The role of megalin in the neuroprotective properties of Transthyretin

Renata Filipa Barbosa Soares Nogueira
Mestrado de Bioquímica
Departamento de Química e Bioquímica
2014

Orientador
Professora Doutora Maria João Mascarenhas Saraiva, Professora Catedrática,
Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Coorientador
Doutor João Carlos Gomes, Doutorado, Instituto de Biologia Molecular e Celular

U.PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

Porto, _____/_____/_____

Agradecimentos

Agradecimentos

À Fundação da Ciência e Tecnologia (FCT) agradeço o financiamento ao projecto, mas a minha aprendizagem passou por muitas pessoas que aqui devem ser mencionadas.

À professora Maria João agradeço a orientação e a confiança pelo meu trabalho. A maneira encantadora como fala de ciência deu me muitas vezes o entusiasmo necessário para nunca desistir e perceber de que como o mundo microscópio é fantástico. É para mim uma referência de empenho e trabalho.

Ao João, por tudo o que me ensinou, os conselhos, a preocupação e a paciência (que desenvolveu em mim). Obrigada por confiares no meu trabalho, somos de facto uma equipa fantástica.

Ao Carlitos pela óptima disposição e a amizade. Parte da beleza estética deste trabalho passa pelas suas mãos. À Cristina pela palavra amiga e o conforto nos tempos mais difíceis. À Andrea por estar sempre disposta a ajudar. À Sustete, à Di, à Anabela e ao Paul, pelo ambiente familiar criado no laboratório, bem como toda ajuda e opiniões.

Aos meus colegas de mestrado pelo companheirismo, em especial à Cristiana por se ter revelado uma óptima confidente e amiga.

À Paulinha e à Tânia do CCGen pelo profissionalismo e simpatia.

Ao Fábio pela paciência incondicional, por estar sempre disposto a ouvir os meus desabafos, por me conhecer tão bem. Ao fim deste ano deviam no mínimo reconhecerem lhe uma licenciatura em bioquímica. És me muito importante!

À Isabel e a Filipa, as minhas bioquímicas favoritas. Sem dúvida das melhores coisas que o curso me pode oferecer foi a vossa amizade.

Porque a família somos nós que escolhemos, o meu muito obrigado pelo todo o carinho e conforto que encontrei na minha família adoptada ☺ Em especial, à minha mãe Laurinha pelas palavras certas, uma mãe tem sempre razão.

À Nocas e ao Tó pela cumplicidade indiscritível de irmãos. Ao Bequinhas, pela alegria contagiante duma criança que nos faz esquecer todos os problemas.

À minha avó, pela sua personalidade segura e teimosa, mas com uma sensibilidade única. Obrigada por todos os ensinamentos, conselhos e confidências. Serei sempre a tua “primogénita”.

À minha mãe, a única pessoa capaz de me levar para o mundo dos sonhos, onde tudo é possível. A que tem sempre a palavra encorajadora, a que mais acredita em mim. Nada disto seria possível sem ti... Amo-te incondicionalmente!

Abstract

Abstract

Transthyretin (TTR) is a plasma protein, known to transport the thyroid hormone thyroxine (T4) and retinol-binding protein (RBP)-retinol complex. The main sources of TTR in the plasma and cerebrospinal fluid (CSF) are the liver and choroid plexus, respectively. In peripheral nervous system, TTR enhanced nerve regeneration and neurite outgrowth in dorsal root ganglion neurons and megalin was proposed as a mediator receptor. Regarding central nervous system, TTR null mice showed to have an infarct area bigger than wild type mice after ischemic condition. Moreover, TTR levels were upregulared in mouse hippocampus after traumatic brain injury, in CSF of rat subjected to a stroke model and also in patients with severe head injury. More recently, clinical studies reported that young acute ischemic stroke patients have levels of serum TTR significantly lower than normal control groups. Additionally, elevated levels of TTR seem to be a marker of good prognosis in cerebral infarction, suggesting a neuroprotective action for TTR.

Given all of these findings, the main goal of our study is to investigate the neuritogenic and neuroprotective proprieties of TTR in hippocampal neuronal cultures and the molecular mechanism involved in these actions. This work demonstrates that TTR promotes neurite outgrowth and neuroprotection over dendritic extensions of neurons in physiological and in ischemic pathological conditions, respectively. Furthermore, these TTR actions were megalin receptor dependent and involved Erk 1/2, Akt and CREB, signaling pathways possibly through a Src/TrK transactivation mechanism.

Since, stroke is one the major causes of mortality worldwide and there is no effective therapy, this study unveils a new possible therapeutic target to explore.

Key words: neuroprotection, neurite outgrowth, transthyretin, megalin, hippocampal neurons

Resumo

Resumo

A transtirretina (TTR) é uma proteína do plasma, conhecida por transportar a hormona da tiroide tiroxina (T4) e o complexo retinol e proteína da ligação do retinol (RBP). As principais fontes da TTR no plasma e no líquido cefalorraquidiano são o fígado e o plexo coroide, respetivamente. No sistema periférico nervoso, a TTR mostrou melhorar a regeneração nervosa e o crescimento de neurites em neurónios da raiz dorsal e a megalina foi proposta como o recetor mediador. Num estudo direcionado para o sistema nervoso central, ratinhos deficientes em TTR mostraram ter uma área de enfarte maior do que ratinhos do tipo selvagem, após condição de isquemia. Além disso, os níveis de TTR foram sob regulados no hipocampo de ratinho após um dano cerebral traumático, no líquido cefalorraquidiano de ratos sujeitos a um modelo de AVC e também em pacientes com grave traumatismo craniano. Mais recentemente, estudos clínicos relataram que jovens pacientes com AVC isquémico agudo têm níveis de TTR significantemente mais reduzidos do que grupos de controlo normais. Adicionalmente, elevados níveis de TTR parecem ser um marcador de bom prognóstico, sugerindo a ação neuroprotectora da TTR.

Dadas todas estas constatações, o principal objetivo do nosso estudo é investigar as propriedades neuritogénicas e neuroprotectoras da TTR em culturas de neurónios do hipocampo e o mecanismo molecular envolvido nessas ações. Este trabalho mostra que TTR promove o crescimento de neurites e a neuroproteção das extensões dendríticas dos neurónios em condições fisiológicas e patológicas de isquémica, respetivamente. Para além disso, estas ações da TTR mostraram ser dependentes do recetor de megalina e envolvem as cascatas de sinalização Erk 1/2, Akt e CREB, possivelmente através da Src/mecanismo de transactivação da TrK.

Uma vez que o AVC é uma das principais causas de mortalidade em todo o mundo e não há nenhuma terapia eficaz, este estudo revela um novo alvo terapêutico possível para explorar.

Palavras-chave: neuroproteção, crescimento de neurites, transtirretina, megalina, neurónios do hipocampo

Table of Contents

Table of Contents

Table of Figures

Table of Figures

Abbreviations

Abbreviations

Å - Ångström

AD - Alzheimer's disease

Aβ- amyloid beta

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid

CNS - central nervous system

CREB - cAMP response element-binding protein

Da – Daltons

DNA - deoxyribonucleic acid

DRG - dorsal root ganglion

CSF - cerebrospinal fluid

EDTA - ethylenediamine tetraacetic acid

EGF - epidermal growth factor

EGTA - ethylene glycol tetraacetic acid

Erk 1/2 - Extracellular signal-regulated protein kinases 1 and 2

FAP - Familial amyloid polyneuropathy

FBS - fetal bovine serum

GSK3 - glycogen synthase kinase-3

HBSS - Hank's Balanced Salt Solution

HSF1 - heatshock transcription factor 1

IL-1β - interleukin 1β

IGF-I - insulin-like growth factor I

IGF-IR - insulin-like growth factor receptor I

IgG – immunoglobulins

Kb – kilobases

K_D- dissociation constant

Low-density lipoprotein (LDL)

LRP1 - low density lipoprotein-related protein 1

mGluR1 - metabotropic glutamate receptor 1

MT - metallothionein

MOPS - 3-(N-morpholino) - propanesulfonic acid

mRNA – messenger ribonucleic acid

NF-kB - nuclear factor kappa-light-chain-enhancer of activated B cells

NMDA - N-methyl-D-aspartate

PAGE - polyacrylamide gel electrophoresis

PBS - phosphate buffered saline

PC12 - cell line derived from a pheochromocytoma of the rat adrenal medulla

PCR - polymerase Chain Reaction

PD - Parkinson's disease

PDGF - platelet-derived growth factor

pMCAO - permanent middle cerebral artery occlusion

PNS - peripheral nervous system

RAGE - advanced glycation end products

RAP - receptor-associated protein

RBP - retinol binding protein

RNS - reactive nitrogen species

ROS - reactive oxygen species

SDS - sodium dodecyl sulfate

SH2 - Src-homology-2

SH3 - Src-homology-3

Wt - wild type

T4 - thyroxine

TBS-T - tris-buffered saline Tween-20

TLP - transthyretin-like proteins

TNFα - tumour necrosis factor α

TrK - tyrosine kinase

TTR - transthyretin

TTR KO - transthyretin knockout

CHAPTER I

General introduction

General introduction

Cerebral Ischemia

Stroke is one of the major causes of mortality in Portugal (George, 2012) and the second cause of death worldwide with 6.7 million of obits in 2012 according to the World Health Organization (2012). There are two kinds of stroke: the hemorrhagic stroke and the ischemic stroke. Ischemic strokes are more frequent and constitute 87% of all cases and are caused by a transient or permanent reduction in cerebral blood flow that is restricted to the territory of a major brain artery. Generally, reduction in flow results from occlusion of a cerebral artery by an embolus or local thrombosis. Despite the dimension of the disease, the cellular pathogenesis of hypoxic-ischemic brain damage is not totally known, and until now there is no effective therapy.

Cerebral blood flow reduction impairs delivery of substrates, particularly oxygen and glucose, essential for brain tissues to obtain energy by oxidative phosphorylation (Martin et al., 1994). This energy depletion results in the loss of membrane potential, and consequently, neurons and glia depolarize and release K^+ and glutamate (Katsura et al., 1994). In addition, the lack of energy prevents the reuptake of excitatory amino acids at the synapse, leading to accumulation of glutamate in the extracellular space (Rossi et al., 2000). Under these conditions, there is overactivation of synaptic and extrasynaptic glutamate receptors, namely NMDA, AMPA, kainate and also metabotropic glutamate receptor 1 (mGluR1) receptors. Glutamatergic overstimulation, a phenomenon known as excitotoxicity, contributes to neuronal degeneration in many acute CNS diseases, including ischemia, trauma, and epilepsy, and may also play a role in chronic diseases, such as amyotrophic lateral sclerosis (ALS), Huntington's, Parkinson's and Alzheimer's disease. This excitotoxicity results in Ca^{2+}, Na^+ and Cl^- overload into neurons, combined with a lower significant efflux of K^+ (Choi, 1992; Arundine and Tymianski, 2003). Increased influx of cations into cells is followed by passive entry of water, resulting in edema in the infarct zone.

Furthermore, the increased calcium concentration activates a series of enzymes, including protein kinase C, proteases, phosphatases, phospholipases, neuronal nitric oxide synthase, and xanthine oxidase and overproduction of proteolytic enzymes, lipid peroxidation, reactive oxygen species and reactive nitrogen species formation (Emerit et al., 2004). Oxidative stress changes energy metabolism and damage to mitochondria (Beal, 1992). Finally, these events trigger apoptosis, also known as programmed cell death (figure I).

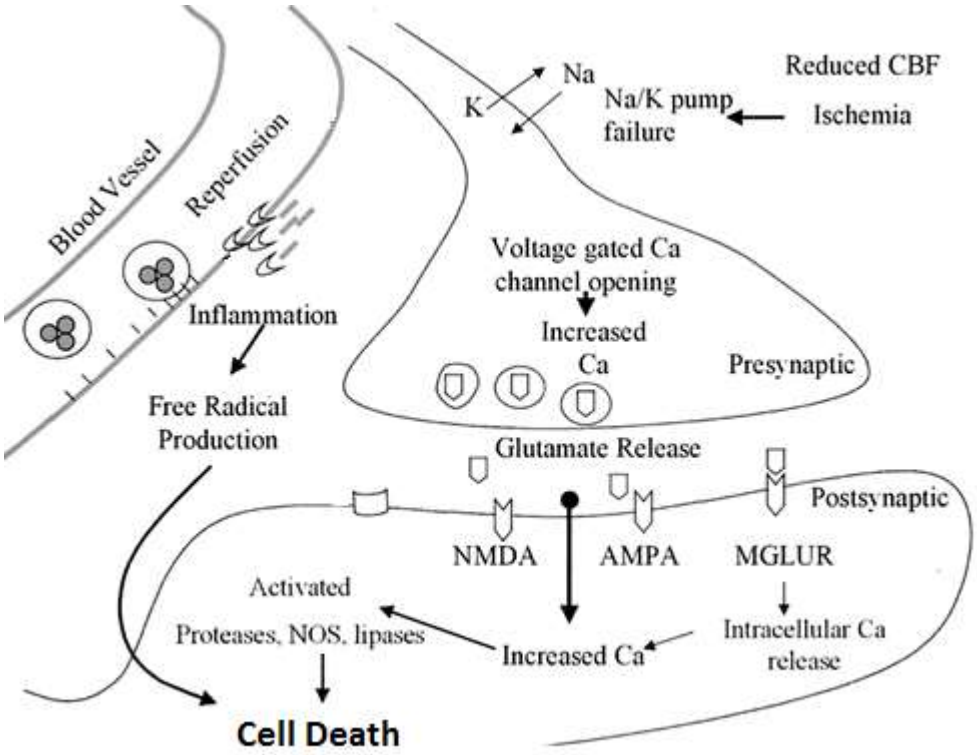

Figure I.Schematic representation of the intracellular ischemic cascade. Flow reduction as consequence of ischemic stroke leads presynaptic neuron depolarization, opening sodium (Na) and potassium (K) channels. Consequently, glutamate is released to the presynaptic cleft and it overstimulates its postsynaptic receptors (NMDA, AMPA and mGluR). Overstimulation increases intracellular calcium concentration, activating proteases, lipases and the formation of reactive molecules. The set of these events triggers cell death apoptosis. [Adapted from (Danton and Dietrich, 2004)]

The cells located in the damaged core region will never be repolarized, but if these cells are in penumbra, the area between the core and the well irrigated, where some perfusion occurs and supply of neurotrophic factors are preserved, recovery of membrane potential and homeostasis happens (Hossmann, 1996).

However, over time and without treatment, the penumbra can progress to an infarct zone similar to the core, due to expansion of excitotoxicity from the core to the rest of the penumbra and through spreading of plasma membrane depolarizations (peri-infarct depolarizations), inflammation and apoptosis (figure II).

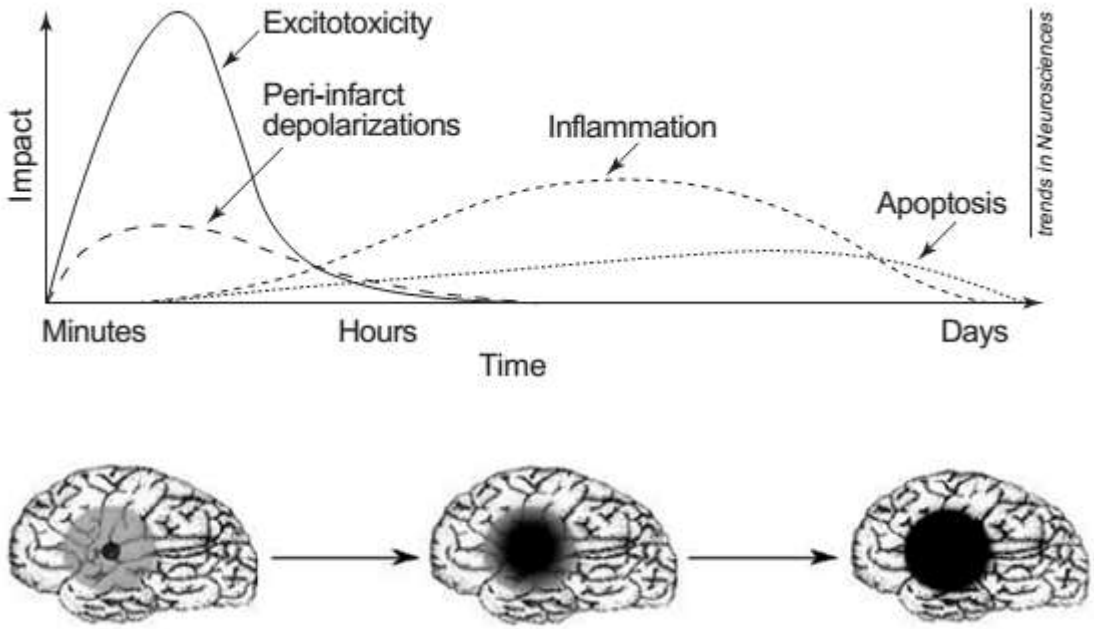

Figure II. Cascade of damaging events as a result of focal ischemic stroke. The ischemic stroke is followed by a sequence of events over the time, responsible by expansion from damaged core (dark) to penumbra (light grey), such as excitotoxicity, peri-infarct depolarization, inflammation process and lastly, apoptosis. The x-axis represents the evolution of the cascade over time and the y-axis illustrates the impact of each event of the cascade on final outcome. [Adapted from (Dirnagl et al., 1999; Brouns and De Deyn, 2009)]

Previous work from Santos et al reported that TTR null mice have significant increase in infarct area as compared to wild type mice, after ischemic conditions. It is thus very important to investigate TTR molecular mechanism related to neuroprotection in the central nervous system, particularly under ischemic conditions.

Transthyretin

In 1942, a study made reference the one “X component, a fraction with a mobility slightly greater than that of albumin” which appeared in electrophoresed human and rabbit blood serum samples (Seibert and Nelson, 1942). In parallel, Kabat et al. described a similar finding in electrophoresed human cerebrospinal fluid (CSF) samples (Kabat et al., 1942). During many years, this CSF- and plasma-circulating protein was designated by prealbumin, but in 1981 the Nomenclature Committee of the International Union of Biochemisty suggested the term “TransThyRetin” (1981), to translate the main physiological roles of this protein, which are the plasma transport of

the thyroid hormone thyroxine (T4) and of retinol (vitamin A) through binding to retinol binding protein (RBP).

Transthyretin (TTR) is found in a large number of vertebrate species including mammals, marsupials, birds, reptiles, amphibians and teleost fish, revealing to be an evolutionarily conserved protein (Schreiber and Richardson, 1997; Power et al., 2000). More recently, homologous sequences to TTR were discovered, known as transthyretin-like proteins (TLPs), in bacteria, nematods and plants. In *Escherichia coli* and *Caenorhabditis elegans* TLPs form homotetramers, like TTR, but do not bind T4 (Eneqvist et al., 2003).

Structure of TTR

The first X-ray crystal structure of human TTR was determined at 1.8 Å resolution by Colin Blake et al. in 1971 (Blake et al., 1971). It was shown to be a 54 980 Daltons (Da) homotetrameric protein, each monomer has 13 745 Da and is constituted by 127 amino acids (Kanda et al., 1974).

The monomer is composed by 8 antiparallel β-strands (A through H), linked by seven loops and a small α-helix of nine residues located at the end of β-strand E, which result in a classic β-barrel conformation. The β-strand form an inner and outer β-sheets (DAGH and CBEF) separated by about 10 Å (figure III-a). The N- and C-terminal regions of each monomer are composed by 10 and 5 unorganized residues, respectively.

The numerous hydrogen bonds formed between the β-strands F and H of each monomer result in a strong dimer. The tetramer is formed by hydrophobic and hydrophilic interactions between the AB loop of one monomer and the H strands of the two primed monomers (figure III-b), although these interactions are much weaker than those formed in dimer (Blake et al., 1974). The strong and extensive interactions formed in dimer suggest that this is the basic unit of transthyretin structure comparatively to the monomeric or tetrameric form.

The homotetrameric structure of native TTR has a globular shape and forms a central hydrophobic channel with two binding sites for T4 (Blake et al., 1974), which exhibit negative cooperativity, thus only one molecule of T4 is transported by TTR (Andrea et al., 1980).

RBP binds to TTR to form a very stable complex, preventing RBP filtration and degradation of TTR in kidney and also serves as retinol transport (Goodman, 1984; Noy et al., 1992). TTR tetramer has four RBP-binding sites, two in each dimer at the protein's surface. However, TTR only can bind two molecules of RBP due to the

steric hindrance, but in physiological conditions, just one molecule of RBP is transported (Monaco et al., 1995; van Bennekum et al., 2001). Moreover, RBP binding to TTR is not affected by T4 binding (Raz and Goodman, 1969).

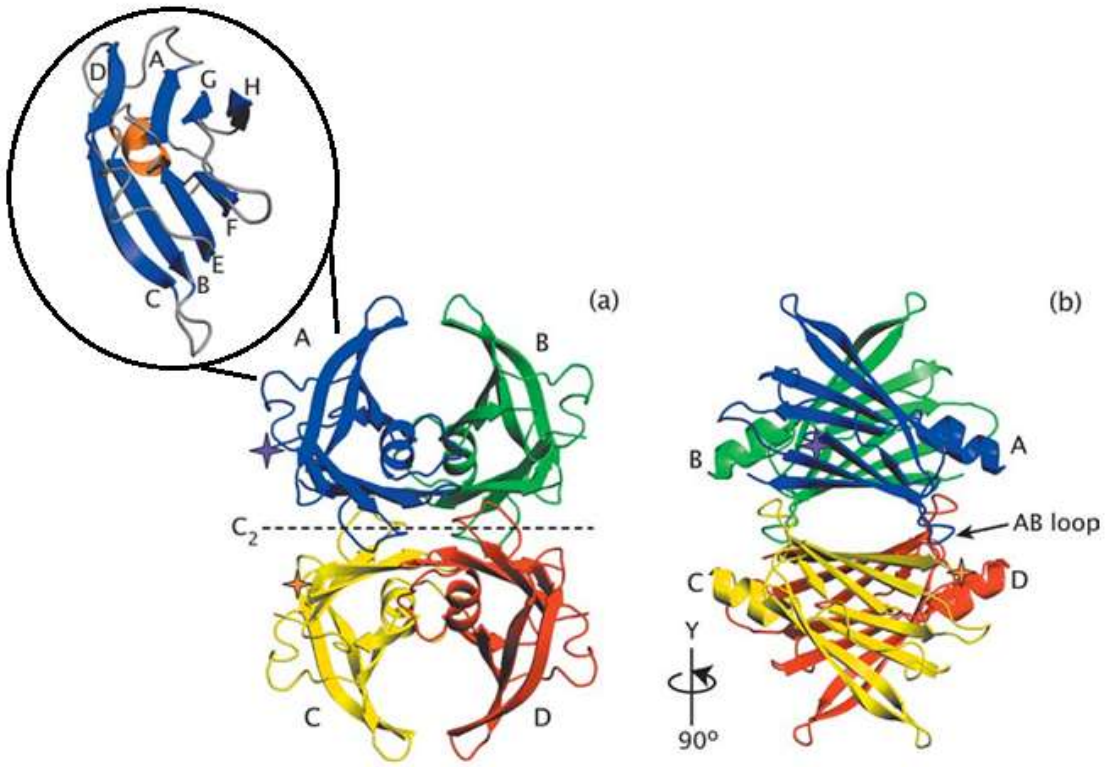

Figure III. The structure of transthyretin. (a) Transthyretin is a homotetrameric protein and each monomer is composed by 8 antiparallel β-strands (A through H), linked by seven loops and a small α-helix. The β-strand form an inner and outer β-sheets (DAGH and CBEF) separated by about 10 Å. (b) The tetramer with a globular shape is formed by hydrophobic and hydrophilic interactions between the AB loop of one monomer and the H strands of the two primed monomers. [Adapted from (Foss et al., 2005)]

Transthyretin expression

TTR is mainly synthesized by liver (Felding and Fex, 1982) and the choroid plexus, a secretory structure which is part of the blood-brain barrier (Aleshire et al., 1983), being the sources of TTR in the plasma and CSF, respectively.

The meninges are also a source of TTR in the nervous system, but in minor amount than the choroid plexus (Blay et al., 1993).

Gerhard Schreiber et al. reported that, the rat choroid plexus contained eleven times more transthyretin mRNA compared to liver per gram wet tissues and that synthesis of TTR was thirteen times faster than the liver (Schreiber et al., 1990). In CSF, TTR concentration ranges from 5-20 mg/L (Vatassery et al., 1991) and

represents about 25% of the total CSF protein content (Aldred et al., 1995). In adult plasma, transthyretin levels reach concentrations of 174-420 mg/L, but after the fifth decade begin to decrease (Stabilini et al., 1968; Benvenga et al., 1986; Li and Buxbaum, 2011).

TTR levels are reduced in conditions of malnutrition or chronic inflammation (Navab et al., 1977; Dickson et al., 1982), being used as a preferential nutritional/inflammatory parameter (Ingenbleek and Young, 1994). Furthermore, TTR levels are lower in patients with transthyretin-associated amyloidosis and at-risk individuals (Skinner et al., 1985), as well as, in young acute ischemic stroke patients (Gao et al., 2011).

It has been shown that TTR is produced in other sites in mammals such as the pancreatic islets of Langerhans (Kato et al., 1985; Jacobsson et al., 1989) and in minor extension in the heart, skeletal muscle, spleen (Soprano et al., 1985), visceral yolk sac endoderm (Soprano et al., 1986), retinal pigment epithelium (Cavallaro et al., 1990) and trophoblast of human placenta (McKinnon et al., 2005).

Regarding its expression in brain, this subject has caused discussion between several authors. The presence of TTR mRNA has been reported in diverse brain areas, such as cortex, hippocampus or cerebellum (Carro et al., 2002; Stein and Johnson, 2002; Buxbaum et al., 2008; Li et al., 2011), suggesting that these tissues secreted TTR. It was reported that the transthyretin gene is also expressed in dorsal root ganglia (Murakami et al., 2008), but this result was disclaimed in the same year (Sousa and Saraiva, 2008) as false positive results by contamination from adjacent meninges (Sousa et al., 2007a).

Transthyretin receptors

In 1988, Makover et al demonstrated that 36-38% of total body TTR degradation occurred in liver, 12-15% in muscle and 8-10% in skin. A minor proportion of body TTR, about 1-8%, is degraded in kidneys, adipose tissue, testes and gastrointestinal tract (GI). In the examined tissues of the nervous system evidence of TTR degradation were not evident. The organs with the highest rate of TTR degradation, per gram of wet weight, were the liver and the kidney (Makover et al., 1988).

Internalization of TTR is not fully understood, but several studies suggest that occurs by a receptor-mediated mechanism.

Studies in hepatomas and primary hepatocytes suggest that TTR uptake is mediated by cellular receptors. TTR internalization demonstrated to be affected by its

ligands, with a 70% decrease for the TTR-RBP complex and a 20% increase for TTR bound to T4. Moreover, it was showed that different TTR mutants display differences in uptake, suggesting that TTR structure is important to receptor binding. TTR hepatic uptake was inhibited by the receptor-associated protein (RAP), a ligand for all members of low-density lipoprotein receptor (LDLr) family; however, no member of LDLr-family was identified to mediate TTR uptake in the liver. Thus, a new unidentified RAP-sensitive receptor to TTR internalization in the liver was proposed (Sousa and Saraiva, 2001).

In contrast, TTR endocytosis in the kidney was inhibited by RAP and megalin, an endocytic multi-ligand receptor of the LDL receptor family expressed on the apical surfaces of epithelium of renal proximal tubules, was identified as first receptor involved in TTR uptake. In addition to this, megalin deficient mice did not present TTR accumulated in lysosomes of renal tubules comparatively to the control animals. TTR binding to megalin was not influenced by its ligands, T4 and RBP (Sousa et al., 2000b).

Regarding the peripheral nervous system, the megalin-TTR interaction was also described, which effects of neurite outgrowth and nerve regeneration; TTR internalization is megalin-dependent in sensory neurons from dorsal root ganglion (Fleming et al., 2009).

The receptor for advanced glycation end products (RAGE), a member of the immunoglobulin superfamily of cell surface molecules, was shown to interact with TTR. In familial amyloidotic polyneuropathy (FAP) tissues, RAGE levels were increased and the binding of aggregated TTR to RAGE induced the activation of the transcription factor NF-kB, mediating an inflammatory and apoptotic response. In vivo, TTR binding to RAGE, in peripheral nerve of FAP patients, leads to the neurodegeneration, but the TTR ability to bind RAGE is lost when TTR interacts with RBP (Sousa et al., 2000a).

Ligand blotting and cross linking experiments performed in ependymoma cells, constituents of the brain cerebrospinal barrier, evidenced the presence of a 100 kDa receptor. Receptor binding of TTR suggests a potential mechanism for the delivery of T4 within the central nervous system (Kuchler-Bopp et al., 2000). This finding was confirmed by the presence of an approximately 115-kDa TTR-binding membrane protein in chicken oocytes, where TTR was detected in clathrin-coated vesicles (Vieira et al., 1995). However, none of these putative TTR receptors was identified. More recently, TTR was shown to upregulate the insulin-like growth factor receptor I (IGF-IR) levels in hippocampal neuronal cultures, through nuclear translocation of receptor (Vieira et al., 2014), suggesting that TTR binds to IGF-IR. Since this receptor as an approximate size of 100KDa and is also highly enriched in the choroid plexus, we can

speculate that this could be the non-identified receptor by Kuchler at the time. However, the detailed characteristics of this binding still need further studies.

TTR null mice: a tool to study the role of TTR

In 1992, Episkopou and colleagues produced a transthyretin knockout (TTR KO) mice strain. For this, the mouse TTR gene was disrupted using the technique of gene targeting in embryonic stem cells. The MC1neo expression cassette was introduced into the second exon of a 5.9-kb genomic mouse TTR gene fragment that carries exons 1-3. TTR null animals shown to be viable and fertile were also phenotypically similar to wild-type and heterozygous littermates. However, these transgenic mice had no detectable plasma retinol and had depressed levels of thyroxine (Episkopou et al., 1993).

The TTR KO mouse model has become an important tool to evidence not only the physiological role of TTR as a transporter of T4 and RBP-retinol, but also to study the importance of TTR in nervous system.

Concerning the study of the nervous system, TTR KO mice revealed, in behavioral studies, less immobility and increased activity in the forced swim and in the locomotor activity test, when compared to WT animals, leading the authors to propose that lack of TTR is associated with increased exploratory activity and reduced signs of depressive-like behavior. Moreover, TTR null mice showed higher levels of noradrenaline in the limbic forebrain (Sousa et al., 2004), indicating that TTR may modulate the noradrenergic system.

In TTR KO mice the gene of peptidylglycinea-amidating monooxygenase (PAM), an enzyme essencial in the process of amidated neuropeptide maturation, is upregulated in dorsal root ganglion (DRG), sciatic nerve, hippocampus, cortex and spinal cord. Consequently, these mice have increased levels of NPY (Nunes et al., 2006), the major amidated neuropeptide and with antidepressant properties, corroborating the finding that TTR KO mice are less depressed (Sousa et al., 2004).

Additionally, older TTR null mice present a sensorimotor impairment as compared to wild type (Wt) mice although any morphological difference has been found in sciatic nerves or in cerebellum of both strains. Nerve regeneration was also affected by lack of TTR, since TTR KO mice presented a decreased regenerative capacity after sciatic nerve injury (Fleming et al., 2007). Nevertheless, this phenotype was recovered when TTR was expressed locally in the nerve of TTR KO mice (Fleming et al., 2009). Furthermore, TTR KO mice present decreased levels of myelinated and unmylinated

axons (Fleming et al., 2007) and a compromised retrograde transport (Fleming et al., 2009).

Using a panel of behavioral tests designed to study cognitive performance, such as the Barnes maze and the Morris water maze, young/adult TTR KO mice displayed a defect in spatial learning and in memory as compared to Wt animals (Sousa et al., 2007b; Brouillette and Quirion, 2008; Buxbaum et al., 2008). In agreement with this notion is the fact that Wt mice also have a decrease in cognitive performance over the years, at the same time that TTR levels in CSF are being reduced (Sousa et al., 2007b).

In vitro, TTR has also the capacity of inducing neurite outgrowth in TTR KO DGR neurons and PC12 cells. This effect of TTR is independent of its ligands, as it is also triggered by I84S TTR, a TTR variant which has very low affinity for both T4 and RBP (Fleming et al., 2007). Neuritogenic activity of TTR in DRG neurons depends on its internalization, a process that is clathrin dependent and megalin-mediated. Mice deficient in megalin had a similar decrease in nerve regeneration comparatively to the TTR KO mice, suggesting that megalin and TTR may act in the same pathway (Fleming et al., 2009).

More recently, it was demonstrated that young/adult TTR KO mice have decreased levels of insulin-like growth factor receptor I in hippocampus (Vieira et al., 2014), an receptor able to protect from apoptosis (Kooijman, 2006; Annunziata et al., 2011)

So, we can conclude that the lack of TTR in physiological conditions impairs several aspects of nervous system. Accordingly, the development of gene therapies for FAP that propose the silencing/reducing the whole production of TTR (Benson et al., 2006) should be discussed with more caution.

TTR in neurodegenerative diseases

In pathological cases TTR is highly associated with familial amyloidotic polyneuropathy (FAP), but over time it has been linked with several other neuropathologies such as Guillain–Barré syndrome, Alzheimer's disease, frontotemporal dementia and Parkinson's disease. More recently, TTR role begins to be studied in cerebral ischemia, the pathology focused in our research project.

Familial amyloid polyneuropathy

Familial amyloid polyneuropathy is an autosomal dominant disease described for the first time by Andrade in patients of the Northern region of Portugal (Andrade,

1952). This neurodegenerative disease is associated with mutations in TTR, resulting in the deposition of TTR amyloid fibrils, particularly in the peripheral nervous system (PNS). The clinical features associated with the disease are early impairment of temperature, pain sensation in the feet, autonomic dysfunction leading to paresis, malabsorption and emaciation. The symptoms start between the ages of 20 and 35 leading to death within 10-15 years (Dyck and Lambert, 1969; Sousa and Saraiva, 2003). The most frequent mutation in FAP patients is the substitution of a valine residue for a methionine at position 30 (TTR V30M) (Saraiva et al., 1984). However, more than 100 TTR mutations have been related with amyloid deposition, with predominance of the PNS and/or the heart (Saraiva, 2001).

The origin of TTR deposition in FAP is unknown, but structural studies suggest that amyloid formation by TTR is triggered by tetramer dissociation to a compact non-native monomer, which can originate instable thermodynamic monomeric species with a high tendency for ordered aggregation into amyloid fibrils through specific intermolecular contacts (Quintas et al., 2001).

Alzheimer´s disease

Alzheimer´s disease (AD) is the most common cause of dementia worldwide and it is clinically characterized by an initial memory decline, which progressively leads to the impairment of cognitive functions, till death. This neurodegenerative disease exhibits extraneuronal amyloid deposits composed by aggregates of Aβ peptide. The aggregates result from the proteolytic cleavage of the amyloid precursor protein and intraneuronal neurofibrillary tangles constituted by aggregates of abnormally hyperphosphorylated tau protein (Goedert and Spillantini, 2006).

In AD patients, TTR levels were found to be significantly reduced in CSF (Elovaara et al., 1986) and in plasma, comparatively to the control group (Han et al., 2011). Moreover, TTR levels in CSF were negatively correlated with the degree of dementia in AD (Riisoen, 1988) and with the abundance of amyloid plaques (Merched et al., 1998). Recently, it was reported that TTR is decreased in serum of mild-cognitive impairment (MCI) and of AD patients in comparison with non-demented controls (Ribeiro et al., 2012), suggesting TTR as an early biomarker of AD.

In a transgenic mouse model for AD, a reduction of TTR plasma levels in early stages of AD as compared to non-transgenic littermates was also observed, but a contradictory effect is verified in later stages (Oliveira et al., 2011); the genetic reduction of TTR accelerates Aβ deposition (Choi et al., 2007).

Studies showed that TTR is able to bind Aβ aggregates, suggesting that TTR has a chaperone-like mechanism to perform its neuroprotective role in Alzheimer´s disease (Buxbaum et al., 2008). Costa and colleagues evidenced that TTR binds soluble, oligomeric and fibrillar forms of Aβ, having also the ability to inhibit and to disrupt Aβ fibrils (Costa et al., 2008a) and to cleave Aβ in multiple positions (Costa et al., 2008b). Also, it was proposed that TTR contributes not only to the maintenance of Aβ levels within a normal range, but also to clearance from the brain, preventing the accumulation of Aβ peptides.

Taken together these evidences and the fact that overexpression of human TTR decreased Aβ deposition and improved cognition in APP23 mice (Buxbaum et al., 2008), makes TTR an interesting therapeutic target.

Parkinson's disease

Parkinson's disease (PD) is a common neurodegenerative movement disorder and the clinic symptoms include motor impairments involving resting tremor, bradykinesia, postural instability, gait difficulty and rigidity. Disease progression involved cognitive (dementia), neuropsychiatric (depression and anxiety) and autonomic (hypotension and constipation) dysfunctions. The pathological features of disease are loss of dopaminergic neurons and presence of Lewy bodies, composed of aggregated α-synuclein and other proteins (Thomas and Beal, 2007; Wirdefeldt et al., 2011).

CSF of patients with Parkinson's disease who underwent adrenal medullary autotransplantation showed a significantly increase in TTR concentration (Abram et al., 1990) and the same finding was confirmed in CSF from a rat model of PD (Rite et al., 2007). More recently, study using two-dimensional differential in gel electrophoresis (2D-DIGE) in postmortem ventricular CSF (V-CSF) from neuropathologically confirmed PD subjects suggested TTR as a biomarker of the disease, since TTR was raised in PD when compared to the normal control group (Maarouf et al., 2012).

Since TTR levels seem to have a correlation with the pathophysiology of Parkinson's disease, studies of TTR role in this neurodegenerative disorder with more detail will be pertinent.

The effect of TTR in cerebral ischemia

In brain, TTR levels have been found to be upregulared in the following ischemic situations: (I) cerebral oligemia in mice, a surgery that results in the blood flow

reduction without acute tissue damage (Liverman et al., 2004); (II) in mouse hippocampus after traumatic brain injury (Long et al., 2003); (III) in CSF of a rat model after transient middle cerebral artery occlusion (Suzuyama et al., 2004) and (IV) in patients with severe head injury (Young et al., 1996). Moreover, TTR excretion in urine of stroke-prone rates before cerebral ischemia was also detected (Sironi et al., 2001). More recently, a clinical study showed that young acute ischemic stroke patients have significantly lower TTR serum levels than normal control groups. Elevated levels of TTR indicate a good prognosis in cerebral infarction (Gao et al., 2011).

Santos et al. used the model of permanent middle cerebral artery occlusion (pMCAO) to induce cerebral ischemia in TTR null mice with impaired heatshock transcription factor 1 (HSF1) to study the possible neuroprotective role of TTR in cerebral ischemia. After pMCAO, $TTR^{-/-}HSF1^{+/-}$ mice showed increased cortical infarction, cerebral edema and microglial-leukocyte response when compared with $TTR^{+/+}HSF1^{+/-}$ mice. In addition, increased transcript levels of TNF-α and IL1-β observed in $TTR^{-/-}HSF1^{+/-}$, suggested that TTR might influence the inflammatory process (Santos et al., 2010).Therefore, this study suggests that TTR contributes to the control neuronal cell death, edema and inflammation, evidencing the possible neuroprotection of TTR in cerebral ischemia.

Megalin, member of LDL-receptor family

Megalin (also known as LRP2, gp330 or gp600) is a giant membrane glycoprotein of 600kDa that is expressed mainly on the apical side of absorptive and secretory epithelial cells. It belongs to the LDL-receptor family, which is characterized by similar structure in ligand binding (complement)-type cysteine-rich repeats, epidermal growth factor (EGF) homology domains consisting of EGF repeats and a YWTD propeller domain, a single membrane-spanning segment and a short cytoplasmic tail that contains various sequence motifs that mediate interactions with cytoplasmic adaptor and scaffolding proteins (Gotthardt et al., 2000; Herz and Beffert, 2000; Herz and Bock, 2002).

The mammalian LDL receptor family is composed by seven core members (LDLR, VLDLR, ApoER2, MEGF7, LRP, LRP-1b, and megalin) and three distantly related receptors (LRP-5, LRP-6, and LR11/SorLA) (Herz and Bock, 2002).

Discovery of megalin

In 1982, Dontscho Kerjaschki and Marilyn Farquhar described a membrane glycoprotein of 330 kDa (gp330) as a pathogenic antigen of Heymann nephritis (Kerjaschki and Farquhar, 1982); two years later it was proposed to be an endocytotic receptor (Kerjaschki et al., 1984).

Claes Juhlin in 1987 produced reactive monoclonal antibodies against parathyroid cells and tubule cells of the kidney (Juhlin et al., 1987), which later were proven to react with a human gp330 homologue (Lundgren et al., 1994).

In 1989, Robert T. McCluskey showed that gp300 is member of LDL-receptor family (Raychowdhury et al., 1989). In 1994, the Farquhar group worked to publish the full-length sequence of rat gp330 (Saito et al., 1994). Afterwards, the human sequence was published, revealing that human gp330 is a protein of 4655 amino acid residues, of which 4398 belonged to the extracellular domain, 23 amino acid residues made up the single transmembrane spanning domain, and 209 amino acid residues the cytoplasmic domain. This sequence also revealed a 25-amino acid residues N-terminal signal peptide sequence (Hjalm et al., 1996).

Regarding the genome, DNA megalin sequences of rat and human have 77% identity; the human megalin gene is located on chromosome 2q24–q31 (Korenberg et al., 1994), containing 79 exons, affording a transcript of 14384 base pairs (Birney et al., 2006).

Megalin structure

The megalin structure is characterized by a large extracellular domain comparatively to short intracellular domains (figure IV). The extracellular domain is composed by four clusters of cysteine-rich complement-type ligand binding repeats, responsible by ligand binding, which are separated by 17 epidermal growth factor (EGF)-like repeats and eight cysteine-poor spacer regions (Saito et al., 1994), which contain YWTD motifs that are involved in the receptor folding (Culi et al., 2004; Lighthouse et al., 2011) and dissociation of their ligands in the acidic endosomal compartment (Jeon et al., 2001). The EGF-precursor like domain can be divided into calcium and non-calcium binding domains and within the YWTD repeat spacer regions there are 19 potential N-linked glycosylation sites. Furthermore, the sequence of human gp330 reveals a potential furin cleavage signal localized between residues 3243 and 4246 (Hjalm et al., 1996).

Megalin contains one transmembrane domain (Marzolo et al., 2003), that is a substrate for the Ɣ-secretase complex (Zou et al., 2004; Biemesderfer, 2006).

The cytoplasmic domain contains two NPxY-motifs and one NPxY like motif (Saito et al., 1994; Hjalm et al., 1996). These motifs of the LDL-receptor family are important by its internalization mediated by clathrin (Chen et al., 1990) and basolateral distribution (Matter et al., 1992; Gan et al., 2002). However, it is not yet clearly defined what is the function of these motifs for megalin.

Megalin also contains one potential SH2-binding motifs (Songyang et al., 1993), one potential dileucine repeat, four potential SH3-domain binding motifs (Yu et al., 1994), three protein kinase C phosphorylation sites (PKC), seven casein kinase II (CK-II) sites and one PDZ-binding motif (Hjalm et al., 1996).

The cytosolic domain of megalin binds various intracellular proteins, namely mitogen activated protein kinase (MAPK) scaffold proteins, JIP-1 and JIP-2 (JNK-interacting proteins, 1 and 2) (Gotthardt et al., 2000), post-synaptic density protein-95 (PSD95)-like membrane-associated guanylate kinase proteins (Larsson et al., 2003), and adaptor-type molecules such as SEMCAP-1 (a transmembrane semaphorin-binding protein) (Gotthardt et al., 2000), Disabled-2 (Dab2) (Oleinikov et al., 2000) and autosomal recessive hypercholesterolaemia protein (ARH) (Nagai et al., 2003).

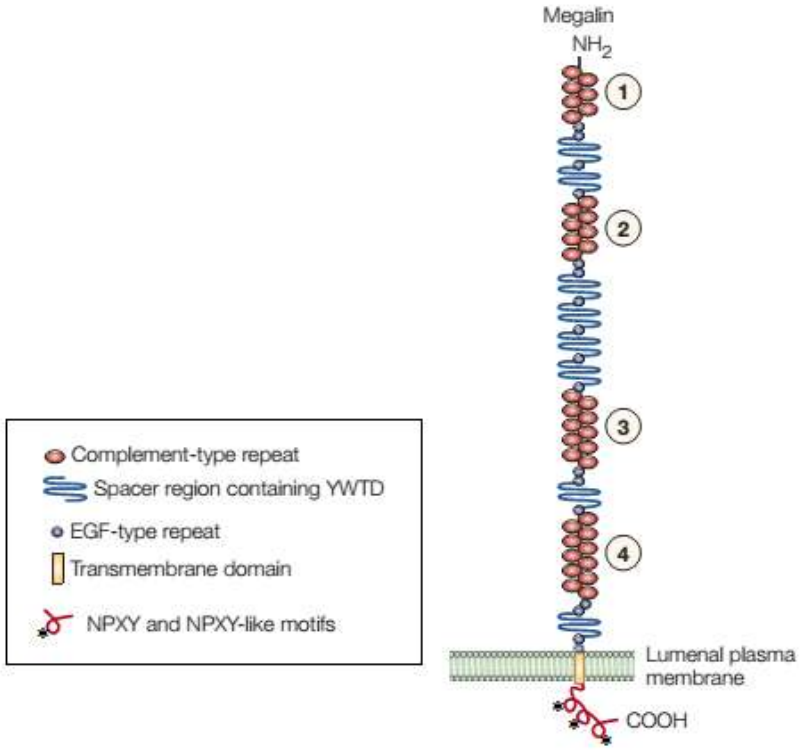

Figure IV. The structure of megalin receptor. Megalin is a transmembrane glycoprotein of approximately 600 kDa composed for a large extracellular domain followed by a transmembrane domain and a small cytoplasmic tail. The extracellular domain is composed by four clusters of cysteine-rich complement-type ligand binding repeats, responsible by ligand binding, which are separated by 17 epidermal growth factor (EGF)-like repeats and eight cysteine-poor spacer regions, which contain YWTD motifs. The cytoplasmic tail is constituted by two NPxY motifs and one NPxY-like motif in addition to several Src-homology-3 (SH3) and one Src-homology-2 (SH2) recognition sites and binds several intracellular proteins involved in signaling cascade. [Adapted from (Christensen and Birn, 2002)]

Megalin signaling

Although only one study reports megalin phosphorylation, the presence of several consensus phosphorylation sites within the cytoplasmic domain strongly suggests that this post translational modification happens and that it may have a relevant role in the receptor's trafficking and signaling.

Studies showed that LRP1 is constitutively phosphorylated by protein kinase A (PKA) at a serine residue, which affects the receptor's endocytosis (Li et al., 2001). Moreover, the extracellular molecules binding to LRP1, such as PDGF, induce tyrosine phosphorylation of its cytoplasmic tail, resulting in the recruitment Src tyrosine kinases (Loukinova et al., 2002). Other example is reelin that binds to extracellular domain of lipoprotein receptors, the apoER2 and VLDL-R, resulting in tyrosine phosphorylation of Dab1, an adaptor protein that interacts with NPxY motifs in both receptor tails, and activatation of Src family of kinases (Arnaud et al., 2003; Bock and Herz, 2003). Also LRP6, involved in the canonical Wnt signaling pathway, is phosphorylated in PPPSP motifs, present within the carboxyl terminus of its cytoplasmic tail, by glycogen synthase kinase-3 (GSK3) and casein kinase-I (CK-I) in response to ligand binding (Tamai et al., 2004; Zeng et al., 2005).

A study using in situ-mutagenesis described that megalin phosphorylation by GSK3 is critically dependent on a PPPSP motif and its function is related to the control of megalin recycling from the endosomes (Yuseff et al., 2007).

Ligands and tissues distribution

Megalin expression occurs mainly in the apical surface of absorptive or secretory epithelial cells. Firstly, megalin was described in parathyroid cells and tubule cells of the kidney (Juhlin et al., 1987), but its expression extends in a wide variety of adult tissues, namely mammary epithelia, thyroid follicular cells and the ciliary body of the eye (Lundgren et al., 1997). It is found in the intestinal brush border (Yammani et al., 2001), the male reproductive tract (Van Praet et al., 2003), uterus and oviduct (Argraves and Morales, 2004), gallbladder epithelium (Erranz et al., 2004), type II pneumocytes of the lung (Chatelet et al., 1986; Lundgren et al., 1997) and yolk sac (Lundgren et al., 1997).

In central nervous system (CNS), megalin was shown to be express in choroid plexus (Chun et al., 1999), ependymal cells of the lateral ventricles (Gajera et al., 2010), oligodendrocytes (Wicher et al., 2006), astrocytes (Bento-Abreu et al., 2008), and neurons, including retinal ganglion cells (Fitzgerald et al., 2007), cortical neurons

(Chung et al., 2008), cerebellar granule neurons (Ambjorn et al., 2008) and DRG neurons of the PNS (Fleming et al., 2009).

Megalin is known to interact with several ligands and some of them are shown in table I, including transthyretin (Sousa et al., 2000b).

Vitamin-binding proteins	**Other carrier proteins**
Transcobalamin–vitamin B12 Vitamin-D-binding protein Retinol-binding protein	Albumin Lactoferrin Haemoglobin Odorant-binding protein Transthyretin
Lipoproteins	**Hormones and hormone precursors**
Apolipoprotein B Apolipoprotein E Apolipoprotein J/clusterin Apolipoprotein H	Parathyroid hormone Insulin Epidermal growth factor Prolactin Thyroglobulin
Drugs and toxins	**Immune- and stress-response-related proteins**
Aminoglycosides Polymyxin B Aprotinin Trichosanthin	Immunoglobulin light chains PAP-1 β2-microglobulin
Enzymes and enzyme inhibitors	**Others**
PAI-1 PAI-1–urokinase PAI-1–tPA Pro-urokinase Lipoprotein lipase Plasminogen β-amylase β1-microglobulin Lysozyme	RAP Ca^{2+} Cytochrome c

Table I. Ligands that bind megalin. [Adapted from (Christensen and Birn, 2002)]

RAP binding to megalin

RAP is a 39-kDa protein located in the endoplasmic reticulum, that has been shown to bind megalin with high affinity (K_D =8nM) (Kounnas et al., 1992). Orland et al described for the first time that RAP binds to the second of the forth clusters of ligand-binding repeats of megalin (amino acids 1111–1210) (Orlando et al., 1997). However,

a more recent report suggests that megalin have additional binding site for RAP (McCarthy et al., 2002).

It was demonstrated that RAP inhibits premature binding of ligands to several of the ligand-binding motifs (Iadonato et al., 1993; Bu and Rennke, 1996) of the LDL-receptor family members, including megalin (Kounnas et al., 1992), and assists in the proper folding of the receptors (Bu and Rennke, 1996). In addition, it was proposed that RAP is involved in biosynthetic processing and trafficking of megalin, being crucial for its function (Kounnas et al., 1992; Birn et al., 2000).

The ability of RAP to inhibit binding of most ligands to the LDL-receptor family has made it an extraordinary tool for physiological and cellular studies.

Megalin deficient mice

Megalin knockout mice have a low survival rate (1/50), because during the perinatal phase they die due to cranial midline defects that include cleft palate and holoprosencephaly, and immediately after birth respiratory complications lead to death. Holoprosencephaly is a set of brain malformations that include forebrain fusion, a common ventricular system and lack of the olfactory bulb (Willnow et al., 1996). Studies in mice with megalin brain conditional KO show that this receptor is important to the development for the ventral telencephalon, since it exerts functions as a receptor for signaling proteins and morphogens such as Shh and Bone Morphogenetic Protein 4 (BMP4) (McCarthy et al., 2002; Spoelgen et al., 2005).

Surviving megalin deficient mice develop low molecular weight proteinuria (Leheste et al., 1999), since molecules cannot be reabsorbed by megalin in the renal proximal tubule. These viable and fertile mice also suffer insufficiency in plasma vitamins, hypocalcemia and severe bone disease, probably due to inability of 25-(OH) vitamin D3-DBP complex to be uptaken by megalin in the proximal tubule (Leheste et al., 2003). This clinical status evidence the crucial role of megalin in vitamin and calcium homeostasis in the renal proximal tubule (Christensen and Willnow, 1999). In 2001, an human case of possible deficiency in megalin, who suffered from holoprosencephaly, pulmonary insufficiency, absent circulating vitamin D metabolites, mild albuminuria and loss of vitamin D-binding protein in urine was reported (Muller et al., 2001).

There are two syndromes associated with mutated megalin, Donnai-Barrow and facio-oculo-acoustico-renal (FOAR) syndromes and both are characterized by agenesis of the corpus callosum, developmental delay, proteinuria, hearing loss and ocular abnormalities (Pober et al., 2009). Therefore, these defects reveal the importance of megalin during development in organs such as brain, eye, ear and kidney.

Megalin and the brain

The expression of megalin in CNS and the several ligands able to bind to this receptor was previously reported. Many studies have shown that some of these ligands have a potential role in neuronal survival and regeneration, revealing megalin as a likely mediator of CNS protection.

In Alzheimer's disease, Aβ peptide, produced by amyloidogenic processing of the amyloid precursor protein (APP), can form complexes with different megalin ligands such as clusterin/apoJ (Zlokovic et al., 1996; Hammad et al., 1997; Nuutinen et al., 2009) and apoE (Zlokovic et al., 1996; Bell et al., 2007), facilitating Aβ clearence. In addition, it was shown that the IGF-I receptor interacts with the transmembrane region of megalin, whereas the perimembrane domain of megalin is required for IGF-I internalization. Furthermore, internalization of IGF-I is increased with inhibition of a GSK3 site within the Src homology 3 domain of the C-terminal region of megalin, modeling Alzheimer amyloidosis (Bolos et al., 2010). Additionally, megalin also regulates the transport of leptin in the choroid plexus by transcytosis (Dietrich et al., 2008) and it is known that leptin levels are involved in the decreased activity of β-secretase or BACE (Marwarha et al., 2010), a limiting the step of Aβ formation.

Megalin levels are decreased with age and in AD patients (Odera et al., 2007; Dietrich et al., 2008), indicating that the neuroprotective function of this receptor is also reduced. In choroid plexus, megalin enhanced the Aβ clearance induced by IGF-I and is involved in IGF-I transport into the brain (Carro et al., 2005).

It has been shown that metallothionein (MT) binds to megalin and exerts several functions in the brain through this receptor. In retinal ganglion cells (RGC), MT binding to megalin promotes neurite extension and it was proposed by some authors that this effect is a result of signal pathways activation by the NPxY motifs of megalin's cytoplasmic tail (Fitzgerald et al., 2007). Both molecules, MT and EmtinB, have been able to bind megalin and LRP1 and induce neurite outgrowth and survival in cerebellar granule neurons cultures, by activation of extracellular signal-regulated kinase, protein kinase B, and cAMP response element binding protein (Ambjorn et al., 2008). Megalin mediates astrocytic metallothioneins transport into neurons, resulting in a regenerative action (Chung et al., 2008).

In PNS, the neurite outgrowth and nerve regeneration mediated by TTR is megalin-dependent (Fleming et al., 2009).

However, pathways involved in the neuroaction mediated by megalin and its ligands require further study.

Chapter II

Objectives

Objectives

The main goal of this research project is to study the effect of TTR in neuronal hippocampal cultures in physiological and ischemic pathological conditions. The molecular mechanisms involved in these effects will be also addressed. Therefore, we propose to:

- Search for the neuritogenic activity of TTR in the central nervous system
- Explore signaling pathways activated by TTR
- Evaluate if TTR has a neuroprotective role towards dendrites and/or axons
- Investigate if the neuritogenic and neuroprotective effects, as well the signaling pathways activated by TTR involve the LDL receptor family
- Clarify if the TTR neuritogenic and/or neuroprotective action are mediated by the megalin receptor

CHAPTER III

Material and methods

Materials and methods

Animals

Mice were handled according to European Union and National rules. Wild type (Wt), TTR knockout (TTR KO) (Episkopou et al., 1993) and $Meg^{+/-}$ TTR KO were used. $Meg^{+/-}$ TTR KO (129/Sv background) were obtained from the offspring of TTR KO and megalin heterozygous breeding pairs [$Meg^{+/-}$, kindly provided by Dr. Thomas Willnow, Max-Delbrueck Center for Molecular Medicine, Berlin, Germany]. All animals were maintained under a 12 hours light/dark cycle and fed with regular rodent's chow and tap water ad libitum. Genotypes were determined from tail extracted genomic DNA, using the primers: 5'-CAT-ATC-TTG-GAA-ATA-AAG-CGA-3' and 5'-GAC-CAT-TTG-GCA-GCC-AAG-G-3' for megalin gene; 5'-CAT-ATC-TTG-GAAATA-AAG-CGA-3' and 5'-GAT-TGG-GAA-GAC-AAT-AGC-AGG-CAT-3' for MC1neo cassette gene.

TTR production and purification

Recombinant mouse TTR was produced in a bacterial expression system using *Escherichia coli BL21* (Furuya et al., 1991) and purified as previously described (Almeida 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 et al., 1951).

GST-RAP expression and purification

Expression of the plasmid pGEX-2T with RAP cDNA fused with GST [kindly provided by Dr. Joaquin Herz, Department of Molecular Genetics, University of Texas, United States of America (Herz et al., 1991)] was induced by treating *an Escherichia coli BL21* culture in the exponential phase of growth (A600nm 0.8 –2) with 0.5mM isopropyl-D-thiogalactoside (BIORON) for 30 min at 30°C. To extract and purify the protein, with an apparent molecular mass of 62 kDa in a SDS PAGE gel, an affinity chromatography on glutathione Sepharose 4B (GE Healthcare) was used. Cleared bacterial extract was applied in the pre-rinsed column with PBS. After several washes, 5mM reduced glutathione in Tris-HCl, pH 8.0 was used to elute GST-RAP. In order to use the recombinant protein in neuronal cultures, a protocol for bacterial endotoxins removal was performed.

Endotoxin removal

To remove endotoxins from recombinant proteins, 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 and GST-RAP were individually applied to the column and incubated during 1 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).

Primary neuronal cultures

Primary cultures of mouse hippocampal neurons were prepared from the hippocampus of E17-E18 Wt and TTR KO mice embryos. The hippocampi were treated with trypsin (1.5mg/mL, 10 minutes at 37ºC) in Ca^{2+} and Mg^{2+} free HBSS (Hank's Balanced Salt Solution). After washes in HBSS supplemented with 10% FBS and HBSS only, cells were mechanically dissociated. Hippocampal cultures were maintained in serum-free Neurobasal medium supplemented with B27, glutamate (25 mM), glutamine (0.5mM) and gentamicin (0.12mg/ml). All culture media and supplements used were from GIBCO (Life Technologies, USA).

Cells were cultured at a density of 85 000 cells/cm^2 and 53 000 cells/cm^2 on poly-D-lysine-coated (Sigma, 150k-300k MW) six-well microplates (MW6) or glass coverslips. For Western blot and neuroprotection experiments a density 85 000 cells/cm^2 was used. Neurite outgrowth experiments were performed with less density (53 000 cell/cm^2) to allow a clear trace of the neurites of each neuron. Cells used to western blot and neuroprotection experiments were kept at 37ºC in a humidified incubator of 5% CO_2/95% air, for 7–8 days in vitro (DIV), the time required for maturation of hippocampal neurons (Brewer et al., 1993)

Neurite outgrowth assay

Hippocampal neurons from Wt, TTR KO and $Meg^{+/-}$ TTR KO embryos were cultured at a density of 53 000 cells/cm^2 on poly-D-lysine-coated (Sigma) glass coverslips. Recombinant mouse TTR (55 µg/ml or 300 µg/ml) with or without RAP (350 µg/mL) or K252a (200nM, Enzo Life Sciences) were added to cell culture medium immediately after plating. Cells were kept at 37ºC in a humidified incubator of 5% CO_2/95% air for 24hours. Cells were fixed with 4% paraformaldehyde/4% sucrose; immunofluorescence was performed, using as primary antibody anti-Map-2 (1:700, Abcam).

Neuroprotection assay

Hippocampal neurons from TTR KO and Meg$^{+/-}$ TTR KO embryos were cultured at a density of 90 000 cells/cm^2 and 80 000 cells/cm^2 on poly-D-lysine-coated (Sigma) six-well microplates(MW6) for Western blot or on glass coverslips, respectively and maintained at 37ºC in a humidified incubator of 5% CO_2/95% air for 7-8 DIV. Cultured hippocampal neurons were subjected to excitotoxic stimulation with glutamate (125 µM glutamate, 20 min) and further incubated in culture conditioned medium with recombinant mouse TTR (55µg/mL or 300µg/mL) during 14 hours after toxic stimulus for immunocytochemistry assay or only 4 hours for western blot analysis.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde/4% sucrose and permeabilized with 0.3% Triton X-100 in PBS. Neurons were then incubated with 5% BSA in PBS 0.1% Tween 20, for 1h at 37°C, to block nonspecific staining, and then incubated with primary antibody anti-Map-2 (1:700, Abcam), overnight at 4°C. Cells were then washed five times with PBS 0.1% Tween 0.5% BSA and incubated with secondary antibody, anti-rabbit Alexa Fluor 488 (1:1000, Life Technologies), for 1h at 37°C and protected from light. After washing, cells were stained with the fluorescent dye Hoeschst 33342 (0.5µg/mL) during 10 minutes at room temperature. Coverslips were mounted on glass slides with Dako fluorecescent mounting medium (Dako) and visualized by a Widefield Fluorescent Microscope (Zeiss Axio Imager Z1). Photos were taken randomly with the objective of 20x, in order to cover the whole coverslip and have isolated neurons.

Neurite measure analysis

Morphological measurements of neuronal neuritis were performed using the plugin NeuronJ from the ImageJ software (Meijering et al., 2004). Number, sum, minimum, mean and maximum length of neurites per cell were the analyzed parameters. At least 50 cells were counted in each condition of each experiment and the experiments were repeated at least 4 times.

Western blot

Hippocampal neuron cultures were washed twice with ice-cold PBS. Cells were then lysed with lysis buffer containing 20 mM MOPS, 2mM EGTA, 5mM EDTA, 30mM sodium fluoride, 60mM β-glycerophosphate, 20mM sodium pyrophosphate, 1mM sodium orthovanadate , 1% Triton X-100 and supplemented with 1mM DTT, 1mM PMSF and 1x protease inhibitors mixture (GE Healthcare).

After centrifugation at 16,100 x g for 10 min at 4°C, protein in the supernatants was quantified using the Bradford method, and samples were denatured with 4x concentrated denaturing buffer (227.8mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 4.4% SDS, 200mM DTT, 44.4% glycerol, 3mM sodium orthovanadate, and 0.02% bromophenol blue) and boiled for 5 minutes.

Protein samples were separated by SDS-PAGE in 7.5% or 12% polyacrylamide gels, except samples where megalin was the target, 6% tris-acetate gels were used. The proteins were transferred to a nitrocellulose Hybond-C membrane (GE Healthcare), using a wet system Mini Trans-Blot® Cell (BioRad).

Membranes were incubated 1 hour at room temperature in blocking buffer, 10% BSA in tris-buffered saline Tween-20 (TBS-T), and then incubated overnight a 4ºC with primary antibodies diluted in 5% BSA in TBS-T, namely rabbit polyclonal Erk1/2 (1:1000, Cell Signaling), p-Erk1/2 (Thr202/Tyr204) (1:1000, Cell Signaling), Akt (1:1000, Cell Signaling), p-Trk (Tyr490) (1:1000, Cell Signaling), Map-2 (1:1000, Abcam), mouse TTR antibody (produced against recombinant mouse TTR, 1:500), rabbit monoclonal p-CREB (Ser133) (1:1000, Cell Signaling), p-Akt (Ser473) (1:1000, Cell Signaling), p-Src family (Tyr416) (1:1000, Cell Signaling), TrkB (1:1000, Cell Signaling), mouse monoclonal Tau (1:1000, Cell Signaling), and mouse polyclonal α-tubulin (1:10000, Sigma). Membranes were then incubated with anti-rabbit IgG-HRP (1:5000; Binding Site) or anti-mouse IgG-HPR (1:2500; Binding Site), during 1 hour at room temperature. Blots were developed using Immun-Star WesternC Chemiluminescent kit (BioRad) and exposed to either ECL Hyperfilm (GE Healthcare) or ChemiDoc™ XRS+ System (BioRad) using Image Lab™ Software.

Quantitative analyses were performed using the Quantity one or Image Lab™ software (BioRad).

Conditioned medium concentration

Conditioned medium from a hippocampal neuron culture at density of 90 000 cells/cm^2 on poly-D-lysine-coated (Sigma) six-well microplates (MW6) was centrifuged at 16,100 x g for 10 min at 4°C and the supernatant was collected for a dialysis membrane with 6-8 K molecular weight cutoff (MWCO). The sample was dialyzed overnight at 4°C and lyophilized until it reached a volume of approximately 80µL. The lyophilized product was denatured with 4x concentrated denaturing buffer and incubated for 5 min at 95°C. The sample was separated by SDS-PAGE in 12% polyacrylamide and a western blot was performed with a primary antibody against mouse TTR (produced against recombinant mouse TTR, 1:500).

Immunoprecipitation

Hippocampal neuron cultures from TTR KO were washed twice with ice-cold PBS. The cells were then lysed with IPB buffer containing 20mM Tris (pH 7.0), 100mM NaCl, 2mM EDTA, 2mM EGTA, 50mM sodium fluoride, 60mM β-glycerophosphate, 20mM sodium pyrophosphate, 1mM sodium orthovanadate, 1% Triton X-100 and supplemented with 1mM PMSF and 1x protease inhibitors mixture (GE Healthcare).

After centrifugation at 16,100 x g for 10 min at 4°C, the supernatant was collected, pre-rinsed in IPB buffer protein-A sepharose beads (GE Healthcare) for 1h at 4°C and centrifuged again to collect the supernatant. The sample was incubated with 2µL of megalin antibody (Abcam) at 4°C overnight and then pre-rinsed in IPB buffer protein-A sepharose beads for 2h at 4°C. The resin with the immunoprecipitate was then washed 5 times with IPB buffer at 1,000 x g for 5 min, denatured with 4x concentrated denaturing buffer and incubated for 5 min at 95ºC. After centrifugation at 16,100 x g for 10 min, the supernatant was collected and separated in a 6% Tris-acetate gel and western blot performed using a primary antibody anti-megalin (1:1000, Abcam).

Statistical analysis

Quantitative data are presented as Mean ± SEM. Statistical analysis was carried out using Graphpad Prism 6 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. ****$p<0.0001$, ***$p < 0.001$, ** $p <0.01$, and * $p < 0.05$.

CHAPTER IV

Results

Results

Different cell morphology between genetic models

Cognitive performance studies showed that TTR null mice have impaired spatial learning and memory as compared to Wt animals (Sousa et al., 2007b; Brouillette and Quirion, 2008; Buxbaum et al., 2008). The difference between these strains was also observed in motor studies, wherein TTR KO mice displayed less immobility and increased activity in the forced swim and in the locomotor activity test, when compared to WT animals, suggesting that lack of TTR is associated with increased exploratory activity and reduced signs of depressive-like behavior (Sousa et al., 2004) .

These observations lead us to search for neuronal morphological changes between TTR KO and Wt mice, in serum free primary neuronal cultures.

In physiological conditions, after 1 day in vitro, hippocampal neurons from TTR KO mice exhibit similar neurite outgrowth as Wt mice. Nevertheless TTR KO mice seem to have a tendency for low number of neuritis (figure 1A), followed by significant increase in the minimum length parameter of the neurites (figure 1C).

In addition to these strains, another genetic model was included, a megalin deficient mice model with TTR null background, since megalin is a known receptor for TTR. Because megalin knockout mice are not viable due to problems associated with brain malformation, only heterozygotes for megalin were used.

We observed that hippocampal neuronal cultures from $Meg^{+/-}$ TTR KO display a significant reduction of sum length and neurites number when compared to TTR KO and Wt (figure 1A and 1B). However, $Meg^{+/-}$ TTR KO has a significant increase in the minimum length parameter of neurites in relation to TTR KO and Wt (figure 1C). These results suggest that cultures with less neurite number are followed by a bigger minimum length of the neurites, revealing a weaker neural network and less extensive neurite sprouting.

So, TTR absence in neuronal cultures, leads to a less developed neuronal network, that is even more acute when there is also a megalin deficiency.

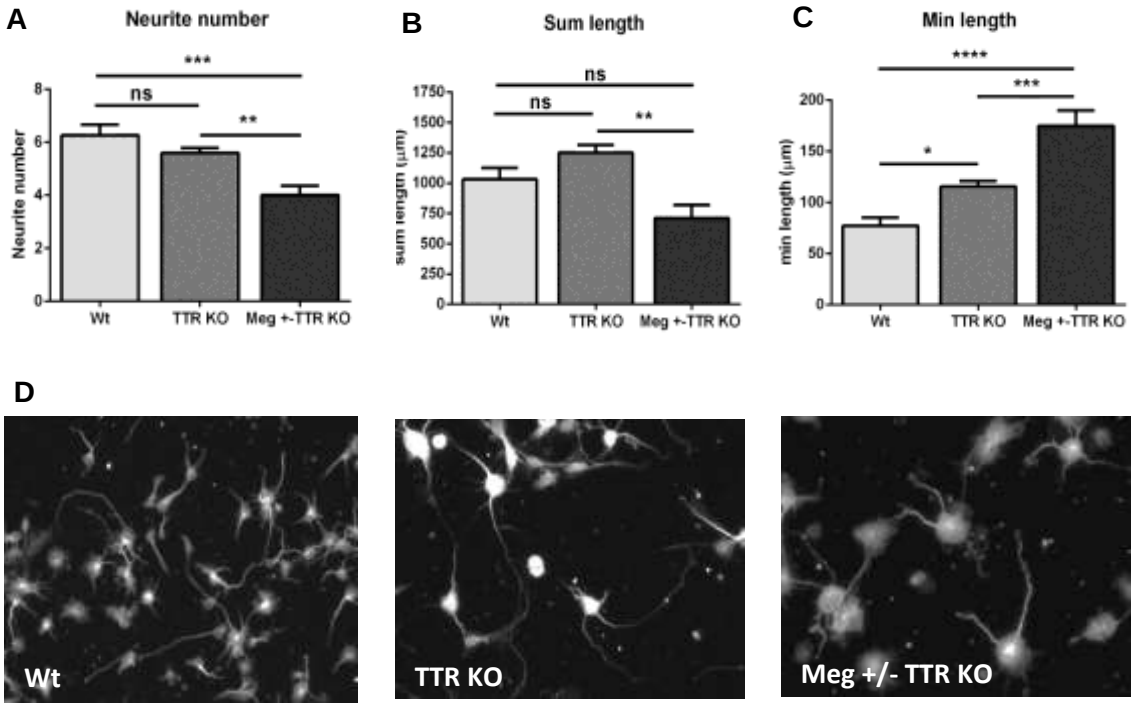

Figure 1. Different cell morphology between genetic models. **A,** Neurite number, **B,** sum length and **C,** minimum length of Wt, TTR KO and Meg$^{+/-}$ TTR KO hippocampal neuronal cultures in physiological conditions (24h in vitro). **D,** Representative images of Wt, TTR KO and Meg$^{+/-}$ TTR KO hippocampal neuronal cultures in physiological conditions. Data represents the means +- SEM of five independent experiments. ns, not significant; *p< 0.05 ; **p<0.01; ***p< 0.001; ****p<0.0001 in one-way ANOVA, with Bonferroni's post test.

TTR is not synthetized by hippocampal neuronal cultures

Since the presence of TTR has been reported in diverse brain areas, such as cortex, hippocampus or cerebellum (Carro et al., 2002; Stein and Johnson, 2002; Buxbaum et al., 2008; Li et al., 2011), several authors proposed that these tissues synthetized TTR. Although other authors shown these to be false positive results by contamination from adjacent choroid plexus cells and meninges (Sousa et al., 2007a), as TTR is absent in the brain parenchyma.

To address the difference of observed phenotype in physiological conditions between TTR KO and Wt cultures, we hypothesized that Wt hippocampal neurons cultures could produce TTR. To evaluate this hypothesis, we checked whether hippocampal cultures in the absence of serum have endogenous TTR, either intracellularly or in the conditioned medium. So, cells lysates with increasing concentrations and concentrated conditioned medium were assayed by western blot for TTR. In the tested conditions, the TTR presence was not observed (figure 2) and recombinant TTR and serum mouse and human were used as positive control.

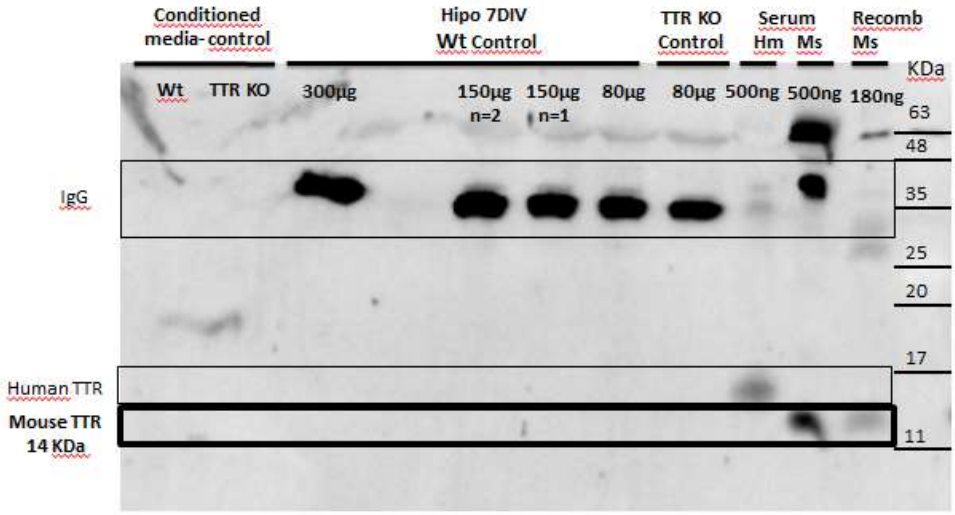

Figure 2. TTR in not synthesized in cultured hippocampal neurons. Western blot image of conditioned media and cells lysates from TTR KO and Wt hippocampal neurons (7-8 DIV) with increasing concentrations. Recombinant TTR and serum of mouse were used as positive controls and serum of human as negative control.

Transthyretin promotes neurite outgrowth in hippocampal neurons cultures

Transthyretin neuritogenic activity was previously demonstrated on PC12 cells and dorsal root ganglion (DRG) of PNS (Fleming et al., 2007). Taken these findings and the fact that TTR can also have an important role in neuroprotection of CNS, the study of TTR neuritogenic effect in hippocampal neurons was addressed.

Primary cultures of hippocampal neurons from TTR KO were prepared and after plating, recombinant mouse TTR at 55µg/mL and 300µg/mL were added to the culture conditioned medium. After 24 hours, cells were fixed and several neurite outgrowth parameters were measured.

We verified that in both concentrations, TTR increases total neurite length and neurite number, in relation to the control situation (figure 3). Therefore, TTR seems to induce neurite outgrowth, contributing to the development of the neuronal network, corroborating previous findings (figure 1) of neuronal morphological analyses from Wt and TTR KO animals.

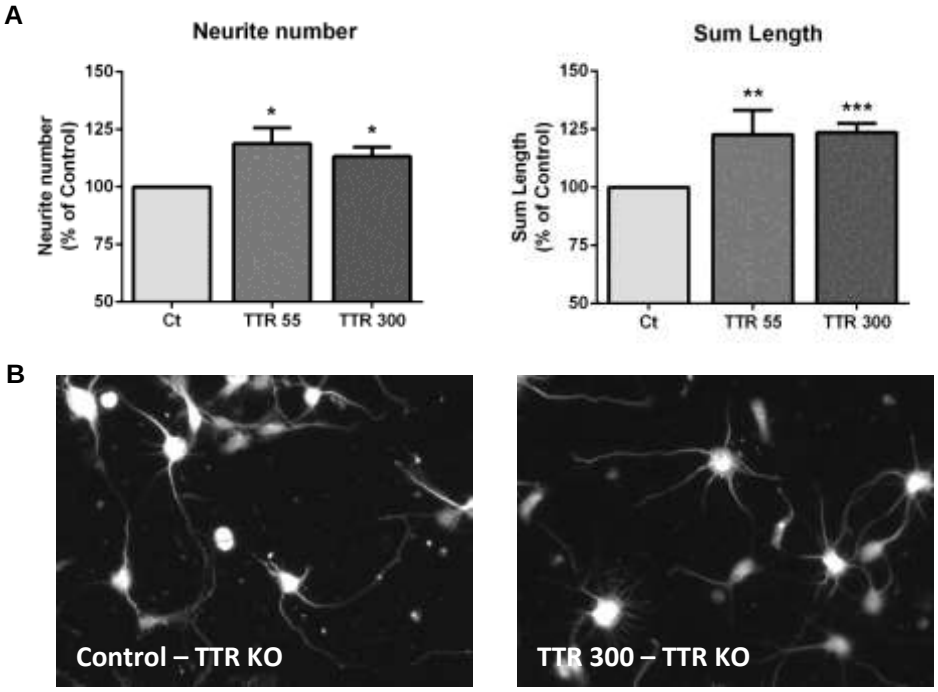

Figure 3. TTR promotes neurite outgrowth in hippocampal neurons. A, neurite number and sum length of TTR KO hippocampal neurons in the presence or absence of TTR at 55µg/mL and 300µg/mL, for 24h. . **B,** Representative images of TTR KO hippocampal neurons in the presence or absence of TTR at 300µg/mL . Data represents the means +- SEM of four independent experiments. ns, not significant; *p< 0.05 ; **p <0.01; ***p< 0.001 in one-way ANOVA, with Bonferroni's post test.

RAP blocks TTR induced neurite growth

Despite the fact that TTR does not have any specific described receptor, studies report that TTR has been shown to interact with different cellular receptors like an unidentified receptor associated protein (RAP)-sensitive receptor in hepatomas (Sousa and Saraiva, 2001) and megalin in renal proximal tubule epithelial cells (Sousa et al., 2000b) and DRG neurons (Fleming et al., 2009).

To understand if neurite outgrowth promoted by TTR is mediated by LDL-receptor family proteins, TTR KO hippocampal neurons were co-incubated with TTR and RAP at 350µg/mL for 24 hours. In this condition, neurite outgrowth promoted by TTR was abolished (figure 4), as reflected in the decrease of neurite number and sum length. Accordingly, this result demonstrates that TTR exerts its neuritogenic activity in hippocampal neuronal cultures through the LDL-receptor family.

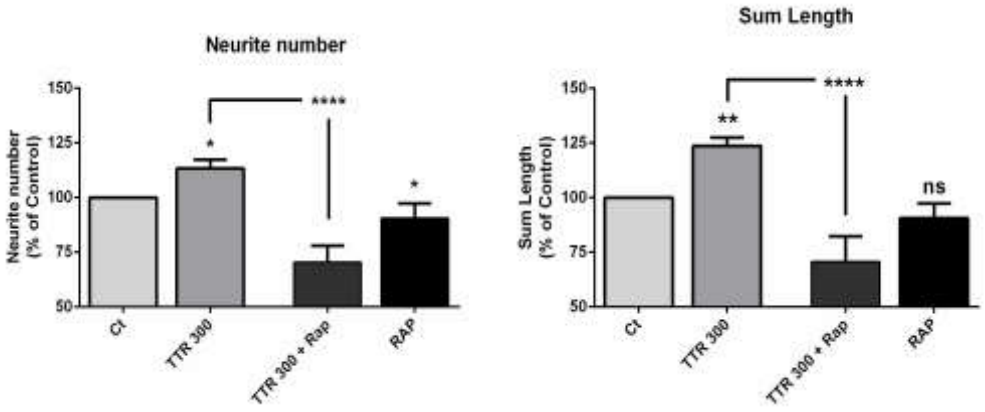

Figure 4. RAP blocks neurite growth promoted by TTR in hippocampal neurons. Neurite number and sum length of TTR KO hippocampal neurons in the presence or absence of TTR 300μg/mL and TTR + RAP for 24h. Data represents the means +- SEM of four independent experiments. ns, not significant; *p< 0.05 ; **p <0.01; ****p< 0.0001 in one-way ANOVA, with Bonferroni's post test.

Megalin is expressed in hippocampal neuronal cultures and is essential to TTR neuritogenic activity

In the literature, megalin, a member of the LDL receptor family and one that binds the receptor-associated protein (RAP), has been identified as a endocytic TTR receptor (Sousa et al., 2000b) and associated with enhancement of neurite size promoted by TTR in DRG neurons (Fleming et al., 2009). Furthermore, in the nervous system, megalin has been described as an important protein for the development of the forebrain (Willnow et al., 1996; Spoelgen et al., 2005) and spinal cord (Wicher and Aldskogius, 2008).

After these evidences, megalin standout as a possible receptor mediating the neurite outgrowth, promoted by TTR. To explore this question, we used hippocampal neuronal cultures from TTR null and megalin heterozygous mice. Neurons from megalin knockout mice are not viable, since these embryos have extensive phenotypic changes, due to progressive neuronal cell death after day 9.5. This phenotype suggests that megalin is required for the normal viability and development of the neuroepithelium (Willnow et al., 1996).

To confirm that hippocampal neurons from TTR KO express megalin, western blot and immunoprecipitation experiments, using an anti-megalin antibody, were performed. As shown in Figure 5, in both methodologies, a band appears at approximately 600 kDa that corresponds to megalin. However, the band presence is more evident by immunoprecipitation method, as expected.

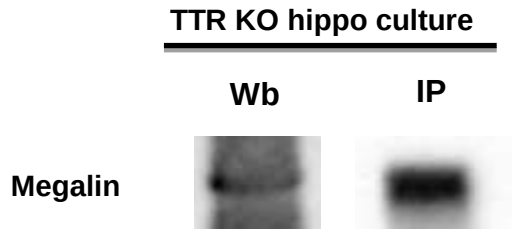

Figure 5. Hippocampal neuronal cultures from TTR KO mice express megalin. Western blot (Wb) and immunoprecipitation (IP) image immunostained against megalin from TTR KO hippocampal neuronal cultures.

When Meg$^{+/-}$ TTR KO hippocampal neurons were incubated with TTR at 55µg/mL and 300µg/mL during 24 hours, improvement of neurite number and size, seen on TTR KO neuronal cultures, was no longer observed in both concentrations (Figure 6A), showing that TTR activity is megalin-dependent.

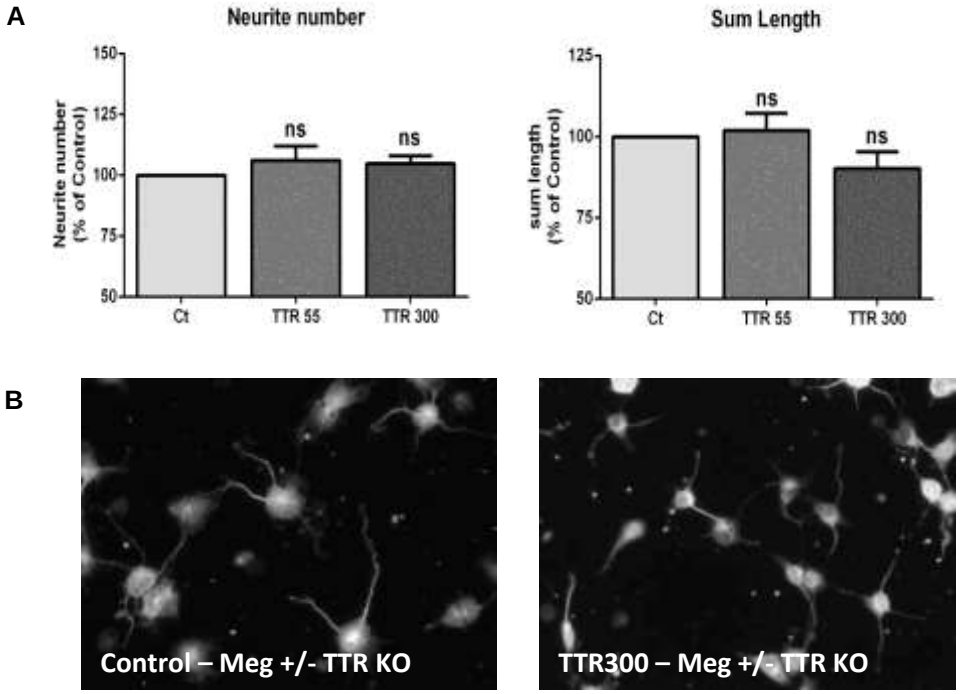

Figure 6. Neurite outgrowth promoted by TTR is megalin-dependent. A, neurite number and sum length of Meg$^{+/-}$ TTR KO hippocampal neurons in absence/presence of TTR at 55µg/mL and 300µg/mL for 24h. **B,** Representative images of Meg$^{+/-}$ TTR KO hippocampal neurons in the absence and presence of TTR at 300µg/mL . Data represents the means +- SEM of four independent experiments. ns, not significant in one-way ANOVA, with Bonferroni's post test.

TTR promotes neuroprotection through megalin

TTR also demonstrated to have a neuroprotective effect in pathological conditions, since TTR can accelerate and enhance nerve regeneration when the sciatic nerve is submitted to injury (Fleming et al., 2007) and TTR null mice have bigger infarct area than wild type mice (Santos et al., 2010) in a model of ischemic stroke.

So, to study whether TTR can be neuroprotective in in-vitro ischemic conditions we simulated cultures hippocampal neurons from KO TTR mice with 125µM glutamate, during 20 minutes. This excitotoxic stimulus leads to an apoptotic neuronal death of about 40% of the neurons, mimicking the penumbra in stroke. After the toxic stimulus, TTR KO hippocampal neurons were incubated with TTR at 300µg/mL during 14 hours. Immunocytochemistry was performed with anti-Map-2 antibody. We observed a clear rise in the number of neuritis and its total length (figure 7A), indicating that TTR administration after excitotoxic stimulus was neuroprotective since more neurites were preserved and/or grown back.

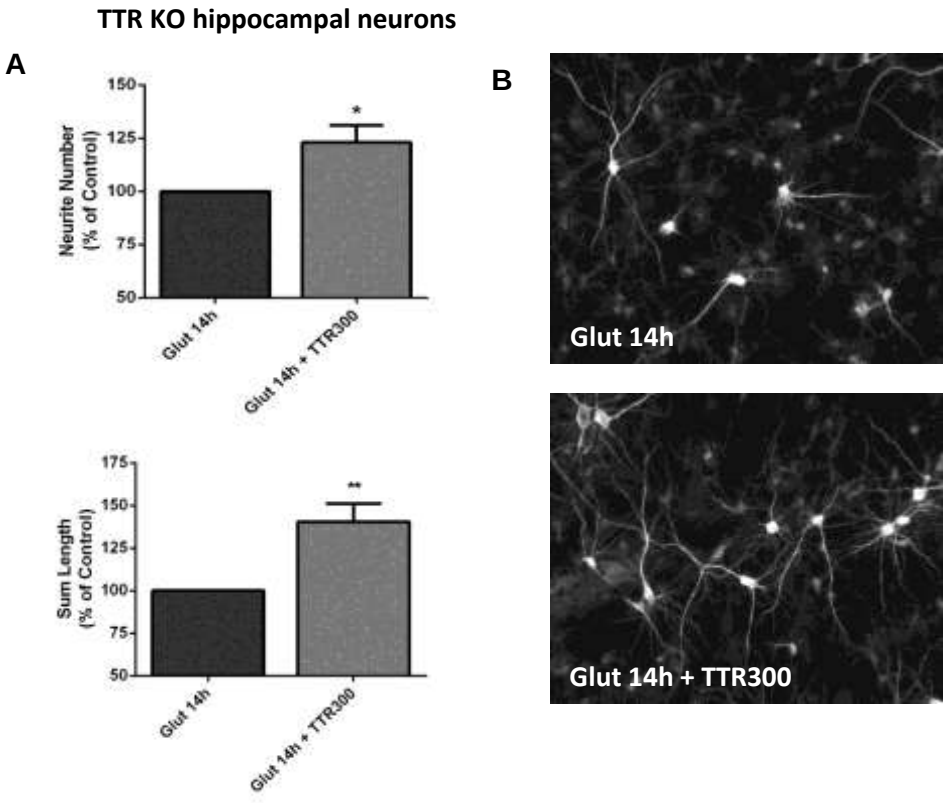

Figure 7. TTR promotes neuroprotection. A, Neurite number and sum length and **B,** its respective representative images of TTR KO hippocampal neurons (7-8 DIV) incubated with TTR at 300µg/mL for 14hours after 125µM glutamate stimulus during 20min. Data represents the means +- SEM of four independent experiments. ns, not significant; *p< 0.05 ; **p <0.01 in Student's t test.

In physiological conditions, megalin has been shown as a receptor responsible for neuritogenic activity of TTR in PNS and in hippocampus (see above). To understand if the neuroprotective activity promoted by TTR was also mediated through megalin receptor, $Meg^{+/-}$ TTR KO primary neuronal cultures were used. When neurons were incubated with TTR during 14 hours after a toxic glutamate stimulus, the increase of both neurite number and sum length parameters promoted by TTR were not observed (figure 8A). Therefore, this result shows that neuroprotection performed by TTR is megalin-dependent.

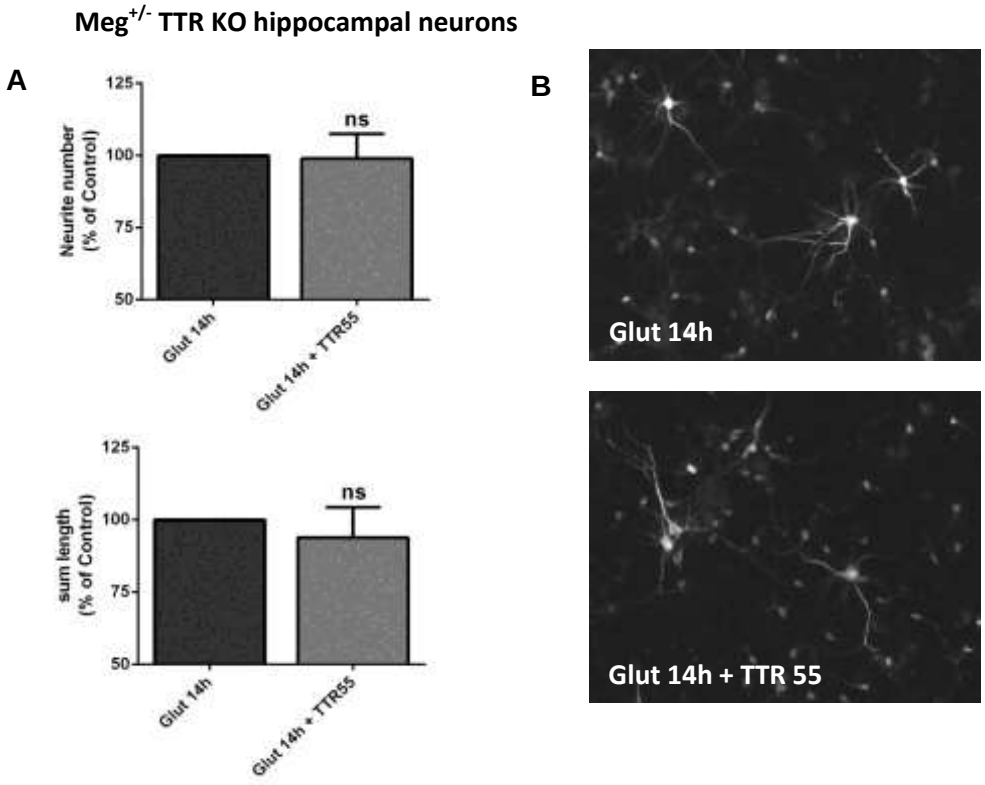

Figure 8. TTR promotes neuroprotection through megalin. A, Neurite number and sum length and **B,** its respective representative images of $Meg^{+/-}$ TTR KO hippocampal neurons incubated with TTR at 300µg/mL for 14hours after 125µM glutamate stimulus during 20min. Data represents the means +- SEM of five independent experiments. ns, not significant in Student's t test.

TTR protects dendrites in hippocampal neurons

To describe if this neuroprotective action is performed over dendrites and/or axons, western blot from TTR KO neurons incubated with TTR at 55µg/mL during 4 hours after glutamate stimulus were performed. Using antibodies against Map-2 (dendritic marker) and Tau (axonal marker), we saw that when TTR was added after glutamate stimulus, Map-2 levels were enhanced, comparatively to the glutamate condition (figure 9A), but Tau levels were not affected (figure 9B). These results

confirm the neuroprotective action observed by immunocytochemistry; furthermore, they show that this effect is specifically over dendritic extensions and not axons.

In addition, since in physiological conditions TTR KO and Wt neuronal cultures exhibit phenotypic differences, changes between the cultures in pathological conditions were also addressed. TTR KO neurons incubated with its conditioned medium during 14 hours after glutamate stimulus showed to have lower Map-2 and Tau levels than Wt (figure 10). This difference reveals that TTR KO surviving neurons (after toxic stimulus) have an impaired recovery and/or a weak neuronal network, comparatively to Wt cultures.

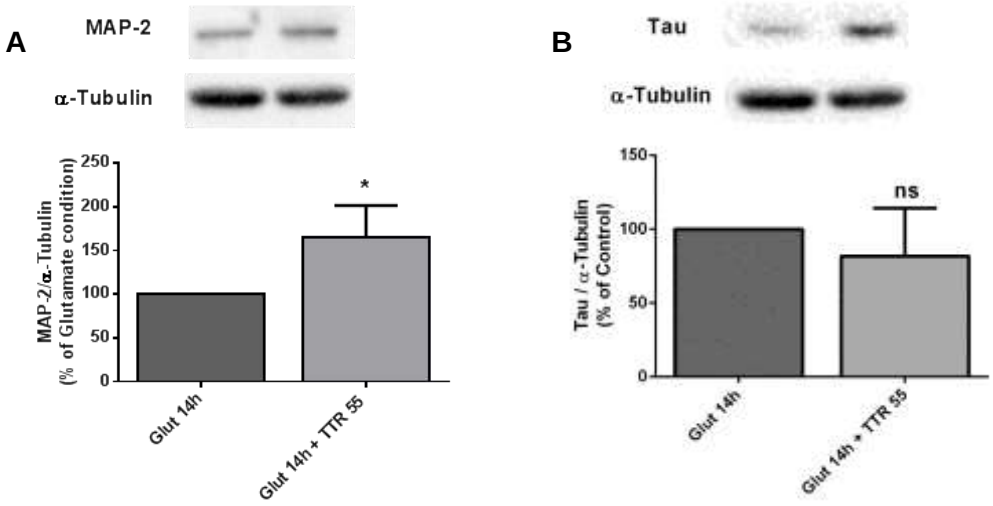

Figure 9. Neuroprotective activity of TTR is performed over dendrites. Western blot quantification of **A,** Map-2 and **B,** Tau from TTR KO hippocampal neurons incubated with TTR at 300µg/mL for 14hours after 125µM glutamate stimulus during 20min. Data represents the means +- SEM of four independent experiments. ns, not significant; *p<0.05 in one-way ANOVA, with in Student's t test.

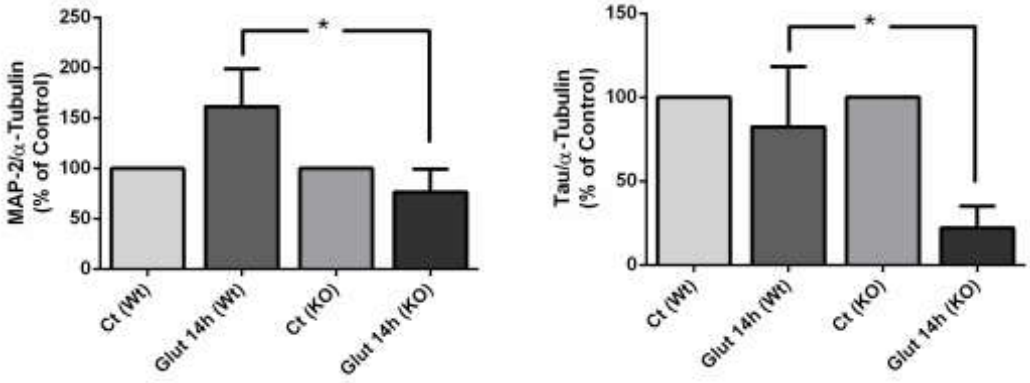

Figure 10. Different levels of Map-2 and Tau between Wt and TTR KO neurons. Western blot quantification of Map-2 and Tau levels from Wt and TTR KO hippocampal neurons in the presence or absence of glutamate stimulus. Data represents the means +- SEM of five independent experiments. *p<0.05 in one-way ANOVA, with Bonferroni's post test.

TTR induces Erk1/2, Akt and CREB phosphorylation through LDL-receptors

Since TTR leads to a change in the neurite phenotype of neurons, it should be activating a series of signaling pathways. So, the signaling pathways involved in neuritogenic action like as Erk1/2 (Perron and Bixby, 1999), Akt (Read and Gorman, 2009) and CREB (Redmond et al., 2002) were investigated. It was observed that TTR stimulus at the concentration 55µg/mL in TTR KO neurons with 7-8 DIV induce a statistically significant increase of Erk1/2 and CREB phosphorylation at 30minutes (Figure 11A and 11B). Besides this, it was also observed that p-Akt was upregulated in the same conditions (Figure 11C).

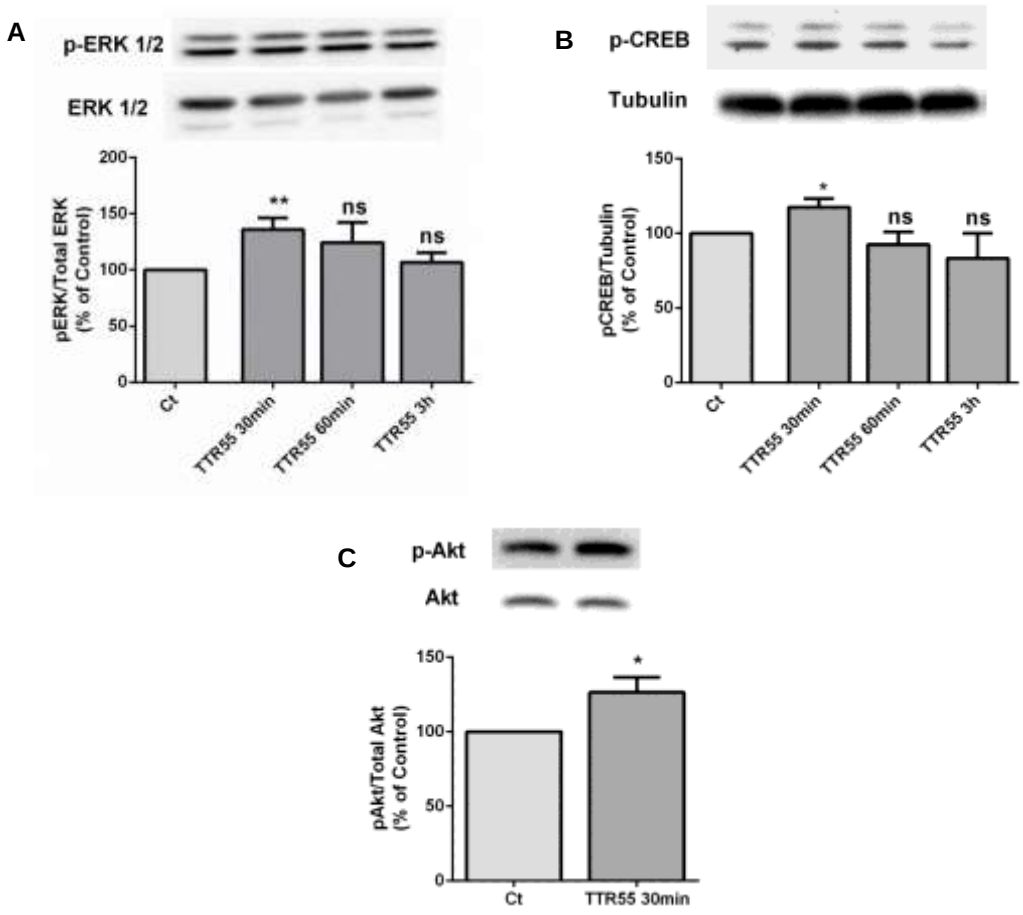

Figure 11. TTR activates p-Erk1/2, p-Akt and p-CREB in TTR KO hippocampal neurons. Western blot quantification of **A,** p-Erk1/2; **B,** p-CREB and **C,** p-Akt from TTR KO hippocampal neurons (7-8 DIV) incubated with TTR at 55µg/mL during 30min, 60min and 3hours. Data represents the means +- SEM of five independent experiments. ns, not significant; *p< 0.05 ; **p <0.01; in one-way ANOVA, with Bonferroni's post test or in Student's t test.

To understand if the activation of these pathways by TTR stimulation is mediated through LDL receptor family, TTR KO neurons were co-incubated with TTR at 300µg/mL and RAP (LDL-receptor family inhibitor) at 350µg/mL for 30 minutes. In this condition, the Erk1/2 and CREB activation by phosphorylation was blocked (figure 12A and 12B), suggesting that TTR neuritogenic activity may occur through the megalin receptor, a member of LDL-receptor family.

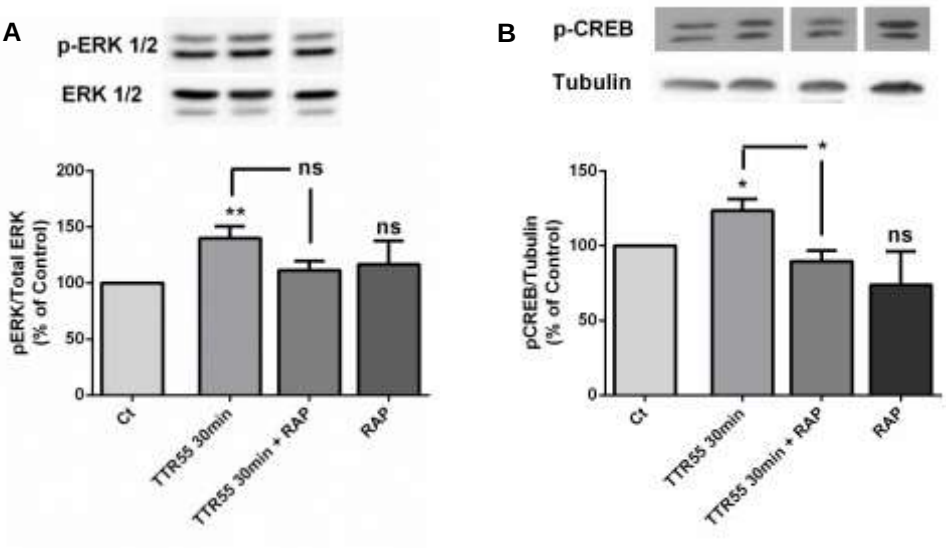

Figure 12. TTR activates p-Erk1/2 and p-CREB through LDL-receptors. Western blot quantification of **A,** p-Erk1/2 and **B,** p-CREB from TTR KO hippocampal neurons in the presence of TTR and TTR + RAP for 30 minutes. Data represents the means +- SEM of four independent experiments. ns, not significant; *p< 0.05 ; **p <0.01; in one-way ANOVA, with Bonferroni's post test

Trk transactivation by Src-family mediated by TTR

In PC12 cells, α-2-macroglobulin binding to LRP1, other member of LDL-family receptor, was shown to transactivate Trk receptors and promote neurite outgrowth (Shi et al., 2009).

Consequently, we tested the hypothesis of neurite outgrowth triggered by TTR, through the megalin receptor, could follow the same mechanism action. A western blot experiment showed that TTR at 55µg/mL stimulus activates Trk receptors by phosphorylation, preferentially at 3 hours in TTR KO hippocampal neuronal cultures (figure 13A).

However, to clarify if neurite outgrowth promoted by TTR is Trk pathway-dependent, primary neurons were treated with TTR at 300µg/mL and K252a (Trk inhibitor) at 200nM. Apart from Trk inhibitor, K252a is also a potent inhibitor of other protein kinases including Protein kinase A (PKA), Protein kinase C (PKC) and Protein

kinase G (PKG) (Kase et al., 1987). K252a inhibited the sum length and the maximum length parameter of the neurites induced by TTR stimulus (figure 14). However, with this concentration, the inhibitor alone could also significantly inhibit the neurite extension (sum and maximum length) compared to control. So, no clear result can yet be taken.

Since in PC12 cells, Trk transactivation is Src family kinase-dependent pathway (Shi et al., 2009), we considered pertinent to search the effect of TTR in these kinases. We found that the phospho-Src family was significantly activated by TTR at 3 hours (figure 13B), like it was observed for the Trk receptor

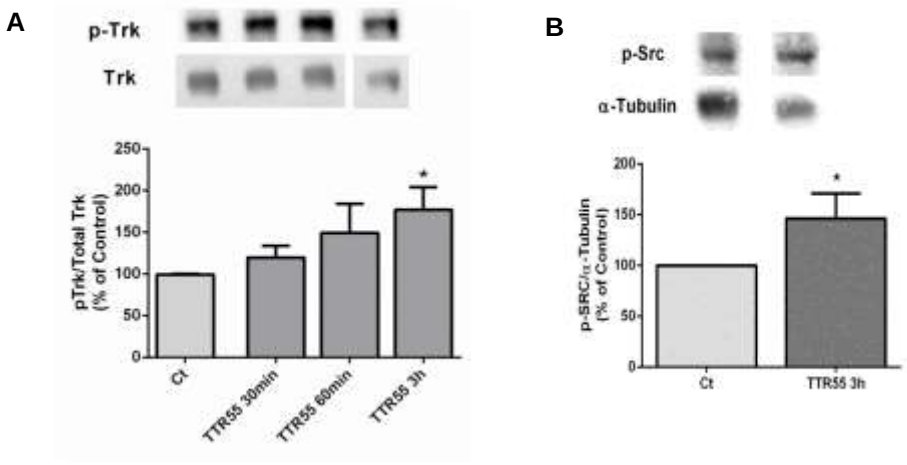

Figure 13. TTR activates p-Trk and p-Src. Western blot quantification of **A,** p-Trk and **B,** p-Src from TTR KO hippocampal neurons in the presence or absence of TTR at 55µg/mL during 30 min, 60min and 3hours**.** Data represents the means +- SEM of four independent experiments. *p< 0.05 in one-way ANOVA, with Bonferroni's post test or in Student's t test.

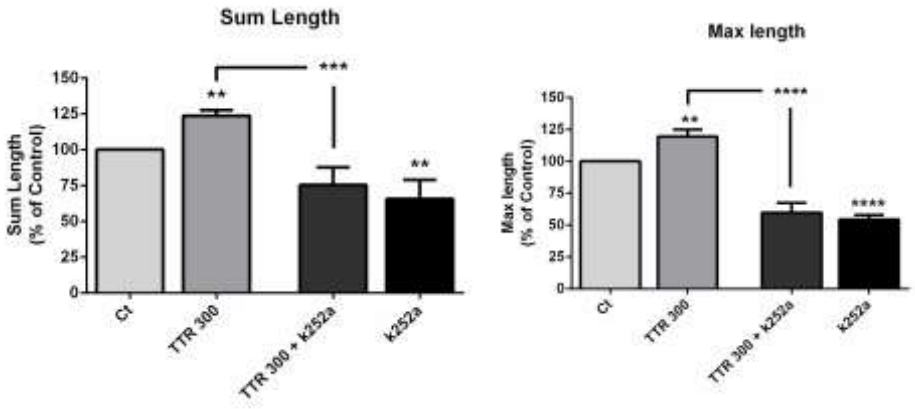

Figure 14. The effect of Trk receptor inhibitor (K252a) in the neurite outgrowth promoted by TTR. Sum length and maximum length of TTR KO hippocampal neurons incubated with TTR at 300µg/mL and TTR + K252a for 30 minutes. Data represents the means +- SEM of three independent experiments. **p <0.01; ***p<0.001; ****p<0.0001 in one-way ANOVA, with Bonferroni's post test

CHAPTER V

Discussion and Perspectives

Discussion and Perspectives

In this work, we show for the first time that TTR promotes neurite outgrowth in hippocampal neurons by activating several signaling pathways through the megalin receptor, a member of LDL-receptor family. In the literature, TTR neuritogenic activity has been reported in DRG neurons from the peripheral nervous system (PNS) and megalin was also suggested as the receptor mediating this action (Fleming et al., 2009).

Since our focus is the role of TTR as a neuroprotective protein in cerebral ischemia, hippocampal neuronal cultures from different genetic models were used, namely wild type (Wt), TTR KO and Meg$^{+/-}$ TTR KO mice. We observed differences in cell morphology in these several genetic models, since TTR knockout mice exhibit a weak neural network as compared to Wt neurons (figure 1A and 1C). In the literature, several studies have shown that Wt and TTR KO mice present some differences, such as motor (Sousa et al., 2004) and cognitive performance (Sousa et al., 2007b; Brouillette and Quirion, 2008; Buxbaum et al., 2008) and nerve regeneration (Fleming et al., 2007).

Some authors have claimed that TTR can be synthesized by neurons (Carro et al., 2002; Stein and Johnson, 2002; Buxbaum et al., 2008; Li et al., 2011), these could explain the phenotypic differences in physiological conditions observed in TTR KO mice, compared to Wt; however we observed that TTR is not produced by hippocampal neuronal cultures (figure 2). Therefore, the endogenous TTR could not explain the results observed. Meg$^{+/-}$ TTR KO showed to have an even weaker neuronal network compared to the TTR KO mice (figure1), so there seems to be a synergetic effect between TTR absence and megalin deficiency. We can speculate that TTR KO mice could also have different megalin expression levels compared to Wt mice. Therefore, as future experiments, the study of the megalin expression levels in the different cultures will be pertinent as it can possibly explain the diverse cell morphology. It will be also interesting to verify if TTR regulates megalin expression.

When cultured neurons were stimulated with TTR in physiologic conditions, there was a clear enhancement of neurite outgrowth in hippocampal neurons (figure 3). This result is in accordance with the observed in DRG neurons, from the PNS (Fleming et al., 2009). In addition, the literature reports non-identified secretory factors from choroid plexus epithelial cells promoted neurite outgrowth in hippocampal neurons (Watanabe et al., 2005), which is in agreement with our finding since the choroid plexus is a major source of TTR in brain (Aleshire et al., 1983).

Moreover, neurite outgrowth induced by a TTR stimulus is blocked in the presence of a LRP-family protein inhibitor (RAP) (figure 4), indicating that LRP receptors are mediators. To clarify if megalin is the member of the LRP receptor family responsible for TTR action, we used neurons from $Meg^{+/-}$ TTR KO embryos, and we verified that the neuritogenic effect was also abolished (figure 6). So, we clarified that neurite outgrowth induced by TTR is megalin-dependent in the hippocampus.

In the nervous system, megalin has been described as an important protein for the development of the forebrain (Willnow et al., 1996; Spoelgen et al., 2005) and spinal cord (Wicher and Aldskogius, 2008). In the last years, it was discovered that megalin is not only expressed in epithelium cells as described initially, but is also expressed in other cell types, namely oligodendrocytes (Wicher et al., 2006), astrocytes (Bento-Abreu et al., 2008), and neurons, including retinal ganglion cells (Fitzgerald et al., 2007), cortical neurons (Chung et al., 2008), cerebellar granule neurons (Ambjorn et al., 2008) and DRG neurons (Fleming et al., 2009). We now describe that hippocampal neurons also express megalin (figure 5) and that the neuritogenic function of TTR depend on this receptor.

Regarding the role of TTR in pathological conditions, TTR levels increase following traumatic brain injury in mouse hippocampus (Long et al., 2003), after transient focal cerebral ischemia in CNS (Suzuyama et al., 2004), in patients with severe closed head injury (Young et al., 1996) and it is secreted in the urine of stroke-prone rats (Sironi et al., 2001). Previous work on TTR null mice indicated that in conditions of nerve injury, TTR deletion delays nerve regeneration (Fleming et al., 2007), and using a permanent middle cerebral artery occlusion (pMCAO), TTR null mice and heterozygous for the heatshock transcription factor 1 ($TTR^{-/-}HSF1^{+/-}$ mice) showed a significant increase in cortical infarction, cerebral edema and the microglial-leukocyte response compared with $TTR^{+/+}HSF1^{+/-}$mice. Moreover, silencing of TTR synthesis in the liver by RNAi had no effect on TTR distribution in the infarct, indicating that the observed TTR infiltration derived from CSF and not from the serum. (Santos et al., 2010).

A clinical study confirms these results, showing that serum pre-albumin (transthyretin) levels were significantly lower in young acute ischemic stroke patients than normal control groups and that elevated levels of serum pre-albumin are indicative of a good prognosis in cerebral infarction (Gao et al., 2011).

These findings raise the putative neuroprotective role of TTR and so we used an in-vitro ischemic model (excitotoxic insult over hippocampal neurons) to try to dissect the molecular basis of this neuroprotection. In cultured neurons from TTR null mice, transthyretin reveals neurite protection through total length and neurite number

following an excitotoxic insult (figure 7) and this effect is mediated by megalin, since the neuroprotection of TTR is abolished in TTR null mice megalin deficient (figure 8).

Additionally, we show that transthyretin exerts its neuroprotection on dendrites and not in axons (figure 9). These results point that TTR preserves some of the neuronal functionality, after an excitotoxic insult, played by the neuronal dendrites. Berliocchi et al have shown that there are different degenerative programs in the cell body and in neurites (Berliocchi et al., 2005), so it would be interesting to study if the neuroprotection promoted by TTR also protects the cell body. Nevertheless, the best neuroprotective strategy is the one that preserves the functional neurons and not just the one that keeps the cell body alive.

Once again, TTR KO mice exhibited differences as compared to Wt, since Map-2 and Tau levels were lower in TTR KO neurons after endotoxic conditions (figure 10). This difference corroborates the weak neuronal network of TTR KO neurons observed in physiological conditions (figure 1A and 1C) and/or reveals impaired recovery comparatively to Wt cultures.

To promote the phenotypic changes observed in hippocampal neurons, TTR has to activate signaling pathways. In literature, several signaling pathways have been associated with neuritogenic action, such as Erk1/2 (Perron and Bixby, 1999), Akt (Read and Gorman, 2009) and CREB (Redmond et al., 2002). We show that TTR upregulates the levels of p-Erk1/2, p-Akt and p-CREB (figure 11), indicating that these will be the signaling pathways probably involved in neurite enhancement. We also observed that activation of Erk1/2 and CREB is mediated by LDL-receptors (figure 12). These pathways also mediate the neuritogenic and neuroprotective action promoted by α2-macroglobulin through LRP-1, another member of LDL-receptor family (Yamauchi et al., 2013). In other cases, the molecular mechanisms involved for neurite outgrowth are also associated to neuroprotection (Ditlevsen et al., 2007; Liu et al., 2014) . For this reason, signaling molecules activated through TTR stimulus could be responsible for the neuritogenic activity, but also to the neuroprotective properties. However, to verify the possible involvement of these pathways in neuroprotection, the study of TTR signaling pathways activated after ischemic conditions is required.

It is described in the literature that Trk receptors when activated by neurotrophic factors are responsible for inducing downstream pathways as p-MAPK and CREB, and consequently, promoting neurite outgrowth, nerve regeneration and cell survival (Heumann, 1994; McAllister et al., 1999). In primary cortical neurons was shown that Fyn tyrosine kinase, a member of Src family, is associated with TrkB (Iwasaki et al., 1998) and it was already reported to be involved in neurite outgrowth (Beggs et al., 1994). In addition, studies showed that Reelin induces dendrite outgrowth through a

lipoprotein receptor-Dab1 signaling pathway (Niu et al., 2004), which is known to activate a nonreceptor tyrosine kinase of the src family (Arnaud et al., 2003; Bock and Herz, 2003).

We observed that TTR stimulus promotes Trk and Src phosphorylation (figure 13), but the hypothesis that TTR binding to megalin transactivates Trk receptors by a Src family kinase-dependent, like Yang Shi et al proposed to LRP1 with its ligands (Shi et al., 2009) was not clarified. In the future, to verify the transactivation of Trk receptors by Src-kinase family, Src inhibitors should be used, to see whether TTR can still activate or not the Trk receptor.

Interestingly, it was described that calcium influx actives Src and Ras and, consequently, Map-kinases resulting in the neurite growth in PC12 cells (Rusanescu et al., 1995). The megalin contains potentially functional motifs including several Src-homology recognition motifs in the cytoplasmic tail (Songyang et al., 1993; Yu et al., 1994). More recently, it was reported that α-2-macroglobulin binding to LRP mediates neurite outgrowth through the effects on intracellular calcium homeostasis and p44/42 MAP kinase activation, leading to the effects on CREB transcription regulation (Qiu et al., 2004).

As mentioned above, we saw that TTR can induce these downstream pathways through the megalin receptor. Given the evidences, another interesting approach study besides the transactivation hypothesis is whether this response is followed by a change of intracellular Ca^{2+} (figure 15). For that, the use of camaleon calcium sensitive probes (Horikawa et al., 2010), through a FRET assay, could be an interesting way to observe changes in intracellular Ca^{2+} after a TTR stimulus. This will also allow to see the kinetics and the place where it occurs (dendrites, axons and/or cell body).

In conclusion, hippocampal neuronal cultures from TTR KO and double TTR and megalin deficient mice exhibit different cell morphology as compared to wild type neurons. Neuritogenic and neuroprotective effects of TTR are megalin-dependent and involve Erk 1/2, Akt and CREB, signaling pathways possibly through a Src/TrK transactivation mechanism.

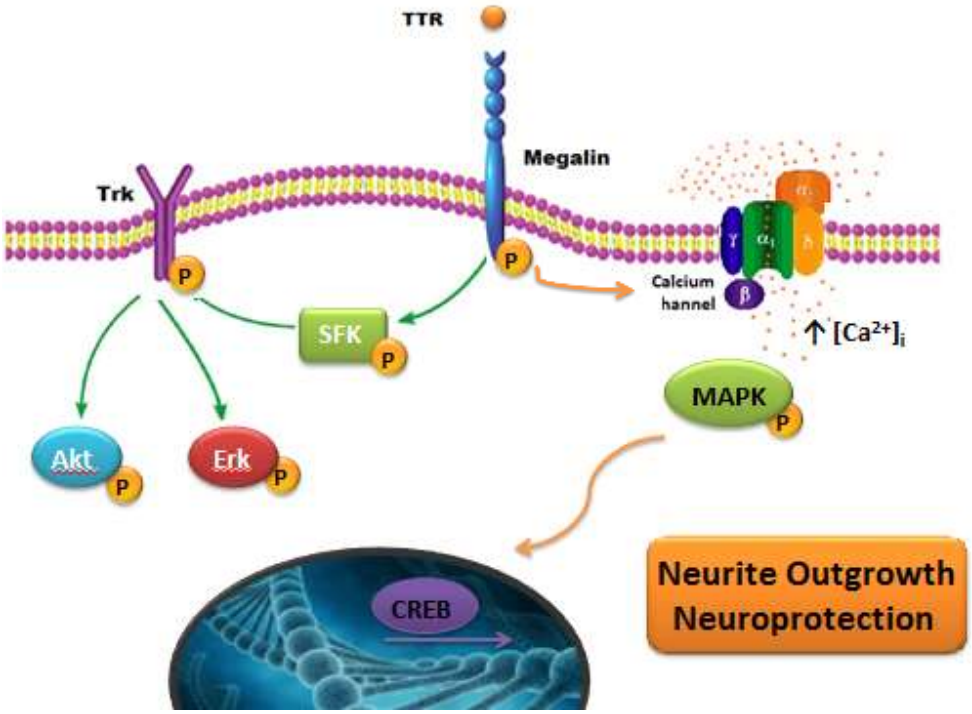

Figure 15. Schematic representation of the possible molecular mechanism induced by TTR. TTR binding to megalin activates Erk 1/2, Akt and CREB, possibly through a Trk receptors transactivation mechanism mediated by a Src family kinase. Increase of Ca^{2+} influx through the activation of Ca^{2+} channels can also be a signaling pathway responsible for neurite outgrowth and neuroprotection promoted by TTR.

CHAPTER VI

References

References

(1981) Nomenclature Committee of IUB (NC-IUB) IUB-IUPAC Joint Commission on Biochemical Nomenclature (JCBN). Newsletter 1981. The Journal of biological chemistry 256:12-14.

(2012) The 10 top causes of death [Internet website]. In: World Health Organization.

Abram SR, Kruskal JB, Allen GS, Burns RS, Parker R, Tulipan N (1990) Alterations in prealbumin concentration after adrenal autotransplantation for Parkinson's disease. Experimental neurology 108:130-135.

Aldred AR, Brack CM, Schreiber G (1995) The cerebral expression of plasma protein genes in different species. Comparative biochemistry and physiology Part B, Biochemistry & molecular biology 111:1-15.

Aleshire SL, Bradley CA, Richardson LD, Parl FF (1983) Localization of human prealbumin in choroid plexus epithelium. J Histochem Cytochem 31:608-612.

Almeida MR, Damas AM, Lans MC, Brouwer A, Saraiva MJ (1997) Thyroxine binding to transthyretin Met 119. Comparative studies of different heterozygotic carriers and structural analysis. Endocrine 6:309-315.

Ambjorn M, Asmussen JW, Lindstam M, Gotfryd K, Jacobsen C, Kiselyov VV, Moestrup SK, Penkowa M, Bock E, Berezin V (2008) Metallothionein and a peptide modeled after metallothionein, EmtinB, induce neuronal differentiation and survival through binding to receptors of the low-density lipoprotein receptor family. Journal of neurochemistry 104:21-37.

Andrade C (1952) A peculiar form of peripheral neuropathy; familiar atypical generalized amyloidosis with special involvement of the peripheral nerves. Brain : a journal of neurology 75:408-427.

Andrea TA, Cavalieri RR, Goldfine ID, Jorgensen EC (1980) Binding of thyroid hormones and analogues to the human plasma protein prealbumin. Biochemistry 19:55-63.

Annunziata M, Granata R, Ghigo E (2011) The IGF system. Acta diabetologica 48:1-9.

Argraves WS, Morales CR (2004) Immunolocalization of cubilin, megalin, apolipoprotein J, and apolipoprotein A-I in the uterus and oviduct. Molecular reproduction and development 69:419-427.

Arnaud L, Ballif BA, Forster E, Cooper JA (2003) Fyn tyrosine kinase is a critical regulator of disabled-1 during brain development. Current biology : CB 13:9-17.

Arundine M, Tymianski M (2003) Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell calcium 34:325-337.

Beal MF (1992) Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? Annals of neurology 31:119-130.

Beggs HE, Soriano P, Maness PF (1994) NCAM-dependent neurite outgrowth is inhibited in neurons from Fyn-minus mice. The Journal of cell biology 127:825-833.

Bell RD, Sagare AP, Friedman AE, Bedi GS, Holtzman DM, Deane R, Zlokovic BV (2007) Transport pathways for clearance of human Alzheimer's amyloid beta-peptide and apolipoproteins E and J in the mouse central nervous system. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 27:909-918.

Benson MD, Kluve-Beckerman B, Zeldenrust SR, Siesky AM, Bodenmiller DM, Showalter AD, Sloop KW (2006) Targeted suppression of an amyloidogenic transthyretin with antisense oligonucleotides. Muscle & nerve 33:609-618.

Bento-Abreu A, Velasco A, Polo-Hernandez E, Perez-Reyes PL, Tabernero A, Medina JM (2008) Megalin is a receptor for albumin in astrocytes and is required for the synthesis of the neurotrophic factor oleic acid. Journal of neurochemistry 106:1149-1159.

Benvenga S, Bartalena L, Antonelli A, Li Calzi L, Di Pasquale G, Trimarchi F, Pinchera A (1986) Radioimmunoassay for human thyroxine-binding prealbumin. Annals of clinical and laboratory science 16:231-240.

Berliocchi L, Fava E, Leist M, Horvat V, Dinsdale D, Read D, Nicotera P (2005) Botulinum neurotoxin C initiates two different programs for neurite degeneration and neuronal apoptosis. The Journal of cell biology 168:607-618.

Biemesderfer D (2006) Regulated intramembrane proteolysis of megalin: linking urinary protein and gene regulation in proximal tubule? Kidney international 69:1717-1721.

Birn H, Fyfe JC, Jacobsen C, Mounier F, Verroust PJ, Orskov H, Willnow TE, Moestrup SK, Christensen EI (2000) Cubilin is an albumin binding protein important for renal tubular albumin reabsorption. The Journal of clinical investigation 105:1353-1361.

Birney E et al. (2006) Ensembl 2006. Nucleic acids research 34:D556-561.

Blake CC, Geisow MJ, Swan ID, Rerat C, Rerat B (1974) Strjcture of human plasma prealbumin at 2-5 A resolution. A preliminary report on the polypeptide chain conformation, quaternary structure and thyroxine binding. Journal of molecular biology 88:1-12.

Blake CC, Swan ID, Rerat C, Berthou J, Laurent A, Rerat B (1971) An x-ray study of the subunit structure of prealbumin. Journal of molecular biology 61:217-224.

Blay P, Nilsson C, Owman C, Aldred A, Schreiber G (1993) Transthyretin expression in the rat brain: effect of thyroid functional state and role in thyroxine transport. Brain research 632:114-120.

Bock HH, Herz J (2003) Reelin activates SRC family tyrosine kinases in neurons. Current biology : CB 13:18-26.

Bolos M, Fernandez S, Torres-Aleman I (2010) Oral administration of a GSK3 inhibitor increases brain insulin-like growth factor I levels. The Journal of biological chemistry 285:17693-17700.

Brewer GJ, Torricelli JR, Evege EK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. Journal of neuroscience research 35:567-576.

Brouillette J, Quirion R (2008) Transthyretin: a key gene involved in the maintenance of memory capacities during aging. Neurobiology of aging 29:1721-1732.

Brouns R, De Deyn PP (2009) The complexity of neurobiological processes in acute ischemic stroke. Clinical neurology and neurosurgery 111:483-495.

Bu G, Rennke S (1996) Receptor-associated protein is a folding chaperone for low density lipoprotein receptor-related protein. The Journal of biological chemistry 271:22218-22224.

Buxbaum JN, Ye Z, Reixach N, Friske L, Levy C, Das P, Golde T, Masliah E, Roberts AR, Bartfai T (2008) Transthyretin protects Alzheimer's mice from the behavioral and biochemical effects of Abeta toxicity. Proceedings of the National Academy of Sciences of the United States of America 105:2681-2686.

Carro E, Trejo JL, Gomez-Isla T, LeRoith D, Torres-Aleman I (2002) Serum insulin-like growth factor I regulates brain amyloid-beta levels. Nature medicine 8:1390-1397.

Carro E, Spuch C, Trejo JL, Antequera D, Torres-Aleman I (2005) Choroid plexus megalin is involved in neuroprotection by serum insulin-like growth factor I. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:10884-10893.

Cavallaro T, Martone RL, Dwork AJ, Schon EA, Herbert J (1990) The retinal pigment epithelium is the unique site of transthyretin synthesis in the rat eye. Investigative ophthalmology & visual science 31:497-501.

Chatelet F, Brianti E, Ronco P, Roland J, Verroust P (1986) Ultrastructural localization by monoclonal antibodies of brush border antigens expressed by glomeruli. II. Extrarenal distribution. The American journal of pathology 122:512-519.

Chen WJ, Goldstein JL, Brown MS (1990) NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. The Journal of biological chemistry 265:3116-3123.

Choi DW (1992) Excitotoxic cell death. Journal of neurobiology 23:1261-1276.

Choi SH, Leight SN, Lee VM, Li T, Wong PC, Johnson JA, Saraiva MJ, Sisodia SS (2007) Accelerated Abeta deposition in APPswe/PS1deltaE9 mice with hemizygous deletions of TTR (transthyretin). The Journal of neuroscience : the official journal of the Society for Neuroscience 27:7006-7010.

Christensen EI, Willnow TE (1999) Essential role of megalin in renal proximal tubule for vitamin homeostasis. Journal of the American Society of Nephrology : JASN 10:2224-2236.

Christensen EI, Birn H (2002) Megalin and cubilin: multifunctional endocytic receptors. Nature reviews Molecular cell biology 3:256-266.

Chun JT, Wang L, Pasinetti GM, Finch CE, Zlokovic BV (1999) Glycoprotein 330/megalin (LRP-2) has low prevalence as mRNA and protein in brain microvessels and choroid plexus. Experimental neurology 157:194-201.

Chung RS, Penkowa M, Dittmann J, King CE, Bartlett C, Asmussen JW, Hidalgo J, Carrasco J, Leung YK, Walker AK, Fung SJ, Dunlop SA, Fitzgerald M, Beazley LD, Chuah MI, Vickers JC, West AK (2008) Redefining the role of metallothionein within the injured brain: extracellular metallothioneins play an

important role in the astrocyte-neuron response to injury. The Journal of biological chemistry 283:15349-15358.

Costa R, Goncalves A, Saraiva MJ, Cardoso I (2008a) Transthyretin binding to A-Beta peptide--impact on A-Beta fibrillogenesis and toxicity. FEBS letters 582:936-942.

Costa R, Ferreira-da-Silva F, Saraiva MJ, Cardoso I (2008b) Transthyretin protects against A-beta peptide toxicity by proteolytic cleavage of the peptide: a mechanism sensitive to the Kunitz protease inhibitor. PloS one 3:e2899.

Culi J, Springer TA, Mann RS (2004) Boca-dependent maturation of beta-propeller/EGF modules in low-density lipoprotein receptor proteins. The EMBO journal 23:1372-1380.

Danton GH, Dietrich WD (2004) The search for neuroprotective strategies in stroke. AJNR American journal of neuroradiology 25:181-194.

Dickson PW, Howlett GJ, Schreiber G (1982) Metabolism of prealbumin in rats and changes induced by acute inflammation. European journal of biochemistry / FEBS 129:289-293.

Dietrich MO, Spuch C, Antequera D, Rodal I, de Yebenes JG, Molina JA, Bermejo F, Carro E (2008) Megalin mediates the transport of leptin across the blood-CSF barrier. Neurobiology of aging 29:902-912.

Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trends in neurosciences 22:391-397.

Ditlevsen DK, Kohler LB, Berezin V, Bock E (2007) Cyclic guanosine monophosphate signalling pathway plays a role in neural cell adhesion molecule-mediated neurite outgrowth and survival. Journal of neuroscience research 85:703-711.

Dyck PJ, Lambert EH (1969) Dissociated sensation in amylidosis. Compound action potential, quantitative histologic and teased-fiber, and electron microscopic studies of sural nerve biopsies. Archives of neurology 20:490-507.

Elovaara I, Maury CP, Palo J (1986) Serum amyloid A protein, albumin and prealbumin in Alzheimer's disease and in demented patients with Down's syndrome. Acta neurologica Scandinavica 74:245-250.

Emerit J, Edeas M, Bricaire F (2004) Neurodegenerative diseases and oxidative stress. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 58:39-46.

Eneqvist T, Lundberg E, Nilsson L, Abagyan R, Sauer-Eriksson AE (2003) The transthyretin-related protein family. European journal of biochemistry / FEBS 270:518-532.

Episkopou V, Maeda S, Nishiguchi S, Shimada K, Gaitanaris GA, Gottesman ME, Robertson EJ (1993) Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid hormone. Proceedings of the National Academy of Sciences of the United States of America 90:2375-2379.

Erranz B, Miquel JF, Argraves WS, Barth JL, Pimentel F, Marzolo MP (2004) Megalin and cubilin expression in gallbladder epithelium and regulation by bile acids. Journal of lipid research 45:2185-2198.

Felding P, Fex G (1982) Cellular origin of prealbumin in the rat. Biochimica et biophysica acta 716:446-449.

Fitzgerald M, Nairn P, Bartlett CA, Chung RS, West AK, Beazley LD (2007) Metallothionein-IIA promotes neurite growth via the megalin receptor. Experimental brain research 183:171-180.

Fleming CE, Saraiva MJ, Sousa MM (2007) Transthyretin enhances nerve regeneration. Journal of neurochemistry 103:831-839.

Fleming CE, Mar FM, Franquinho F, Saraiva MJ, Sousa MM (2009) Transthyretin internalization by sensory neurons is megalin mediated and necessary for its neuritogenic activity. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:3220-3232.

Foss TR, Kelker MS, Wiseman RL, Wilson IA, Kelly JW (2005) Kinetic stabilization of the native state by protein engineering: implications for inhibition of transthyretin amyloidogenesis. Journal of molecular biology 347:841-854.

Furuya H, Saraiva MJ, Gawinowicz MA, Alves IL, Costa PP, Sasaki H, Goto I, Sakaki Y (1991) Production of recombinant human transthyretin with biological activities toward the understanding of the molecular basis of familial amyloidotic polyneuropathy (FAP). Biochemistry 30:2415-2421.

Gajera CR, Emich H, Lioubinski O, Christ A, Beckervordersandforth-Bonk R, Yoshikawa K, Bachmann S, Christensen EI, Gotz M, Kempermann G, Peterson AS, Willnow TE, Hammes A (2010) LRP2 in ependymal cells regulates BMP signaling in the adult neurogenic niche. Journal of cell science 123:1922-1930.

Gan Y, McGraw TE, Rodriguez-Boulan E (2002) The epithelial-specific adaptor AP1B mediates post-endocytic recycling to the basolateral membrane. Nature cell biology 4:605-609.

Gao C, Zhang B, Zhang W, Pu S, Yin J, Gao Q (2011) Serum prealbumin (transthyretin) predict good outcome in young patients with cerebral infarction. Clinical and experimental medicine 11:49-54.

George F (2012) [Causes of deaths in Portugal and challenges in prevention]. Acta medica portuguesa 25:61-63.

Goedert M, Spillantini MG (2006) A century of Alzheimer's disease. Science (New York, NY) 314:777-781.

Goodman DS (1984) Vitamin A and retinoids in health and disease. The New England journal of medicine 310:1023-1031.

Gotthardt M, Trommsdorff M, Nevitt MF, Shelton J, Richardson JA, Stockinger W, Nimpf J, Herz J (2000) Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. The Journal of biological chemistry 275:25616-25624.

Hammad SM, Ranganathan S, Loukinova E, Twal WO, Argraves WS (1997) Interaction of apolipoprotein J-amyloid beta-peptide complex with low density lipoprotein receptor-related protein-2/megalin. A mechanism to prevent pathological accumulation of amyloid beta-peptide. The Journal of biological chemistry 272:18644-18649.

Hammond JB, Kruger NJ (1988) The bradford method for protein quantitation. Methods in molecular biology (Clifton, NJ) 3:25-32.

Han SH, Jung ES, Sohn JH, Hong HJ, Hong HS, Kim JW, Na DL, Kim M, Kim H, Ha HJ, Kim YH, Huh N, Jung MW, Mook-Jung I (2011) Human serum transthyretin levels correlate inversely with Alzheimer's disease. Journal of Alzheimer's disease : JAD 25:77-84.

Herz J, Beffert U (2000) Apolipoprotein E receptors: linking brain development and Alzheimer's disease. Nature reviews Neuroscience 1:51-58.

Herz J, Bock HH (2002) Lipoprotein receptors in the nervous system. Annual review of biochemistry 71:405-434.

Herz J, Goldstein JL, Strickland DK, Ho YK, Brown MS (1991) 39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. The Journal of biological chemistry 266:21232-21238.

Heumann R (1994) Neurotrophin signalling. Current opinion in neurobiology 4:668-679.

Hjalm G, Murray E, Crumley G, Harazim W, Lundgren S, Onyango I, Ek B, Larsson M, Juhlin C, Hellman P, Davis H, Akerstrom G, Rask L, Morse B (1996) Cloning and sequencing of human gp330, a Ca(2+)-binding receptor with potential intracellular signaling properties. European journal of biochemistry / FEBS 239:132-137.

Horikawa K, Yamada Y, Matsuda T, Kobayashi K, Hashimoto M, Matsu-ura T, Miyawaki A, Michikawa T, Mikoshiba K, Nagai T (2010) Spontaneous network activity visualized by ultrasensitive Ca(2+) indicators, yellow Cameleon-Nano. Nature methods 7:729-732.

Hossmann KA (1996) Periinfarct depolarizations. Cerebrovascular and brain metabolism reviews 8:195-208.

Iadonato SP, Bu G, Maksymovitch EA, Schwartz AL (1993) Interaction of a 39 kDa protein with the low-density-lipoprotein-receptor-related protein (LRP) on rat hepatoma cells. The Biochemical journal 296 (Pt 3):867-875.

Ingenbleek Y, Young V (1994) Transthyretin (prealbumin) in health and disease: nutritional implications. Annual review of nutrition 14:495-533.

Iwasaki Y, Gay B, Wada K, Koizumi S (1998) Association of the Src family tyrosine kinase Fyn with TrkB. Journal of neurochemistry 71:106-111.

Jacobsson B, Collins VP, Grimelius L, Pettersson T, Sandstedt B, Carlstrom A (1989) Transthyretin immunoreactivity in human and porcine liver, choroid plexus, and pancreatic islets. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 37:31-37.

Jeon H, Meng W, Takagi J, Eck MJ, Springer TA, Blacklow SC (2001) Implications for familial hypercholesterolemia from the structure of the LDL receptor YWTD-EGF domain pair. Nature structural biology 8:499-504.

Juhlin C, Holmdahl R, Johansson H, Rastad J, Akerstrom G, Klareskog L (1987) Monoclonal antibodies with exclusive reactivity against parathyroid cells and tubule cells of the kidney. Proceedings of the National Academy of Sciences of the United States of America 84:2990-2994.

Kabat EA, Moore DH, Landow H (1942) AN ELECTROPHORETIC STUDY OF THE PROTEIN COMPONENTS IN CEREBROSPINAL FLUID AND THEIR RELATIONSHIP TO THE SERUM PROTEINS. The Journal of clinical investigation 21:571-577.

Kanda Y, Goodman DS, Canfield RE, Morgan FJ (1974) The amino acid sequence of human plasma prealbumin. The Journal of biological chemistry 249:6796-6805.

Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C, Sato A, Kaneko M (1987) K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. Biochemical and biophysical research communications 142:436-440.

Kato M, Kato K, Blaner WS, Chertow BS, Goodman DS (1985) Plasma and cellular retinoid-binding proteins and transthyretin (prealbumin) are all localized in the islets of Langerhans in the rat. Proceedings of the National Academy of Sciences of the United States of America 82:2488-2492.

Katsura K, Kristian T, Siesjo BK (1994) Energy metabolism, ion homeostasis, and cell damage in the brain. Biochemical Society transactions 22:991-996.

Kerjaschki D, Farquhar MG (1982) The pathogenic antigen of Heymann nephritis is a membrane glycoprotein of the renal proximal tubule brush border. Proceedings of the National Academy of Sciences of the United States of America 79:5557-5561.

Kerjaschki D, Noronha-Blob L, Sacktor B, Farquhar MG (1984) Microdomains of distinctive glycoprotein composition in the kidney proximal tubule brush border. The Journal of cell biology 98:1505-1513.

Kooijman R (2006) Regulation of apoptosis by insulin-like growth factor (IGF)-I. Cytokine & growth factor reviews 17:305-323.

Korenberg JR, Argraves KM, Chen XN, Tran H, Strickland DK, Argraves WS (1994) Chromosomal localization of human genes for the LDL receptor family member glycoprotein 330 (LRP2) and its associated protein RAP (LRPAP1). Genomics 22:88-93.

Kounnas MZ, Argraves WS, Strickland DK (1992) The 39-kDa receptor-associated protein interacts with two members of the low density lipoprotein receptor family, alpha 2-macroglobulin receptor and glycoprotein 330. The Journal of biological chemistry 267:21162-21166.

Kuchler-Bopp S, Dietrich JB, Zaepfel M, Delaunoy JP (2000) Receptor-mediated endocytosis of transthyretin by ependymoma cells. Brain research 870:185-194.

Larsson M, Hjalm G, Sakwe AM, Engstrom A, Hoglund AS, Larsson E, Robinson RC, Sundberg C, Rask L (2003) Selective interaction of megalin with postsynaptic density-95 (PSD-95)-like membrane-associated guanylate kinase (MAGUK) proteins. The Biochemical journal 373:381-391.

Leheste JR, Rolinski B, Vorum H, Hilpert J, Nykjaer A, Jacobsen C, Aucouturier P, Moskaug JO, Otto A, Christensen EI, Willnow TE (1999) Megalin knockout mice as an animal model of low molecular weight proteinuria. The American journal of pathology 155:1361-1370.

Leheste JR, Melsen F, Wellner M, Jansen P, Schlichting U, Renner-Muller I, Andreassen TT, Wolf E, Bachmann S, Nykjaer A, Willnow TE (2003)

Hypocalcemia and osteopathy in mice with kidney-specific megalin gene defect. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 17:247-249.

Li X, Buxbaum JN (2011) Transthyretin and the brain re-visited: is neuronal synthesis of transthyretin protective in Alzheimer's disease? Molecular neurodegeneration 6:79.

Li X, Masliah E, Reixach N, Buxbaum JN (2011) Neuronal production of transthyretin in human and murine Alzheimer's disease: is it protective? The Journal of neuroscience : the official journal of the Society for Neuroscience 31:12483-12490.

Li Y, van Kerkhof P, Marzolo MP, Strous GJ, Bu G (2001) Identification of a major cyclic AMP-dependent protein kinase A phosphorylation site within the cytoplasmic tail of the low-density lipoprotein receptor-related protein: implication for receptor-mediated endocytosis. Molecular and cellular biology 21:1185-1195.

Lighthouse JK, Zhang L, Hsieh JC, Rosenquist T, Holdener BC (2011) MESD is essential for apical localization of megalin/LRP2 in the visceral endoderm. Developmental dynamics : an official publication of the American Association of Anatomists 240:577-588.

Liu J, He J, Huang L, Dou L, Wu S, Yuan Q (2014) Neuroprotective effects of ginsenoside Rb1 on hippocampal neuronal injury and neurite outgrowth. Neural regeneration research 9:943-950.

Liverman CS, Cui L, Yong C, Choudhuri R, Klein RM, Welch KM, Berman NE (2004) Response of the brain to oligemia: gene expression, c-Fos, and Nrf2 localization. Brain research Molecular brain research 126:57-66.

Long Y, Zou L, Liu H, Lu H, Yuan X, Robertson CS, Yang K (2003) Altered expression of randomly selected genes in mouse hippocampus after traumatic brain injury. Journal of neuroscience research 71:710-720.

Loukinova E, Ranganathan S, Kuznetsov S, Gorlatova N, Migliorini MM, Loukinov D, Ulery PG, Mikhailenko I, Lawrence DA, Strickland DK (2002) Platelet-derived growth factor (PDGF)-induced tyrosine phosphorylation of the low density lipoprotein receptor-related protein (LRP). Evidence for integrated co-receptor function betwenn LRP and the PDGF. The Journal of biological chemistry 277:15499-15506.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. The Journal of biological chemistry 193:265-275.

Lundgren S, Hjalm G, Hellman P, Ek B, Juhlin C, Rastad J, Klareskog L, Akerstrom G, Rask L (1994) A protein involved in calcium sensing of the human parathyroid and placental cytotrophoblast cells belongs to the LDL-receptor protein superfamily. Experimental cell research 212:344-350.

Lundgren S, Carling T, Hjalm G, Juhlin C, Rastad J, Pihlgren U, Rask L, Akerstrom G, Hellman P (1997) Tissue distribution of human gp330/megalin, a putative Ca(2+)-sensing protein. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 45:383-392.

Maarouf CL, Beach TG, Adler CH, Shill HA, Sabbagh MN, Wu T, Walker DG, Kokjohn TA, Roher AE (2012) Cerebrospinal fluid biomarkers of neuropathologically diagnosed Parkinson's disease subjects. Neurological research 34:669-676.

Makover A, Moriwaki H, Ramakrishnan R, Saraiva MJ, Blaner WS, Goodman DS (1988) Plasma transthyretin. Tissue sites of degradation and turnover in the rat. The Journal of biological chemistry 263:8598-8603.

Martin RL, Lloyd HG, Cowan AI (1994) The early events of oxygen and glucose deprivation: setting the scene for neuronal death? Trends in neurosciences 17:251-257.

Marwarha G, Dasari B, Prasanthi JR, Schommer J, Ghribi O (2010) Leptin reduces the accumulation of Abeta and phosphorylated tau induced by 27-hydroxycholesterol in rabbit organotypic slices. Journal of Alzheimer's disease : JAD 19:1007-1019.

Marzolo MP, Yuseff MI, Retamal C, Donoso M, Ezquer F, Farfan P, Li Y, Bu G (2003) Differential distribution of low-density lipoprotein-receptor-related protein (LRP) and megalin in polarized epithelial cells is determined by their cytoplasmic domains. Traffic (Copenhagen, Denmark) 4:273-288.

Matter K, Hunziker W, Mellman I (1992) Basolateral sorting of LDL receptor in MDCK cells: the cytoplasmic domain contains two tyrosine-dependent targeting determinants. Cell 71:741-753.

McAllister AK, Katz LC, Lo DC (1999) Neurotrophins and synaptic plasticity. Annual review of neuroscience 22:295-318.

McCarthy RA, Barth JL, Chintalapudi MR, Knaak C, Argraves WS (2002) Megalin functions as an endocytic sonic hedgehog receptor. The Journal of biological chemistry 277:25660-25667.

McKinnon B, Li H, Richard K, Mortimer R (2005) Synthesis of thyroid hormone binding proteins transthyretin and albumin by human trophoblast. The Journal of clinical endocrinology and metabolism 90:6714-6720.

Meijering E, Jacob M, Sarria JC, Steiner P, Hirling H, Unser M (2004) Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. Cytometry Part A : the journal of the International Society for Analytical Cytology 58:167-176.

Merched A, Serot JM, Visvikis S, Aguillon D, Faure G, Siest G (1998) Apolipoprotein E, transthyretin and actin in the CSF of Alzheimer's patients: relation with the senile plaques and cytoskeleton biochemistry. FEBS letters 425:225-228.

Monaco HL, Rizzi M, Coda A (1995) Structure of a complex of two plasma proteins: transthyretin and retinol-binding protein. Science (New York, NY) 268:1039-1041.

Muller D, Ankermann T, Stephani U, Kirschstein M, Szelestei T, Luft FC, Willnow TE (2001) Holoprosencephaly and low molecular weight proteinuria: the human homologue of murine megalin deficiency. American journal of kidney diseases : the official journal of the National Kidney Foundation 37:624-628.

Murakami T, Ohsawa Y, Sunada Y (2008) The transthyretin gene is expressed in human and rodent dorsal root ganglia. Neuroscience letters 436:335-339.

Nagai M, Meerloo T, Takeda T, Farquhar MG (2003) The adaptor protein ARH escorts megalin to and through endosomes. Molecular biology of the cell 14:4984-4996.

Navab M, Mallia AK, Kanda Y, Goodman DS (1977) Rat plasma prealbumin. Isolation and partial characterization. The Journal of biological chemistry 252:5100-5106.

Niu S, Renfro A, Quattrocchi CC, Sheldon M, D'Arcangelo G (2004) Reelin promotes hippocampal dendrite development through the VLDLR/ApoER2-Dab1 pathway. Neuron 41:71-84.

Noy N, Slosberg E, Scarlata S (1992) Interactions of retinol with binding proteins: studies with retinol-binding protein and with transthyretin. Biochemistry 31:11118-11124.

Nunes AF, Saraiva MJ, Sousa MM (2006) Transthyretin knockouts are a new mouse model for increased neuropeptide Y. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 20:166-168.

Nuutinen T, Suuronen T, Kauppinen A, Salminen A (2009) Clusterin: a forgotten player in Alzheimer's disease. Brain research reviews 61:89-104.

Odera K, Goto S, Takahashi R (2007) Age-related change of endocytic receptors megalin and cubilin in the kidney in rats. Biogerontology 8:505-515.

Oleinikov AV, Zhao J, Makker SP (2000) Cytosolic adaptor protein Dab2 is an intracellular ligand of endocytic receptor gp600/megalin. The Biochemical journal 347 Pt 3:613-621.

Oliveira SM, Ribeiro CA, Cardoso I, Saraiva MJ (2011) Gender-dependent transthyretin modulation of brain amyloid-beta levels: evidence from a mouse model of Alzheimer's disease. Journal of Alzheimer's disease : JAD 27:429-439.

Orlando RA, Exner M, Czekay RP, Yamazaki H, Saito A, Ullrich R, Kerjaschki D, Farquhar MG (1997) Identification of the second cluster of ligand-binding repeats in megalin as a site for receptor-ligand interactions. Proceedings of the National Academy of Sciences of the United States of America 94:2368-2373.

Perron JC, Bixby JL (1999) Distinct neurite outgrowth signaling pathways converge on ERK activation. Molecular and cellular neurosciences 13:362-378.

Pober BR, Longoni M, Noonan KM (2009) A review of Donnai-Barrow and facio-oculo-acoustico-renal (DB/FOAR) syndrome: clinical features and differential diagnosis. Birth defects research Part A, Clinical and molecular teratology 85:76-81.

Power DM, Elias NP, Richardson SJ, Mendes J, Soares CM, Santos CR (2000) Evolution of the thyroid hormone-binding protein, transthyretin. General and comparative endocrinology 119:241-255.

Qiu Z, Hyman BT, Rebeck GW (2004) Apolipoprotein E receptors mediate neurite outgrowth through activation of p44/42 mitogen-activated protein kinase in primary neurons. The Journal of biological chemistry 279:34948-34956.

Quintas A, Vaz DC, Cardoso I, Saraiva MJ, Brito RM (2001) Tetramer dissociation and monomer partial unfolding precedes protofibril formation in amyloidogenic transthyretin variants. The Journal of biological chemistry 276:27207-27213.

Raychowdhury R, Niles JL, McCluskey RT, Smith JA (1989) Autoimmune target in Heymann nephritis is a glycoprotein with homology to the LDL receptor. Science (New York, NY) 244:1163-1165.

Raz A, Goodman DS (1969) The interaction of thyroxine with human plasma prealbumin and with the prealbumin-retinol-binding protein complex. The Journal of biological chemistry 244:3230-3237.

Read DE, Gorman AM (2009) Involvement of Akt in neurite outgrowth. Cellular and molecular life sciences : CMLS 66:2975-2984.

Redmond L, Kashani AH, Ghosh A (2002) Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. Neuron 34:999-1010.

Ribeiro CA, Santana I, Oliveira C, Baldeiras I, Moreira J, Saraiva MJ, Cardoso I (2012) Transthyretin decrease in plasma of MCI and AD patients: investigation of mechanisms for disease modulation. Current Alzheimer research 9:881-889.

Riisoen H (1988) Reduced prealbumin (transthyretin) in CSF of severely demented patients with Alzheimer's disease. Acta neurologica Scandinavica 78:455-459.

Rite I, Arguelles S, Venero JL, Garcia-Rodriguez S, Ayala A, Cano J, Machado A (2007) Proteomic identification of biomarkers in the cerebrospinal fluid in a rat model of nigrostriatal dopaminergic degeneration. Journal of neuroscience research 85:3607-3618.

Rossi DJ, Oshima T, Attwell D (2000) Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature 403:316-321.

Rusanescu G, Qi H, Thomas SM, Brugge JS, Halegoua S (1995) Calcium influx induces neurite growth through a Src-Ras signaling cassette. Neuron 15:1415-1425.

Saito A, Pietromonaco S, Loo AK, Farquhar MG (1994) Complete cloning and sequencing of rat gp330/"megalin," a distinctive member of the low density lipoprotein receptor gene family. Proceedings of the National Academy of Sciences of the United States of America 91:9725-9729.

Santos SD, Lambertsen KL, Clausen BH, Akinc A, Alvarez R, Finsen B, Saraiva MJ (2010) CSF transthyretin neuroprotection in a mouse model of brain ischemia. Journal of neurochemistry 115:1434-1444.

Saraiva MJ (2001) Transthyretin mutations in hyperthyroxinemia and amyloid diseases. Human mutation 17:493-503.

Saraiva MJ, Birken S, Costa PP, Goodman DS (1984) Amyloid fibril protein in familial amyloidotic polyneuropathy, Portuguese type. Definition of molecular abnormality in transthyretin (prealbumin). The Journal of clinical investigation 74:104-119.

Schreiber G, Richardson SJ (1997) The evolution of gene expression, structure and function of transthyretin. Comparative biochemistry and physiology Part B, Biochemistry & molecular biology 116:137-160.

Schreiber G, Aldred AR, Jaworowski A, Nilsson C, Achen MG, Segal MB (1990) Thyroxine transport from blood to brain via transthyretin synthesis in choroid plexus. The American journal of physiology 258:R338-345.

Seibert FB, Nelson JW (1942) Electrophoretic study of the blood protein response in tuberculosis. J Biol Chem 143 (1):29-38.

Shi Y, Mantuano E, Inoue G, Campana WM, Gonias SL (2009) Ligand binding to LRP1 transactivates Trk receptors by a Src family kinase-dependent pathway. Science signaling 2:ra18.

Sironi L, Tremoli E, Miller I, Guerrini U, Calvio AM, Eberini I, Gemeiner M, Asdente M, Paoletti R, Gianazza E (2001) Acute-phase proteins before cerebral ischemia in stroke-prone rats: identification by proteomics. Stroke; a journal of cerebral circulation 32:753-760.

Skinner M, Connors LH, Rubinow A, Libbey C, Sipe JD, Cohen AS (1985) Lowered prealbumin levels in patients with familial amyloid polyneuropathy (FAP) and their non-affected but at risk relatives. The American journal of the medical sciences 289:17-21.

Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, et al. (1993) SH2 domains recognize specific phosphopeptide sequences. Cell 72:767-778.

Soprano DR, Soprano KJ, Goodman DS (1986) Retinol-binding protein and transthyretin mRNA levels in visceral yolk sac and liver during fetal development in the rat. Proceedings of the National Academy of Sciences of the United States of America 83:7330-7334.

Soprano DR, Herbert J, Soprano KJ, Schon EA, Goodman DS (1985) Demonstration of transthyretin mRNA in the brain and other extrahepatic tissues in the rat. The Journal of biological chemistry 260:11793-11798.

Sousa JC, Cardoso I, Marques F, Saraiva MJ, Palha JA (2007a) Transthyretin and Alzheimer's disease: where in the brain? Neurobiology of aging 28:713-718.

Sousa JC, Marques F, Dias-Ferreira E, Cerqueira JJ, Sousa N, Palha JA (2007b) Transthyretin influences spatial reference memory. Neurobiology of learning and memory 88:381-385.

Sousa JC, Grandela C, Fernandez-Ruiz J, de Miguel R, de Sousa L, Magalhaes AI, Saraiva MJ, Sousa N, Palha JA (2004) Transthyretin is involved in depression-like behaviour and exploratory activity. Journal of neurochemistry 88:1052-1058.

Sousa MM, Saraiva MJ (2001) Internalization of transthyretin. Evidence of a novel yet unidentified receptor-associated protein (RAP)-sensitive receptor. The Journal of biological chemistry 276:14420-14425.

Sousa MM, Saraiva MJ (2003) Neurodegeneration in familial amyloid polyneuropathy: from pathology to molecular signaling. Progress in neurobiology 71:385-400.

Sousa MM, Saraiva MJ (2008) Transthyretin is not expressed by dorsal root ganglia cells. Experimental neurology 214:362-365.

Sousa MM, Yan SD, Stern D, Saraiva MJ (2000a) Interaction of the receptor for advanced glycation end products (RAGE) with transthyretin triggers nuclear transcription factor kB (NF-kB) activation. Laboratory investigation; a journal of technical methods and pathology 80:1101-1110.

Sousa MM, Norden AG, Jacobsen C, Willnow TE, Christensen EI, Thakker RV, Verroust PJ, Moestrup SK, Saraiva MJ (2000b) Evidence for the role of megalin in renal uptake of transthyretin. The Journal of biological chemistry 275:38176-38181.

Spoelgen R, Hammes A, Anzenberger U, Zechner D, Andersen OM, Jerchow B, Willnow TE (2005) LRP2/megalin is required for patterning of the ventral telencephalon. Development (Cambridge, England) 132:405-414.

Stabilini R, Vergani C, Agostoni A, Agostoni RP (1968) Influence of age and sex on prealbumin levels. Clinica chimica acta; international journal of clinical chemistry 20:358-359.

Stein TD, Johnson JA (2002) Lack of neurodegeneration in transgenic mice overexpressing mutant amyloid precursor protein is associated with increased levels of transthyretin and the activation of cell survival pathways. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:7380-7388.

Suzuyama K, Shiraishi T, Oishi T, Ueda S, Okamoto H, Furuta M, Mineta T, Tabuchi K (2004) Combined proteomic approach with SELDI-TOF-MS and peptide mass fingerprinting identified the rapid increase of monomeric transthyretin in rat cerebrospinal fluid after transient focal cerebral ischemia. Brain research Molecular brain research 129:44-53.

Tamai K, Zeng X, Liu C, Zhang X, Harada Y, Chang Z, He X (2004) A mechanism for Wnt coreceptor activation. Molecular cell 13:149-156.

Thomas B, Beal MF (2007) Parkinson's disease. Human molecular genetics 16 Spec No. 2:R183-194.

van Bennekum AM, Wei S, Gamble MV, Vogel S, Piantedosi R, Gottesman M, Episkopou V, Blaner WS (2001) Biochemical basis for depressed serum retinol levels in transthyretin-deficient mice. The Journal of biological chemistry 276:1107-1113.

Van Praet O, Argraves WS, Morales CR (2003) Co-expression and interaction of cubilin and megalin in the adult male rat reproductive system. Molecular reproduction and development 64:129-135.

Vatassery GT, Quach HT, Smith WE, Benson BA, Eckfeldt JH (1991) A sensitive assay of transthyretin (prealbumin) in human cerebrospinal fluid in nanogram amounts by ELISA. Clinica chimica acta; international journal of clinical chemistry 197:19-25.

Vieira AV, Sanders EJ, Schneider WJ (1995) Transport of serum transthyretin into chicken oocytes. A receptor-mediated mechanism. The Journal of biological chemistry 270:2952-2956.

Vieira M, Gomes JR, Saraiva MJ (2014) Transthyretin Induces Insulin-like Growth Factor I Nuclear Translocation Regulating Its Levels in the Hippocampus. Molecular neurobiology.

Watanabe Y, Matsumoto N, Dezawa M, Itokazu Y, Yoshihara T, Ide C (2005) Conditioned medium of the primary culture of rat choroid plexus epithelial (modified ependymal) cells enhances neurite outgrowth and survival of hippocampal neurons. Neuroscience letters 379:158-163.

Wicher G, Aldskogius H (2008) Megalin deficiency induces critical changes in mouse spinal cord development. Neuroreport 19:559-563.

Wicher G, Larsson M, Fex Svenningsen A, Gyllencreutz E, Rask L, Aldskogius H (2006) Low density lipoprotein receptor-related protein-2/megalin is expressed in oligodendrocytes in the mouse spinal cord white matter. Journal of neuroscience research 83:864-873.

Willnow TE, Hilpert J, Armstrong SA, Rohlmann A, Hammer RE, Burns DK, Herz J (1996) Defective forebrain development in mice lacking gp330/megalin. Proceedings of the National Academy of Sciences of the United States of America 93:8460-8464.

Wirdefeldt K, Adami HO, Cole P, Trichopoulos D, Mandel J (2011) Epidemiology and etiology of Parkinson's disease: a review of the evidence. European journal of epidemiology 26 Suppl 1:S1-58.

Yamauchi K, Yamauchi T, Mantuano E, Murakami K, Henry K, Takahashi K, Campana WM (2013) Low-density lipoprotein receptor related protein-1 (LRP1)-dependent cell signaling promotes neurotrophic activity in embryonic sensory neurons. PloS one 8:e75497.

Yammani RR, Seetharam S, Seetharam B (2001) Cubilin and megalin expression and their interaction in the rat intestine: effect of thyroidectomy. American journal of physiology Endocrinology and metabolism 281:E900-907.

Young B, Ott L, Kasarskis E, Rapp R, Moles K, Dempsey RJ, Tibbs PA, Kryscio R, McClain C (1996) Zinc supplementation is associated with improved neurologic recovery rate and visceral protein levels of patients with severe closed head injury. Journal of neurotrauma 13:25-34.

Yu H, Chen JK, Feng S, Dalgarno DC, Brauer AW, Schreiber SL (1994) Structural basis for the binding of proline-rich peptides to SH3 domains. Cell 76:933-945.

Yuseff MI, Farfan P, Bu G, Marzolo MP (2007) A cytoplasmic PPPSP motif determines megalin's phosphorylation and regulates receptor's recycling and surface expression. Traffic (Copenhagen, Denmark) 8:1215-1230.

Zeng X, Tamai K, Doble B, Li S, Huang H, Habas R, Okamura H, Woodgett J, He X (2005) A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. Nature 438:873-877.

Zlokovic BV, Martel CL, Matsubara E, McComb JG, Zheng G, McCluskey RT, Frangione B, Ghiso J (1996) Glycoprotein 330/megalin: probable role in receptor-mediated transport of apolipoprotein J alone and in a complex with Alzheimer disease amyloid beta at the blood-brain and blood-cerebrospinal fluid barriers. Proceedings of the National Academy of Sciences of the United States of America 93:4229-4234.

Zou Z, Chung B, Nguyen T, Mentone S, Thomson B, Biemesderfer D (2004) Linking receptor-mediated endocytosis and cell signaling: evidence for regulated intramembrane proteolysis of megalin in proximal tubule. The Journal of biological chemistry 279:34302-34310.