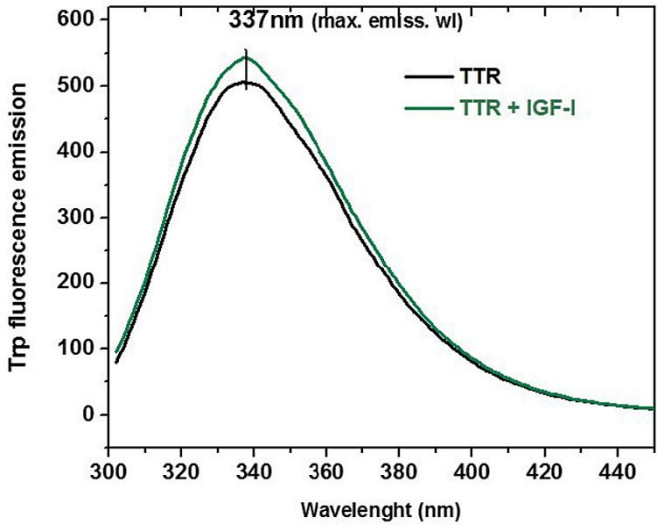


**Figure 9**. Tryptophan fluorescence emission spectra of TTR (black line) and TTR plus IGF-I (green line).

Hydrophobic surface

To verify if incubation of TTR with IGF-I results in alterations in the surface hydrophobicity of the protein, we have probed ANS (1-anilinonaphthalene-8-sulfonic acid) emission. This hydrophobic fluophore is well recognized to bind to exposed hydrophobic patches in a protein, and therefore is an excellent probe to evaluate perturbations on the hydrophobic core of TTR in the presence of IGF-I (Hawe, Sutter et al. 2008). Free ANS is scarcely fluorescent and presents an emission max. at around 520 nm in aqueous neutral solution (Gasymov and Glasgow 2007). However, a typical blue shift to around 480 nm, as well as an increase in intensity occurs upon its binding to hydrophobic surfaces in a protein (Cardamone and Puri 1992). ANS incubation with TTR, results in a positive increment in fluorescence intensity and a blue shift to 470 nm (Figure 10, black line), hence suggesting significant binding of ANS to TTR alone. Interestingly, a ≈30% intensity increase in ANS fluorescence emission was observed upon TTR incubation with IGF-I (Figure 10, green line), whereas IGF-I alone does not bind ANS (Figure 10, gray line). This observed increase in the hydrophobic surface of TTR in the presence of IGF-I suggests possible alterations on the overall packing of the native structure.

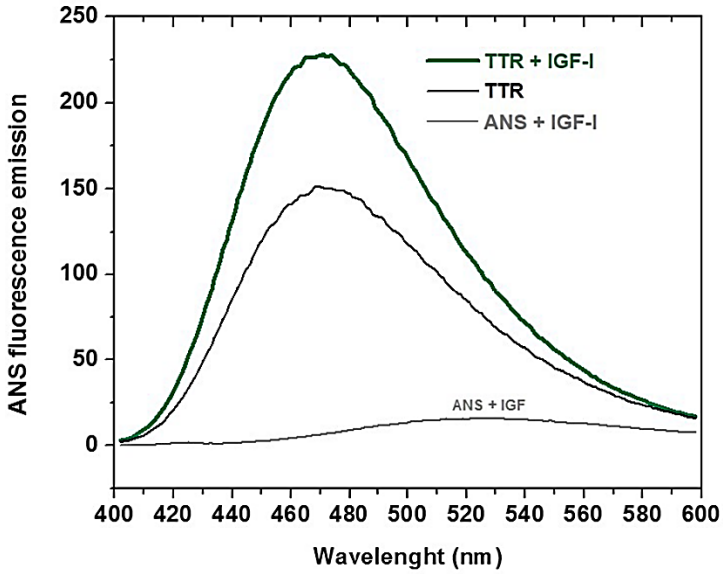


**Figure 10**. ANS fluorescence emission spectra of TTR (black line), TTR in the presence of IGF-I (green line) and IGF-I (gray line).

**Synergistic effect of TTR and IGF-I protects HT22 from glutamate-induced cell death**

IGF-I has been shown as a neuroprotective peptide against several stimulus, including amyloid-β toxicity and neurotoxic injury (Torres-Aleman 2000), being Akt a key molecule on the prosurvival effect of IGF-I (Dudek, Datta et al. 1997; Kulik, Klippel et al. 1997). It is well known that excess of glutamate input is involved in many pathological conditions as epilepsia, stoke, brain trauma, among others (Beal 1992). Glutamate inhibits Akt (Chalecka-Franaszek and Chuang 1999) and attenuates IGF-I signaling (Garcia-Galloway, Arango et al. 2003).TTR has been described as a neuroprotective molecule in cerebral ischemia (Santos, Lambertsen et al. 2010), a pathological condition related with glutamate excitotoxicity. To understand if the synergistic effect of TTR and IGF-I on IGF-IR activation could protect cells against glutamate-induced excitotoxicity, HT22 cells (an immortalized mouse hippocampal cell line) were used. This cell line is an excellent model for oxidative stress by glutamate since is defective on functional ionotropic glutamate receptors (Maher and Davis 1996), which leads to decreased intracellular levels of cysteine and glutathione, resulting in an increased in ROS accumulation and elevated $Ca^{2+}$ influx, generating oxytosis (Tan, Schubert et al. 2001). Oxytosis cell death is characterized by biochemical changes identical to apoptosis with morphological changes similar to necrosis. The oxidative pathway of glutamate in these cells also occur *in vivo*,

therefore cells are a good model to approach oxidative stress in neuronal cells (Rossler, Giehl et al. 2004). As HT22 cells do not synthetize TTR, they are a good model to study TTR protection against oxidative stress.

HT22 cells were exposed to 5mM of glutamate during 24h, which produced pronounced cell death. Pre-treatment with IGF-I during 30 minutes before glutamate exposure, increased cell survival; pre-treatment with TTR plus IGF-I, lead to higher increase in survival when compared with cells treated just with IGF-I (Figure 11).

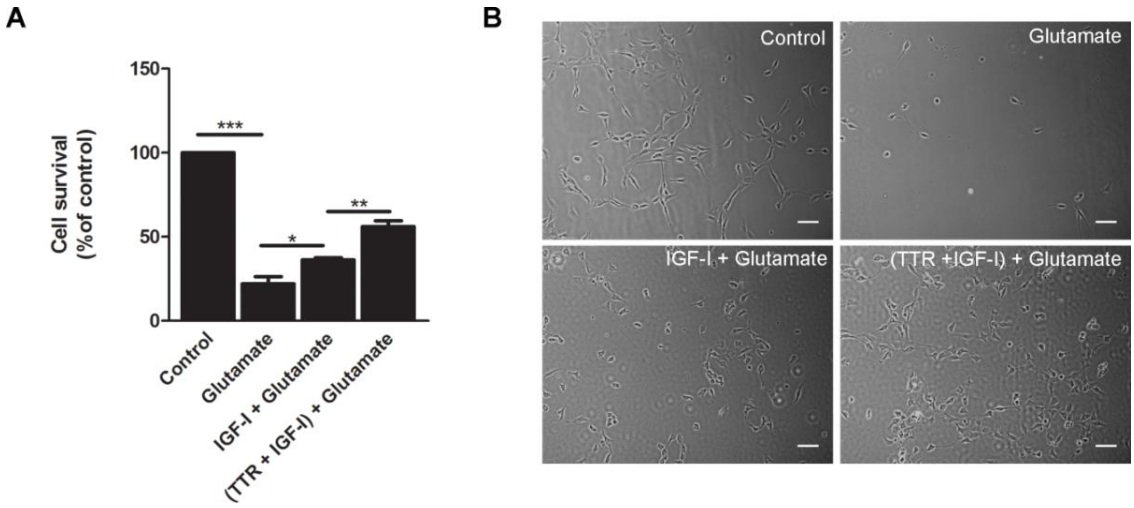


**Figure 11.** Synergistic effect of TTR and IGF-I protects HT22 from glutamate-induced cell death. HT22 cells were pre-incubated during 20 minutes with IGF-I or TTR plus IGF-I, followed by incubation with 5mM of glutamate for 24h. (A) Cell survival assay. (B) Cell morphology by phase-contrast microscopy. Scale bar = 10μm. Data represents the means +- SEM of four independent experiments. Error bars represent SEM. *$p$ < 0.05; **$p$ < 0.01; ***p< 0.001 in one-way ANOVA, with Bonferroni's post test.

To analyze if protection from cellular death by TTR plus IGF-I could be related to increased activation of IGF-IR signaling pathways, western blots to pIGF-IR and pAkt under different conditions were performed. As above described for NIH3T3 cells, TTR plus IGF-I induced increased activation of IGF-IR and Akt when compared with IGF-I alone. TTR alone do not activate IGF-IR signaling pathway in HT22 cells (Figure 12).

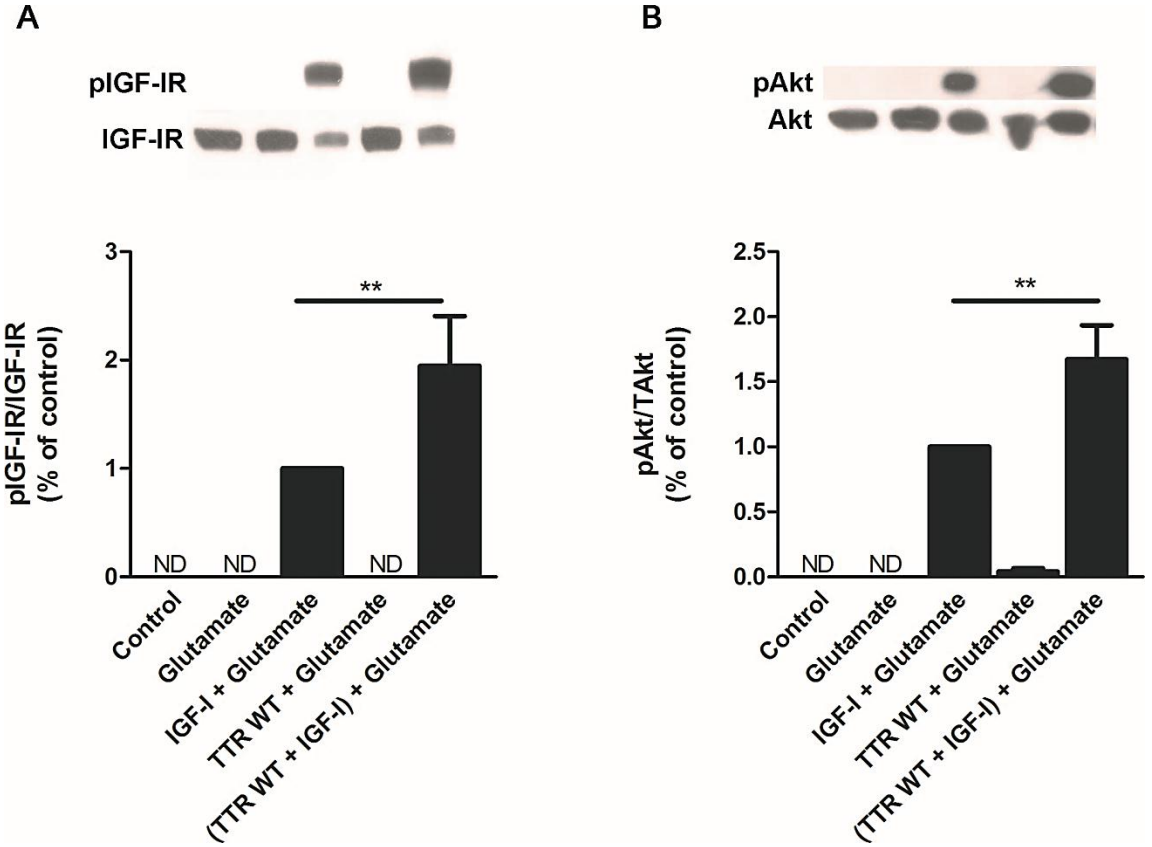


**Figure 12**. Western blot analysis and respective quantification and of phosphorylated IGF-IR (A) and Akt (B).HT22 cells were pre-incubated during 20 minutes with either IGF-I or TTR alone and with TTR plus IGF-I, followed by incubation with 5mM of glutamate for 24h. Data represents the means +- SEM of four independent experiments. Error bars represent SEM. ; $^{**}p < 0.01$; $^{*}p < 0.05$ in one-way ANOVA, with Bonferroni's post test. ND: not detected.

One of the targets of activated Akt, that regulates cell survival is a transcription factor from a subfamily of Forkhead factors denoted FoxO (Forkhead box, class O). FoxO1 is a downstream effector of PI3K/Akt pathway induced by IGF-I in PC12, hippocampal and cortical neurons. (Gan, Zheng et al. 2005). Akt phosphorylates $Ser^{253}$, $Ser^{315}$ and $Thr^{32}$ of FoxO that shifts from the nucleus to cytoplasm, preventing expression of several apoptotic genes (Biggs, Meisenhelder et al. 1999; Brunet, Bonni et al. 1999).

Analysis of pFoxO by immunofluorescence revealed that when cells were pre-incubated with TTR plus IGF-I, as compared to IGF-I alone, pFoxO intensity was stronger being more localized in cytoplasm (Figure 13). These results corroborate the notion that TTR synergistically amplifies IGF-I action as a cell survaival molecule and protects cells from oxidative stress caused by glutamate.

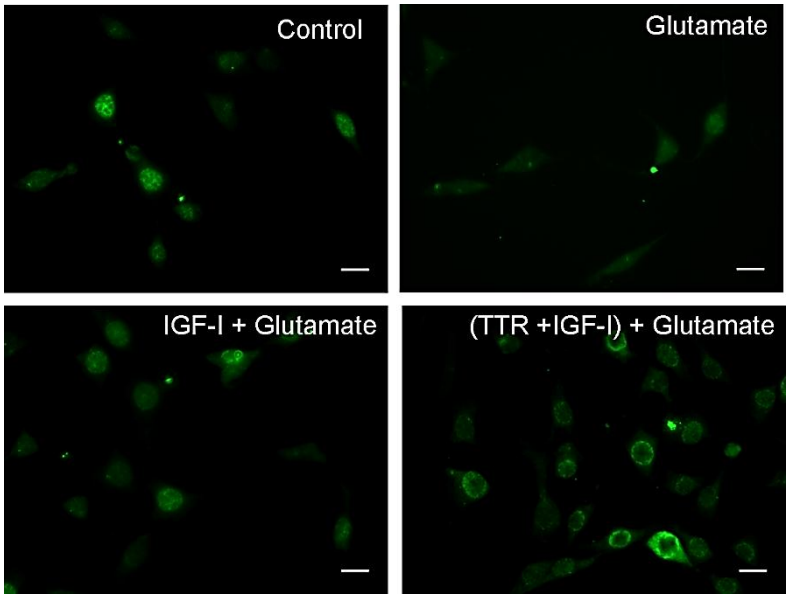


**Figure 13**. pFoxO immunofluorescence of HT22 cells. Representative images of three independent experiments of pFoxO immunofluorescence of HT22 cells pre-incubated 20 minutes with IGF-I or TTR plus IGF-I before 5mM glutamate stimulation for 24 hours. Scale bar = 10μm.

## Discussion

Although earlier studies described in the introduction suggest that TTR and IGF-I could be related in neuroprotection, this is the first report that demonstrates a direct synergistic effect of TTR in IGF-IR activation and signaling pathway thereof.

Analysis of pIGF-IR and pAkt in the hippocampus of TTR null mice showed downregulation of these molecules as compared with age matched wild type littermates. Absence of TTR induced reduced signaling through the IGF-I receptor. To better understand the molecular mechanism that was behind this finding, activation studies were performed in NIH3T3 cell line. Interestingly, TTR alone did not induce any activation of Akt, a pivotal intermediate in the IGF-IR signaling cascade. However, TTR affects Akt activation only when simultaneously added with IGF-I.

Both insulin and IGF-I receptors belong to the tyrosine kinase receptor family. These receptors are structurally similar, being formed by two extracellular $\alpha$-subunits, covalently linked to two transmembrane $\beta$-subunits. The tyrosine domain is localized in the $\beta$-subunit. There are also hybrid receptors composed by half of IGF-IR and half of IR. Due to its similarity, cross talk in terms of ligand binding exists. IGF-I binds with highest affinity to IGF-IR, although it can also be recognized by a hybrid receptor and, to a lesser extent, by the IR (Annunziata, Granata et al. 2011; Arnaldez and Helman 2012; Fernandez and Torres-Aleman 2012). Through different approaches, including the use of fibroblastic R- cells and N-terminal sequencing of IGF-I, we show highly improbable that TTR signaling properties on the IGF-IR pathway occur via the IR or by cleavage of IGF-I. Furthermore, immunoprecipitation assays evidenced that TTR binds to IGF-IR, and biophysical studies demonstrated rearrangements in secondary structure and increase in hydrophobic surface of TTR in the presence of IGF-I. Further studies are warranted using additional methodologies to determine binding affinities and sites of interaction of TTR with these new ligands.

The main source of IGF-I present in CSF is blood. Traffic of IGF-I from blood to CSF is megalin-mediated (Carro, Spuch et al. 2005) or through endocytosis of IGF-IR (Geary, Rosenfeld et al. 1989) which is very abundant in choroid plexus (Davidson, Bohannon et al. 1990). Both receptors increase the amount of IGF-I, derived from blood, in CSF (Carro, Spuch et al. 2005). Once in CSF, IGF-I can diffuse to periventricular areas such as the hippocampus or hypothalamus to be transported to distant areas, as cerebellum; the transport occurs through interaction with insulin-like growth factor binding proteins (IGFBPs). It is interesting that synthesis of TTR in brain only occurs in choroid plexus where the expression of IGF-IR is also more pronounced when compared with

other brain tissues. Megalin, one of the entries of IGF-I in brain, is also a receptor to TTR. The observed effect of TTR on IGF-IR signaling in the hippocampus, only occurred *in vitro* in the presence of IGF-I. Probably, *in vivo*, this interaction also exists, since IGF-I is present in CSF. Analysis of IGF-I levels in plasma (the main source of IGF-I in CSF) showed no differences between TTR wild type and TTR null mice. Worth mentioning is the control of IGF-I bioavailability by IGFPBs that bind IGF-I strongly than IGF-IR does (Rapp, Deger et al. 1988; Le Roith, Bondy et al. 2001). Further studies should be performed to verify if TTR can also impact on IGFBP structure and IGF-I regulation.

Interestingly, in older animals, activation of IGF-IR was similar in TTR null mice and wild type littermates. Decrease of CSF TTR levels with age, previously described (Sousa, Marques et al. 2007) might trigger compensatory mechanisms that attenuate the effect of TTR on the IGF-I axis observed in young animals.

Though serum TTR can cross blood brain barrier (BBB), TTR effect on IGF-IR signaling in hippocampus must be related to CSF TTR produced by the choroid plexus. Further studies need to be performed to assess the relative contribution of blood and CSF TTR on the IGF-IR cascade.

Excitotoxicty is a common pathogenic pathway to several neurodegenerative diseases (Dong, Wang et al. 2009). IGF-I activation of Akt is a neuroprotective mechanism against glutamate induced apoptosis in different cell types such as dorsal root ganglion (DRG) neurons (Liu, Cai et al. 2012) and oligodendrocytes (Ness and Wood 2002). Akt mediates protection against glutamate also in HT22 cells (Koh 2007). IGF-I prevents neuronal cell death caused by glutamate, but TTR together with IGF-I induced a stronger effect on IGF-IR activation, Akt and FoxO when compared with IGF-I alone, leading to more efficient suppression of cell death. Whether this effect extends to other neuronal types or glia is a matter of further investigation.

IGF-I administration has been beneficial for many neurodegenerative diseases. In brain ischemia, although low serum levels of IGF-I are associated with lower levels of brain damage, if IGF-I administration occurs after ischemia, the peptide becomes neuroproptective (Guan, Bennet et al. 2003). CSF TTR protects neurons from cell death in brain ischemia (Santos, Lambertsen et al. 2010). It is tempting to believe that the synergistic effect of these proteins, could improve neuroprotection in cerebral brain ischemia. We hypothesized that IGF-I administration together with pharmacological agents to increase CSF TTR are of added value in ischemia pre-conditioning.

Patients with AD have decreased levels of TTR in CSF (Serot, Christmann et al. 1997) and in plasma (Ribeiro, Santana et al. 2012), as reduced levels of serum IGF-I (Watanabe, Miyazaki et al. 2005). Decrease of these molecules in AD is associated with increased pathology. Administration of serum IGF-I in Tg2576 mice, increased TTR levels

in brain leading to increase in Aβ clearance, reducing pathology (Carro, Trejo et al. 2002); however this effect was not observed by all authors working on the field (Lanz, Salatto et al. 2008). The tight connection between TTR and IGF-I in several neuropathologies, highlight their significance in neuroprotection. Synergy between TTR and IGF-I described in this work could be a novel strategy to overcome, or at least, delay the phenotype of these diseases, a subject that deserves further attention.

In summary, our work described for the first time a synergy between TTR and IGF-I and their protective role in glutamate-induced toxicity. This protection occurs through activation of IGF-IR signaling cascades, pointing out a new role of TTR as a signaling transducing molecule.

## Acknowledgements

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# CHAPTER II

# Transthyretin increases transcription of Insulin-like growth factor receptor

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## Abstract

Transthyretin (TTR) is the carrier protein of $T_4$ and retinol through the retinol-binding protein (RBP). Besides these properties, it has a neuroprotective role in several contexts such as Alzheimer Disease (AD) and cerebral ischemia. Activation of insulin-like growth factor receptor (IGF-IR) pathways and increased levels of TTR are associated with absence of neurodegeneration in anAD mouse model. In the present study we verified that young/adult TTR null mice had decreased levels of IGF-IR in hippocampus, but not in choroid plexus when compared with age matched controls. *In vitro* studies, in NIH3T3 cell line, showed that TTR regulates IGF-IR transcription. The data demonstrated that TTR can induce neurite outgrowth in primary hippocampal neurons. In summary, the results provide evidence of new role of TTR as a transcription inducer of IGF-IR.


**Key words:** transcription, hippocampus, neurites, regulation

## Introduction

Transthyretin (TTR) is a 55,000-Dalton homotretrameric protein, carrier of thyroxine ($T_4$) (Woeber and Ingbar 1968) and retinol (vitamin A) through the retinol-binding protein (RBP), both in plasma and the cerebrospinal fluid (CSF) (Kanai, Raz et al. 1968). It is mainly synthetized by liver and by choroid plexus, being secreted to blood and CSF, respectively (Aleshire, Bradley et al. 1983; Soprano, Herbert et al. 1985). In brain, the only source of TTR is the choroid plexus and represents 25% of total CSF protein (Aldred, Brack et al. 1995). Besides its carrier properties, TTR has also been described as a neuroprotective molecule. TTR prevents Aβ toxicity (Costa, Goncalves et al. 2008) and modulates Aβ brain levels (Oliveira, Ribeiro et al. 2011). The neuroprotective role of TTR is extended to other pathologies besides Alzheimer disease (AD); in cerebral ischemia, CSF TTR enhances survival of endangered neurons (Santos, Lambertsen et al. 2010), and under nerve injury conditions TTR improves nerve regeneration (Fleming, Saraiva et al. 2007).

Insulin-like growth factors (IGFs) are a family of polypeptides that have important functions in development, cell differentiation, plasticity and survival of the nervous system (reviewed in (Benarroch 2012)). Most biological actions of IGF-I are mediated through type I IGF receptor (IGF-IR). IGF-IR is a ubiquitously glycoprotein that consists of two extracellular α-domains and two transmembrane β-domains, linked by disulfide bonds (Rubin and Baserga 1995; Adams, Epa et al. 2000; Navarro and Baserga 2001). The ligand binding domain is located in the α-subunit and the tyrosine kinase domain is located in the intracellular region of the β-subunit. Upon ligand binding, two main downstream pathways are activated by IGF-IR, namely MAPK/Ras-Raf-Erk and PI3K/Akt/mTor pathway (Peruzzi, Prisco et al. 1999; Arnaldez and Helman 2012). Physiological responses to IGF-IR tyrosine kinase activation are diverse and include differentiation, proliferation, protection from apoptosis and neurite outgrowth (Kim, Leventhal et al. 1997; Shelton, Steelman et al. 2004; Sosa, Dupraz et al. 2006; Arnaldez and Helman 2012).

In an AD mouse model, administration of IGF-I induced clearance of Aβ from brain, hypothesized to occur through the regulation of Aβ transport proteins such as albumin and TTR (Carro, Trejo et al. 2002). Absence of neurodegeneration in the same mice model was hypothesized to be related to increased TTR levels and activation of growth factors signaling pathways (Stein and Johnson 2002). A synergistic effect of TTR and IGF-I on IGF-IR signaling pathway was previously described and *in vitro* studies also demonstrated that TTR binds IGF-IR (previous chapter). Taken together, these findings suggest a strong

connection between TTR and IGF-IR. The main objective of this work is to dissect the relation between these molecules, clarifying their importance at biological level. For that purpose, *in vivo* and *in vitro* studies were performed.

## Materials and Methods

### Animals

The number of mice handled for this research was approved by the Institutional and National General Veterinary Board Ethical Committees according to National and European Union rules. Three and nine-month-old TTR wild type ($^{+/+}$) and TTR knockout ($^{-/-}$) (Episkopou, Maeda et al. 1993), in 129/svJ background were obtained from the littermate offspring of heterozygous breeding pairs. The animals were maintained under a 12-h light/dark cycle and fed with regular rodent's chow and tap water *ad libitum*. Genotypes were determined from tail extracted genomic DNA, using primers for the detection of exon 2 of TTR (which is disrupted in $TTR^{-/-}$ by insertion of neomycin resistance gene) as previously described (Episkopou, Maeda et al. 1993).

Mice were sacrificed with a lethal injection of a premixed solution containing ketamine (75mg/Kg) plus medetomidine (1mg/Kg). Hippocampus and choroid plexus were dissected and frozen at -80ºC.

Efforts were made to minimize pain and distress; all animal experiments were carried out in accordance with the European Communities Council Directive.

### TTR production and purification

Recombinant TTR was produced in a bacterial expression system using *Escherichia coli* BL21 (Furuya, Saraiva et al. 1991) and purified as previously described (Almeida, Damas et al. 1997). Briefly, after growing the bacteria, the protein was isolated and purified by preparative gel electrophoresis after ion-exchange chromatography. Protein concentration was determined using the Lowry method (Lowry, Rosebrough et al. 1951).

### Endotoxin removal

To remove endotoxin, a polymixin B column (Thermo Scientific) was used. Briefly, the column was regenerated with 1% sodium deoxycholate (Sigma) and washed with pyrogen-free buffer to remove detergent. Recombinant TTR was applied to column and incubated during one hour at room temperature. Aliquots of pyrogen-free buffer were added and the flow-through was collected. Protein concentration was determined by the Bradford method (Hammond and Kruger 1988).

### Cell culture

NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS), 100μg/L streptomycin, 100U/mL penicillin, 300 μg/mL of L-glutamine and maintained at 37ºC in a humidified incubator of 5% $CO_2$ /95% air. Once cells reached 80% confluency, cells were serum starved, rinsed with phosphate buffered saline (PBS) and then stimulated with TTR (55μg/mL) in the presence or absence of α-amanitin (Sigma, 10μg/mL) at 37 ºC.

Primary cultures of mouse hippocampal neurons were prepared from the hippocampus of E18-E19 $TTR^{-/-}$ mice embryos. The hippocampi were treated with trypsin (0.5mg/mL, 15 minutes at 37ºC) in $Ca^{2+}$ and $Mg^{2+}$ HBSS (Hank's Balanced Salt Solution) free. The hippocampi were then washed in HBSS supplemented with 10% FBS. After centrifugation at 140_$g_{av}$, for 1 min, cells were washed in HBSS and mechanically dissociated. Hippocampal cultures were maintained in serum-free Neurobasal medium supplemented with B27, glutamate (25 mM), glutamine (0.5 mM) and gentamicin (0.12 mg/ml). Cells were kept at 37ºC in a humidified incubator of 5% $CO_2$/95% air, for 7–8 days, the time required for maturation of hippocampal neurons (Brewer, Torricelli et al. 1993).

All culture media and supplements used were from GIBCO.

**Western blot analysis**

Cultured cells and hippocampus were homogenized in lysis buffer containing 20 mM MOPS, 2mM EGTA, 5mM EDTA, 30mM sodium fluoride, 60mM β-glycerophosphate, 20mM sodium pyrophosphate, 1mM sodium orthovanadate , 1mM phenylmethylsulphonyl fluoride, 1% Triton X-100 and 1x protease inhibitors mixture (GE Healthcare). Total protein concentration was determined using the Bradford method. 50μg of protein were applied and separated by 10% SDS-PAGE and transferred to a nitrocellulose Hybond-C membrane (GE Healthcare), using a wet system. Membranes were dried, blocked one hour at room temperature in blocking buffer, 5% BSA in phosphate-buffered saline Tween-20 (PBST), and then incubated overnight a 4ºC with primary antibodies diluted in blocking buffer, namely rabbit polyclonal IGF-IR (1:1,000; Cell Signaling), β-actin (1:5000, Sigma) and α-tubulin (1:10,000, Sigma). Membranes were then incubated with anti-rabbit IgG-HRP (1:10,000; Binding Site) and anti-mouse IgG-HPR (1:5000; Binding Site), during 1 hour at room temperature. Blots were developed using Immun-Star WesternC Chemiluminescent kit (BioRad) and exposed to ECL Hyperfilm (GE Healthcare). Quantitative analyses were performed using the ImageJ software.

**Reverse Transcriptase – Polymerase Chain Reaction**

Total RNA was isolated using Trizol reagent (Invitrogen). First-strand cDNA was synthesized using the Superscript II kit (Invitrogen). PCR was performed with the following oligonucleotides to IGF-IR: 5'-TCTTGGATGCGGTGTCCAATAAC-3' and 5'-AGGTTGTGTTGTCGTCCGGTGTG-3'; for mouse β-actin: 5'-CTCTTTGATGTCACGCACGATTTC-3' and 5'-GTGGGCCGCTCTAGGCACCAA-3'.
Ethidium bromide-stained gels were scanned using GENE FLASH syngene bio imaging equipment. The results were analyzed using the ImageJ software.

**Neurite outgrowth assay**

Hippocampal neurons from $TTR^{-/-}$ embryos were isolated under the above described conditions, plated on poly-D-lysine-coated glass coverslips at a density of $5x10^4$ cells/$cm^2$. TTR (55μg/ml and 300μg/ml), IGF-I (100ng/ml) and TTR plus IGF-I were added to cell culture medium immediately after plating. Cells were maintained in culture during 24 hours. Medium was removed, cells fixed with 4% paraformaldehyde and immunofluorescence to anti-MAP2 (1:800, Abcam) performed.

**Neurite outgrowth analysis**

Morphological measurements of neurite outgrowth were performed using the plugin NeuronJ from the ImageJ software (Meijering, Jacob et al. 2004). Neurites from 24 hours were stained with anti-MAP2. Number, sum length and maximum length of neurites per cell were the analyzed parameters. At least 80 cells were counted in each experiment and the experiments were repeated 4 times.

**Immunofluorescence**

$TTR^{-/-}$ embryonic hippocampal neurons were grown under the above described conditions. Cells were washed with PBS, fixed with 4% paraformaldehyde during 10 minutes at room temperature (RT), permeabilised 15 minutes with 0.25% Triton X-100/PBS, blocked for 30 minutes at 37ºC in PBS containing 4% of FBS and 1% bovine serum albumin (BSA) and then incubated overnight at 4ºC with rabbit polyclonal MAP2 (1:800, Abcam). After washing with PBS, cells were incubated with secondary antibody, anti-rabbit 488 (1:1,000; Molecular Probes), 30 minutes at room temperature and protected from light. After washing, cells were stained with the fluorescent dye Hoeschst 33342 (0.5μg/mL). Coverslips were mounted on glass slides with Dako fluorecescent mounting medium (Dako) and visualized by a Widefield Fluorescent Microscope (Zeiss Axio Imager Z1).

### Statistical analysis

Quantitative data are presented as Mean ± SEM. Statistical analysis was carried out using Graphpad Prism 5 software. Differences among groups were analyzed by one-way ANOVA (followed by Bonferroni's Multiple Comparison Test), comparisons between two groups were made by Student's *t* test. *P* values of lower than 0.05 were considered significant. ***$p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

## Results

**Hippocampus of TTR$^{-/-}$ animals have decreased levels of IGF-I receptor.**

In the previous chapter it was demonstrated that TTR influenced IGF-IR signaling in the hippocampus. To understand the relationship of TTR and IGF-IR in this brain area, hippocampus of TTR$^{-/-}$ and TTR$^{+/+}$ mice from different ages, were homogenized and analyzed by western blot to IGF-IR. At 3 months of age, TTR$^{-/-}$ animals had decreased levels of IGF-IR when compared with age matched wild type littermates (Figure 1A). However, this difference was only observed in young animals. 9 months TTR$^{-/-}$ mice presented the same levels of IGF-IR when compared with age matched TTR$^{+/+}$ littermates (Figure 1B).

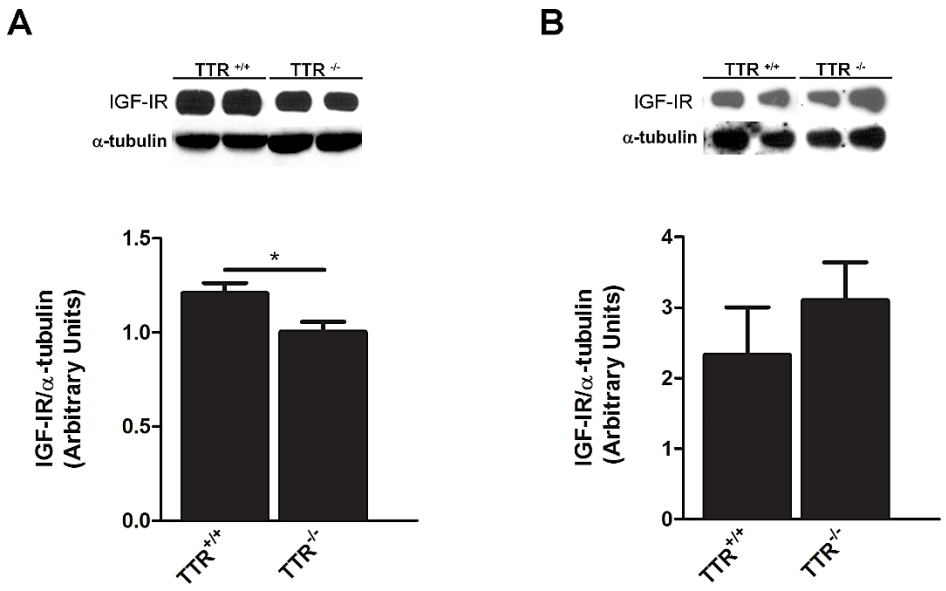


**Figure 1**: Hippocampus of young TTR$^{-/-}$ mice have decreased levels of IGF-IR. Representative images of western blot analysis and quantitative charts of IGF-IR levels in: (A) hippocampus samples of 3 months TTR$^{+/+}$ (n=5) and TTR$^{-/-}$ (n=4) mice; (B) hippocampus samples of 9 months TTR$^{+/+}$ (n=4) and TTR$^{-/-}$ (n=5) animals. Results are presented as average ± SEM. Error bars represent SEM. * $p < 0.05$ in a Student's t test.

**Levels of IGF-IR in choroid plexuses of TTR$^{-/-}$ animals are similar to TTR wild type littermates**.

Choroid plexus (CP) is the site of TTR synthesis in brain, and also one of the sites where IGF-IR is most abundant (Fernandez and Torres-Aleman 2012), so it became relevant to evaluate IGF-IR levels in this tissue. Western blot analysis of CP of 3 months

animals demonstrated that IGF-IR levels are very similar between $TTR^{+/+}$ and $TTR^{-/-}$ mice (Figure 2).

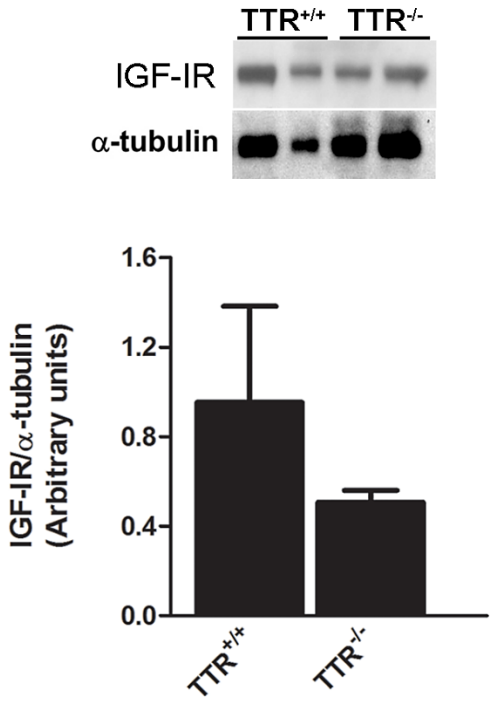


**Figure 2.** Young TTR null mice have similar levels of IGF-IR in choroid plexuses when compared TTR wild type littermates**.** Representative image of western blot analysis and quantitative charts of IGF-IR levels of choroid plexus samples of $TTR^{+/+}$ (n=4) and $TTR^{-/-}$ (n=4) animals at 3 months of age. Results are presented as average ± SEM. Error bars represent SEM.

**TTR regulates IGF-IR at transcriptional level.**

In order to clarify how TTR influenced IGF-IR levels in the hippocampus, cellular studies were performed in NIH3T3 cells. Recombinant endotoxin free human TTR (55μg/mL) was added to NIH3T3 cells for 6h under serum free conditions. Western blot analysis of whole cell extracts showed that in the presence of TTR, IGF-IR levels increased approximately 50% when compared with controls without added TTR (Figure 3).

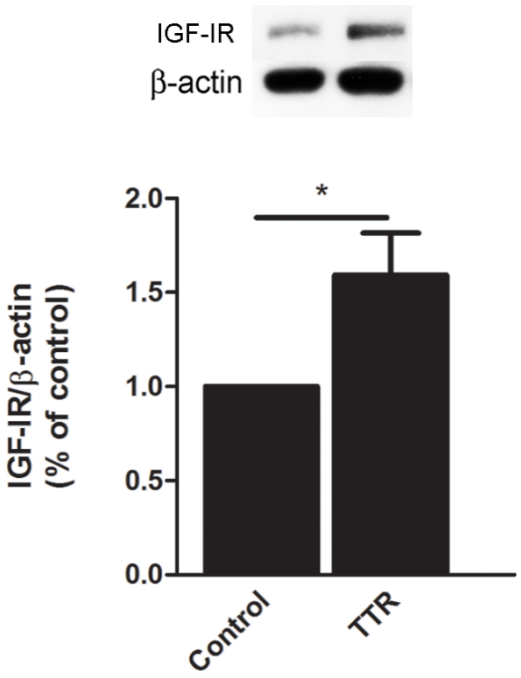


**Figure 3.** TTR increases IGF-IR protein levels. Representative image and respective chart of western blot analysis of IGF-IR in NIH3T3 cells incubated with TTR for 6 hours. Data represents the means +- SEM of four independent experiments. Error bars represent SEM. *p <0,05 in a Student's t test.

To discern if the role of TTR occurred at the transcriptional level, semi-quantitative RT-PCR of IGF-IR was performed. For that purpose, fibroblasts were incubated with TTR (55μg/mL) during 4h under serum free conditions and RNA was extracted from cells. PCR to IGF-IR and β-actin was performed; the analyses of IGF-IR/β-actin ratios demonstrated that levels of IGF-IR increased 30% in cells that had been exposed to TTR when compared with controls (Figure 4).

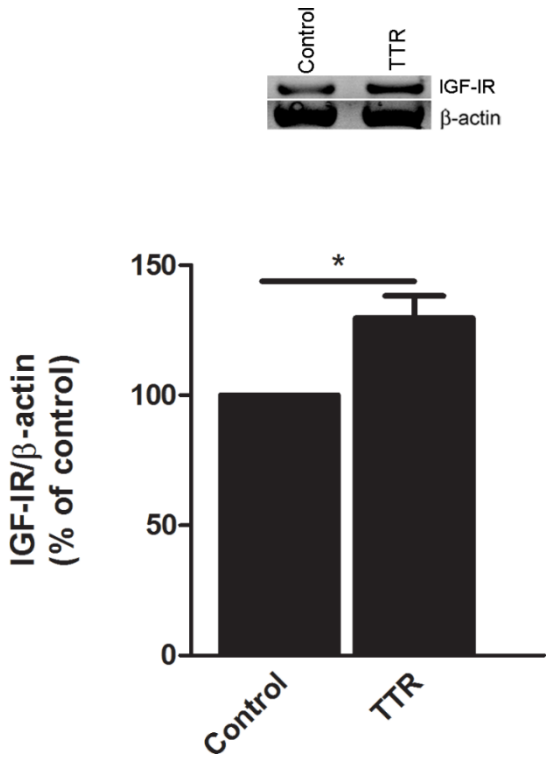


**Figure 4.** TTR increases transcription of IGF-IR. Semi-quantitative RT-PCR of NIH3T3 cells exposed to TTR during 4 hours. Data represents the means +- SEM of three independent experiments. Error bars represent SEM. *p < 0,05 in a Student's t test.

These results suggested that TTR influences transcription of IGF-IR. To confirm this effect, α-amanitin (inhibitor of RNA polymerase) was added to the cells in the presence or absence of TTR (55μg/mL). After 6 hours, cells were lysed and whole cell extract was separated by SDS-PAGE. Western blot analysis showed that when in the presence of α-amanitin, TTR had no longer effect on the regulation of IGF-IR levels (Figure 5). Taken together, these results demonstrated for the first time that TTR up-regulates IGF-IR transcription.

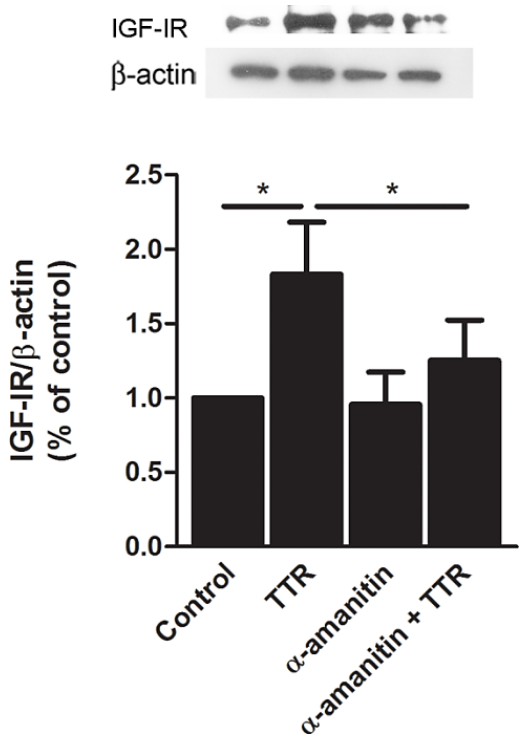


**Figure 5**. TTR increases transcription of IGF-IR. Western blot analysis of IGF-IR when exposed to TTR for 6 hours in the presence or absence of α-amanitin. Data represents the means +- SEM of five independent experiments. Error bars represent SEM. *p < 0,05; in one-way ANOVA, with Bonferroni's post test.

**TTR enhances hippocampal neurite outgrowth.**

Transthyretin neuritogenic activity was previously demonstrated on PC12 cells and dorsal root ganglion (DRG) (Fleming, Saraiva et al. 2007). Internalization of TTR, mediated by megalin, was required to induce increased neurites number and length (Fleming, Mar et al. 2009). Primary cultures of hippocampal neurons from $TTR^{-/-}$ embryos were prepared and different concentrations of TTR were added to culture medium; after 24 hours, cells were fixed and several parameters of neurite outgrowth were measured. Total neurite length was increased when TTR was added to hippocampal neurons in a dose response manner, when compared with the control situation. Neurite number and neurite maximum length per cell were also increased when TTR was added to cells. Although only at concentrations of 300 μg/mL the increase was statistically different, at lower TTR concentration a trend in increased neurite number and maximum length was observed (Figure 6).

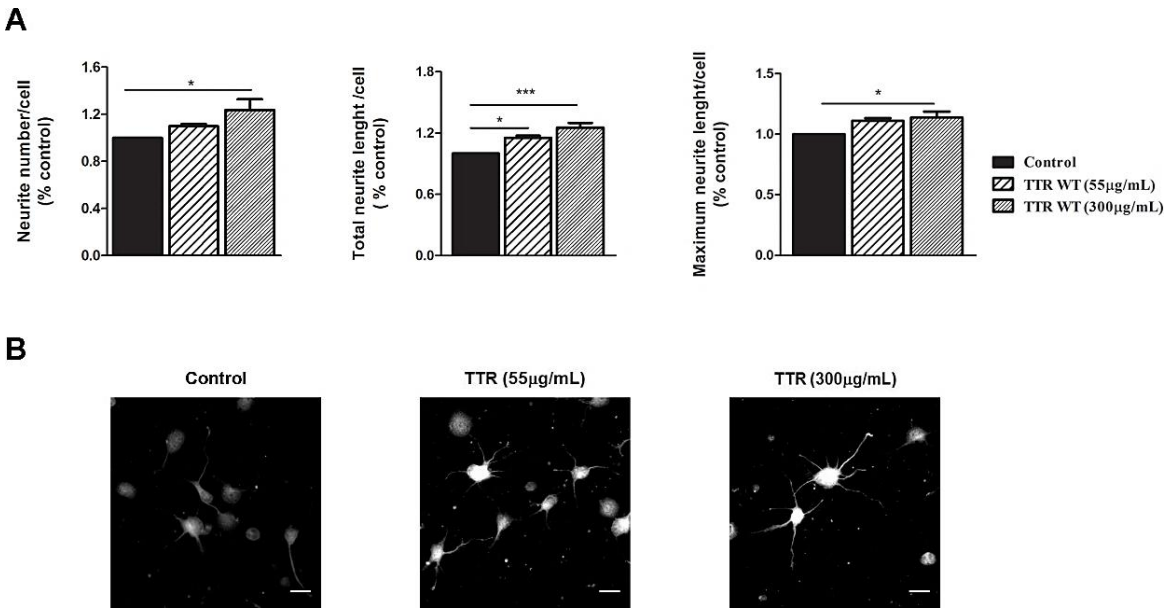


**Figure 6**. TTR induces hippocampal neurite outgrowth. (A) Neurite number, maximum length and total length per cell of $TTR^{-/-}$ hippocampal neurons in the presence of different concentrations of TTR for 24h. (B) Representative images of $TTR^{-/-}$ hippocampus neurons in the presence of TTR for 24 hours. Data represents the means +- SEM of four independent experiments. Error bars represent SEM. ***$p$ < 0.001, **p <0,01. *p<0.05 in one-way ANOVA, with Bonferroni's post test.

Several reports describe the effect of IGF-I on neurite outgrowth in many cellular types; IGF-I promotes neurite outgrowth in chick bulbospinal neurons (Salie and Steeves 2005), SH-SY5Y cells (Kim, Leventhal et al. 1997), myenteteric plexus (Mulholland, Romanchuk et al. 1992), organotypic cultures of DRG (Xiang, Ding et al. 2011) and is essential to initiate axon specification in hippocampal neurons (Sosa, Dupraz et al. 2006). We previously reported that TTR and IGF-I interact up-regulating the IGF-IR signaling pathway (chapter I); as TTR and IGF-I individually display neuritogenic roles among different cell types, it was reasonable to speculate that the interaction of TTR and IGF-I could enhance neurite outgrowth on hippocampal neurons. For that reason hippocampal neurons from $TTR^{-/-}$ embryos were incubated with IGF-I or TTR alone and TTR plus IGF-I, and analysis of different parameters of neurite outgrowth were performed. Neurites number, maximum length and total length per cell were not different from control situation when IGF-I alone or TTR plus IGF-I were added to culture medium (Figure 7). It is plausible that in the absence of TTR, neurons might not grow in the best conditions, affecting IGF-I action which is not recovered by exogenous addiction of TTR.

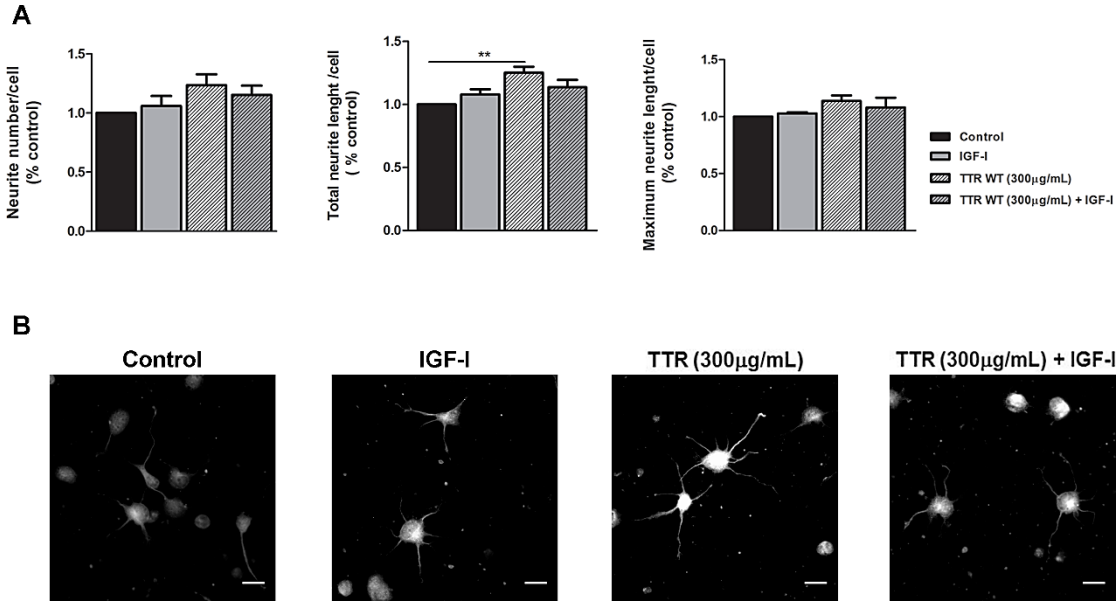


**Figure 7.** Synergistic effect of TTR plus IGF-I do not increase hippocampal neurite outgrowth of $TTR^{-/-}$ neurons. (A) Neurite number, maximum length and total length per cell of $TTR^{-/-}$ hippocampal neurons in the presence of IGF-I, TTR and TTR plus IGF-I for 24h. (B) Representative images of $TTR^{-/-}$ hippocampus neurons in the presence of IGF-I, TTR and TTR plus IGF-I. Data represents the means +- SEM of four independent experiments. Error bars represent SEM. **p <0,01 in one-way ANOVA, with Bonferroni's post test.

## Discussion

This is the first report that describes TTR as a positive regulator of IGF-IR levels. The synergistic effect of TTR and IGF-I on activation of IGF-IR signaling pathway was observed in NIH3T3 cells and mouse hippocampal samples (previous chapter). Although TTR together with IGF-I induced up-regulation of pIGF-IR and pAkt molecules, TTR by itself was not capable to activate these molecules. In this work we evaluated the role of TTR, not in the activation of the IGF-IR signaling pathway, but on IGF-IR levels. Analysis of IGF-I receptor levels in hippocampal samples showed that TTR null mice had decreased levels when compared with TTR wild type littermates at 3 months of age. The same analysis in 9 months animals did not reveal any differences. The presence of TTR in CSF is mainly derived from synthesis and secretion by choroid plexus. TTR levels in CSF decreases with age: 18 months animals had a 30% reduction in CSF TTR when compared with 5 months animals (Sousa, Marques et al. 2007). It is reasonable to speculate that the decrease of TTR could be a factor to abolish difference in IGF-I receptor levels between TTR wild type and TTR null mice at nine months.

As in CP tissue IGF-IR levels were similar between wild type and TTR null animals; we hypothesized that the difference observed in hippocampal IGF-IR levels between TTR wild type and TTR null mice could be due to the action of exogenous circulating CSF TTR, which might be modified from freshly secreted TTR; besides molecular differences between cell types. We excluded the hypothesis of a possible synthesis of TTR in hippocampus, since the residual levels of this that were found by some authors in hippocampus can be attributed to choroid plexus contamination during experimental procedures (Sousa, Cardoso et al. 2007).

In cell culture, with the $\alpha$-amanitin experiments, we showed for the first time that TTR regulates transcriptional activity of IGF-IR.

IGF-I decreases IGF-IR mRNA in muscle and neuroblastome cell lines. The decrease was attributable to transcriptional activity and not due to changes in mRNA stability (Hernandez-Sanchez, Werner et al. 1997). On the other hand, *in vivo*, increased postnatal levels of IGF-I have been associated to lower levels of IGF-IR mRNA (Werner, Woloschak et al. 1989). We have analysed IGF-I levels in plasma and did not find any differences between TTR wild type and TTR null mice (previous chapter), indicating that regulation of IGF-IR levels by TTR is unlikely related to IGF-I levels.

IGF-IR is a tyrosine kinase receptor that upon stimulation by its ligands induces autophosphorylation of the receptor leading to activation of Akt that prevents cells from apoptosis. Interestingly, Akt activation increases IGF-IR levels, revealing an autocrine

regulation (Knuefermann, Lu et al. 2003). Previous studies showed that hippocampus of TTR null mice had decreased levels of signaling molecules downstream IGF-IR when compared with TTR wild type littermates. Comparing those results with the decreased levels of IGF-IR reported now, we can speculate that regulation of IGF-IR by TTR *in vivo* could encompass activation of Akt. However, our *in vitro* studies with TTR, by itself, did not induce Akt activation, which might indicate that TTR regulation of IGF-IR levels do not occur through this pathway.

TTR null mice are healthy and fertile, although they present lower levels of plasma retinol and thyroid hormone (Episkopou, Maeda et al. 1993). Some studies associate retinol and thyroxine with IGF-IR levels. Retinol deficiency was associated with increased in IGF-IR expression in some tissues of Japanese quail (Fu, Noguchi et al. 2001), whereas administration of retinoic acid up-regulates IGF-I receptors in lungs (Ruttenstock, Doi et al. 2011). IGF-IR levels can be regulated by thyroid hormone in the pituitary gland (Matsuo, Yamashita et al. 1990) as well as in cardiac tissue (Araujo, Enzveiler et al. 2007). Analyzing the results obtained with *in vivo* samples, we could not exclude the effect of TTR ligands on IGF-IR levels. Although, in *in vitro* experiments, increased IGF-IR protein and mRNA levels were obtained under serum free conditions and in the presence of recombinant TTR, where no ligands were present. Taken together these results suggested that up-regulation of IGF-IR levels is dependent of TTR and not due to the action, so far described, of the ligands transported by this protein.

Several transcription factors are described as regulators of IGF-IR transcription. Sp1 and E2F1 are examples of transcription factors that are potent transactivators of the *IGF-IR* gene (Beitner-Johnson, Werner et al. 1995; Schayek, Bentov et al. 2010), whereas breast cancer gene-1 *(BRCA1*), p53 and Wilms' tumor protein-1 (WT1) are negative regulators of *IGF-IR* gene (Sarfstein, Maor et al. 2006). IGF-IR can be a transcriptipon factor also. Despite the fact that it is a transmembrane receptor, IGF-IR can be found in nucleus. It translocates to this cellular compartment following clathrin-mediated endocytosis, process that is regulated by IGF levels (Chen and Roy 1996; Aleksic, Chitnis et al. 2010). It can regulate the *IGF-IR* gene, binding to the *IGF-IR* promoter increasing its expression (Sarfstein, Pasmanik-Chor et al. 2012). The binding of TTR to IGF-IR (as demonstrated by pull down assays in the previous chapter) could influence its migration to nucleus, increasing its expression. Further studies need to be performed to better elucidate the mechanism by which TTR increases IGF-IR levels, namely if it involves internalization of the protein.

TTR effect on neurite outgrowth had been shown in PC12 cells and DRGs (Fleming, Saraiva et al. 2007) and demonstrated to require TTR internalization via megalin. We reported here that TTR promotes neuritogenic activity in $TTR^{-/-}$ hippocampal

neurons. The presence of megalin in these neurons has to be investigated, since in DRGs the neuritogenic effect of TTR is megalin-mediated. Different mechanisms for TTR internalization might exist in different cells, a subject under investigation in the laboratory.

IGF-I is an inducer of neurite outgrowth among cellular types; however, it did not influence neurite outgrowth on $TTR^{-/-}$ hippocampal neurons. It has to be investigated whether $TTR^{-/-}$ embryonic neurons do not respond to IGF-I stimulus. Probably in $TTR^{-/-}$ neurons other molecules that influence IGF-I neuritogenicity might be altered and addition of exogenous TTR is not sufficient to induce neurite outgrowth in the presence of IGF-I. Similar studies in $TTR^{+/+}$ hippocampal neurons should be performed to solve this question.

Despite the synergist effect of TTR and IGF-I on activation of IGF-IR signaling via Akt pathway, TTR and IGF-I together did not have a synergistic effect on neurite outgrowth in $TTR^{-/-}$ hippocampal neurons. This fact could be explained by the fact that IGF-I-mediated neurite outgrowth requires mitogen-activated protein kinase activation (Kim, Leventhal et al. 1997), and the synergistic effect of TTR and IGF-I was observed mainly on the Akt pathway.

Interestingly, in the presence of IGF-I, TTR increase on neurite outgrowth was abolished. This finding suggests that TTR neuritogenic role on hippocampal neurons is IGF-I independent; a statement that needs confirmation with hippocampal neurons from wild type animals, as above pointed out.

Apoptotic neuronal cell death is characteristic from neurodegenerative disorders and IGF-IR role is becoming more relevant to protect from apoptosis (Kooijman 2006; Annunziata, Granata et al. 2011). Increased IGF-IR levels induced by TTR, described here for the first time, is an important finding that might be very useful to control IGF-IR levels in many different pathological situations.

## Acknowledgements

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# CHAPTER III

# Transthyretin regulates 14-3-3ζ protein levels

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## Abstract

Transthyretin (TTR) is the carrier protein of thyroxine ($T_4$) and retinol in plasma and cerebrospinal fluid (CSF) and has been described also as a neuroprotective molecule. 14-3-3 proteins are very important in many cellular processes, being their absence related with deficits in memory and learning. The analysis of the relationship between these two proteins is the main objective of this work. We found that hippocampus of young TTR null mice presented lower levels of 14-3-3ζ protein, but no changes in gene expression when compared to TTR wild type littermates were noted. Cellular studies ascribed this finding to increased degradation of 14-3-3ζ in lysosomes in the absence of TTR, increasing autophagy.


**Keywords**: 14-3-3, transthyretin, lysosomes, degradation, brain, neurons.

## Introduction

Transthyretin (TTR) is a 55kDa homotetrameric protein mainly synthetized in liver and choroid plexus (Aleshire, Bradley et al. 1983; Soprano, Herbert et al. 1985), being up-regulated by sex hormones (Goncalves, Alves et al. 2008; Quintela, Goncalves et al. 2011). It is the carrier protein of thyroxine ($T_4$) (Woeber and Ingbar 1968) and retinol through the binding protein (RBP) in plasma and cerebrospinal fluid (CSF) (Kanai, Raz et al. 1968). Besides its effects on the transport of retinol and $T_4$, TTR has been described as associated with high density lipoproteins (HDL) (Nakamura, Tanaka et al. 1996). It has also been shown to have neuroprotective properties described in several contexts. Studies in TTR null mice revealed that absence of TTR reduces signs of depressive- like behavior (Sousa, Grandela et al. 2004), increases the levels of neuropeptide Y (NPY) (Nunes, Saraiva et al. 2006) and delays nerve regeneration in nerve injury conditions (Fleming, Saraiva et al. 2007). TTR has also a protective role in Alzheimer's disease (AD) (Choi, Leight et al. 2007), and is able to modulate brain Aβ levels (Oliveira, Ribeiro et al. 2011). In cerebral ischemia, CSF TTR influences the survival of endangered neurons (Santos, Lambertsen et al. 2010).

Aiming to dissect differential expression/post-translational modifications between wild type and TTR null mice, two-Dimensional (2D) electrophoresis studies were initially performed; among other differences, the hippocampus of TTR null mice displayed lower levels of 14-3-3ζ protein when compared to TTR wild type littermate mice, which prompted further studies to confirm this finding.

14-3-3 proteins are a family of highly conserved acidic proteins representing 1% of the total amount of brain protein (Boston, Jackson et al. 1982). Seven isoforms of 14-3-3 proteins are identified in mammalian cells (β,η, γ, τ, ζ, ε, σ) (Martin, Patel et al. 1993), each of them encoded by different genes; just σ and τ/θ isoforms are non-neuronally expressed (Hermeking 2003).

14-3-3 proteins can interact with more than 400 molecules through phospho-serine/phospho-threonine residues (Bustos 2012), a key regulatory mechanism in cell biology. These proteins had emerged as essential factors in many biological processes such as signal transduction, cell-cycle regulation, cell survival, cellular trafficking, cytoskeletal organization, protein synthesis, redox-regulation, protein folding with consequences in neurodegenerative and neurodevelopmental disorders (Berg, Holzmann et al. 2003; Kjarland, Keen et al. 2006; Steinacker, Aitken et al. 2011; Foote and Zhou 2012).

Several studies revealed that the absence of 14-3-3ζ induces a deficit in memory and learning (Skoulakis and Davis 1996; Philip, Acevedo et al. 2001; Cheah, Ramshaw et al. 2012); on the other hand, young TTR null mice present spatial memory impairment (Sousa, Marques et al. 2007). Thus, it is important to analyze the relationship between these two proteins in the central nervous system (CNS), particularly in the hippocampus. In the present report we describe the regulation of 14-3-3ζ protein levels by TTR, studied both in mice and in cell culture experiments.

## Material and Methods

### Animals

Mice were handled according to European Union and National rules. Three, six and twelve-month-old TTR wild type ($^{+/+}$) and TTR knockout ($^{-/-}$) (Episkopou, Maeda et al. 1993), in a 129/svJ background were obtained from the littermate offspring of heterozygous breeding pairs. The animals were maintained under a 12h light/dark cycle and fed with regular rodent's chow and tap water *ad libitum*. Genotypes were determined from tail extracted genomic DNA, using primers for the detection of exon 2 of TTR (which is disrupted in $TTR^{-/-}$ by insertion of neomycin resistance gene) as previously described (Episkopou, Maeda et al. 1993). Mice were sacrificed with a lethal injection of a premixed solution containing ketamine (75mg/Kg) plus medetomidine (1mg/Kg). Half brain was fixed in 4% neutral buffered formalin, embedded in paraffin and the remaining half portion was dissected and frozen at -80ºC.

All efforts were made to minimize pain and distress; all animal experiments were carried out in accordance with the European Communities Council Directive.

### Cell culture

The AF5 rat mesencephalic cell line (kindly given by Dr. William Freed, Baltimore) was grown in DMEM (Dulbecco's modified Eagle's medium) high glucose/F12 with 10% fetal bovine serum (FBS) at 37ºC in a humidified incubator of 5% $CO_2$ /95% air. At 90% confluence, FBS was withdrawn and medium supplemented with 10% of $TTR^{+/+}$ or $TTR^{-/-}$ mouse serum during 18 hours; in some experiments MG132 (Calbiochem), proteasome inhibitor, and chloroquine (Sigma), lysosome inhibitor, were added for 30 minutes before serum withdrawn.

Primary cultures of mouse hippocampal neurons were prepared from the hippocampus of E18-E19 $TTR^{-/-}$ mice embryos. The hippocampi were treated with trypsin (0.5mg/mL, 15 minutes at 37ºC) in $Ca^{2+}$ and $Mg^{2+}$ HBSS (Hank's Balanced Salt Solution) free. The hippocampi were then washed in HBSS supplemented with 10% FBS. After centrifugation at 140_$g_{av}$, for 1 min, cells were washed in HBSS and mechanically dissociated. Hippocampal cultures were maintained in serum-free Neurobasal medium supplemented with B27, glutamate (25 mM), glutamine (0.5 mM) and gentamicin (0.12 mg/ml). Cells were kept at 37ºC in a humidified incubator of 5% $CO_2$/95% air, for 7-8 days, the time required for maturation of hippocampal neurons (Brewer, Torricelli et al. 1993). All culture media used were from GIBCO.

**Two-Dimensional electrophoresis studies**

Cold acetone precipitates from hippocampal extracts of $TTR^{+/+}$ and $TTR^{-/-}$ littermate mice were prepared and treated with 2D Clean Up Kit (GE Healthcare). Protein pellets were dissolved in 7 M urea, 2 M thioure and 0.5% Pharmalyte pH 3 to 10 (GE Healthcare) containing buffer. Protein concentration was determined using the Bradford protein assay (Bio Rad). Approximately 100μg of protein were applied to Immobiline DryStrips (18 cm, pH 3-10 NL (nonlinear), GE Healthcare), isoelectric focusing performed on an IPGphor system (GE Healthcare). The proteins in the IPG strips in were then subsequently separated on the second dimension in a SDS-PAGE gel under reducing conditions.

The fixed gels were then silver stained. Digital images of the 2D PAGE maps were acquired using a gel scanner. PDQuest Image Analysis software was used to identify differentially regulated proteins; the procedure was performed twice in duplicated samples. Differential spots were excised and MALDI mass spectroscopic analysis was performed on a PerSeptive Voyager mass spectrometer.

**Immunohistochemistry**

Brain samples were fixed in 4% neutral buffered formalin and embedded in paraffin. For immunohistochemistry, brains were serially sectioned into 4μm thick sections in a microtome (Microm HM335E). Sections were deparafinated in xylol and dehydrated in a descendent alcohol series. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide in methanol for 20 minutes; slices were then blocked in blocking solution (4% FBS and 1% BSA) 1h hour at 37ºC in a humidified chamber. Primary antibody used was rabbit polyclonal anti-14-3-3ζ (1:200, Santa Cruz Biotechnology). Antibody binding was visualized by a biotin-extravidin-peroxidase kit (Sigma-Aldrich) using as substrate 3-amino-9-ethyl carbaxole (AEC), (Sigma). Slides were counterstained with hematoxylin (Merck).

**Western blot analysis**

Frozen tissue and cultured cells were homogeneized in lysis buffer containing 20 mM MOPS, 2mM EGTA, 5mM EDTA, 30mM sodium fluoride, 60mM β-glycerophosphate, 20mM sodium pyrophosphate, 1mM sodium orthovanadate , 1mM phenylmethylsulphonyl fluoride, 1% Triton X-100 and 1x protease inhibitors mixture (GE Healthcare). Total protein concentration was determined using the Bradford method. 50 μg of protein were applied and separated by SDS-PAGE and transferred to a nitrocellulose Hybond-C membrane (GE Healthcare), using a wet system. The membrane was blocked one hour at room temperature in blocking buffer, 5% nonfat dry milk in phosphate-buffered saline

Tween-20 (PBST), and then incubated overnight a 4ºC with primary antibodies diluted in blocking buffer, namely rabbit polyclonal 14-3-3ζ (1:1,000; Santa Cruz Biotechnology), rabbit polyclonal 14-3-3β (1:500, Santa Cruz Biotechnology ), rabbit polyclonal LC3 (1:1000; Cell Signaling), mouse monoclonal anti-polyubiquitinated conjugates (1:1000, Enzo Life Sciences), mouse α-tubulin (1:10,000; Sigma), mouse monoclonal anti-β-actin (1:5,000; Sigma) and rabbit polyclonal antibodies for 14-3-3γ (1:2,000), and 14-3-3η (1:1,000) (kindly provided by Dr. Alastair Aitken, Edinburgh). Membranes were then incubated with horseradish peroxidase (HRP)-labeled secondary antibodies, namely anti-rabbit IgG-HRP (1:10,000; Binding Site) or anti-mouse IgG-HRP (1:5000; Binding Site) during 1 hour at room temperature. The blots were developed using the ECL PlusTM Western blotting reagents (GE Healthcare) and exposed to Hyperfilm ECL (GE Healthcare). Quantitative analyses were performed using the ImageJ software. Results are shown as the ratio of 14-3-3ζ, and β-actin or α-tubulin signals.

**Reverse Transcriptase – Polymerase Chain Reaction**

Total RNA was isolated using Trizol reagent (Invitrogen). First-strand cDNA was synthesized using the Superscript II kit (Invitrogen). PCR was performed with the following oligonucleotides to mouse 14-3-3ζ: 5'-TGAGCAGGGAGCTGAGCTGTC3-' and 5'-GTTGCGAAGCATTGGGGATCAAGA-3'; for mouse β-actin: 5'-CTGTTTGATGTCACGCACGAT-3' and 5'-GTGGGCCGCTCTAGGCACCAA-3'. For rat amplification of 14-3-3ζ in AF5 cells, primers used were: 5'-CGCCACCCACTCCGGACACAGAATA-3', 5'-TCTGGCTGCGAAGCATTGGGGA-3' and for rat β-actin: 5'-CCACCATCACACCCTGGTGCC-3'and 5'-GTCGAGTCCGCGTCCACCC-3'.

Ethidium bromide-stained gels were scanned using GENE FLASH syngene bio imaging equipment. The results were analyzed using the ImageJ software.

**Statistical analysis**

Quantitative data are presented as Mean ± SEM. Statistical analysis was carried out using Graphpad Prism 5 software. Differences among groups were analyzed by one-way ANOVA (followed by Bonferroni's Multiple Comparison Test), comparisons between two groups were made by Student's *t* test. *P* values of lower than 0.05 were considered significant.

## Results

### Hippocampus of TTR$^{-/-}$ animals have decreased 14-3-3ζ protein levels

To unravel differences between TTR$^{-/-}$ and TTR$^{+/+}$ mice hippocampus homogenates from 5 month old mice were analyzed by 2D electrophoresis. One of the main spots that presented striking differences was identified as the 14-3-3ζ isoform, which clearly had lower levels in the hippocampus of TTR$^{-/-}$ mice when compared with wild type littermates (Figure 1).

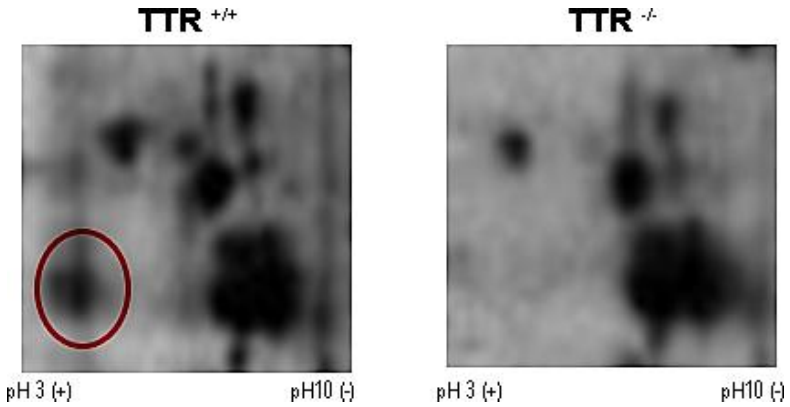


**Figure 1.** Silver stained two dimensional gel electrophoresis of hippocampus homogenates in the 3-10 pH range. Left panel: representative 2D gel from TTR$^{+/+}$ mice; Right panel: representative 2D gel from TTR$^{-/-}$ mice. The spot highlightened by a red circle was identified as 14-3-3ζ.

To further confirm the results obtained in 2D, western blots for 14-3-3ζ of hippocampus of TTR$^{+/+}$, TTR heterozygous ($^{+/-}$) and TTR$^{-/-}$ mice of the same age were performed. The 14-3-3ζ levels in TTR$^{-/-}$ mice were significantly decreased when compared with TTR$^{+/+}$ animals; in turn, levels in heterozygote mice were lower when compared with TTR$^{+/+}$ animals and significantly increased as compared to TTR$^{-/-}$ mice, presenting intermediate levels between TTR$^{+/+}$ and TTR$^{-/-}$ (Figure 2).

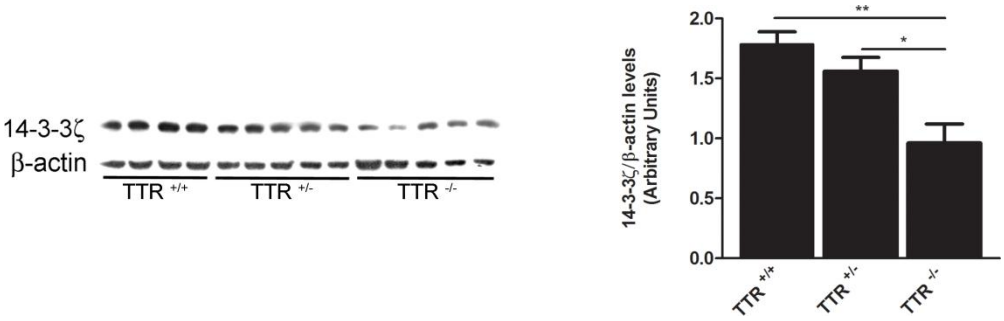


**Figure 2**. Hippocampus of TTR null mice had decreased levels of 14-3-3ζ when compared with heterozygous and TTR wild type animals. Western blot analysis and quantitative charts of 14-3-3ζ in hippocampus samples at 5 months from TTR $^{+/+}$ (n=4), TTR $^{+/-}$ (n=5) and TTR $^{-/-}$ (n=5). Data represents the means +- SEM. Error bars represent SEM.**p<0,01, *p < 0,05; in one-way ANOVA, with Bonferroni's post test.

Analysis of other brain areas such as cerebellum, hypothalamus, choroid plexus, did not reveal any effect of the absence of TTR on 14-3-3ζ levels, showing that this effect is specific for the hippocampus (Figure 3).

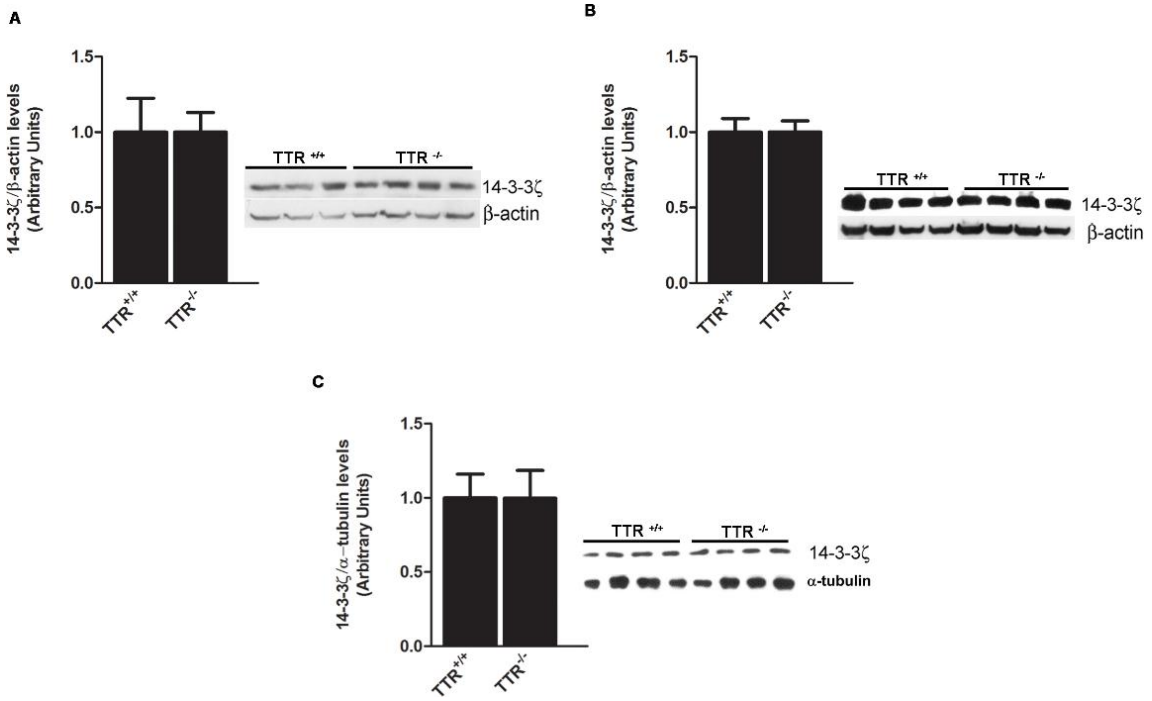


**Figure 3**. No difference on 14-3-3ζ levels between TTR wild type and TTR null mice in several brain areas. Western blot and chart quantification of 14-3-3ζ in cerebellum (A), hypothalamus (B) and choroid plexus of TTR$^{-/-}$ (n=4) when compared to TTR$^{+/+}$ (n=4) littermate mice. Data represents the means +- SEM. Error bars represent SEM.

The effect of TTR in 14-3-3ζ protein levels is age dependent in a transcriptional independent manner. It has been described that TTR CSF levels are decreased with age (Chen, Athauda et al. 2005; Sousa, Marques et al. 2007). To evaluate if the effect of TTR in 14-3-3ζ levels is age dependent, immunohistochemistry of brain and western blot analysis of hippocampus was performed in $TTR^{+/+}$ and $TTR^{-/-}$ mice at 3, 6 and above 12 months of age.

Western blot analysis of hippocampus showed a 50% reduction in 14-3-3ζ protein levels in $TTR^{-/-}$ mice when compared with $TTR^{+/+}$ animals, both at 3 and 6 months of age; no difference was observed between the two genotypes in older mice (Figure 4).

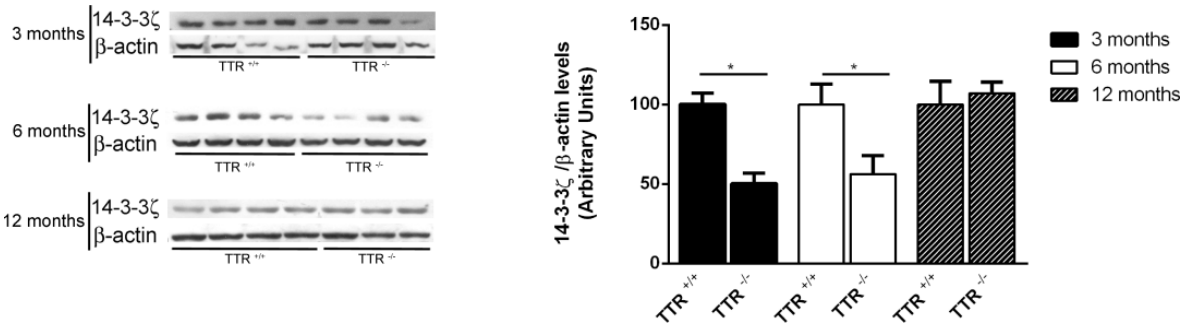


**Figure 4.** Absence of TTR correlates with decreased levels of 14-3-3ζ in hippocampus in an age dependent manner. Western blot analysis ( left panel) and quantification (right panel) of 14-3-3ζ levels in hippocampus of TTR wild type and TTR null mice at 3, 6 and 12 months of age. Data represents the means +- SEM. Error bars represent SEM. *p < 0,05 in Student's t test.

This result was corroborated by immunohistochemistry; in fact at 3 months $TTR^{-/-}$ mice presented clear decreased levels of 14-3-3ζ in the hippocampus when compared with $TTR^{+/+}$ mice. This difference is still clear until 6 months but is abolished in animals older than 12 months (Figure 5).

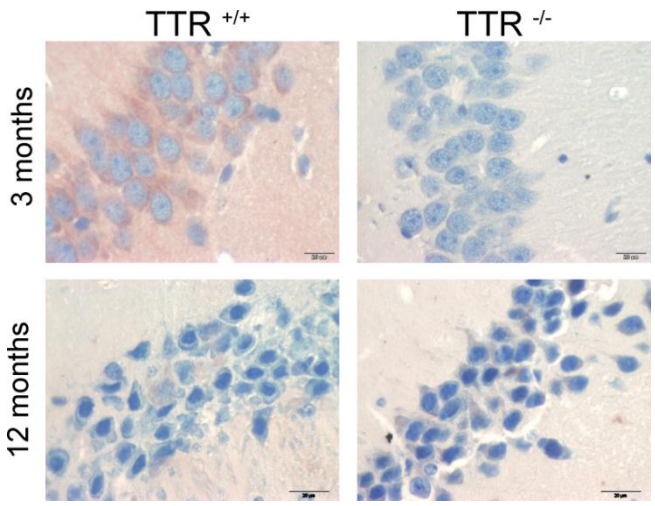


**Figure 5**. Immunohistochemistry to 14-3-3ζ at 3 and 12 months in the hippocampus (CA1 region) of $TTR^{-/-}$ mice and $TTR^{+/+}$ mice. 14-3-3ζ was visualized by a biotin-extravidin-peroxidase kit using AEC as substrate. Slides were counterstained with hematoxylin. Scale bar = 20μm.

Despite the fact that 14-3-3 isoforms are highly conserved, each isoform has different and specific functions. To verify the effect of TTR on γ, η and β isoforms, western blot of hippocampus of 3 months animals were performed. No differences were observed in these 14-3-3 isoforms (Figure 6).

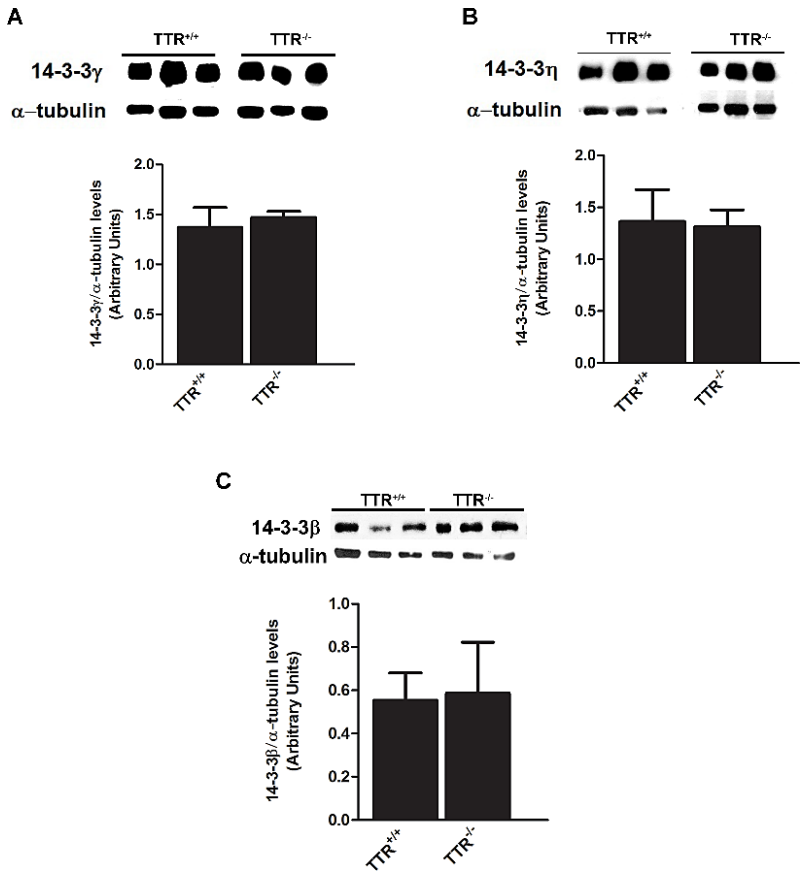


**Figure 6**. TTR does not influence hippocampus levels of other 14-3-3 γ, η and β isoforms. Western blot analysis and quantification of 14-3-3γ (A), η (B) and β (C) levels in hippocampus of $TTR^{-/-}$ (n=3) mice when compared to $TTR^{+/+}$ littermates (n=3) at 3 months of age. Data represents the means +- SEM. Error bars represent SEM.

In order to assess if the influence of TTR on 14-3-3ζ levels occurs at the transcriptional level, semi-quantitative RT-PCR analysis for 14-3-3ζ was performed for hippocampus of $TTR^{+/+}$ and $TTR^{-/-}$ animals at 3, 6 and 12 months respectively. No differences in 14-3-3ζ transcription were observed, between the two genotypes at the analyzed ages (Figure 7).

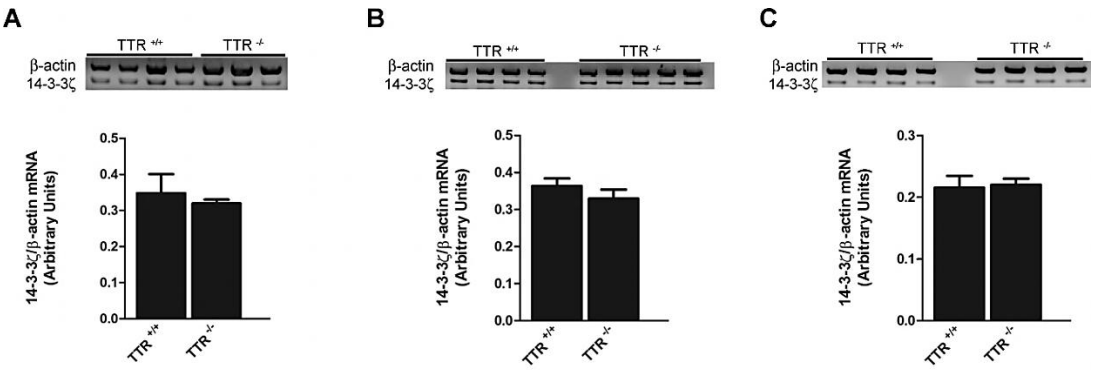


**Figure 7**.TTR does not influence 14-3-3ζ at transcriptional level. Semi-quantitative RT-PCR analysis of 14-3-3ζ in hippocampus of TTR wild mice and TTR null mice at 3 (A), 6 (B) and 12 (C) months of age. Number of animals per group are shown in figure. Data represents the means +- SEM. Error bars represent SEM.

14-3-3ζ protein levels are down-regulated in hippocampal neurons in the absence of serum TTR. To evaluate the effect of TTR on 14-3-3ζ levels in cultured neurons, hippocampal neurons from $TTR^{-/-}$ embryonic mice were isolated and exposed to sera collected either from $TTR^{+/+}$ and $TTR^{-/-}$ mice, during 75 minutes at 37ºC. Western blot analysis revealed that 14-3-3ζ levels decrease in cells treated with $TTR^{-/-}$ serum when compared with $TTR^{+/+}$ serum (Figure 8A). No significant differences were observed in 14-3-3ζ transcription in the presence/absence of TTR, as the mRNA levels of 14-3-3ζ did not change significantly (Figure 8B).

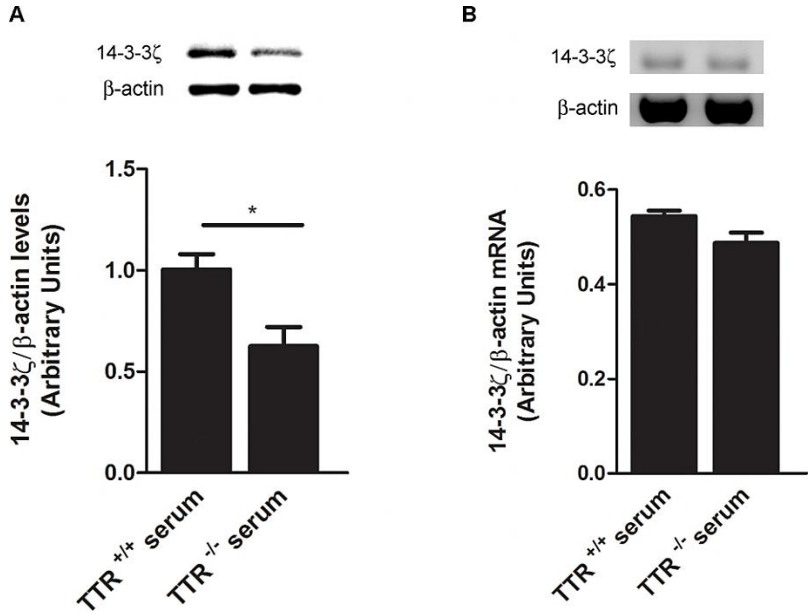


**Figure 8**. 14-3-3ζ levels were significantly lower in hippocampal neurons exposed to $TTR^{-/-}$ serum when compared with neurons exposed to $TTR^{+/+}$ serum. (A) Representative image of western blot in neurons

cultured in $TTR^{-/-}$ serum when compared with neurons in the presence of $TTR^{+/+}$ serum and respective quantification (lower blots). (B) Semi-quantitative RT-PCR analysis of 14-3-3ζ expression under the same conditions. Data represents the means +- SEM of three independent experiments. Error bars represent SEM. *$p$ < 0.05 in a Student's t test.

### TTR influences 14-3-3ζ degradation

To better understand the mechanism by which absence of TTR decreases 14-3-3ζ levels, AF5 cells were used as a cell model. These cells do not synthetize TTR and have been used addressing the behavior of 14-3-3ζ to excitotoxicity in neuroprotection studies (Chen, Lee et al. 2007).

AF5 cells were cultured during 18 hours with $TTR^{+/+}$ or $TTR^{-/-}$ serum to replace FBS. Western blot analysis displayed highly decreased levels of 14-3-3ζ when cells grew in $TTR^{-/-}$ serum as compared to cells exposed to $TTR^{+/+}$ serum (Figure 9A). As observed in the above described studies with cultured hippocampal neurons, no differences were found in 14-3-3ζ at the transcriptional level in cells grown under the two different conditions (Figure 9B). This indicates that the effect of TTR on 14-3-3ζ protein levels occurs post-transcriptionally.

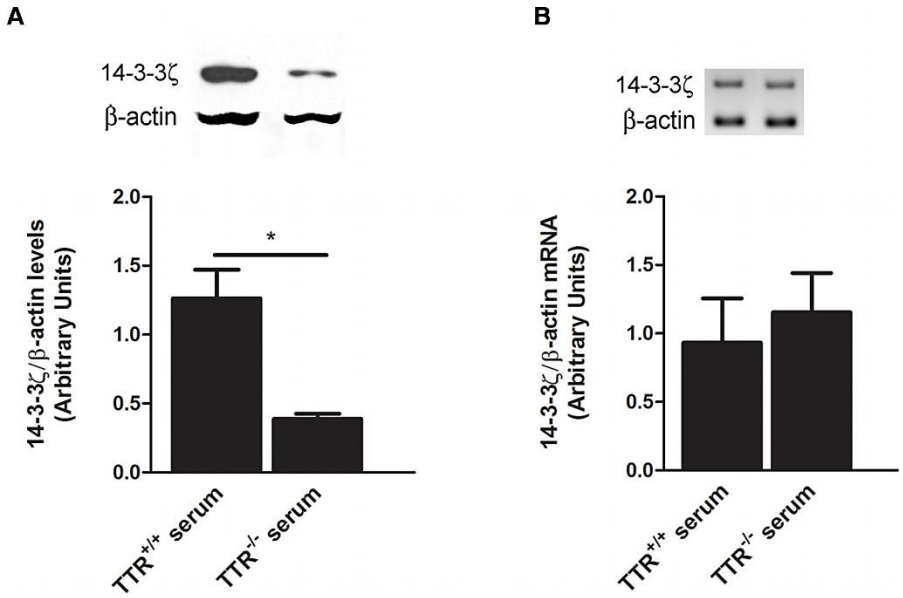


**Figure 9.** 14-3-3ζ levels were significantly decreased in AF5 cells exposed to $TTR^{-/-}$ serum when compared with cells exposed to $TTR^{+/+}$ serum. No differences were observed at transcritional level. (A) Representative image of 14-3-3ζ western blot analysis and respective quantification of AF5 cells cultured in the presence of $TTR^{-/-}$ and $TTR^{+/+}$ serum. Data represents the means +- SEM of three independent experiments (B) Semi-quantitative RT-PCR analysis of 14-3-3ζ expression under the same conditions. Data represents the means +- SEM of four independent experiments. Error bars represent SEM. *$p$ < 0.05 in a Student's t test.

We reasoned that the decrease of 14-3-3ζ protein levels in the absence of TTR might be due to active degradation of the protein. The majority of intracellular proteins are degraded by proteasome or lysosome pathways (Qiao and Zhang 2009). To assess if TTR is involved in the degradation process of 14-3-3ζ by the proteasome, AF5 cells were exposed to 10 μM of the proteasome inhibitor MG132. The inhibition of the proteasome did not invert the effect triggered by $TTR^{-/-}$ serum, indicating that the proteasome is not involved in the 14-3-3ζ differential degradation (Figure 10A). To confirm the effectiveness of MG132, a western blot to polyubiquitinated proteins were performed. When MG132, proteasome is inhibited and an increase in polyubiquitinated proteins is observed (Figure 10B).

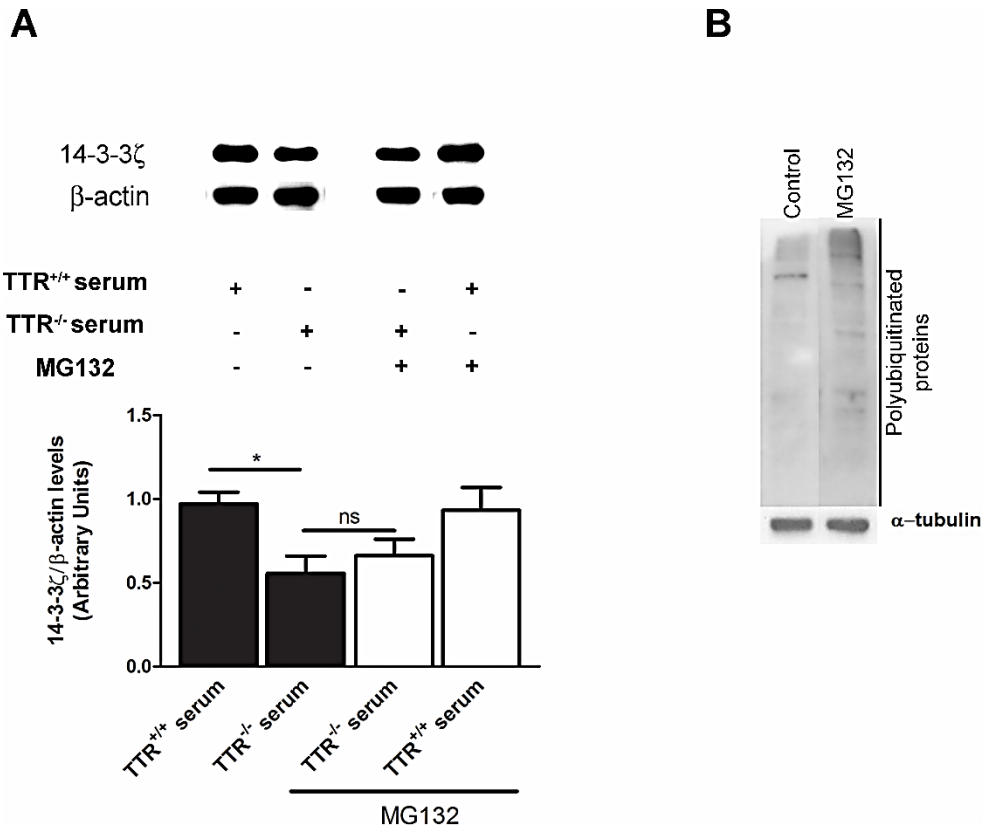


**Figure 10**. Absence of TTR does not induce degradation of 14-3-3ζ by proteasome. AF5 cells treated with $TTR^{+/+}$ or $TTR^{-/-}$ serum during 18 hours. (A) Western blot analysis of 14-3-3ζ in the presence or absence of MG132. Data represents the means +- SEM of six independent experiments. (B) Western blot analysis of polyubiquitinated in the presence or absence of MG132. Error bars represent SEM. *$p$ < 0.05 in one-way ANOVA, with Bonferroni's post test.

To evaluate if 14-3-3ζ was differentially degraded by the lysosome, AF5 cells were exposed to 100μM of lysosome inhibitor chloroquine. Use of chloroquine in cells treated

with TTR$^{-/-}$ serum did not induce any difference in 14-3-3ζ levels when compared with the same situation without the inhibitor, as shown in figure 11. However inhibition of lysosome abolishes the differential effect of TTR on 14-3-3ζ levels. These results suggest that TTR controls 14-3-3ζ degradation by the lysosome.

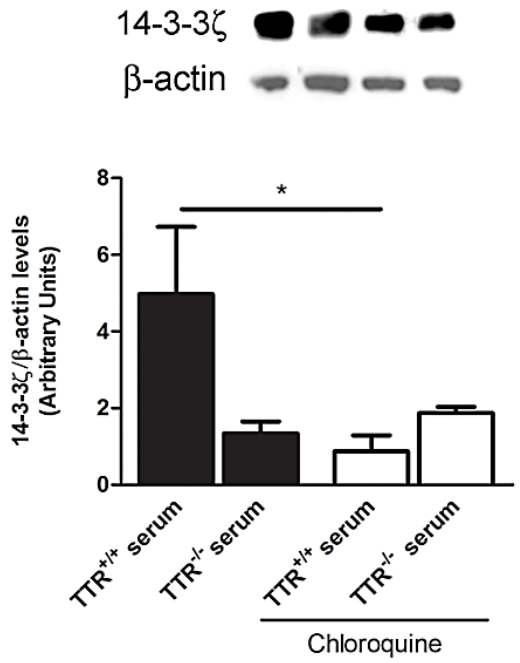


**Figure 11**. TTR influences lysosomal degradation of 14-3-3ζ. Western blot analysis of 14-3-3ζ in the presence or absence of chloroquine. Data represents the means +- SEM of three independent experiments. Error bars represent SEM. *$p$ < 0.05 in one-way ANOVA, with Bonferroni's post test.

Autophagy, also referred as programmed cell death type II, is characterized by the presence of autophagosomes that fuse with lysosomes for degradation of sequestered material. Autophagy marker Light Chain 3 (LC3), involved in autophagosome formation, undergoes post-translational modification in autophagy. LC3 exists in two forms two cellular forms, LC3-I (18 kDa) and LC3-II (16 kDa). During autophagy LC3-I is converted in LC3-II, being the amount of LC3-II a marker of this process. 14-3-3ζ is a negative regulator of autophagic activity (Pozuelo-Rubio 2011; Pozuelo-Rubio 2011). When AF5 cells were exposed to TTR$^{-/-}$ serum, the levels of LC3-II were significantly increased when compared with cells grown in the presence of serum TTR (Figure 12). The lower 14-3-3ζ levels that are associated with the absence of TTR induced increased autophagy.

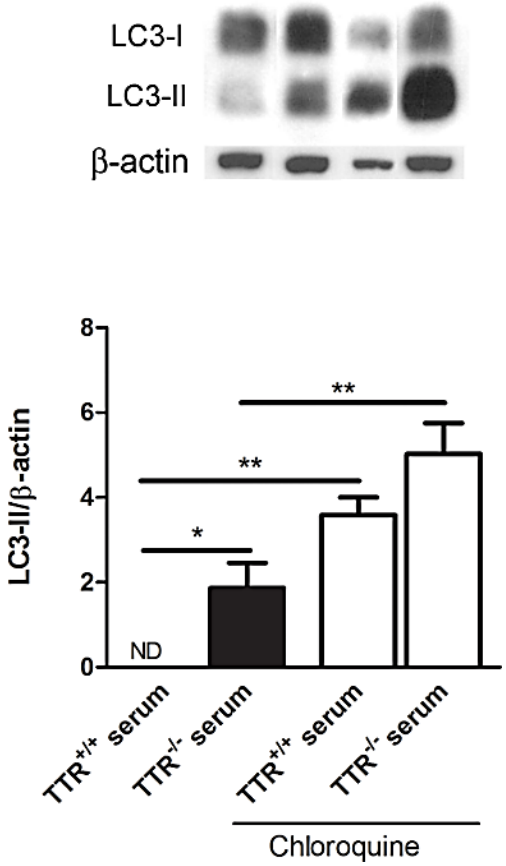


**Figure 12**. TTR influences autophagy. LC3-II western blot analysis (upper panel) and quantification of the LC3-II/β-actin ratio (lower panel). Data represents the means +- SEM of three independent experiments. Error bars represent SEM. *$p < 0.05$; **$p < 0.01$ in one-way ANOVA, with Bonferroni's post test.

## Discussion

The present study demonstrates for the first time that TTR regulates 14-3-3ζ protein levels. Immunohistochemistry and western blot studies showed reduced levels of 14-3-3ζ in the hippocampus of TTR null mice as compared to TTR wild type animals. This finding occurs in young/adult animals (3 and 6 months, respectively) and is not observed in older animals (>12 months). 14-3-3 proteins have been described as decreased with aging. 14-3-3 gamma and epsilon had decreased levels in adult brain when compared with neonatal brain (Fountoulakis, Hardmaier et al. 2000). 14-3-3θ, 14-3-3γ, and 14-3-3ζ expression levels decreased with increased of aging (VanGuilder, Yan et al. 2010). It is well known that TTR levels in CSF decrease with age; thus 18-month-old mice present a 30% decrease of TTR levels in CSF when compared with 5 months animals (Sousa, Marques et al. 2007). So the decrease of TTR in the CSF of old animals could be responsible for the abolishment of differences on 14-3-3ζ levels between TTR wild type and TTR null mice. Several important neurobiological processes are associated with advancing age such as increased oxidative stress, decrease metabolism, protein synthesis and trafficking. Cognitive decline is strongly associated with decrease expression in synaptic activity-dependent proteins, which 14-3-3θ is included (VanGuilder, Farley et al. 2011).

TTR in CSF is mainly derived from synthesis and secretion by the choroid plexus. Synthesis of TTR by the hippocampus has not been clearly demonstrated. While some authors claim that hippocampal and cortical neurons can synthetize TTR, other authors suggest that this synthesis might be due to choroid plexus contamination in the experimental procedures (Sousa, Cardoso et al. 2007). To overcome this issue and avoid misleading conclusions, hippocampal neurons from TTR null mice and AF5 cell lines which do not synthesize TTR were used. This way, a possible contribution of endogenous TTR was ruled out. Instead, exogenous TTR contributed to regulate disposal of 14-3-3ζ at the lysosomal compartment. Internalization of TTR by neurons is a well documented process, not only in primary cultures (Nunes, Saraiva et al. 2006) but also in cell lines of neuronal origin (Divino and Schussler 1990), as well as uptake by a variety of other cell types (Fleming, Mar et al. 2009).

In neurons, 14-3-3 proteins exist in cytoplasm, in mitochondria, microsomes and also in the nucleus (Schindler, Heverin et al. 2006; Heverin, Brennan et al. 2012). Decreased 14-3-3γ and ζ isoforms were detected in rats, in microsomes of hippocampus, after damaging seizures. As the levels of these proteins do not increase in other cellular fractions, proteolysis has been the mechanism proposed to explain this reduction. In this

type of evoked seizures, it is known that 14-3-3 is proteolyzed by caspase 3 during apoptosis (Schindler, Shinoda et al. 2004; Schindler, Heverin et al. 2006). When cortical neurons cultures are exposed to necrotic agents, 14-3-3ζ is released to the medium culture, being considered a surrogate marker of acute brain damage (Siman, McIntosh et al. 2004). Although TTR null mice had decreased levels of 14-3-3ζ, absence of TTR does not induce cellular death in hippocampal slices cultures, when compared with wild type animals (Nunes, Montero et al. 2009). The decreased levels of 14-3-3ζ found in the absence or lower TTR levels (as in the case of heterozygote animals) are highly unlikely related to cellular death.

14-3-3 proteins interact with more than 400 molecules and their action is based mostly on altering the conformation of the targets, physical occlusion of sequence-specific or structural features and scaffolding (Bridges and Moorhead 2005). The interaction of 14-3-3 proteins with their binding partners occurs, mostly, through phospho-serine/phospho-threonine residues. There are three binding motifs namely, RSXpSXP, RXϕXpSXP and -pS/pT $X_{1-2}$-COOH , where pS represents phospho-serine, ϕ is an aromatic or aliphatic amino acid and X is any amino acid (Yaffe, Rittinger et al. 1997). The binding motif at the C-terminal is less common. (Ganguly, Weller et al. 2005). It is important to note that not all interactions are phosphorylation dependent. Analysis of TTR sequence did not reveal any of the binding motifs above described, which is suggestive of the effect of TTR on 14-3-3ζ levels might not necessarily encompass a direct structural interaction. Alternatively, TTR might bind another molecule, either at the membrane, in the endocytic or lysosomal compartment (where TTR is degraded) resulting in activation of an intermediate molecule that interacts/stabilizes 14-3-3ζ. The physical interaction of TTR with the most abundant lysosomal protein, LAMP-1, was previously described (Chang, Hua et al. 2004). Although that study was performed with LAMP-1 in circulation, it is reasonable to speculate that this interaction could also occur within cells, and regulates lysosomal activity, in particular of 14-3-3ζ, preventing degradation. In fact, inhibition of lysosome abolished the effect of TTR in this organelle. These are possibilities that need to be discerned and the subject of future studies.

The specificity of TTR to regulate 14-3-3ζ but not other isoforms as the effect only in hippocampus also merits future investigation.

At this point we attribute TTR effect on autophagy to regulation of 14-3-3ζ levels in view of the well known negative regulation of autophagy through the bind to hVps34 (the class III phosphatidylinositol-3-kinase) (Pozuelo-Rubio 2011; Pozuelo-Rubio 2011).

TTR regulation of autophagy becomes a pivotal subject of study; retinoic acid, one of the main molecules transported by TTR, promotes autophagosome maturation

(Rajawat, Hilioti et al. 2010), through the cation-independent mannose-6-phosphate receptor (Rajawat, Hilioti et al. 2011). However, studies on retinoic acid in TTR null mice do not address retinoic acid in the hippocampus.

In physiological terms, decreased 14-3-3ζ levels can impact in learning and memory. In Drosophila, the *14-3-3zeta* gene named Leonardo, is highly expressed in adult mushroom bodies, centers of insect learning and memory, and mutant flies have a deficit in memory and learning (Skoulakis and Davis 1996; Philip, Acevedo et al. 2001). 14-3-3ζ -deficient mice present the same type of deficits observed in the Drosophila studies, as well as hyperactivity and disrupted sensorimotor gating. This mice model also exhibits abnormal neuronal migration and glutamatergic synapse formation, as well functional disrupted mossy fiber circuit (Cheah, Ramshaw et al. 2012). Young/adults TTR null mice display spatial reference memory impairment when compared with age matched controls (Sousa, Marques et al. 2007). The difference disappears in older animals. Correlating the phenotype of young/adult TTR null mice with the reduced capacity observed in 14-3-3ζ-deficient mice to learn and memorize, it is tempting to speculate that memory impairment observed in adult TTR null mice as compared to wild type age matched controls, can relate to reduced levels of 14-3-3ζ in the hippocampus. Future biochemical and genetic studies should address this hypothesis.

In conclusion, our study showed that the absence of TTR decreases 14-3-3ζ protein levels in the hippocampus. The reduced levels of 14-3-3ζ found *in vitro* and *in vivo*, are attributable to increased degradation in the lysosomal compartment. Increase in autophagy is observed in the absence of TTR, probably by the reduced levels of 14-3-3ζ.

**Acknowledgements**

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# CONCLUSIONS AND PERSPECTIVES

In this work we identified, for the first time, TTR role as a signaling molecule through IGF-IR. It was proved that TTR not only binds IGF-IR but also that increases its expression at transcriptional level. Hippocampus of young TTR null mice display lower levels of IGF-IR likely independently of IGF-I levels, since no difference was observed in serum IGF-I levels of TTR wild type and TTR null animals.

In the present study we approached only the hypocampus, but will search for decreased IGF IR in the brain of young TTR null mice by immunohistochemistry, followed by mRNA and protein levels in other brain regions.

*In vitro* studies showed that TTR could also activate IGF-IR and signaling molecules of the IGF-IR pathway only in the presence of IGF-I. Upon IGF-I binding, secondary structure rearrangements of TTR occurred increasing the hydrophobic surface of TTR which could explain the synergistic effects of TTR and IGF IR on activation of the IGF-IR pathway. Two main signaling cascades are activated upon ligand binding to IGF-IR: PI3/Akt and Ras/Erk. It was proved that TTR and IGF-I had a synergistic effect on activation of PI3/Akt signaling pathway. We have searched for the synergistic effect in the Ras/Erk cascade, but found no evidence for TTR action.

Through *in vitro* studies, we present strong evidence for the protective role of TTR in CNS, taking glutamate excitoxicity as the paradigm. Glutamate is the most abundant neurotransmitter, principal responsible for synaptic plasticity, learning and memory among other cognitive functions. However, at elevated concentrations it induces overactivation of glutamate receptors, increasing ions influx into the cell. Calcium overload results in activation of enzymes that degrade proteins, membranes and nucleic acids leading to cell death. So far, our studies suggest TTR protection in cell death and/or neuritogenic effect, by different mechanisms, i.e., whereas TTR protection in cell death is clearly related to TTR-IGF-I synergy (not excluding other stil unknown co-factors), it is too early to ascertain if the same mechanisms operate in dendrites, which might involve TTR effect on transcription of IGF IR or other stil unknown genes/cascades.

Glutamate excitotoxicity has been demonstrated in several diseases such as stroke, AD, ALS, epilepsy, Huntington and Parkinson diseases and brain trauma (Dong, Wang et al. 2009; Mehta, Prabhakar et al. 2013). The protective role of IGF-I against glutamate excitotoxic insults is observed in a several cell types: oligodendrocytes (Ness and Wood 2002), motor neurons (Vincent, Mobley et al. 2004) and DRG (Zhang, Wang et al. 2010; Liu, Cai et al. 2012). We demonstrated that the synergistic effect of TTR and IGF-I protected HT22 from glutamate-induced cell death through activation of PI3K/Akt cascade. Brain ischemia it is a pathology characterized by glutamate overload. Therapeutic effect of IGF-I on ischemia had been previously demonstrated (Guan, Bennet

et al. 2003). A recent study showed that administration of IGF-I before MCAO reduced volume lesion, and stimulates pro-survival signaling of IGF-IR (pIGF-IR and pAkt). The same study also showed that injection of IGF-I at 60 minutes after MCAO could also reduce the injured area (Sun, Meng et al. 2012). The synergistic effect of TTR and IGF-I, observed in this work, could enhance the neuroprotective roles of IGF-I namely on ischemia where both molecules have protective roles, but independently of each other.

Up-regulation of IGF-IR was reported in the hippocampal area after global ischemia, which could be explained as a compensatory mechanism (Bergstedt and Wieloch 1993). TTR effects on increasing IGF-IR total levels could be a helpful mechanism in recovery from traumatic brain ischemia. From now on, TTR effects on IGF-IR signaling should be taken in consideration, in new studies, to overcome glutamate toxicity in ischemia.

In case of AD pathology, some controversial data concerning IGF-IR signaling role exists. Some authors suggest administration of IGF-I protect animals from Aβ toxicity and the blockade of IGF-IR induced neurological disease (Carro, Trejo et al. 2002; Carro, Trejo et al. 2006), while others claim that reduced IGF-I signaling delay AD pathology in mice (Cohen, Paulsson et al. 2009). A negative feedback signaling between systemic IGF-I and intracerebral IGF-I is suggested to explain these contradictory results. Some studies corroborate this hypothesis: mutations on IGF-IR gene in centenarians females are associated with increased serum levels of IGF-I and reduced activity of the receptor, suggesting a role of IGF-IR pathway in lifespan (Suh, Atzmon et al. 2008). However, it was shown that circulating IGF-I regulates hippocampal IGF-I protein levels without affecting IGF-I or IGF-IR gene transcription in this brain area, but regulating gene expression associated with vascular development, learning and memory (Yan, Mitschelen et al. 2011). Either in ischemic and AD pathologies, the actions of IGF-I seem to be condition dependent: if before injury, in animal mice models, attenuated IGF-I signaling seems to be neuroprotective, after injury IGF-I have a protective role (Fernandez and Torres-Aleman 2012). Further studies need to be performed in order to clarify and evaluate how synergistic effect of TTR and IGF-I on IGF-IR signaling could be helpful under these pathological conditions.

TTR regulation of 14-3-3ζ hippocampal levels described in the last chapter was a very interesting finding. 14-3-3 proteins are involved in several biological processes, interacting with more than 400 molecules. Hippocampus of TTR null mice presented lower levels of 14-3-3ζ when compared with wild type littermates. Regulation of 14-3-3ζ levels seems to occur at the lysosomal compartment, although further studies should be performed to clarify this issue. Regulation of 14-3-3ζ in hippocampus could explain the

impairment memory observed in TTR null mice, since 14-3-3ζ-deficient mice present cognitive and learning deficits, abnormal neuronal migration and glutamatergic synapse formation and functional disrupted mossy fiber circuit (Cheah, Ramshaw et al. 2012). In the absence of TTR increased autophagy is observed, a fact that was related with decreased levels of 14-3-3ζ in the absence of TTR, as 14-3-3ζ is a negative regulator of autophagic process through the bind to hVps34 (the class III phosphatidylinositol-3-kinase) (Pozuelo-Rubio 2011; Pozuelo-Rubio 2011). Regulation of 14-3-3ζ levels by TTR is isoform specific.

At least three isoforms of 14-3-3 proteins (β, ε and ζ) can bind IGF-IR through phosphorylation of serine residues ($Ser^{1272}$ and $Ser^{1283}$) in the C-terminal domain of the receptor. (Craparo, Freund et al. 1997; Furlanetto, Dey et al. 1997). However, 14-3-3ζ and IGF-IR are related in signaling, TTR effect on each one of them seems to be independent.

In summary, this work revealed important TTR functions on aspects that had never been described before: (i) increase of IGF-I receptor levels; (ii) synergistic effect in activation of the IGF-I receptor signaling cascade leading to protection from glutamate toxicity and (iii) modulation of autophagy through regulation of 14-3-3ζ levels.

# APPENDIX

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# ABREVIATIONS

2D - two-Dimensional

Å - Ångström

AD - Alzheimer disease

AEC - substrate 3-amino-9-ethyl carbaxole

ALS - acid labile subunit

ANS - 1-anilinonaphthalene-8-sulfonic acid)

Apo - apolipoprotein

APP - amyloid precursor protein

Aβ - amyloid *beta*

Bad - Bcl-XL/Bcl-2- associated death promoter

BBB - blood brain barrier

Bp - base pairs

BRCA1 - breast cancer gene-1

BSA - bovine serum albumin

C/EBP - CAAAT/enhancer binding protein

CNS - central nervous system

CP - choroid plexus

CREB - cAMP response element-binding protein

CSF - cerebrospinal fluid

CSK - Src kinase

CTF – C-terminal fragment

Da – Daltons

DEAE - diethylaminoethyl cellulose

DMEM - Dulbecco's modified Eagle's medium

DNA - deoxyribonucleic acid

DNA-PK - DNA-dependent protein kinase

DRG - dorsal root ganglion

ECM - extracellular matrix

EDTA - ethylenediamine tetraacetic acid

EGFR - epidermal growth factor receptor

EGTA - ethylene glycol tetraacetic acid

Erk - extracellular signal related kinase

FAP - familial amyloidotic polyneuropathy

FBS - fetal bovine serum

FKHR - forkhead in rhabdomyosarcoma

FoxO - forkhead box O

GH - growth hormone
GI - gastrointestinal tract
GRB - growth factor receptor-bound protein
GSK3 - glycogen synthase kinase 3
HBSS - Hank's Balanced Salt Solution
HDL - high-density lipoprotein
HNF - hepatocyte nuclear factor
HRP - horseradish peroxidase
HSF - heatshock factor
IGFBP - insulin-like growth factor binding protein
IGF-I - insulin-like growth factor I
IGF-IR - IGF-I receptor
IgG - immunoglobulins
IR - insulin receptor
IRS - insulin-receptor substrate
JNK - c-Jun $NH_2$-terminal kinase
kb – kilobases
$K_D$ - dissociation constant
LC3 - light chain 3
LDL - low-density lipoprotein
LRP2 - low density lipoprotein-related protein 2
M6P/IGF-IIR - mannose-6-phosphate/IGF-II receptor
MAPK - mitogen-activated protein kinase
MOPS - 3-(N-morpholino) - propanesulfonic acid
mRNA – messenger ribonucleic acid
mTOR - mammalian target of rapamycin
NMDA - N-methyl-D-aspartate
NMR - nuclear magnetic resonance
NPY - neuropepetide Y
ORF - open reading frame
PAGE - polyacrylamide gel electrophoresis
PAM - peptidylglycine $\alpha$-amidating monooxygenase
PBS - phosphate buffered saline
PBST - phosphate-buffered saline Tween-20
PCR - polymerase Chain Reaction
PDK - phosphoinositide-dependent kinase

PI3K - phosphatidylinositol-3-kinase

PKB - protein kinase B

PKC - protein kinase C

pMCAO - permanent middle cerebral artery occlusion

PNS - peripheral nervous system

RAP - receptor-associated protein

RBP - retinol-binding protein

RBPR2 - RBP4 receptor-2

ROS - reactive oxygen species

RPE - retinal pigment epithelium

Rsk - ribosomal S6 kinase

RT - room temperature

RT-PCR- reverse transcriptase - polymerase chain reaction

RXR - retinoid X receptor

sAPP - soluble APP

SDS - sodium dodecyl sulfate

SEM - standard error

SH2 - Src-homology-2

Shc - Src homology domain C

$T_3$ - triiodothyronine

$T_4$ – thyroxine

TBG - thyroxin-binding globulin

TH - thyroid hormones

THBP - thyroid-hormones binding protein

TLPs - transthyretin-like proteins

TNF - tumor necrosis factor

TTR - transthyretin

TUDCA - tauroursodeoxycholic acid

UTR - untranslated region

UV-CD - ultra violet- circular dichroism

WT - wild type

WT1 - Wilm’s tumor 1