UNIVERSIDADE DA BEIRA INTERIOR
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Transthyretin and metallothioneins affect amyloid-beta metabolism and are regulated by glucocorticoids in choroid plexus

Ana Isabel de Jesus Martinho

Tese para obtenção do Grau de Doutor em
Biomedicina
(3° ciclo de estudos)

Orientador: Prof. Doutora Cecília Reis Alves dos Santos
Co-Orientadores: Prof. Doutora Isabel Maria Theriaga Mendes Varanda Gonçalves
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Health Sciences

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Thesis for obtaining the Doctor Degree in
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Aos meus pais

Por me terem ensinado a perseguir os meus sonhos!

"Determination, courage and self-confidence are the key factors for success. In spite of obstacles and difficulties, if we have firm determination, we can work them out..."

Dalai Lama

Preface

This PhD thesis is based on the experimental work carried out at the Health Sciences Research Centre (CICS), University of Beira Interior (UBI), Covilhã, Portugal, since May 2007. It is based on three studies, resulted from the PhD project, two of which are published and one is submitted, in peer–reviewed journals. In the thesis, the original studies are referred by the numerals II, III and IV. The experimental work mentioned in Paper II (Chapter 3) was partially realized at the Molecular Neurobiology Group of the Institute for Molecular and Cellular Biology (IBMC), Porto, Portugal.

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List of Papers

Papers included in the thesis:

I. **NEUROPROTECTIVE AND NEUROREGENERATIVE PROPERTIES OF METALLOTHIONEINS**
Santos CRA, Martinho A, Quintela T and Goncalves I
IUBMB Life (2012) 64(2):126-135.

II. **HUMAN METALLOTHIONEINS 2 AND 3 DIFFERENTIALLY AFFECT AMYLOID-BETA BINDING BY TRANSTHYRETIN**
Martinho A, Gonçalves I, Cardoso I, Almeida MR, Quintela T, Saraiva MJ and Santos CRA
FEBS J (2010) 277:3427-3436.

III. **STRESS AND GLUCOCORTICOIDS INCREASE TRANSTHYRETIN EXPRESSION IN RAT CHOROID PLEXUS *VIA* MINERALOCORTICOID AND GLUCOCORTICOID RECEPTORS**
Martinho A, Gonçalves I, Costa M and Santos CRA
J Mol Neurosci (2012) 48(1):1-13.

IV. **GLUCOCORTICOIDS REGULATE METALLOTHIONEIN-1/2 IN RAT CHOROID PLEXUS: EFFECTS ON APOPTOSIS**
Martinho A, Gonçalves I and Santos CRA
Mol Cell Biochem (2012) (submitted).

Papers not included in the thesis:

V. **COMBINED MOLECULAR DIAGNOSIS OF B-CELL LYMPHOMAS WITH T(11;14)(Q13;Q32) OR T(14;18)(Q32;Q21) USING MULTIPLEX- AND LONG DISTANCE INVERSE-POLYMERASE CHAIN REACTION**

Vieira L, Martinho A, Antunes O, Silva E, Ambrósio AP, Geraldes MC, Nascimento R, Silva C, Pereira JM, Júnior EC and Jordan P

Diagn Mol Pathol (2008) 17(2):73-81.

VI. **PROGESTERONE ENHANCES TRANSTHYRETIN EXPRESSION IN THE RAT CHOROID PLEXUS *IN VITRO* AND *IN VIVO* VIA PROGESTERONE RECEPTOR**

Quintela T, Gonçalves I, Martinho A, Alves CH, Saraiva MJ, Rocha P and Santos CR

J Mol Neurosci (2011) 44(3):152-158.

VII. **AN ESTROGEN RESPONSIVE ELEMENT IN DISTAL PROMOTER OF HUMAN TRANSTHYRETIN GENE ENHANCES ITS TRANSCRIPTION UPON ERα AND/OR ERβ TRANSACTIVATION**

Martinho A, Gonçalves I and Santos CRA

(*in prep*).

List of Scientific Communications

Scientific communications during the doctoral work:

I. **TRANSTHYRETIN RESPONDS TO STRESS IN RATS**
Martinho A, Gonçalves I, Veloso I, Santos CRA
The 10th International Conference on Alzheimer's & Parkinson's Diseases, AD/PD. March 2011, Barcelona - Spain.

II. **REGULATION OF TRANSTHYRETIN BY CORTISOL IN THE CHOROID PLEXUS, LIVER AND CEREBROSPINAL LIQUID OF RATS**
Martinho A, Gonçalves I, Veloso I, Santos CRA
XVII National Congress of Biochemistry. December 2010, Porto - Portugal.

III. **REGULATION OF TRANSTHYRETIN IN THE CHOROIDS PLEXUS OF RATS BY CORTISOL**
Veloso I, Martinho A, Gonçalves I, Santos CRA
V Annual CICS Symposium. July 2010, Covilhã - Portugal.

IV. **HUMAN METALLOTHIONEINS 2 AND 3 HAVE OPPOSITE EFFECTS ON THE ABILITY OF TRANSTHYRETIN TO BIND AMYLOID BETA**
Martinho A, Gonçalves I, Cardoso I, Almeida M, Saraiva MJ and Santos CRA
11th International Geneva/Springfield Symposium on Advances in Alzheimer Therapy. March 2010, Geneva - Switzerland.

V. **TRANSTHYRETIN INTERACTS WITH METALLOTHIONEIN 3 AND IMPROVES ITS BINDING TO AMILOID-BETA PEPTIDE**
Martinho A, Gonçalves I, Cardoso I, Almeida MR, Saraiva MJ, Santos CRA
IV Annual CICS Symposium. July 2009, Covilhã - Portugal.

VI. **INTERACTION BETWEEN TRANSTHYRETIN AND METALLOTHIONEINS AND ITS ROLE IN AMYLOID BETA DEPOSITION**
Martinho A, Gonçalves I, Cardoso I, Almeida MR, Saraiva MJ, Santos CRA
XVI National Congress of Biochemistry. October 2008, Azores - Portugal.

Table of Contents

Thesis Overview

This thesis is structured in six main chapters. The first chapter consists in a concise literature review which includes: a short description of choroid plexus (CP) and its main functions; a characterization of the proteins included in the study, transthyretin (TTR) and metallothioneins (MTs), particularly the isoforms 1/2 and 3 (MT-1/2 and MT-3) (**Paper I**), mainly focused on their neuroprotective actions; and, a brief description of hormones, namely glucocorticoids, with emphasis on their neurological roles. The second chapter contains the global aims established for the development of this work. The third, fourth and fifth chapters present the results obtained during the course of the PhD work that were published or submitted as original research papers, organized as follows:

The third chapter comprises the **Paper II** – *Human metallothioneins 2 and 3 differentially affect amyloid-beta binding by transthyretin*. This paper shows that TTR and MT-3 interact and the TTR-MT-2 and TTR-MT-3 interactions affect the TTR binding to amyloid-beta (Aß) peptide, decreasing or increasing it, respectively. Thus, it is suggested that the effects of these interactions in Aß metabolism could be relevant in Alzheimer's disease (AD) context.

The fourth chapter includes the **Paper III** – *Stress and glucocorticoids increase transthyretin expression in rat choroid plexus via mineralocorticoid and glucocorticoid receptors*. The work presented in this chapter shows the up-regulation of TTR expression, particularly in CP, promoted by glucocorticoids. Furthermore, according to the overall effects of TTR and stress/glucocorticoids in AD, a down-regulation of TTR expression would be expectable, which was not observed. Thus, it is suggested that the increased TTR expression promoted by glucocorticoids, particularly in CP, is not sufficient by itself to inhibit the deleterious effects of these hormones in other brain regions and in the overall AD pathophysiology.

The fifth chapter encloses the **Paper IV** - *Glucocorticoids regulate metallothionein-1/2 expression in rat choroid plexus: Effects on apoptosis*. Here, it is showed that the expression of MT-1/2 is regulated by glucocorticoids, in liver and CP, in a gender-, tissue- and time exposure dependent manner. In addition, a decrease in the apoptosis in CP cells, upon incubation with anti-MT-1/2 antibody, is demonstrated, indicating that glucocorticoids have protective roles in CP.

Finally, the sixth chapter summarizes the general conclusions and future perspectives achieved with this project, regarding the interactions between TTR and MT-2/MT-3 and their effects in TTR-Aß binding and the role of glucocorticoids in the regulation of TTR and MT-1/2 expressions, particularly in CP.

Resumo Alargado

Os plexus coroideus (CP) são estruturas cerebrais muito vascularizadas, que se projectam para o interior dos ventrículos e constituem a barreira entre o sangue e o líquido cefalorraquidiano (CSF). Têm como principal função a secreção de CSF. Para além disso, durante o desenvolvimento e o envelhecimento, participam na síntese, secreção e regulação dos compostos biologicamente activos do CSF, na manutenção da biodisponibilidade de metais no cérebro e na remoção dos seus compostos tóxicos, protegendo-o contra insultos neurotóxicos. Por estes motivos, os CP são estruturas essenciais para a manutenção da homeostasia no cérebro.

A transtiretina (TTR) é uma proteína homotetramérica maioritariamente produzida pelos hepatócitos e pelas células epiteliais dos CP (CPECs) e secretada, respectivamente, para a circulação sanguínea e para o CSF, no qual, corresponde a cerca de 25% do seu conteúdo proteico total. Inicialmente, a TTR foi descrita como sendo uma proteína transportadora de hormonas tiróideias, principalmente a tiroxina, e, indirectamente, de retinol (vitamina A), através da sua ligação à proteína de ligação do retinol. Nas últimas décadas, outras importantes funções têm sido atribuídas à TTR: foi demonstrado que a TTR é a principal proteína de ligação ao péptido beta-amilóide (Aß) no CSF, que é uma molécula chave na doença de Alzheimer (AD), actuando como seu sequestrador, impedindo a sua agregação e/ou deposição e promovendo a sua eliminação. Esta propriedade parece ter implicações clínicas já que na AD, a expressão de TTR se encontra diminuida.

As metalotioneínas (MTs) são proteínas polivalentes, de ligação a metais com funções antioxidantes e anti-inflamatórias amplamente descritas. Em mamíferos, existem quatro isoformas descritas (MT-1 a MT-4). Para além das funções já mencionadas, vários autores têm atribuído às isoformas 1/2 e 3 outras acções fisiológicas ao nível do sistema nervoso central (CNS), nomeadamente: a inibição de mecanismos pró-apoptóticos principalmente através da captação de espécies reactivas de oxigénio; o aumento da sobrevivência celular; a regeneração de tecidos; e, o controlo dos níveis de iões metálicos envolvidos na agregação do péptido Aß. Estudos anteriores mostraram que as MTs expressas no cérebro se encontram diferencialmente reguladas em várias doenças neurodegenerativas, sendo que, contrariamente à MT-3, as isoformas MT-1 e MT-2, são altamente induzidas pelo *stress*. De facto, numerosos agentes, tais como metais, citocinas, agentes oxidantes, hormonas e uma variedade de outras moléculas promotoras de *stress* fisiológico e/ou psicológico são reguladores eficientes da expressão de MT-1/2 em algumas regiões do cérebro.

A TTR e as MTs intervêm em numerosas vias metabólicas e desempenham diversas funções ao nível do CNS, sendo, por isso, consideradas proteínas bastante relevantes no seu funcionamento. Um estudo prévio realizado pelo nosso grupo mostrou que a TTR interage com a MT-2, com efeitos desconhecidos ao nível das funções isoladas de cada proteína. Assim, numa primeira abordagem, investigou-se a ocorrência de interacção entre a TTR e a MT-3, uma isoforma predominantemente expressa no cérebro, e estudou-se o efeito das interacções TTR-MT-2/MT-3

na ligação da TTR ao Aß. A interacção entre a TTR e a MT-3 foi caracterizada através de ensaios de *two-hybrid* em leveduras, ensaios de saturação *in vitro*, ensaios de co-imunoprecipitação e ensaios de co-imunolocalização. Para avaliar os possíveis efeitos destas interacções na ligação da TTR ao Aß, realizaram-se ensaios de competição *in vitro*. Da análise dos resultados obtidos concluiu-se que a TTR interage com a MT-3 com uma constante de dissociação (K_d) de 373,7 ± 60,2 nM e que a ligação da TTR à MT-2 ou à MT-3 afecta o metabolismo do Aß. Especificamente, observou-se que, a MT-2 desfavoreceu a ligação da TTR ao Aß, enquanto a MT-3 potenciou esta mesma ligação. Para além disso, os ensaios de co-imunolocalização mostraram que ambas as proteínas co-localizam com o retículo endoplasmático das CPECs, sobretudo na região perinuclear, sugerindo que as MTs, tal como a TTR, são secretadas, podendo estas interacções ocorrer dentro e fora destas células. Este estudo mostrou-se bastante relevante na medida em que serviu não só para a identificação de um novo ligando da TTR como também para mostrar que, na presença de MT-2 ou MT-3, a ligação TTR-Aß é afectada. Assim, deste estudo emergiram novas perspectivas para a clarificação dos mecanismos implicados na ligação da TTR ao Aß na presença de MT-2 e/ou MT-3 e surgiu a necessidade de esclarecer se os efeitos das interacções TTR-MT-2 e TTR-MT-3 na ligação TTR-Aß também ocorrem, e são relevantes, *in vivo*, em modelos animais de AD e, em caso afirmativo, identificar especificamente os mecanismos envolvidos.

Numerosos estudos têm identificado o *stress* como um factor ambiental, directa e/ou indirectamente, relacionado com várias doenças neurodegenerativas. O *stress* provoca aumentos dos níveis de glucocorticóides que, por sua vez, são capazes de gerar respostas adequadas aos seus agentes indutores, participando na neuroprotecção e neuroregeneração. Estudos prévios mostraram que altos níveis de glucocorticóides, induzidos pelo *stress* crónico, regulam a expressão de várias moléculas, incluindo a TTR e as MT-1/2 em algumas regiões do cérebro, e condicionam diversas neuropatologias como a perda de memória, a depressão, o *stress* fisiológico e a AD. No entanto, nos CP, não existe qualquer estudo no que respeita à regulação da expressão de TTR e MT-1/2 pelos glucocorticóides, nos quais podem gerar desequilíbrios na homeostasia dos metais e conduzir ao aumento do *stress* oxidativo e da apoptose nas suas células, podendo danificar a sua estrutura.

Com base nos efeitos do *stress* ao nível do CNS e o papel dos CP na sua homeostasia, bem como o facto de TTR, MT-1/2 e receptores de glucocorticóides e mineralocorticóides (GR e MR, respectivamente) serem expressos pelas células dos CP, pesquisou-se a presença de elementos responsivos dos glucocorticóides (GREs) nos genes da TTR e da MT-1/2. Os resultados revelaram a presença de GREs em ambos os genes, sugerindo que ambas são proteínas potencialmente reguladas por estas hormonas. Neste contexto, estudou-se o efeito dos glucocorticóides na regulação da expressão de TTR e de MT-1/2, particularmente ao nível dos CP. Para tal, realizaram-se estudos *in vitro* incubando culturas primárias de CPECs e células de uma linha celular de CP de rato (RCP) com hidrocortisona, uma forma sintética do cortisol. Para além disso, estudou-se ainda a possibilidade desta regulação ocorrer através dos seus receptores, tendo-se procedido à incubação de células RCP com hidrocortisona, na presença e na ausência de antagonistas dos receptores GR e/ou MR (mifepristona/RU486 e spironolactona,

respectivamente). Paralelamente, foram ainda efectuados estudos *in vivo* em ratos adultos (*Wistar Han*), machos e fêmeas, nos quais se provocou um aumento dos níveis de glucocorticóides, através da indução de um *stress* psicossocial (populacional) agudo ou crónico, e, posteriormente, se avaliou a expressão de TTR (fígado, CP e CSF) e de MT-1/2 (fígado e CP) ao nível da proteína e do mRNA por *Western blot* e PCR em tempo real, respectivamente. A análise dos resultados obtidos mostrou que a hidrocortisona promoveu o aumento da expressão de TTR em culturas celulares de RCP e CPECs, via GR e MR, dado que na presença de pelo menos um dos antagonistas destes receptores, este efeito não se verificou. Nas experiências de indução de *stress* psicossocial agudo ou crónico foi observado um consistente aumento na expressão de TTR no fígado, CP e CSF, em machos e em fêmeas, tendo esse aumento sido máximo nos machos após o *stress* agudo. De um modo geral, concluiu-se que o *stress*, com associados aumentos nos níveis de glucocorticóides circulantes, regula positivamente a expressão TTR, via GR e MR, particularmente ao nível dos CP.

Nas experiências realizadas *in vitro* para testar a hipótese da expressão de MT-1/2 ser regulada pelos glucocorticóides, observou-se que a hidrocortisona induziu a expressão de MT-1/2 quer em células RCP quer em CPECs via GR e MR, dado que, também para a MT-1/2, a incubação com antagonistas de GR e/ou MR anulou este efeito. Os estudos *in vivo* mostraram um aumento consistente na expressão de MT-1/2 ao nível do fígado e CP, nos ratos machos e fêmeas, após a indução de um *stress* psicossocial crónico. O mesmo foi observado no fígado, após a indução do *stress* agudo. Pelo contrário, a indução de um *stress* psicossocial agudo, provocou efeitos distintos entre sexos ao nível da expressão de MT-1/2 nos CP, já que se observou uma sobrexpressão de MT-1/2 nas fêmeas, e o efeito oposto nos machos, sugerindo um papel preponderante do género, com consequentes diferenças nas respostas ao mesmo estímulo. Neste estudo foi ainda testada a hipótese da regulação da expressão de MT-1/2 pelos glucocorticóides ter implicações na apoptose. Para tal, realizaram-se ensaios de citometria de fluxo para determinação dos níveis de apoptose nas células RCP, decorrentes do estímulo da expressão de MT-1/2 pelos glucocorticóides. A análise dos resultados mostrou uma redução nos níveis de apoptose para cerca de metade (de 17,8% para 9,7%) na presença de MT-1/2, tendo este efeito sido revertido pela pré-incubação com anticorpo anti-MT-1/2. Assim, a sobrexpressão de MT-1/2 após a incubação com glucocorticóides diminuiu a apoptose das células de CP e, neste sentido, os glucocorticóides poderão promover a manutenção da integridade deste tecido e, por conseguinte, serem neuroprotectores nestas estruturas. De um modo geral, concluiu-se que a expressão de MT-1/2 nos CP é regulada pelos glucocorticóides, com implicações na apoptose, sendo esta regulação dependente do sexo e da duração do estímulo.

As doenças neurodegenerativas encontram-se associadas a perturbações em numerosas vias, moléculas e suas interacções complexas. Uma dada molécula ou estímulo pode ser potenciador ou inibidor de uma determinada patologia dependendo de muitos outros aspectos, e alguns resultados contraditórios têm surgido ao longo dos anos. Estudos anteriores mostraram que níveis sistematicamente elevados de glucocorticóides perturbam a homeostasia do cérebro e estão, geralmente, associados a demências. No entanto, outros autores mostraram que estes aumentos

também podem ser benéficos na medida em que podem prevenir o aparecimento/ progressão de algumas doenças neurodegenerativas e ter efeitos anti-inflamatórios em determinadas regiões do cérebro.

De um modo geral, sugere-se que os efeitos neuroprotectores promovidos pelos glucocorticóides ao nível dos CP podem não ser suficientes, por si, para prevenir os efeitos adversos promovidos pelos elevados níveis de glucocorticóides noutras regiões do cérebro, na expressão de outras moléculas ou na progressão global de uma patologia. De facto, de acordo com os resultados obtidos, a regulação positiva da expressão de TTR e de MT-1/2, promovida pelos glucocorticóides, confere propriedades neuroprotectoras aos CP. No entanto, esta poderá originar também uma maior biodisponibilidade de TTR e MT-1/2 e favorecer a ocorrência de interacções TTR-MT-2 que, por sua vez, poderão contribuir para a acumulação de Aß ao desfavorecerem a sua interacção com a TTR. Assim, o benefício *versus* dano de uma dada molécula numa determinada neuropatologia dependerá sempre do seu equilíbrio sendo o seu efeito final, o resultado dos seus impactos directo, na patologia, e indirecto, em outras moléculas também elas intervenientes.

Em suma, os resultados acima descritos mostraram a ocorrência de uma interacção entre a TTR e a MT-3 e salientaram a importância da ocorrência de interacções TTR-MT-2 e TTR-MT-3 na ligação da TTR ao Aß. Para além disso, mostrou-se a relevância dos glucocorticóides na regulação da expressão de TTR e MT-1/2, particularmente ao nível dos CP.

Este trabalho não relaciona directamente os efeitos dos glucocorticóides na expressão de TTR ou de MT-1/2 com qualquer patologia específica. No entanto, a TTR e as MTs, bem como os glucocorticóides, são moléculas chave em diversas neuropatologias, como a AD, e, a TTR e a MT-2 ou MT-3 interagem e afectam o metabolismo do Aß tornando-se, por isso, proteínas bastante promissoras para a investigação nesta área. Nesta perspectiva, estudos futuros que envolvam indução de *stress*/administração de glucocorticóides em modelos animais de AD cruzados com TTR knockout (*KO*) e/ou MT-1/2 e/ou MT-3 *KO*, com determinação dos níveis de Aß no cérebro e análise do desempenho cognitivo, serão cruciais para a clarificação do papel destas moléculas no metabolismo do Aß e na apoptose e, consequentemente, na progressão de certas doenças neurodegenerativas, como a AD.

Palavras-chave

Transtiretina, Metalotioneínas, Plexus coroideu, Glucocorticóides, Péptido beta-amilóide, Regulação, Expressão.

Abstract

The choroid plexus (CP), localized within brain ventricles, constitutes the barrier between the blood and the cerebrospinal fluid (CSF) and is an essential structure for the maintenance of brain homeostasis. It participates in the synthesis, secretion and regulation of the CSF and various biologically active compounds, in the maintenance of central nervous system (CNS) metal bioavailability and in the removal of brain toxic compounds, protecting it against neurotoxic insults.

Transthyretin (TTR) is a homotetrameric protein mostly produced and secreted by the liver to the peripheral circulation and by the CP epithelial cells (CPECs) to the CSF. The TTR major functions are the transport of thyroid hormones, principally thyroxin, and, indirectly, retinol. Furthermore, in the last decades, it has also been shown that TTR acts as an amyloid-beta (Aß) peptide scavenger, a key molecule in Alzheimer's disease (AD), preventing its aggregation and/or deposition and promoting its clearance. Actually, the non-mutated form of TTR has been identified as the main Aß binding protein in the CSF.

Metallothioneins (MTs) are multipurpose proteins with widely described metal binding, antioxidant and anti-inflammatory properties. In mammals, four distinct isoforms had been identified (MT-1 to MT-4). In addition, other physiological actions have also been attributed to MTs: inhibition of pro-apoptotic mechanisms, enhancement of cell survival and tissue regeneration. Previous studies showed that the expression of brain MTs is regulated in several neurodegenerative disorders, as AD, and MT-1/2, contrarily to MT-3, are highly inducible by physiological and psychological stress.

TTR and MTs act in several metabolic pathways, especially within the CNS, where they play major roles in its homeostasis. Previously, our group showed that TTR interacts with MT-2, an ubiquitous isoform of the MTs, with unknown effects on the functions of each protein. Thus, we firstly investigated whether it also interacts with MT-3, an isoform predominantly expressed in the brain, and studied the role of MT-2 and MT-3 in human TTR–Aß binding. The TTR–MT-3 interaction was characterized by yeast two-hybrid assays, saturation-binding assays, co-immunolocalization and co-immunoprecipitation assays. Moreover, the effect of MT-2 and MT-3 in TTR–Aß binding was assessed by competition-binding assays. The results demonstrated that TTR interacts with MT-3 with a dissociation constant (K_d) of 373.7 ± 60.2 nM. Also, TTR-MT-2 interaction diminished the TTR–Aß binding, whereas MT-3 enhanced the binding of TTR to Aß, most likely promoting its degradation. Furthermore, both proteins co-localized with the endoplasmic reticulum of CPECs, indicating that, as TTR, MTs may also be secreted and interactions between TTR and MTs might occur inside and outside these cells.

Stress is related with neurodegenerative disorders because it raises glucocorticoid levels, which generate adequate responses to stressors and regulate key molecules, as TTR and MT-1/2, in some brain regions, participating in neuroprotection and neuroregeneration. However, in CP, nothing is known concerning the regulation of TTR and MT-1/2 expressions by glucocorticoids.

In silico analyses of TTR and MT-1/2 genes identified glucocorticoid responsive elements (GREs) in both genes. Also, CP expresses TTR, MTs (isoforms 1, 2 and 3) and glucocorticoid and mineralocorticoid receptors (GR and MR, respectively) turning it into a likely glucocorticoid responsive tissue. Thus, we hypothesized that TTR and MT-1/2 could be regulated by glucocorticoids within the CP. We investigated the regulation of TTR expression in response to hydrocortisone in a rat choroid plexus (RCP) cell line and in primary cultures of CPECs. In addition, the effect of psychosocial stress induction in TTR expression was analyzed in rat liver, CP and CSF. The results showed that hydrocortisone up-regulated TTR expression in RCP and CPEC cultures, and, this effect was suppressed upon addition of GR and/or MR antagonists, suggesting the involvement of these receptors in this regulatory mechanism. Moreover, induction of psychosocial stress increased TTR expression in liver, CP and CSF of animals subjected to acute or chronic stress conditions, showing that stress up-regulates TTR expression, particularly in CP.

To test the hypothesis that MT-1/2 expression in CP could also be regulated by glucocorticoids, with implications in apoptosis, we performed analogous experiments to those described above. Data obtained showed that hydrocortisone up-regulated MT-1/2 expression in RCP cells and CPECs and the incubation with GR and/or MR antagonists abrogated this effect. In addition, comparing to controls, the incubation of RCP cells with hydrocortisone diminished the ratio of apoptotic/late apoptotic cells from 17.8% to 9.7% and, this effect was abolished by the addition of an anti-MT-1/2 antibody. Thus, the up-regulation of MT-1/2 expression after incubation with glucocorticoids diminished apoptosis in CP and in this regard, glucocorticoids may beneficiate CP integrity and therefore become neuroprotective. *In vivo* studies showed that induction of chronic psychosocial stress increased MT-1/2 expression in liver and CP of male and female rats. A similar pattern was observed after acute stress in liver. Interestingly, in CP, induction of acute stress caused different effects between genders as, in females, it promoted an up-regulation of MT-1/2 expression, while a down-regulation was observed in males, indicating a distinct regulation gender-dependent and, suggesting distinct readjustment in response to stress between males and females in this structure. Generally, results showed that glucocorticoids regulate MT-1/2 expression in rat CP, time-, tissue- and gender-dependently, with implications in apoptosis.

Numerous pathways and molecules and its complex interactions are involved in neurodegenerative disorders. In line with this, we suggest that the putative and isolated neuroprotective effects promoted by glucocorticoids in CP may not be sufficient *per se* to prevent the negative effects promoted by high levels of cortisol in other brain regions, other molecules or in the overall progression of a disease. Taken together, our results suggest that the up-regulation of TTR and MT-1/2 in CP, promoted by glucocorticoids, also result in a higher bioavailability of these proteins that may potentiate the occurrence of TTR-MT-2 interactions, which negatively affect the Aß clearance and, consequently, AD. Future studies will be crucial for clarification of the role of these molecules and their interactions with Aß metabolism and apoptosis, which may have far reaching effects in various neurodegenerative disorders, as AD.

Keywords

Transthyretin, Metallothioneins, Choroid Plexus, Glucocorticoids, Amyloid-beta peptide, Regulation, Expression.

List of Figures

Figure 8: Overview of steroidogenesis. Cholesterol provides the substrate for *de novo* steroidogenesis. StAR (steroidogenic acute regulatory protein) mediates cholesterol delivery to the inner mitochondrial membrane and P450scc (cholesterol side-chain cleavage enzyme) in the adrenal glands, gonads and rodent placenta. The enzymes that mediate all reactions are indicated in italics and the major tissues for each reaction are boxed. Abbreviations include Preg (pregnenolone), P (progesterone), DOC (deoxycorticosterone), ZF (*zona fasciculata*), ZR (*zona reticularis*), ZG (*zona gomerulosa*), 3β-HSD (3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase), P450c17α (17α-hydroxylase/17,20-lyase), DHEA (dehydroepiandrosterone) and 17β-HSD (17β-hydroxysteroid dehydrogenase). Adapted from Lavoie and King 2009.

List of Acronyms

11-HSD	11ß-hydroxysteroid dehydrogenase
17ß-HSD	17ß-hydroxysteroid dehydrogenase
3ß-HSD	3ß-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase
3ß-HSD2	3ß-hydroxysteroid dehydrogenase 2
aa	Amino acid
Aß	Amyloid-beta
A. C.	Carbonic anhydrase
ACTH	Adrenocorticotrophic hormone
AD	Alzheimer's disease
Ade	Adenine
ALS	Amyotrophic lateral sclerosis
apo	Apolipoprotein
apoA-I	Apolipoprotein A-I
APP	Amyloid precursor protein
AQP	Aquaporins
AR	Androgen receptor
Ara-C	Cytosine arabinoside
ATP	Adenosine triphosphate
BCSFB	Blood CSF barrier
bp	Base pairs
BSA	Bovine serum albumin
C/EBP	CCAAT/ enhancer binding protein
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
CBG	Corticosteroid-binding globulin
cDNA	Complementary DNA
Chr	Chromosome
Cl^-	Chloride ion
CNS	Central nervous system
Co-IP	Co-immunoprecipitation
CORT	Corticosterone
CP/CPs	Choroid plexus / Choroid plexuses
CPEC/CPECs	Choroid plexus epithelial cell / Choroid plexus epithelial cells
CRH	Corticotrophin releasing hormone
CSF	Cerebrospinal fluid
Cu^{2+}	Copper ion

CycA	Cyclophilin A
CYP11A	Cholesterol side-chain cleavage enzyme
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DHEA	Dehydroepiandrosterone
DHT	5α-dihydrotestosterone
DMEM	Dulbecco's modified eagle medium
DNA	Desoxyribonucleic acid
dNTP/dNTP's	Deoxynucleotide Triphosphate / Deoxynucleotide Triphosphates
DOC	11-deoxycorticosterone
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
E. coli	*Escherichia coli*
E2	Estradiol / 17β-estradiol
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
Emtins	Peptides derived from M-1/2 domains
ERα	Estrogen alpha receptor
ERβ	Estrogen beta receptor
FAP	Familial amyloid polyneuropathy
FBS	Fetal bovine serum
Fe^{2+}	Iron ion
FITC	Fluorescein isothiocyanate
FSH	Follicule-stimulating hormone
FTD	Frontotemporal dementia
GR/GRs	Glucocorticoid receptor / Glucocorticoid receptors
GRE/GREs	Glucocorticoid responsive element / Glucocorticoid responsive elements
HBS	Hormone-binding site
HCl	Hydrochloric acid
HCO^{3-}	Bicarbonate ion
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
hMT/hMTs	Human MT / Human MTs
HNF	Hepatocyte nuclear factors
HPA	Hypothalamic-pituitary-adrenal
HPT	Hypothalamic-pituitary-thyroid
HSF1	Heat shock factor 1
HSPs	Heat shock proteins

hTTR	Human TTR
K^+	Potassium ion
kb	Kilobase
KCl	Potassium chloride
K_d	Dissociation constant
kDa	Kilodaltons
KO	Knockout
LDL	Low density lipoprotein
Leu	Leucine
LH	Luteinizing hormone
MAPK	Mitogen activated protein kinase
$MgCl_2$	Magnesium chloride
MR/MRs	Mineralocorticoid receptor / Mineralocorticoid receptors
mRNA	Messenger RNA
MT/MTs	Metallothionein/Metallothioneins
MT1/MT-1	Metallothionein 1
MT2/MT-2	Metallothionein 2
MT1/2/MT-1/2	Metallothionein 1 and 2
MT3/MT-3	Metallothionein 3
MT4/MT-4	Metallothionein 4
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
Na^+	Sodium ion
NaCl	Sodium chloride
NO	Nitric oxide
NPY	Neuropeptide Y
ORFs	Open reading frames
P	Progesterone
P40c21	21-hydroxilase
P450c11	11ß-hydroxylase
P450c17	17α-hydroxyprogesterone
P450c17α	17α-hydroxylase/17,20-lyase
P450scc	P450 side chain cleavage
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PD	Parkinson's disease
PI	Propidium iodide
PMSF	Phenylmethylsulfonyl fluoride
PNS	Peripheral nervous system
POR	P450 oxireductase

PR	Progesterone receptor
PREG	Pregnenolone
PVDF	Polyvinylidene difluoride
RAP	Receptor-associated protein
RBP	Retinol-binding protein
RCP	Rat choroid plexus cell line
rMT/rMTs	Rat MT / Rat MTs
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rTTR	Rat TTR
RU486	Mifepristone
S. cerevisiae	*Saccharomyces cerevisiae*
SAPPα	Soluble (non-amyloidogenic) APP
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SHs	Steroid hormones
Spiro	Spironolactone
SSHs	Sex steroid hormones
StAR	Steroidogenic acute regulatory protein
T	Testosterone
T3	Triiodothyronine / 3,5,3'-triiodothyronine
T4	Thyroxine / 3,3',5,5'-tetraiodothyronine
TBG	Thyroxine-binding globulin
TBS	Tris buffered solution
THs	Thyroid hormones
TRH	Thyrotropin-releasing hormone
Trip	Tryptophane
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol / tris(hydroxymethyl)aminomethane
TSH	Thyrotropin / Thyroid-stimulating hormone
TTR	Transthyretin
Wt	Wild-type
Zn^{2+}	Zins ion
ß2m	Beta-2 microglobulin

Chapter 1: General Introduction

Overview

The increase of the life expectancy, particularly in developed countries, has been contributed to the raise of the incidence of several neurodegenerative disorders. Currently, the central nervous system (CNS) disorders became the most common form of dementia, particularly in aging, affecting millions of people. Regarding this issue, several brain structures and regions have been associated with various neuropathologies and deregulation of numerous metabolic pathways therein have been described as contributors to the onset and/or progression of various disorders, including the Alzheimer's disease (AD). Among these structures is the choroid plexus (CP), a pivotal brain structure in the maintenance of the overall CNS homeostasis. Furthermore, there are also several molecules related with brain disorders with well, but not fully, described outcomes. For example, the amyloid-beta (Aß) peptides, transthyretin (TTR), some isoforms of metallothioneins (MTs) and many steroid hormones (SHs), including glucocorticoids, are relevant players in various neuropathologies, as AD. Also, both TTR and MTs (isoforms 1, 2 and 3) are expressed in CP and are regulated by various SHs.

Therefore, in this chapter we introduce and describe several concepts that were the basis for the development of the research work.

Choroid Plexus

1. Introduction

In the central part of the human brain there are four cavities, interconnected with each other and with the subarachnoid space and the central canal, known as ventricles: two lateral ventricles located centrally in each of the cerebral hemispheres; and, a third and fourth ventricles, sited below the lateral ventricles. The CP is a brain structure found within both lateral, third and fourth ventricles (Emerich et al. 2005b), thus in a total of four. It constitutes the main site of cerebrospinal fluid (CSF) production, and forms a unique interface between the blood and the CSF - blood-CSF barrier (BCSFB) (Ghersi-Egea and Strazielle 2001). In addition to the secretion of CSF, CP also acts in the onset/progression of various CNS disorders and plays other key functions mainly related with brain homeostasis, development, detoxification and aging.

2. Basic Structure and Morphology

The CP is a highly phylogenetic conserved structure that develops and becomes active early during embryogenesis (Dziegielewska et al. 2001). It is a lobulated structure formed by a continuous and single layer of epithelial cells that surround a central core of extremely vascularized connective tissue. The CP contains a ramified and complex vascular network, which provides a 4 to 7 times higher irrigation comparing to other brain tissues (Emerich et al. 2005b). It has a leaf-like structure which floats in the CSF and is attached to the ependyma by a thin stalk. CP differentiates from the ependymal cells lining the ventricular walls. As represented in figure 1, the CP epithelial cells (CPECs) constitute a simple cuboidal epithelium, supported by the basal lamina (Lipari and Lipari 2008; Wheater's 2007). These modified ependymal cells have abundant villi with a brush border of microvilli in the apical side and numerous infoldings in the basal side, which vastly expand the contact area between the cell cytoplasm and the CSF (Del Bigio 1995; Keep and Jones 1990a; Keep and Jones 1990b). The apical side contacts directly with CSF, which fills the interior of the ventricles, and the basal side contacts with the inner stroma and numerous fenestrated blood capillaries, dendritic cells, macrophages and fibroblasts. In the apical apical side (ventricular side) are also present the epiplexus cells (Kolmer's cells) that are attached to the microvilli in the apical side of these epithelial modified ependymal cells and are referred as intraventricular macrophages (Fig. 1) (Pietzsch-Rohrschneider 1980; Schwarze 1975). Adjacent CPECs are juxtaposed by the tight junctions present in the apical side (Engelhardt and Sorokin 2009) (Fig. 2), which restrict the passage of molecules and ions through the BCSFB (Brightman and Reese 1969).

Intracellularly, each CPEC contains a large central spherical nucleus and an abundant cytoplasm, with numerous mitochondria, prominent Golgi apparatus and smooth endoplasmic reticulum and high number of vesicles with lisossomal features that ensure its large secretory activity, particularly the secretion of CSF (Smith et al. 2004; Emerich et al. 2005b).

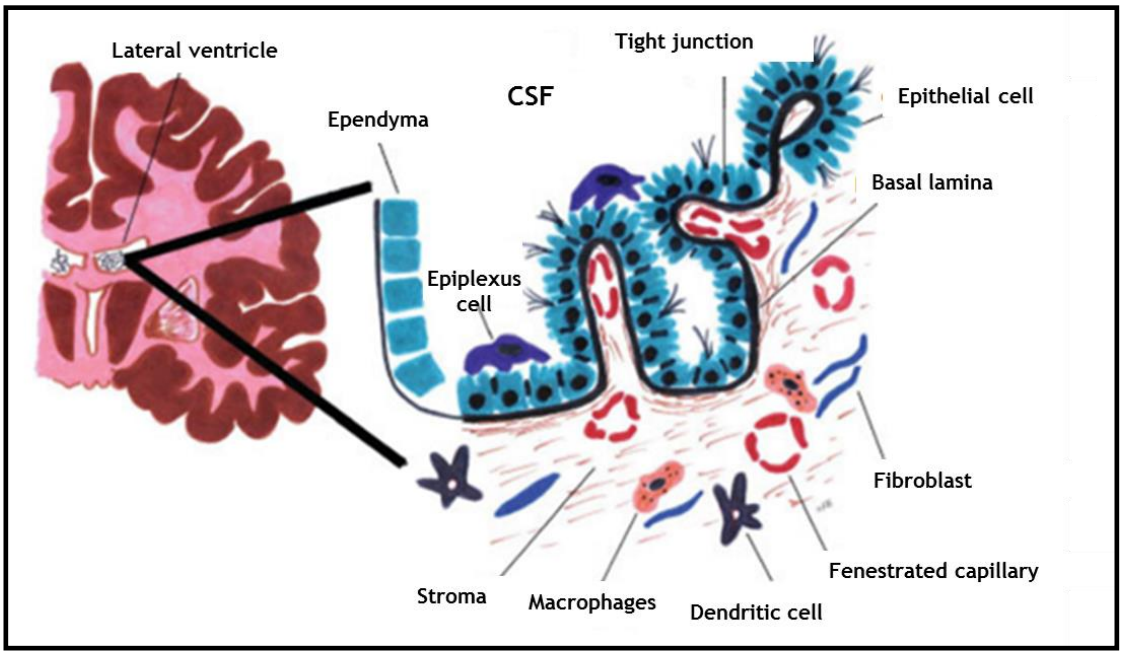

Figure 1: Morphological representation of the choroid plexus (CP) within the lateral ventricle of the human brain. Adapted from Emerich et al. 2005b.

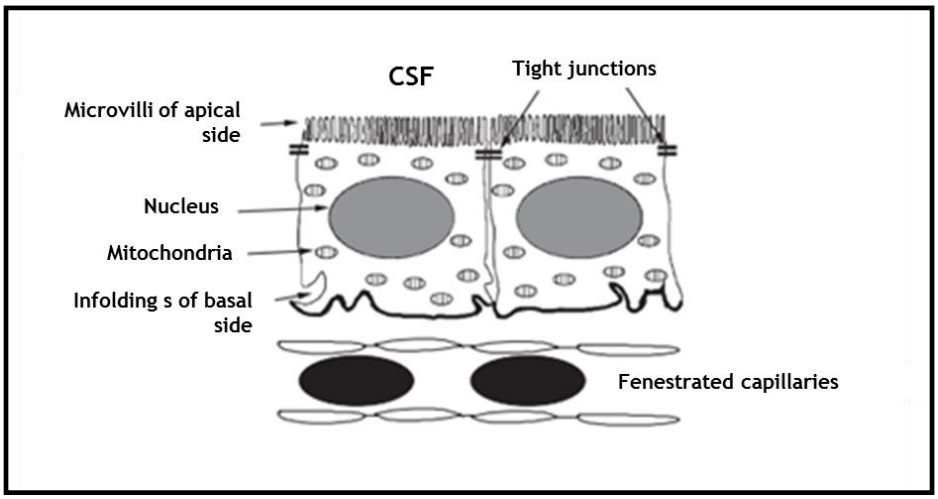

Figure 2: Morphological representation of the tight junctions between the CP epithelial cells (CPECs). Adapted from Smith et al. 2004.

3. Biological Functions

The CP epithelium forms the BCSFB and the structural and functional integrity of this barrier is crucial to the homeostasis of the internal environment of the CNS as it controls the passage of molecules and cells from the blood into the CSF through specific transporters and receptors

(Marques et al. 2011). The functions of CP-CSF system were firstly restricted to provide physical protection to the brain and to facilitate the removal of brain metabolites through the bulk drainage of CSF. In the last years, these functions had been extended as it was showed that the CP-CSF system also plays major roles during the development, homeostasis and repair of the CNS (Chodobski and Szmydynger-Chodobska 2001; Redzic et al. 2005). Despite the most well-recognized function of CP is the synthesis and secretion of the CSF (Speake et al. 2001), it is also responsible for nutrition and protection of the brain, by renewing the CSF and providing micronutrients, neurotrophins, neuropeptides and growth factors. Previous studies showed that CP participates in the synthesis, secretion and regulation of numerous biologically active compounds (Chodobski and Szmydynger-Chodobska 2001), some of them implicated in neuroprotection and neurorepair processes (Li et al. 2002; Emerich et al. 2005a; Itokazu et al. 2006;). It also regulates the biochemical environment of the brain, protecting it from the accumulation of toxic products of its metabolism (Chodobski and Szmydynger-Chodobska 2001; Redzic and Segal 2004; Emerich et al. 2005b). CP maintains the extracellular milieu of the brain through the control of the chemical exchange between the CSF and brain parenchyma (Engelhardt et al. 2001; Engelhardt and Sorokin 2009) and is highly sensitive to any alterations in the CSF composition. Indeed, it limits the access of blood substances to the cerebral compartment and serves as a unique source of essential elements to the CNS (Nilsson et al. 1992; Zheng et al. 2001). CP also facilitates the transport of thyroid hormones (THs) from blood to CSF through TTR, which is also synthesized in the choroidal epithelia (Schreiber et al. 1990; Southwell et al. 1993); participates in the maintenance of the homeostasis of the essential metal ions, as iron (Mesquita et al. 2012); and, mediates some repair processes following trauma and acute neurotoxic insults through a complex detoxification system (Gao and Meier 2001; Ghersi-Egea and Strazielle 2001). In addition, CP possesses numerous receptors involved in the inflammatory process, suggesting that these structures may have major roles in the relationship between the CNS and the peripheral immune system (Petito and Adkins 2005). Also, CP cells exhibit several receptors for various neurotransmitters, growth factors and hormones (Chodobski and Szmydynger-Chodobska 2001), such as progesterone (PR) (Quadros et al. 2007), estrogen alpha (ERα) and beta (ERß) (Hong-Goka and Chang 2004; Quintela et al. 2009), androgen (AR) (Alves et al. 2009) and glucocorticoid (GR) and mineralocorticoid receptos (MR) (Amin et al. 2005; Gomez-Sanchez 2010; Sinclair et al. 2007; Sousa et al. 1989), evidencing that CP is a target tissue for, at least some, steroid hormones (Quintela et al. 2008; Quintela et al. 2009; Quintela et al. 2011). Taken together, this variety of functions suggests that even modest changes in the CP can have far-reaching effects. Also, changes in the anatomy and physiology of the CP have been linked with aging and various CNS disorders.

3.1 Production of Cerebrospinal Fluid

CP produces and secretes the vast majority (70-90%) of the CSF, which fills the ventricles, the subarachnoid space and the spinal cord (Speake et al. 2001). In humans, there is about 150-270 mL CSF and new one is constantly formed at a rate of approximately 600 mL/24 h (Kohn et al.

Wright 1978) allowing its replacement, 3 to 4 times per day (Wright 1978).

The CSF is a slightly viscous, clear and colorless aqueous solution, with a pH of ~7.5 (Johanson et al. 2008) and constitutes the major part of the extracellular fluid of the CNS. It serves as an intracerebral transport medium for numerous nutrients, neurotransmitters, neuroendocrine molecules and few cells, such as leucocytes (Pollay 2010). As mentioned, the vast majority of the CSF is produced by CP cells, under neuroendocrine modulation (Nilsson et al. 1992; Weaver et al. 2004). Additionally, small amounts of CSF (10-30%) have extrachoroidal sources, namely the blood vessels, the pia-arachnoid and ependymal lining of the ventricular system and the pia-glial membrane (Johanson et al. 2008). As represented in figure 3, the mechanism of CSF choroidal production and secretion is an active process, mainly driven by the carbonic anhydrase (A.C.) and the Na^+/K^+ ATPase. It comprises the net transport of sodium (Na^+), chloride (Cl^-), potassium (K^+) and bicarbonate (HCO^{3-}) ions and water from plasma to CPECs (on the basal membrane) and then, to CSF (on the apical side). Briefly, Na^+ ions are transported across the CPECs and pushed outside of the CP cells to the CSF. These ions attract HCO^{3-} and Cl^- ions, changing the osmotic gradient and then, the CSF, draws water across the CPECs apical membrane, through osmosis or specific water channels as aquaporins (AQP). Glucose, and HCO^{3-} and Na^+ ions are transported out of the blood capillaries by other processes (Fig. 3) (Johanson et al. 2008; Marques et al. 2011).

Despite some differences, it is generally considered that the composition of the CSF is similar to blood plasma. Even though, under physiological conditions, the CSF levels of some of its components differ slightly from those in plasma, as it: contains approximately 99% water (compared to the 92% water plasma content); exhibits few cells; has lower levels of proteins, glucose and calcium (Ca^{2+}), K^+ and HCO^{3-} ions (Johanson et al. 2008); and, presents higher contents of folate (Serot et al. 2001), Cl- and magnesium ions.

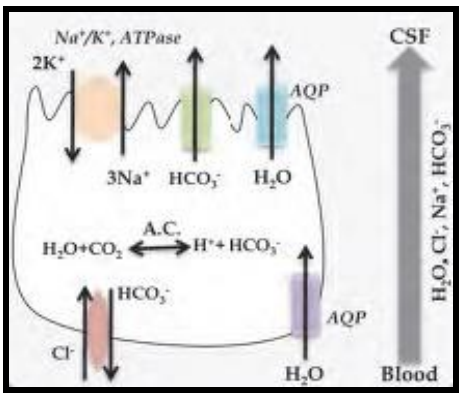

Figure 3: Schematic representation of the cerebrospinal fluid (CSF) production and secretion in the CPECs. Adapted from Marques et al. 2011.

The major functions of the CSF include: the buoyancy and intracranial volume adjustment that confer physical support to the brain; the buffering of brain extracellular fluid ions and other solutes; the drainage and clearance of catabolites; and, the transport of several peptides

and neurotransmitters into various brain regions (Segal 2000), including injured regions and neural stem cells in periventricular regions, promoting tissue recovery and stimulating neurogenesis (Johanson et al. 2004). Moreover, the CSF also protects the brain against ischemia and high acute blood pressure and, improves neuronal survival (Watanabe et al. 2005), by mechanisms not yet fully understood. Therefore, the conservation of the equilibrium in the volume and composition of the CSF, through the blood brain barrier and BCSFB, is crucial for the maintenance of the brain homeostasis.

4. Choroid Plexus, Aging and Neurological Disorders

The CSF homeostasis is mediated mainly by CP and, during aging, significant changes occur in the morphological structure and metabolism of CP, with effects on its normal functions. Furthermore, the CP-CSF system also becomes less able to regulate the brain interstitial fluid (Johanson et al. 2004). In fact, with advancing age: the CPECs acquire irregular shapes and their basal membrane become flattened, losing about 10 to 12% in height (Serot et al. 2000); the stroma surrounding the cells also undergoes a thickening due to the deposition of collagen fibers and hyaline bodies; accumulation of numerous intracellular cytoplasmic inclusions - the Biondi rings - and lipofuscin vacuoles occur in the CPECs cytoplasm (Jane et al. 2005; Kiktenko 1986; Wen et al. 1999); the blood vessels that support the CP cells suffer calcification; and, the basal lamina, thickens and fragments. Furthermore, the overall enzymatic and metabolic activities of CP cells decrease as well as their capacity to remove toxic compounds; the number of co-carriers of Na^+, K^+ and Cl^- ions and Na^+/K^+ ATPase reduce; the number of cytochrome c oxidase in CPECs increase, providing changes in mitochondrial respiratory chain, and consequent decreases in the ATP production (Ferrante et al. 1987); and, the synthesis of biologically active substances, as TTR, decrease and its delivery becomes imbalanced (Emerich et al. 2005b). Also, the CSF choroidal secretion decreases (Preston, 2001).

All these modifications cause dramatic alterations in CP cells and, consequently, in CP functions with profound implications in the overall brain homeostasis that may contribute to the age-related cognitive and motor decline observed in several neurological disorders (Emerich et al. 2005b), as in AD (Mesquita et al. 2012). In line with this, various evidences support the hypothesis that imbalances in the CP functions during aging intensify the pathogenesis of AD (Serot et al. 2000; Serot et al. 2003). Authors confirmed that these alterations in aged CP are exacerbated in AD patients (Krzyzanowska and Carro 2012).

Transthyretin

1. Introduction

TTR is a protein firstly observed in the cerebrospinal fluid (CSF) (Kabat et al. 1942) and then in the serum (Siebert and Nelson 1942). It was initially named prealbumin, as it migrates ahead of albumin during electrophoresis (Kabat et al. 1942). After, it was designated thyroxine-binding prealbumin due to its binding to THs (Ingbar 1958). Only in 1981 this designation changed definitely to transthyretin, which reflects the two major TTR physiological roles: transporter of THs, mainly thyroxine (T4) (Woeber and Ingbar 1968), and, indirectly, retinol, through its binding to retinol-binding protein (RBP) complexed with retinol (Goodman 1985).

2. Gene Structure

The gene encoding human TTR is a single copy, localized in the long arm of chromosome 18 (Chr 18) (Tsuzuki et al. 1985) in the q11.2-q12.1 region (Sparkes et al. 1987) (Fig. 4a). It encompasses about 7.0 kilobases (kb), containing four exons of 95, 131, 136 and 253 base pairs (bp) and three introns of 934, 2090 and 3308 bp (Fig. 4b) (Sasaki et al. 1985).

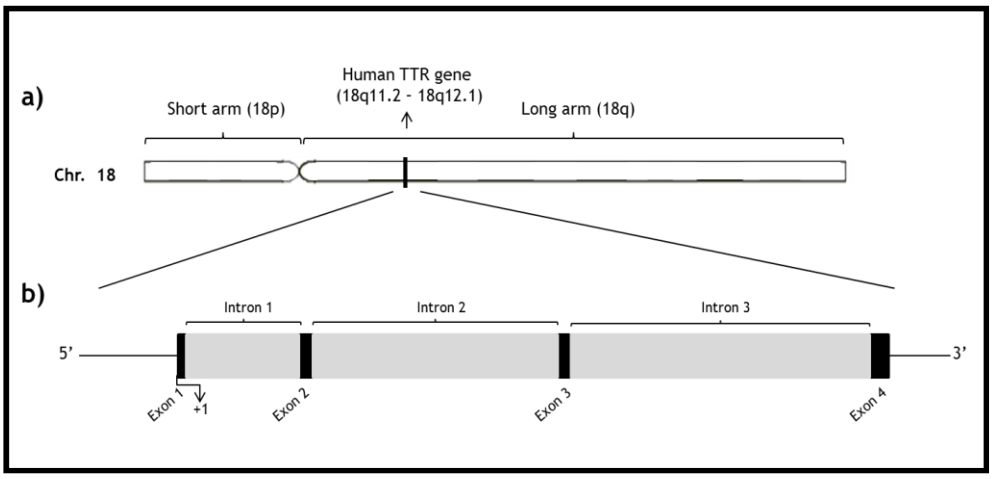

Figure 4: Representation of the human transthyretin (TTR) gene localization and structure: **a)** *Locus* of TTR on chromosome 18 (18q11.2-18q12.1). **b)** Structure of TTR gene. Black boxes represent the exons 1, 2, 3 and 4 and the grey boxes represent the introns 1, 2 and 3.

Exon 1 encodes a leader peptide of 20 amino acids (aa), which are removed post-translationally, and 3 aa of the mature protein; exons 2, 3 and 4 encode the 4-47, 48-92 and 93-127 aa residues of the mature TTR, respectively (Sakaki and Sasaki 1985; Tsuzuki et al. 1985). In the 5' flanking region upstream the transcription initiation site, several consensus sequences were identified: a TATA box sequence at position -30 to -24 bp, a CAAT box sequence from -101 to -96 bp, a GC-rich region (of approximately 20 bp), Alu sequences and several hormone responsive

consensus sequences. For instance, in its proximal promoter, two overlapping sequences homologous to glucocorticoid responsive elements (GREs) were present at positions -224 and -212 bp. In the 3'-untranslated region, downstream the coding sequence, a polyadenylation signal (AATAAA) was identified at 123 bp (Sasaki et al. 1985; Tsuzuki et al. 1985; Wakasugi et al. 1985). Two independent open reading frames (ORFs) are present in the first and third introns, although it is well established that, *in vivo,* these ORFs are not expressed independently, or in part, as functional transcripts (Soares et al. 2003).

Phylogenetically, TTR gene is highly conserved. Both mouse and rat TTR genes are composed by 4 exons and 3 introns, as in human, and, the DNA sequence of the coding region of the mouse TTR gene exhibits 90 and 82% homology with the rat and the human genes, respectively. Moreover, the highest homology between mouse, rat and human TTR was observed in the promoter region, around -190 bp to the cap site (Costa et al. 1986b; Fung et al. 1988).

3. Gene Regulation

Previous studies carried out on mice showed that TTR gene is controlled by two major regulatory regions located in the 5' flanking region: a promoter proximal sequence at -50 to -150 bp and a distal enhancer sequence at -1.86 to -1.96 kb. These regulatory sequences are conserved in humans. In both regions, several putative regulatory sites were identified: DNA binding sites for liver-specific nuclear factors, as the hepatocyte nuclear factors 1, 3 and 4 (HNF-1, HNF-3 AND HNF-4) and CCAAT/enhancer binding proteins (C/EBP) families (Costa et al. 1986a; Costa et al. 1990). In human TTR gene, these regulatory regions were proved to be sufficient to regulate its hepatic expression (Costa et al. 1986a; Yan et al. 1990). Concerning TTR expression in CP, less is known, and the few studies performed suggested that TTR is differentially regulated between liver and CP, thus involving different transcription factors (Nagata et al. 1995; Yan et al. 1990). In fact, while a shorter sequence (<1 kb) upstream the mRNA cap site is sufficient to drive TTR expression in the liver, in CP, the presence of a further upstream sequence (>3 kb) is required (Nagata et al. 1995; Yan et al. 1990). Studies performed in transgenic mice showed that the 600 bp sequence upstream of the TTR gene is enough for the TTR expression in liver and yolk sac (Yan et al. 1990) whereas sequences of approximately 6 kb upstream the coding sequence are necessary for the total tissue specific synthesis and quantitatively normal expression (Nagata et al. 1995).

4. Protein Structure

X-ray crystallography and diffraction studies determined the three dimensional structure of TTR protein (Blake et al. 1971) and revealed a 55 kDa tetramer, composed of four identical subunits

assembled around a central channel. Each monomer contains 127 amino acids brought together in a ß-sandwich structure organized in two extensive ß-sheets, each composed of 4 ß-strands (DAGH and CBEF) (Fig. 5a). The strands are 7-8 residues long, except the strand D that has only 3 residues. With the exceptions for strands A and G, strands interact in an anti-parallel fashion arranged in a topology analogous to the classical Greek key barrel. Each monomer exhibits a single α-helix segment of 9 residues located at the end of ß-strand E (75-83 residues), which connects to the F strand (Blake et al. 1978). The strands DAGH mold the channel surface and the strands CBEF define the external surface of the monomer in the tetrameric structure. A stable dimer is formed by two monomers, A and B or C and D, linked by antiparallel hydrogen bonds between chains F and H of two ß-sheets in each monomer (Fig. 5b) (DAGHH'G'A'D' and CBEFF'E'B'C'). Two dimers form a tetramer through dimer-dimer contacts. The contact region between dimers is weaker than between the two monomers and linked by hydrogen bonds and hydrophobic contacts between the AB loop from one dimer and the strand H from the other, forming the functional tetramer (Fig. 5c). These loop's interactions are involved in stabilizing the AB to CD quaternary interface and in defining the outer boundary of the hormone-binding sites (HBS) (Blake et al. 1978; Foss et al. 2005). The quaternary structure of TTR has a globular protein shape whose overall size is 70 Å x 55 Å x 50 Å (Blake et al. 1978).

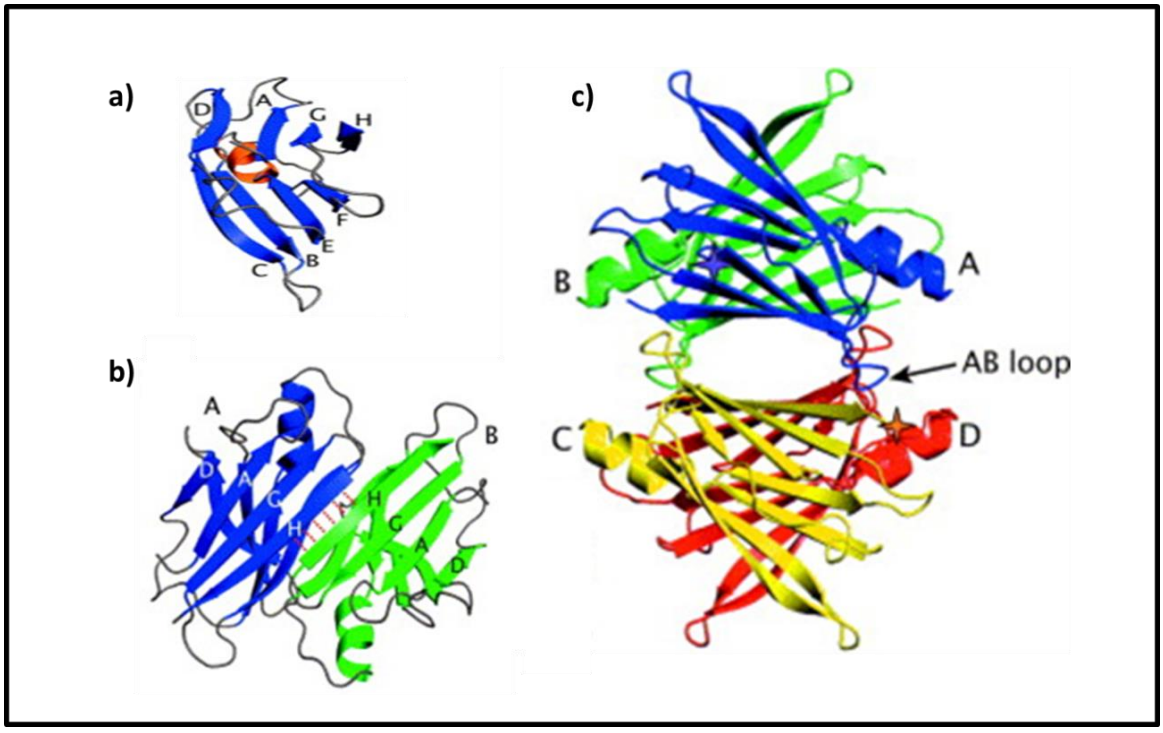

Figure 5: Representation of wild-type (wt) ribbon TTR structure: **a)** Monomer with its beta-strands labeled. **b)** Dimer AB showing the beta-sheet formed by lateral association of the HH' strands (red dashes). **c)** Tetramer structure with each monomer distinguished by different colors (subunit A - blue, subunit B - green, subunit C - yellow, subunit D - red). The AB loop of subunit A is indicated by an arrow. Adapted from Foss et al. 2005.

As mentioned, structurally, the four monomers that constitute the TTR tetramer form an open, large, central and hydrophobic channel - the HBS – where the binding sites for T4, and other hormones and small molecules, are located (Blake et al. 1978).

5. Expression and Regulation

Transcription of the TTR gene results in an approximately 700 bp mRNA containing a 5' untranslated region of 26-27 nucleotides, a coding region of 441 nucleotides, a 3' untranslated region of 145-148 nucleotides and a poly(A) tail (Mita et al. 1984; Soprano et al. 1985). TTR is synthesized as a precursor with a larger molecular weight, the pre-transthyretin, which includes a signal peptide at the N-terminal region that is cleaved upon TTR translocation to the endoplasmic reticulum (Soprano et al. 1985).

TTR is mainly synthesized by hepatocytes in the liver (Dickson et al. 1985) and secreted to the peripheral circulation, where its concentration ranges from 20 to 40 mg/dL (Vatassery et al. 1991). TTR plasma concentrations varies with age: newborns have about a half of that in healthy adults (Vahlquist et al. 1975) and declines after age 50 (Ingenbleek and De Visscher 1979). TTR is also synthesized by CPECs in the CNS (Soprano et al. 1985) and secreted unidirectionally into the CSF (Aleshire et al. 1983; Herbert et al. 1986), where its concentration ranges from 2 to 4 mg/dL, and represents approximately 20 to 25 % of the total protein content in the CSF (Weisner and Roethig 1983). It was previously reported that 1 g of CP contained about 25 times larger amounts of TTR mRNA than 1 g of liver (Dickson et al. 1985), showing the very active synthesis of TTR in this tissue.

Liver and CP are responsible for up to 90 % of the total TTR amount. Nevertheless, despite some controversy, various authors also described small amounts of TTR in pancreas (islets of Langerhans) (Kato et al. 1985), heart, muscles, stomach, spleen (Soprano et al. 1985), human placenta (McKinnon et al. 2005), human scalp skin and hair follicles (Adly 2010), retinal pigment epithelium in the eye (Cavallaro et al. 1990) and meninges (Blay et al. 1993), in various animal models.

Studies showed that in the course of human embryonic development, TTR is firstly detectable in the fetal blood since the 8th week of gestation (Jacobsson 1989), in the tela choroidea, the precursor of CP and, later, in the liver (Harms et al. 1991) and pancreas (Jacobsson 1989). Furthermore, others observed TTR mRNA in endodermal cells of the visceral yolk sac, tela choroidea and hepatocytes since the 10th day of gestation (Murakami et al. 1987).

In evolutionary terms, TTR synthesis occurs in fish (Santos and Power 1999), reptiles, birds and mammalian ancestors (Richardson et al. 1994). In the first, TTR is mainly produced in liver, while in reptiles it is mostly synthesized by CP (Achen et al. 1993) and, in birds and mammals, it occurs in both tissues (Harms et al. 1991), suggesting that TTR has a common fish ancestor (Santos and Power 1999) or its expression occurred first in CP and later in liver (Schreiber et al. 1993).

TTR has been implicated in acute stressful conditions (Ingenbleek and Young 1994) and is involved in stages of stress response (Bernstein et al. 1989). It is well demonstrated that regulation of TTR expression in liver is different from CP. Studies showed that in liver, TTR levels are decreased, to a minimum of 25%, during chronic inflammation or malnutrition conditions due to the release of cytokines, which bind to hepatocyte receptors that express transcriptional factors potentially involved in the inhibition of TTR transcription (Dickson et al. 1982; Wade et al. 1988). Regarding the regulation of TTR expression in CP, less is known and further studies are required. In fact, previous studies showed that: during an acute phase response, rat CP TTR levels are not changed (Blay et al. 1993); nicotine increased mRNA TTR levels time- and dose-dependently in rat CP (Li et al. 2000); TTR expression and secretion is increased after administration of leaf extracts of *Gingko biloba* (Watanabe et al. 2001); various sex steroid hormones (SSHs) as 17β-estradiol (E2), 5α-dihydrotestosterone (DHT) and progesterone (P) are up-regulated TTR expression in murine CP (Quintela et al. 2008; Quintela et al. 2009; Quintela et al. 2011); aged rats exhibited increased TTR mRNA levels in CP, after a short-term consumption of omega-3-rich fish oil (Puskas et al. 2003); and, TTR expression is decreased in neonatal CNS of rats subjected to a maternal separation induced stress (Kohda et al. 2006).

6. Metabolism

Several studies performed in various animal models focused on TTR metabolism and turnover but, until now, these mechanisms are poorly understood. It is well established that, in humans, the total body turnover is 250-300 mg/m^2/day (Vahlquist and Peterson 1973) and the biological half-time of TTR is approximately 2-3 days (Socolow et al. 1965), which, in rats, decreases to about 29 h (Dickson et al. 1982). Studies performed in rats showed that several tissues participate in TTR turnover and catabolism (Makover et al. 1988) and established that it mainly occurs in the liver (36-38%), and, at minor levels, in muscles (12-15%) and skin (8-10%) and, residually, in kidneys, adipose tissue, testis and in the gastrointestinal tract (1-8%) (Makover et al. 1988).

The uptake of TTR occurs specifically and saturably (Divino and Schussler 1990) thus, indicating the existence of a receptor-mediated mechanism. Others identified a putative TTR receptor (100 kDa, approximately) on chicken oocytes (Vieira et al. 1995) and on membrane of ependymoma cells (Kuchler-Bopp et al. 2000). In hepatomas and primary hepatocytes, TTR internalization is dependent of other unidentified receptor-associated protein (RAP)-sensitive (Sousa and Saraiva 2001). Besides, only one receptor, megalin, a member of the low density lipoprotein (LDL) receptors, implicated in the renal re-uptake of plasma proteins carriers of lipophilic compounds, was showed to play an active role in uptake of TTR in kidney, preventing its glomerular filtration (Sousa et al. 2000b).

Taken together, previous studies suggest that the uptake and internalization of TTR is a receptor-mediated process (Divino and Schussler 1990) and shares a common pathway with lipoprotein metabolism (Sousa and Saraiva 2001). However, the precise mechanism by which TTR uptake occurs is not yet understood because, until now, no specific TTR receptor was identified.

7. Physiological Functions

TTR plays many functions in organisms. TTR transports RBP and approximately 15% of T4. Also, it, indirectly, transports retinol (vitamin A), which is bound to its specific transporter, RBP, forming the TTR-RBP-retinol complex (Kanai et al. 1968). So, the transport and distribution of THs and retinol, *via* interaction with RBP, in plasma and CSF, are the first main functions attributed to TTR (Hagen and Elliott 1973; Monaco 2000; Raz and Goodman 1969; Schreiber et al. 1995). Presently, it is well known that its actions largely exceed those initially proposed and increasing evidences link TTR to a number of neuropathological conditions as AD, nerve biology and repair and proteolytic activity. In fact, several *in vitro* and *in vivo* studies showed that TTR is also implicated in: regulation of neuropeptide Y (NPY) processing by its cleavage (Liz et al. 2009); depression and exploratory activity by modulation of the noradrenergic system (Sousa et al. 2004); enhancement of nerve regeneration (Liz et al. 2009) through megalin-mediated internalization (Fleming et al. 2007); maintenance of normal cognitive processes during aging by acting on retinoid signaling pathway (Brouillette and Quirion 2008); and, Aß fibrils sequestration through the formation of stable complexes with Aß peptide, preventing its deposition (Costa et al. 2008b; Schwarzman et al. 1994; Stein and Johnson 2002). TTR also cleaves Aß aggregates protecting against its toxicity (Costa et al. 2008a).

7.1 Transport of Thyroxine

THs, T4 and triiodothyronine (T3), are lipophilic hormones synthesized by the thyroid gland and secreted into the bloodstream. They play several main functions in organisms. THs are essential in the normal growth, development and function of the CNS (Thompson 1996), participating in the maintenance of the brain homeostasis (Hulbert 2000). In mammals, most of the THs produced is T4, but T3, which is derived from the deiodination of T4 in the thyroid gland or in the periphery, constitutes the biologically active form (Yamauchi et al. 1999). In humans, the THs are secreted into the peripheral circulation but only a residual percentage of the hormone circulate in a free form (Bartalena 1990). In fact, in humans, more than 99% of THs circulate bound to one of the three major plasma TH carrier proteins: thyroxine-binding globulin (TBG), TTR and albumin and, in rodents, bound to TTR and albumin. These proteins are synthesized and secreted by the liver and are involved in the distribution of THs from their site of synthesis to their locals of action, *via* bloodstream. In rodents and humans, from the three THs distributor proteins mentioned, only TTR is also produced in the brain turning it into the major T4 carrier

in the CSF, transporting about 80% of its total amount (McCammon et al. 2002).

In mammals, TTR also binds T3 but with lower affinity than T4. Human TTR has an intermediate affinity for T4 and T3 (7 x 107 M^{-1} and 1.4 x 107 M-1, respectively), while TBG has the highest and albumin the lowest affinity for these hormones (Loun and Hage 1992).

TTR has two binding sites for T4 located in the central hydrophobic channel in the TTR tetrameric structure (Blacke et al. 1978). Although these two potential binding sites to T4, each located between two of the 4 monomers, under physiological conditions only one binding site is occupied by T4, due to negative cooperativity (Blake et al. 1974; Wojtczak et al. 2001). The T4 binding channel has three main regions: a hydrophobic region formed by the hydroxyl group of Ser112, Ser115, Ser117 and Thr119 residues at the tetramer center, a hydrophobic portion formed by the methyl groups of Leu17, Thr106, Ala108 and Val121 and a charged residues group of Lys15, Glu54 and His56 near the binding channel entrance that form hydrogen bond contacts, which hold T4 in the binding channel (Fig. 6) (Klabunde et al. 2000; Wojtczak et al. 1996).

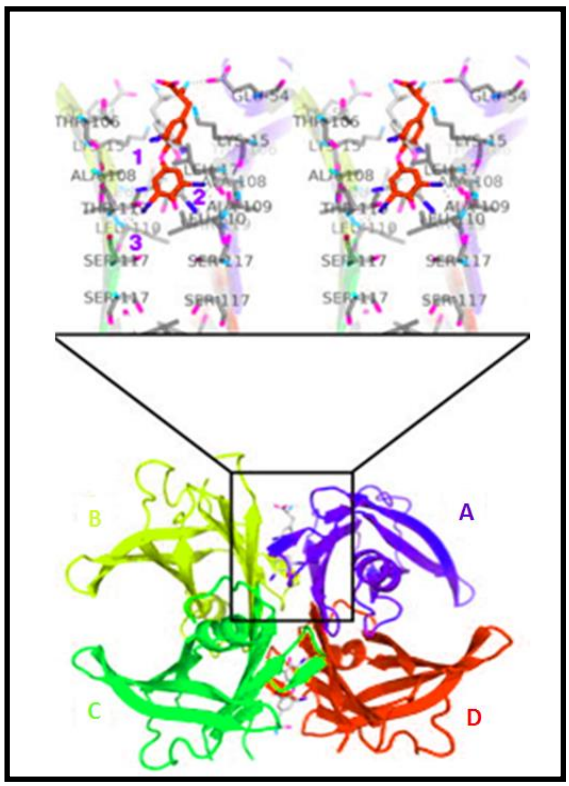

Figure 6: Representation of TTR tetramer binding to thyroxine (T4). The TTR tetramer is shown at the lower panel with the monomers differently colored and named A, B, C or D. The two hormone-binding sites (HBS) can be found at the interface of the AB and CD. One of the sites is showed in detail in the upper panel. The TTR amino acid residues that contact T4 (yellow and its symmetric in grey) are shown as sticks and labeled. Adapted from Trivella et al. 2011.

Due to T4-TTR binding, the last has emerged as a potential mediator of the T4 transport from the blood to tissues, especially into the brain (Dickson et al. 1987), through the BCSFB,

where TTR is the major THs binding protein found (Hagen and Elliott 1973). However, the role of TTR in the delivery of THs to target tissues has been controversial (Palha et al. 2002) and, currently, the more accepted hypothesis is the free hormone hypothesis, which postulates that the free hormone concentration in blood is crucial for its biological activity, rather than the protein-bounded hormone concentration (Mendel 1989; Episkopou et al. 1993). Free THs can enter cells *via* TH transporters located in plasma membrane or by passive diffusion and, the identified membrane bound TH transporters may assist their uptake in specific tissues (Sugiyama et al. 2003). A subsequent study corroborated this hypothesis: in TTR-null mice it was showed that TTR is not crucial for THs entry into the brain or other tissues (Palha et al. 1997), suggesting the presence of an alternative mechanism for T4 metabolism in the absence of TTR and a consequent redundant role of TTR in TH homeostasis (Palha 2002; Sousa et al. 2005), only acting as a storage molecule for THs in plasma and CSF.

7.2 Transport of the Complex Retinol - Retinol-Binding Protein

The TTR tetramer transports retinol in the bloodstream, through the formation of a TTR-RBP-retinol complex (Kanai et al. 1968). RBP is a 21 kDa monomeric protein with 121 aa residues (Rask et al. 1980). It is synthesized and secreted primarily by hepatocytes and constitutes the exclusive retinol transporter in blood. The RBP secretion is stimulated upon its association with retinol, which alters the conformation of the protein (van Bennekum et al. 2001). X-ray crystallography showed the conformational three dimensional structure of TTR-RBP-retinol complex and, although TTR has theoretically four binding sites for RBP (Kopelman et al. 1976), under physiological conditions, only one molecule binds to TTR tetramer. However, *in vitro*, TTR is able to bind to two RBP molecules, each establishing molecular interactions with one of the dimers, blocking the another binding site presented in the other monomer (Fig. 7) (Monaco et al. 1995). Interactions between RBP and TTR are mediated by residues at the entrance of the ligand binding pocket and span across the two TTR dimers. Both RBP- and T4- binding sites are independent from each other (Monaco 2000; Monaco et al. 1995).

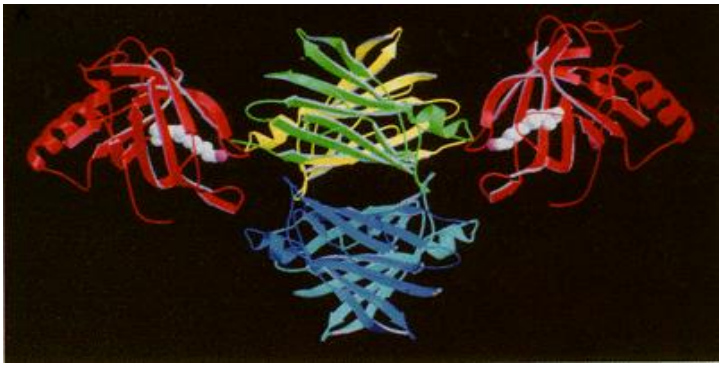

Figure 7: Ribbon representation of the quaternary structure of the *in vitro* complex TTR - Retinol-binding protein (RBP) - retinol complex. TTR tetramer is represented in yellow, green, light blue and blue. The retinol (vitamin A) is represented in grey and the RBP molecules are in red. The center of the TTR channel, the HBS, which bind T4 and other small molecules, is represented empty in the figure (center). Adapted from Monaco et al. 1995.

RBP circulates in the bloodstream bound to TTR only when it is complexed with retinol, as a 1:1 molar protein complex (Goodman 1985), which facilitates its release and protects its glomerular filtration and catabolism (Raz et al. 1970). In turn, RBP constitutes the main mechanism by which cells acquire retinol because RBP protects retinol from oxidation and prevents the retinol plasma insolubility. TTR also binds to retinoic acid but with less affinity than retinol (Smith et al. 1994). Retinol is an essential micronutrient in several functions, namely vision, development, reproduction and cellular differentiation. After retinol is delivered to tissues, the affinity of RBP to TTR becomes reduced and, once in cells, retinol can be stored or metabolized. In plasma, both RBP and retinol prevent TTR misfolding, through stabilization of its tetrameric structure (Raghu and Sivakumar 2004) and the formation of the TTR-RBP-retinol complex may also function as a reservoir of RBP-retinol. Actually, TTR-null mice show a significant increase in the renal filtration of the retinol-RBP complex (van Bennekum et al. 2001) and a consequent decrease in plasma RBP and retinol levels (Episkopou et al. 1993). However, the total retinol levels in the tissues of these animals remains similar to those observed in wild-type (wt) animals, suggesting alternative mechanisms to compensate the loss of retinol delivery to tissues mediated by RBP.

The retinol uptake by cells is controversial and various mechanisms have been proposed: retinol can enter the cells by simple diffusion (Fex and Johannesson 1988); also, there could exist unidentified specific membrane receptors that interact with the complex resulting in its endocytosis (Gjoen et al. 1987); and, a third mechanism, also involving a receptor, but adding a role for cellular RBP as an acceptor of retinol inside cells, suggesting that apo-RBP remains outside the cell (Sivaprasadarao and Findlay 1994). The second and the third proposed mechanisms are the most accepted ones and, the controversy is whether RBP is internalized, or not, during the retinol transfer by endocytosis. Until now, the role of TTR in this process is not yet clarified.

Recently, in bovine retinal pigment epithelium cells, a multitransmembrane domain protein, the STRA6, was identified. Authors suggest that it is a specific membrane receptor for RBP because it binds RBP with high affinity and have strong retinol uptake activity from the retinol-RBP complex, independent from endocytosis (Kawaguchi et al. 2007). Authors also demonstrated that STRA6 is widely expressed in embryonic development and in the adult brain, spleen, thymus, kidney, female genital tract, testis and placenta and at lower quantities in heart, lung and liver.

7.3 Other Functions

As mentioned, TTR has been described as an important molecule that participates in several mechanisms. Beyond those already discussed, TTR plays numerous actions mainly related with various neuropathological conditions, as AD, nerve biology and repair and anti-apoptotic and proteolytic activities.

Previous studies showed that TTR has anti-apoptotic pancreatic activity as it protects ß cells from apolipoprotein C-III-induced apoptosis. Also, the anti-apoptotic activity of TTR was only observed in the tetrameric structure and it was postulated that the TTR monomer

conversion must be involved in ß cells destruction in diabetes type 1 patients. These authors concluded that TTR is a functional component in pancreatic ß cell stimulus-secretion coupling (Refai et al. 2005).

Approximately 1-2% of the plasma TTR circulates bound to high density lipoproteins (HDL) *via* its interaction with apolipoprotein A-I (apoA-I), which forms the TTR-apoA-I complex (Sousa et al. 2000). Liz and coworkers showed that TTR is able to cleave the C-terminus of free and lipidated apoA-I, promoting a decrease in the capacity of HDL to promote cholesterol efflux and to bind to their receptor and an increase in apoA-I amyloidogenic potential. Furthermore, authors demonstrated that the TTR proteotylic activity is slightly compromised when it is complexed with T4 and, it is lost when bound to RBP (Liz et al. 2004; Liz et al. 2007).

Neuropeptide Y (NPY) is involved in several brain mechanisms and TTR is also implicated in its regulation (Nunes et al. 2006). Liz and colleagues (2009), showed that the NPY cleavage between Arg33 and Arg35 aa residues induced neuronal regeneration (Liz et al. 2009).

Finally, it is well known that TTR acts in Aß metabolism as it forms stable complexes with Aß, inhibiting its aggregation/fibril formation and clearance (Costa et al. 2008b; Schwarzman et al. 1994). Morever, TTR had been also described as exerting proteolytic actions in Aß peptide, as it was showed that TTR cleaves the Aß fibrils, generating smaller Aß peptides, less toxic than the full length ones (Costa et al. 2008a; Costa et al. 2008b). This issue is further discussed in the following section (8.2 – Alzheimer's Disease).

8. TTR in Disorders of the Nervous System

The main neurodegenerative disorder associated with TTR is familial amyloid polyneuropathy (FAP) but several studies showed that this protein is also involved in several other neurological diseases since altered TTR levels have been found in various distinct neuronal dysfunctions. Nevertheless, some studies did not completely clarify the TTR function in some pathologies and further research is required.

8.1 Familial Amyloid Polyneuropathology

FAP was firstly described by Andrade and his colleagues in 1952 (Andrade 1952) and mutated TTR was identified as the major protein present in amyloid deposits from FAP patients (Costa et al. 1978). FAP is an autosomal dominant neurodegenerative disorder characterized by the systemic extracellular deposition of mutated TTR aggregates and amyloid fibrils throughout the connective tissue, except in the liver parenchyma and in the brain. It has an unclear origin (Coimbra and Andrade 1971a; Coimbra and Andrade 1971b; Saraiva 2001) and affects particularly the peripheral nervous system (PNS). Dissociation of the TTR tetramer is widely accepted as the

first step in the formation of TTR amyloid fibrils (McCammon et al. 2002). To date, over 100 point mutations of TTR have been identified and, the vast majority is pathogenic. The most common TTR mutation is valine-30-methionine (V30M) and one of the most aggressive is leucine-55-proline (L55P). In FAP, TTR amyloid deposits are diffuse in PNS, involving nerve trunks, plexuses and sensory and autonomic ganglia, and, as a consequence, the axonal degeneration rises, beginning in unmyelinated and low diameter myelinated fibers and ending up in neuronal loss at ganglionic sites (Andrade 1952; Said et al. 1984; Santos et al. 2010; Sousa and Saraiva 2003; Thomas and King 1974). The precise mechanisms underlying TTR amyloid fibril formation are unknown. However, several studies suggest that amyloidogenic mutations destabilize the native TTR tetrameric structure, thereby inducing conformational changes that lead to its dissociation into non-native monomeric intermediates, which aggregate and polymerize in amyloid fibrils (Almeida et al. 2005; Bonifacio et al. 1996; Quintas et al. 1999). These partially unfolded species can, subsequently, self-assemble forming high molecular mass aggregates and protofilaments that accumulate in protofibrils and subsequently form amyloid fibrils (Cardoso et al. 2002; Saraiva et al. 2012).

Currently, three mechanisms gathered more consensus: the conformational hypothesis that defends that rich ß-sheet secondary structure proteins, as TTR, are more prone to form amyloid (Blake et al. 1978) and alterations in its structural conformation (mutations) leads to tetramer dissociation and formation of partially unfolded intermediates as the initiation step in the amyloidogenic cascade (Saraiva 1991); the hypothesis that defends that the amyloid formation could occurs *via* a nucleation-dependent oligomerization that, subsequently promotes the fibril growth (Lansbury 1997); and, a recent hypothesis that defends that the proteolytic property of TTR could trigger fibril formation in TTR peptides or intact protein, by releasing amyloidogenic fragments (Costa et al. 2008a). Other possibility is based on the seeding events because previous studies showed that TTR fragments with a fibrillar conformation induce *in vivo* murine amyloidosis, working as an amyloid enhancing factor (Johan et al. 1998). A recent report suggests that clusterin can influences TTR aggregation but not modulates the toxicity of TTR aggregates nor plays a role in TTR clearance in FAP (Magalhaes and Saraiva 2011; Magalhaes and Saraiva 2012). Others hypothesized that heat shock factor 1 (HSF1) could be involved in FAP pathogenesis as a cell defense mechanism against the presence of TTR extracellular deposits and, the disruption of the heat shock proteins (HSPs) response would exacerbate TTR deposition (Santos et al. 2010). Further, TTR aggregates are able to induce stress responses in tissues affected by its deposition, where an up-regulation of a resident endoplasmic reticulum chaperone BiP, which belongs to the family of HSP70, is observed (Teixeira et al. 2006). Others suggested that amyloid deposits can be noxious to nerve fibers through mechanical and toxic effects by, until now, unidentified processes (Sobue et al. 1990; Sousa and Saraiva 2003).

8.2 Alzheimer's Disease

AD is a neurodegenerative disorder widely abundant in elderly people. Pathological hallmarks of AD include intracellular neurofibrillary tangles consisting of insoluble deposits of

hyper-phosphorylated microtubule-associated tau protein and extracellular amyloid plaques deposition, mainly composed by neurotoxic Aß peptides (Maccioni et al. 2001).

Despite the many pathological characteristics of AD, the most consensual hypothesis that explains the disease process is the amyloid hypothesis. It defends that the gradual accumulation and aggregation of the hydrophobic Aß peptides can directly and indirectly, through a generation of an inflammatory cascade, result in progressive synaptic and neuritic injury, which, subsequently, leads to hyperphosphorylation of tau and formation of neurofibrillary tangles (Hardy and Higgins 1992; Hardy and Selkoe 2002). Indeed, Aß accumulation, oligomerization and deposition within the brain are the main hallmarks in the pathogenesis of AD and, imbalances between its production and clearance results in AD progression. Aß is found in the extracellular fluids of the brain, including CSF, and in the interstitial fluids surrounding neurons and glial cells in brain lobes (Seubert et al. 1992; Vigo-Pelfrey et al. 1993).

- ***Amyloid-beta metabolism and actions of transthyretin***

The Aß peptides (~4kDa) are proteolytic cleavage products of the sequential processing of the amyloid precursor protein (APP) by ß- and γ-secretases. APP is a type I transmembrane protein composed of three domains: a large hydrophilic N-terminal extracellular, a single hydrophobic transmembrane and a small C-terminal cytoplasmic domains (Kang et al. 1987). It is ubiquitously expressed in neuronal and non-neuronal cells. The APP gene is located on chromosome 21 (Goate et al. 1991; Korenberg et al. 1989; Yoshikai et al. 1990) and form different fragments during its intracellular trafficking through alternative splicing (Haass 2004). The main APP isoforms, APP695, APP751 and APP770, contain 695, 751 and 770 aa, respectively. Contrarily to APP695 that is primarily expressed in neurons (Kang and Muller-Hill 1990), both APP751 and APP770 are commonly expressed in many tissues (Zheng and Koo 2006). APP is synthesized in the endoplasmic reticulum and it is transported through the Golgi apparatus to the trans-Golgi-network (TGN) and TGN-derived vesicles to the cell surface to be cleaved or endocytosed and, either back into the TGN (Caporaso et al. 1994) or degraded by lysosomes, where α-, ß- and γ-secretases act (Nunan and Small 2000; Schubert et al. 1991). The cleavage of APP involves a variety of secretases (α-, ß- and γ-secretases) and can occur in two different pathways: a non-amyloidogenic pathway, which prevents the formation of Aß peptides (α- and γ-secretase cleavages), and an amyloidogenic pathway, which generates Aß peptides (ß- and γ-secretase cleavages). The amyloidogenic pathway comprises an initial cleavage by ß-secretase that generates a larger extracellular soluble APP-beta (sAPPß) and a membrane anchored C-terminal end (C99). Then, γ-secretase acts in C99 in the Aß domain generating Aß peptide and the APP intracellular domain. The γ-secretase cleavage site within the C99 is variable and produces a variety of Aß peptides whose sizes range from 39 to 43 aa. Even though, there are two major Aß species, Aß1-40 and Aß1-42. The predominant form is the Aß1-40, whose levels are ten times higher comparing to Aß1-42 (Lorenzo and Yankner 1994). However, Aß1-42 is more prone to aggregate and to form amyloid fibrils (Jarrett et al. 1993), is significantly more neurotoxic and is the major specie accumulated in senile plaques (Tsuzuki et al. 2000). The Aß peptides are

mainly produced by plasma membranes and released to the extracellular space where they can deposit as senile plaques (LaFerla et al. 2007). Although, it can also be deposited as diffuse deposits as an intermediate step in the formation of compact amyloid plaques.
It is clear that APP cleavage takes a central position in AD pathogenesis, as alterations in its processing result in increased Aß peptide generation, which is deposited as amyloid plaques in AD brains (Li et al. 2004). Indeed, aberrant and/or cumulative Aß production, have been postulated to be the main etiological basis of AD.
The clearance of Aß in the human CNS takes place in the extracellular space and is controlled by the brain Aß degradation and its efflux from the CNS to the peripheral circulation through the blood-brain barrier or BCSFB (Zlokovic 2004). This process is mediated by various proteins. In fact, some reports recognized several extracellular proteins, present in CSF, as Aß carriers: α-1-antichymotrypsin (Aksenova et al. 1996), apolipoprotein E (ApoE) (Strittmatter et al. 1993) and J (Apo J) and TTR (Schwarzman et al. 1994; Tsuzuki et al. 1997). The sequestration hypothesis for the Aß clearance emerged. It suggests that normally produced Aß is sequestrated by certain extracellular proteins, thereby preventing amyloid formation and Aß toxicity (Schwarzman and Goldgaber 1996).

Several proteins are involved in Aß production, degradation and clearance (Santos et al. 2011). Among them, TTR has been described as a protective molecule in Aß metabolism (Schwarzman et al. 1994; Serot et al. 1997) because it binds to Aß, forming stable complexes (Mazur-Kolecka et al. 1997) and preventing its aggregation/amyloid formation (Schwarzman et al. 1994). In fact, the non-mutated form of TTR was identified as the major Aß binding protein in the CSF that could decrease the aggregation state of the peptide and its toxicity (Carro et al. 2002; Mazur-Kolecka et al. 1995; Schwarzman et al. 1994). Also, other authors observed that TTR expression is induced in response to an overproduction of Aß peptides (Stein and Johnson 2002; Wu et al. 2006) and, an inverse correlation exist between TTR levels and senile plaques abundance (Elovaara et al. 1986; Merched et al. 1998; Riisoen 1988; Serot et al. 1997), raising to the possibility of an inadequate physiological sequestration of Aß in the CSF/extracellular fluid (Serot et al. 1997). Moreover, lack of amyloid plaques in young AD mouse models is associated with increased levels of TTR whereas neutralization of TTR is associated to increased Aß levels, tau phosphorylation, neuronal loss and apoptosis in the hippocampus (Stein and Johnson 2002; Stein et al. 2004).
Even though, it remains unknown whether the decreased levels of TTR in the CSF are restricted to AD (Chiang et al. 2009) and are a primary or a later event in the disease onset/progression.
An *in vitro* protein-protein interaction study, between TTR and Aß aggregates indicated that TTR is protective due to its capacity to bind toxic or pretoxic Aß aggregates, both intra and extracelularly. Using APP23 transgenic mice model of AD carrying the human TTR (hTTR) gene, authors showed that hTTR overexpression was ameliorative whereas silencing of the endogenous mouse TTR gene accelerated the development of AD phenotype (Buxbaum et al. 2008). Others observed that Aß levels and deposition were higher in the brains of an AD model of transgenic

mice crossed to carriers of a TTR hemizygous deletion (ceAPPswe/PS1ΔE9/TTR+/- mice) compared to age-matched controls (ceAPPswe/PS1ΔE9/TTR+/+ mice) (Choi et al. 2007), without altering APP processing. Likewise, transgenic mice harboring APPswe/PS1ΔE9 transgenes that lead to development of AD, maintained in an enriched environment, resulted in increased TTR expression and notable declines in cerebral Aß levels and amyloid deposits, compared to mice held in standard housing conditions (Lazarov et al. 2005). In *Caenorhabditis elegans* expressing human Aß42, TTR diminished the neurodegeneration prompted by the Aß toxic peptides (Link, 1995). Despite, a recent study in Tg2576 mice concluded that the absence of TTR inhibited Aß deposition (Wati et al. 2009). Besides some controversy, TTR is generally considered as a neuroprotective molecule in AD (Fleming et al. 2009).

Costa and colleagues (2008) confirmed and extended the neuroprotective properties of the soluble APP alpha (sAPPα) produced by the non-amyloidogenic APP cleavage pathway, as they showed that it facilitated the Aß cleavage by TTR (Costa et al. 2008a). Also, Costa and coworkers (2008) showed that TTR decreased the rate of aggregation without affecting the fraction of Aß in the aggregate pool. The authors pointed that the total pool of Aß peptide depends on its production and its clearance, and TTR contributes either to maintenance of the soluble Aß levels and removal of deposited/insoluble Aß in amyloid plaques (Costa et al. 2008b). In fact, TTR is not only able to inhibit Aß fibril formation (Schwarzman and Goldgaber 1996), but also to degrade aggregated forms of Aß peptides through cleavage of Aß peptide, disrupting Aß fibrils and contributing to the maintenance of Aß normal levels (Costa et al. 2008a). The authors identified several cleavage sites that generate smaller Aß peptides, less toxic than the full length ones, which can be eliminated by cells (Costa et al. 2008a). Recently, it was also showed that some metal chelators, as EDTA or 1,10-phenanthroline, abolished TTR proteolytic activity and it could be reversed with Zn^{2+} and Mn^{2+} (Liz et al. 2012), suggesting that TTR act also as a metallopeptidase in Aß aggregates.

The nature of TTR-Aß binding was confirmed by various authors and, it was well established that it occurs in Aß monomers, oligomers and fibrils (Carro et al. 2002; Costa et al. 2008b; Liu and Murphy 2006), with comparable affinities (Costa et al. 2008b). However, the conformation of TTR that binds to Aß species is ambiguous, as some authors showed that TTR tetramer bound mostly to Aß aggregates rather than Aß monomers that may slightly enhance the Aß aggregation, whereas TTR monomers prevented the growth of the Aß aggregates (Du and Murphy 2010). Schwarzman and coworkers (2004) and Costa and colleagues (2008) demonstrated that TTR variants bound differently to Aß, and an inverse correlation between the amyloidogenicity of TTR and affinity to Aß was observed, thus affecting Aß aggregation (Costa et al. 2008b; Schwarzman et al. 2004). In line with this, emerged the hypothesis that mutations in the TTR gene or conformational changes at the protein level, mainly induced by aging, could affect the sequestration properties of TTR. Yet, a populational study in AD patients did not found any correlation between the TTR variants and Aß sequestration (Palha et al. 1996). A very recent study showed that single point mutations (L82A and L110A aa) of TTR gene inhibited its binding to Aß, indicating that Aß binding to TTR is mediated through these hydrophobic residues (Du et

al. 2012). However, the precise mechanisms by which the TTR-Aß interaction occurs and how TTR alters Aß aggregation and clearance are not completely understood.

It is important to note that, although nearly consistent global data regarding the role of TTR in Aß degradation and clearance, some of the contradictory results could be promoted by variables that were not considered or evaluated, as the gender or the animal model. Accordingly, a study performed in APPswe/PS1A246E transgenic mice crossed with TTR-null mice, showed a gender-associated modulation of brain Aß levels and brain sex steroid hormones (SSHs) by TTR, thus suggesting that decreased levels of brain SSHs in female mice with reduced TTR expression may underlie their AD-like neuropathology (Oliveira et al. 2011). More recently, similar results were obtained by Ribeiro and colleagues (2012) where they found decreased plasma TTR levels in mild-cognitive impairment and AD patients, gender-dependent (Ribeiro et al. 2012).

Despite intensive research about the TTR-Aß binding and on the role of TTR in Aß degradation and clearance, further studies are required to disclose the mechanisms that mediate the expression of TTR, particularly in CP and to unravel the whole functions of TTR in Aß metabolism.

8.3 Other Disorders

Nerve regeneration after a peripheral injury involves TTR. Studies conducted in TTR KO mice showed a decreased regenerative capacity after sciatic nerve crush and a slower recovery of locomotor activity and nerve conduction velocity comparing to *wt* mice. Also, *in vitro* studies showed that in the absence of TTR, neurite outgrowth was compromised. So, TTR seems to be the responsible factor for the enhancement of nerve regeneration and, some authors defended that it occurs through megalin-mediated internalization (Fleming et al. 2007). Others showed that TTR-null mice heterozygous for the HSF-1, TTR(-/-) HSF1(+/-), which compromise the stress response, showed a significant increase in cortical infarction, cerebral edema and microglial-leukocyte response compared with TTR(+/+) HSF1(+/-) mice and, the specific silencing of TTR synthesis in the liver by RNAi had no effect on TTR distribution in the infarct. Authors concluded that CSF TTR contributes to control neuronal cell death, edema and inflammation, thereby influencing the survival of endangered neurons in cerebral ischemia (Santos et al. 2010). Furthermore, Fleming and colleagues (2009) observed that the clathrin-dependent megalin-mediated TTR internalization is required in TTR neuritogenic properties and the activity of megalin as a regeneration enhancer is shown to be TTR-dependent (Fleming et al. 2009a).

Authors suggest that the development of memory impairments during aging could be due to decreased levels of TTR (Brouillette and Quirion 2008; Sousa et al. 2007). It was showed that following stimulation in a spatial memory task, TTR is decreased in aged memory-impaired rats when comparing to controls (Bastianetto et al. 2007). Further, in TTR KO mice memory deficits (Brouillette and Quirion 2008) and spatial reference memory impairments are also observed and no longer memory impairment with increasing age is observed (Sousa et al. 2007). Authors also found that lower levels of C/EBP overlap the decreased TTR gene expression in aged-impaired rats, time-dependently, suggesting that alterations in transcription levels of TTR are attributed,

at least in part, to altered C/EBP protein expression. C/EBP is part of the immediate-early gene cascade initiated by cAMP response element binding protein (CREB), which is suggested that following its activation, may regulate downstream effector genes, such as TTR, for the consolidation of new memory (Bastianetto et al. 2007; Taubenfeld et al. 2001).

Other authors also suggested that the maintenance of the normal cognitive abilities is related to the capacity of TTR to, indirectly, transport retinol (Brouillette and Quirion 2008) and deficient levels of TTR may contribute to a poorer cognitive performance associated with normal aging and accelerated in AD (Sousa et al. 2007).

Psychosis is a severe mental disorder usually associated with loss of contact with reality and hallucinations. Common examples of these disorders are schizophrenia and bipolar affective disorder. Previously, it was showed that TTR expression is downregulated in the CSF, plasma and prefrontal cortex of schizophrenic patients when compared to healthy individuals (Huang et al. 2006; Wan et al. 2006). Contrarily, Ruano and coworkers (2007) observed no differences in TTR expression between schizophrenic and healthy individuals (Ruano et al. 2007). TTR has also been linked to bipolar disorder, characterized by recurrent episodes of depression and mania, because its synthesis was found to be decreased following chronic administration of lithium chloride to rats, a stabilizer agent widely used in the treatment of this disease (Pulford et al. 2006).

TTR expression seems to be involved in depression. However, contradictory data has been obtained as some authors described increased TTR levels in CSF from depressed patients, comparing to healthy individuals (Jorgensen 1988) and others the opposite effect (Hatterer et al. 1993), or no differences (Sullivan et al. 1999).

Sousa and coworkers (2004) showed that TTR *KO* mice displayed reduced depressed-like behaviour and increased noradrenaline levels in the limbic forebrain, suggesting the involvement of TTR in depression (Sousa et al. 2004). Nevertheless, the mechanism for TTR involvement in depressive behaviours remains to be unequivocal.

Frontotemporal dementia (FTD) is a neurodegenerative disorder resultant from the atrophy of the frontal and temporal cortex regions, the anterior portion of the cingulate gyrus with a relative sparing of postcentral cortex. Studies revealed that TTR was increased in the CSF of FTD patients compared to controls (Hansson et al. 2004; Ruetschi et al. 2005).

Parkinson's Disease (PD) is characterized by the loss of dopaminergic neurons in the substantia nigra with subsequent decline of striatal dopamine levels (Moore et al. 2005). In this disease, TTR was found to be increased in CSF (Rite et al. 2007).

Amyotrophic Lateral Sclerosis (ALS) is a motor neuron disease characterized by a rapid degeneration of motor neurons in the cerebral cortex, brainstem and spinal cord (Jackson and Bryan 1998). Studies showed that TTR was decreased in CSF of ALS patients comparing to controls (Ranganathan et al. 2005).

Metallothioneins

(Paper I)

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Critical Review

Neuroprotective and Neuroregenerative Properties of Metallothioneins

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Summary

Metallothioneins (MTs) are low-molecular weight cysteine- and metal-rich proteins with unquestionable metal binding capacity, antioxidant and anti-inflammatory properties, and a clear involvement in diverse physiological actions as inhibition of proapoptotic mechanisms, enhancement of cell survival, and tissue regeneration. Concurrent with this wide array of functions, MT-1/2 have been implicated in neuroprotection and neuroregeneration. The zinc binding capacity and antioxidant properties of MTs may account for most of their physiological features in the brain. However, some receptor-mediated actions of MT-1/2 have also been reported recently, a subject to be fully elucidated. This review analyses and updates the current knowledge on the actions of MTs related to neuroprotection and neuroregeneration in an effort to distinguish receptor-mediated actions of MTs from those arising from its zinc binding capacity and its antioxidant properties. © 2011 IUBMB

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Keywords metallothionein 1; metallothionein 2; metallothionein 3; brain; zinc binding; oxidative stress; neuroprotection; neuroregeneration; Alzheimer disease.

INTRODUCTION

Metallothioneins (MTs) were discovered as cadmium binding proteins in equine kidney cortex. MTs is a generic name for a superfamily of low-molecular weight cysteine- and metal-rich proteins with high affinity for divalent metals, such as zinc, cadmium, and copper with four major isoforms, MT-1 to MT-4, identified in humans. They occur in all living organisms from the simplest forms of life, such as prokaryotes to the most complex, such as higher plants and vertebrate animals. It is clear that MTs are multipurpose proteins with unquestionable metal binding, antioxidant, and anti-inflammatory properties. In addition, there is increasing evidence that MT-1 and MT-2 (MT-1/2) and MT-3 display such diverse physiological actions as inhibition of proapoptotic mechanisms, enhancement of cell survival, and tissue regeneration. Concurrent with this wide array of functions, MT-1/2 have been implicated in neuroprotection and neuroregeneration. The zinc binding capacity and the antioxidant properties of MTs may account for most of their physiological features. However, receptor-mediated uptake of MT-1/2 actions have also been reported recently, a subject not yet fully elucidated with impact in neurite outgrowth and neuronal survival, apoptosis, and perhaps regulation of inflammation.

This review analyses and updates the current knowledge on the actions of MTs related to neuroprotection and neuroregeneration in an effort to distinguish receptor-mediated actions of MTs from those arising from its zinc binding and antioxidant properties.

GENE AND PROTEIN STRUCTURE

MTs are a genetically polymorphous protein family with subfamilies, subgroups, and isoforms. Human MTs are encoded by a family of 10 genes consisting of functional isoforms, and the encoded proteins are conventionally subdivided into four groups, designated MT-1 to MT-4. Although a single MT-2A gene encodes the MT-2 protein, there are different subtypes of MT-1 proteins encoded by a set of MT-1 genes (MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, and MT-1X). In humans, all MTs genes are localized on chromosome 16, and the protein coding region is split in three exons separated by two introns (*1*).

Metals, glucocorticoids, cytokines, and a variety of physical stress conditions can induce the expression of MT-1/2 genes. Concurrent with protecting cells from reactive oxygen species (ROS)-mediated damage, MTs transcription is also induced by ROS, and oxidative stress (*2*). MT-1/2 are expressed in all organs, while MT-3 expression has been found in the brain and choroid plexus (CP), reproductive system, kidney, tongue, and

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salivary glands. MT-4 expression is restricted to some stratified squamous epithelia (*3–5*).

MTs have unique structural and functional characteristics. They are small molecular weight proteins, with 6–7 kDa, composed of a single polypeptide chain containing 60 to 68 amino acid residues, characterized by a conserved array of 20 cysteines and the absence of histidine and aromatic amino acids. On metal binding to MT-1/2, the metal-free protein, which has a predominantly disordered structure, folds into a monomeric protein composed of two globular domains: N-terminal β-(residues 1–31) and C-terminal α-(residues 32–61) domains, containing at their cores four and three metal clusters, respectively. The two protein domains are connected by a flexible hinge region composed of a conserved Lys–Lys segment (residues 30 and 31) in the middle of the polypeptide chain. A similar structure has been depicted for the mammalian isoform MT-3 (*6*). MT-3 has 68 amino acids and shares 70% sequence identity to the MT-1/2 isoforms. Its sequence contains two inserts, which are absent in the other MTs: an acidic glutamate rich hexapeptide in the C-terminal region, and a conserved Cys-Pro-Cys-Pro motif between positions 6 and 9 preceded by a Thr residue (*7*).

PRIMARY FUNCTIONS OF MTs

Metal Binding and Antioxidative Properties

Under physiological conditions, MTs bind zinc and copper through the cysteine residues, but these metals can be replaced by others such as cadmium, mercury, silver, platinum, and lead (*7*). The high metal binding capacity confers MTs an essential role in metal cellular homeostasis and heavy metal detoxification, protecting cells and tissues against their toxicity (*8*).

Zinc is the most abundant transition metal in biological systems. It is a structural or catalytic cofactor in hundreds of enzymes and many zinc finger domains and is crucial for the proper assembly of proteins to macromolecular complexes and in zinc transporter proteins (*8*). Due to the high abundance and predominance of zinc in living organisms, MTs, particularly MT-1/2 and MT-3 are primarily zinc binding proteins, serving as a reservoir of cellular zinc, fundamental to the tightly controlled release of zinc in target cells and tissues (*2, 9*). The zinc binding properties of MTs have been thoroughly reviewed by Maret in 2009 (*8*) and are briefly described here. The 20 cysteine residues in MTs bind to seven zinc ions in two zinc/thiolate clusters with different affinities. The thiolate ligands confer redox-activity to the molecule, which has the remarkable capacity of coupling zinc and redox metabolism. Therefore, in the presence of MTs, reducing conditions lower the availability of zinc ions, while oxidizing conditions increase it. As zinc and the redox state of the sulfur donors are linked, the more zinc is bound, the less reactive MTs become available toward thiol-oxidizing or modifying agents. These features demonstrate that the functional structure of MTs depends on the availability of zinc ions and on the redox state of the cellular compartments. The capacity to bind zinc and redox properties, also enable MTs to transport zinc from the cytoplasm to the intermembrane space of mitochondria or to the nucleus. In addition, by reducing ROS, including superoxide, hydrogen peroxide, hydroxyl radicals, and nitric oxide (NO), MTs promote the survival of mitochondrial dysfunctional cells, limiting apoptosis and preserving the mitochondrial membrane potential (*10*).

Metal Detoxification

When exposed to heavy metals, cellular zinc and copper ions bound to MTs are displaced because the affinity of MTs for heavy metals, such as mercury, silver, and cadmium, is much stronger, than that for zinc or copper, as reviewed by Lindeque et al. (*10*). Therefore, binding of these metals to MTs, puts them away from macromolecules preventing, to some extent, their deleterious effects. The higher affinity of MTs for heavy metals is of extreme importance, as it allows them to protect against their toxicity in the brain and in other organs (*11*).

In line with this, transgenic mice over-expressing MT-1/2 are more resistant to cadmium toxicity, and MT-1/2 knockout (KO) mice are more susceptible to this metal (*12*). In MT-1/2 KO mice, exposed to mercury vapor, which is effectively absorbed by inhalation and easily crosses the blood-brain barrier, mercury accumulation in the brain was significantly higher than that registered in wild-type (WT) mice. In these animals, the absence of MT-1/2 was compensated by MT-3 in the storage of mercury (*11*).

FUNCTIONS OF MTs IN THE BRAIN

Expression of MTs in the Central Nervous System

Within the central nervous system (CNS), MT-1/2 are primarily expressed in astrocytes and to a lesser extent in neurons. MT-1/2 are also expressed in CP epithelium, ependymal cells, endothelium, meningeal cells, and microglia. MT-1/2 expression in astrocytes is predominantly cytoplasmic, mitochondrial, and lysossomal, but nuclear detection of MT-1/2 also occurs in response to oxidative stress. Despite having no known signal peptide for secretion, MT-1/2 have been detected in the brain extracellular space and in the cerebrospinal fluid (*3, 13–15*).

Astrocytes also express high levels of MT-3, particularly those in the cortex, brainstem, spinal cord, thalamus, hippocampus, basal forebrain, neocortex, cranial nerve nuclei, olfactory bulb, and cerebellum. In addition, MT-3 is also expressed, in glial cells, monocytes and/or macrophages, and in the epithelial cells of rat CPs. MT-3 immunolabeling is more prominent in the soma and in the fine processes of astrocytes, in association with free ribosomes, rough endoplasmic reticulum, small vesicles, the outer membrane of the mitochondria, and part of the plasma membrane. Neurons also express MT-3, but its immunoreactivity is restricted to a subset of the neuronal population, particularly those from the cerebral cortex, amygdale, and cerebellum. In these cells, MT-3 is localized predominantly

Table 1
Outcomes of brain injury in MTs knockout and transgenic animals

Animals	Injury	Outcome	References
MT-1/2 KO mice	Cortical cryolesion	Impaired wound recovery Increased apoptosis Increased inflammatory response Reduced neuronal survival	(*22*)
	Kainic acid induced seizures	Reduced neuronal survival	(*23*)
	MCAO	Increased neuronal damage	(*24, 25*)
Transgenic MT-1 mice	Brain cryolesion	Reduced oxidative stress Reduced inflammatory response Reduced neuronal apoptosis	(*26*)
	MCAO	Reduced neuronal damage	(*27*)
MT-3 KO mice	Kainic acid induced seizures	Increased neuronal damage	(*28*)
	MCAO	Increased neuronal damage	(*29*)
	Without lesions	Decreased zinc levels	(*28*)
	Cortical cryolesion	Increased expression of neuronal sprouting factor	(*30*)
Transgenic MT-3 mice	Without lesions	Increased zinc levels	(*21*)

Table 2
Effects of the administration of MTs in murine models and in cell cultures

Treatment	Models	Lesion	Outcome	References
MT-1/2	Mice/rats	Traumatic injury	Reduces oxidative stress	(*31–33*)
			Reduces inflammatory response	(*31–33*)
			Increases brain tissue repair	(*31–33*)
			Improves rota-rod performance	(*34*)
	Neuron/astrocyte cell cultures	Oxidative stress	Reduces oxidative stress	(*35, 36*)
			Promotes neurite outgrowth and neuronal survival	(*37, 38*)
		Axonal transection	Promotes neuritic elongation and axonal growth	(*13, 32*)
MT-3	Mice/rats	Stab wounds	Improves or reduces brain repair and neuronal survival, depending on dosage administrated	(*39*)
		No lesion	Decreases neuronal apoptosis of SAMP8 mice	(*40*)
	Glial/neuron cell cultures	Axonal transection	Inhibits neuronal growth and repair	(*17, 41*)
		Oxidative stress	Protects against oxidative stress	(*42–44*)
			Protects against Zn^{2+} toxicity, in combination with Rab3A	(*45*)
		Aβ	Antagonizes neurotoxic effects of Aβ peptide	(*46, 47*)

in the processes including axons and dendrites, in association with microtubules, ribosomes, the outer membrane of the mitochondria, and the plasmalemma (*15–21*).

MTs in Neuroprotection and Neuroregeneration

MT-2. Many of the functions attributed to MTs were elucidated from several studies in KO and transgenic animal models of MTs (Table 1), or depicted from a wide range of studies in which the administration of exogenous MT-1/2 or MT-3, in cell culture experiments and in animal models of disease, has been accomplished (Table 2) causing effects consistent with the roles proposed from studies in KO mice.

Despite MT-1/2 KO mice developing without any major complications or impairments (*12, 48*), several studies have shown that, under physiological challenges, the lack of MT-1/2 has deleterious consequences with implications in resistance to metal toxicity, ROS, compromised brain repair upon injury and cognitive performance.

Initial evidence for the involvement of MT-1/2 in neuroprotection and neuroregeneration was provided by Penkowa and Moos (*49*), who reported that a lesion inflicted to the neonatal

Table 3
Receptor mediated actions of MT-1/2 and peptides derived from its α and β domains

Peptide	Residues	Receptor	Activated pathways	Effects	References
Full length MT-1/2		LDLR	PI3 kinase/AKT MAPK CREB	↑ Neurite outgrowth	(*37, 52*)
			G-protein Phospholipase C Protein kinase A	↑ Neuronal survival	
		Unknown	JAK/STAT Rho	↑GFAP Astrogliosis	(*53*)
EmtinBn	1–14	LDLR		↑ Neuronal survival	(*38*)
EmtinB	15–28	LDLR	PI3 kinase/AKT MAPK CREB	↑ Neurite outgrowth ↑ Neuronal survival	(*37*)
				↓Reduces KA-induced neurodegeneration	(*54*)
EmtinAn	30–43	Unknown		↑ Neurite outgrowth ↑ Neuronal survival	(*38*)
EmtinAc	45–58	LDLR		↑ Neurite outgrowth ↑ Neuronal survival	(*38*)

rat brain induced a transient expression of MT-1/2 in astrocytes. Afterwards, they showed that up on a brain cryolesion, normal mice exhibited microglia/macrophage activation and astrocytosis in the vicinity of the lesion site, the days following the lesion, but 3 weeks later the parenchyma of these animals had regenerated. In contrast, in MT-1/2 KO mice, microglia/macrophages infiltrated the lesion more severely, and 3 weeks afterward these were still present, as well as reactive astrocytosis. At this stage, no signs of wound healing were visible, and apoptosis had increased. This study provided the first indication that MT-1/2 is essential for normal brain wound repair, and that its deficiency compromises neuronal survival up on injury (*22*). Concurrently, MT-1/2 KO mice show reduced neuronal survival during kainic acid (KA) induced seizures (*23*) and after middle cerebral artery occlusion (MCAO) (*24, 25*) than control littermates.

In line with these observations, transgenic mice overexpressing MT-1 show reduced oxidative stress, inflammatory response, and neuronal apoptosis in response to cryolesions (*26*) and show increased brain tissue repair and enhanced protection against MCAO and reperfusion (*27*). Intraperitoneal administration of MT-2 to both normal and MT-1/2 KO mice and rats reproduced these results (*31–33*). Furthermore, a comparison between WT and MT-1/2 KO mice subjected to a cryolesion of the somatosensorial cortex and sacrificed at different time points after perpetration of the lesion, showed a pattern of gene responses consistent with the processes involved in the initial tissue injury and subsequent regeneration of the parenchyma, and confirmed the antioxidant properties of MT-1/2 in response to brain injury. In addition, this study strongly suggested a role of MT-1/2 in postlesion activation of neural stem cells (*50*).

More recent studies give further support to the neuroprotective actions of MT-1/2 as a result of its antioxidant properties; excess dopamine taken up by astrocytes increases MT-1/2 expression protecting dopaminergic neurons from damage through quinone quenching and/or free radical scavenging (*35*); MT-1/2 protect primary cortical neuron/astrocyte cultures obtained from neonatal MT-1/2 KO and WT mice against brain injury due to oxidative stress (*36*).

Corroborating evidence for the neuroregenerative properties of MT-1/2 has also been provided by *in vitro* studies. MT-2A promoted a significant increase in neurite elongation of individual plated rat cortical neurons and promoted reactive axonal growth after transection injury. In this study, MT-2A acted directly on neurons as no astrocytes were present in neuronal cell cultures. These observations were further confirmed using a cortical neuron/astrocyte coculture model, in which wound injury in pure astrocyte cultures resulted in no change in MT-1/2 expression whereas neuronal injury elicited MT-1/2 induction (*13*). Although MT-1/2 are essentially produced in astrocytes, these cells secrete MT-1/2 to the extracellular fluid and intercellular transfer of MT-1/2 from astrocytes to neurons occurs. MT-1/2 can be detected in the extracellular fluid of the injured brain, released by cultured astrocytes in response to external stimuli. Transport of MT-1/2 into neurons is mediated by the receptor megalin (*51*).

Another important group of studies looked deeper into the signaling pathways associated with MT-1/2 neuroprotection and neuroregeneration and identified the regions of the molecules associated with these properties (Table 3).

Exogenous administration of MT-1/2 to cultured astrocytes induces morphological changes and GFAP upregulation

consistent with astrogliosis, but unlike "classical" growth inhibitory astrogliosis, MT-1/2-induced astrogliosis is permissive to neurite outgrowth and is regulated via JAK/STAT and Rho signaling pathways instead of occurring via the mitogen-activated protein kinase (MAPK) pathway (*53*). Full MT-1/2 and a synthetic peptide derived from the C-terminal end of the β-domain of MT-1/2 encompassing residues 15–28 (EmtinB) stimulate neurite outgrowth and promote cell survival in primary cultures of cerebellar granule neurons (CGN) via low-density lipoprotein receptor (LDLR)-mediated endocytosis, most likely via megalin and lipoprotein receptor related protein 1 (LRP-1). The authors identified the C_{19}KCK motif in the middle of the EmtinB sequence that together with the total charge of the peptide was essential for its neurite outgrowth effect. Downstream signaling triggered by full MT-1/2 and EmtinB included activation of phosphatidylinositol (PI3 kinase/AKT) and MAPK pathways and the transcription factor cAMP responsive element binding protein (CREB) (*37*). Consistent with these findings, EmtinB was also effective in reducing KA-induced neurodegeneration in the CA1 region of the brain (*54*). However, promotion of neurite outgrowth can also be induced by MT-1/2 α-domain-derived peptides (EmtinAn, residues 30–43; EmtinAC, residues 45–58) with an efficiency similar to that reported for full-length MT-1/2 and EmtinB. Both EmtinAn and EmtinAc have also antiapoptotic properties, thereby promoting neuronal survival. Another peptide derived from the N-terminus of the MT-1/2 β-domain (EmtinBn, residues 1–14) did not induce neurite outgrowth but had a clear survival-promoting effect. Except for EmtinAn, the neuritogenic and survival promoting effects of these peptides were dependent on the functional integrity of LRP-1 and megalin (*38*). Megalin has also been implicated in the MT-2A stimulation of neurite outgrowth in retinal ganglion cells (*55*). The complexity of the intracellular events underlying MT-1/2-induced neurite outgrowth in cultures of CGN was further analyzed using full MT-1/2 showing that it also requires activation of a heterotrimeric G-protein-coupled pathway but not activation of protein tyrosine kinases or receptor tyrosine kinases (*52*). Activation of phospholipase C was necessary for MT-1/2-induced neurite outgrowth and inhibition of several intracellular protein kinases, such as protein kinase A or C, PI3 kinase, calcium/calmodulin kinase-II, and MAPK, abrogated the MT-1/2 induced neuritogenic response. Moreover, exogenously applied MT-1/2 resulted in a decrease in phosphorylation of intraneuronal kinases known to be implicated in proinflammatory reactions and apoptotic cell death, GSK-3α, Jun, and STAT-3 (*56–58*). Data from behavioral experiments in mice subjected to brain cryoinjury administered full mouse MT-1 or independent α and β domains, alone or together, showed that while all the proteins showed a modest effect in the horizontal ladder beam and in rota-rod tests, only full MT-1 improved the performance of animals in the rota-rod, whereas the α domain alone showed a rather detrimental effect. All these proteins were able to regulate the expression of host-response genes such as GFAP, Mac1, and ICAM, with the β domain more effective than the α domain or even the full MT-1, in some cases. Mutating MT-1 to MT-3 diminished some of the effects caused by normal MT-1, such as the general anti-inflammatory effects of MT-1 and highlighted the importance of the unique primary sequence of MT-3 (*34*).

These studies show that MT-1/2 contains at least three neuritogenic sites, located in the C-terminal of the β-domain, and in the N- and C-terminals of the α-domain, and four survival-promoting sites, two in each domain. Except for the survival promoting effects triggered by the N-terminal of the α-domain, all the other sites of the molecule seem to exert their effects through receptors of the LDLR family. Interestingly, the homology between the two domains of the molecule is only 34.4%, and the only motif identified in Emtin B (C_{19}KCK) essential for the neuritogenic activity of the peptide is only partially represented in the α-domain (cystein-isoleucine-cystein-lysine-CICK), but in EmtinAc the first cysteine has been replaced by a serine, enhancing their differences to serine-isoleucine-cystein-lysine (SICK). Therefore, it might be that the different sites contained in Emtins bind different sites of the LDLR or different receptors of the family, all triggering common intracellular pathways. As the receptors of this family bind several structurally unrelated ligands (*59*), they may as well bind several different regions of MT-1/2. Due to their small size, EmtinB, and most likely all the other Emtins, has the capacity to cross the blood-brain barrier (*54*), while retaining the beneficial properties of full-length MT-1/2 what makes it a promising tool for therapeutic approaches targeting brain lesions.

MT-3. MT-3 was purified and characterized as a growth inhibitory factor abundantly expressed in the normal human brain but greatly reduced in Alzheimer disease (AD) brains, which inhibited survival and neurite formation of cortical neurons *in vitro* (*60*). These effects were shared by recombinant MT-3 underlining its distinct functions from MT-1/2 (*16*).

Several studies performed subsequently further confirmed the growth inhibitory properties of MT-3 in C6-glial cell lines (*17*), in rat embryonic cortical neurons (*41*), and in differentiated catecholaminergic neuronal CATH.a cells treated with dibutyryl cyclic AMP, in which treatment with mouse forebrain extracts induced apoptotic cell death (*61*).

Transgenic mice that overexpress human MT-3 display higher concentration of zinc in brain regions where MT-3 mRNA expression is more prominent, particularly in the cortex, hippocampus, thalamus, brainstem, olfactory bulb, and cerebellum. These animals breed normally and appear to have normal behavior (*21*). MT-3 KO mice are not sensitive to systemic zinc or cadmium exposure, precluding an important role in metal detoxification, and do not display any major neuropathology deficits although an age-related increase in GFAP expression has been detected. Regarding behavioral traits, MT-3 KO mice demonstrate normal spatial learning in the Morris water maze (*28*), and in the novel object recognition test, as well as normal adaptation to novel environments. However, the duration of their social interactions are significantly shorter than that of WT

mice, and their acoustic startle response show diminished prepulse inhibition, also. No differences in locomotor activity or circadian rhythm have been detected in MT-3 KO mice (*62*). Other differences include their enhanced susceptibility to KA-induced seizures which result in greater neuron injury in the CA3 field of hippocampus, compared with control littermates and to MT-3 transgenic mice, which are also more resistant to CA3 neuron injury induced by seizures (*28*), suggesting that MT-3 may also be neuroprotective. In contrast to MT-1/2, there is no evidence for an anti-inflammatory role of MT-3 as no differences in the inflammatory response can be distinguished between WT and MT-3 KO mice (*30*).

Several reports provide a clear indication that MT-3 has relevant antioxidant properties in the CNS. MT-3 prevented glutamate- and NO-induced neurotoxicity in a dose-dependent manner (*42*), it is induced by oxidative stress, particularly in oligodendrocytes and microglia (*63*), and protects cells from H_2O_2-induced production of ROS and DNA damage (*43*). Exogenous administration of MT-3 also prevents the death of differentiated neurons caused by high oxygen exposure, probably resulting from its scavenging of hydroxyl radicals (*44*).

In an *in vitro* cell culture system of rat hippocampal neurons, 300 μM Zn^{2+} readily induced death of hippocampal neuronal cells, which was characterized by massive necrosis and a minor degree of apoptosis. Neither the addition of recombinant MT-3 nor Rab3A alone could rescue these cells from death. However, the combination of MT-3 with Rab3A could significantly enhance the survival of the hippocampal neurons, suggesting that MT-3 may inhibit Zn^{2+}-induced neuronal death via its interaction with Rab3A (*45*). So, whether MT-3 can be neuroprotective or inhibit neuronal growth is a controversial issue.

A study performed by Hozumi et al. may explain some of the controversial results pinpointed earlier as it shows that MT-3 can either promote or inhibit neuronal survival in response to brain damage using a stab wound model. In this experiment, the administration of 3 μM purified rat MT-3 significantly improved brain repair, whereas the administration of 15 μM MT-3 had the opposite effect, showing the importance of analyzing the more adequate dosage for *in vivo* treatments (*39*). In fact, previous studies had already demonstrated that MT-3 promote neuron survival at low concentrations but inhibited it at high concentrations (*46*). The neuronal survival effect of MT-3 seems to be mediated by both the wild type and the N-terminal domain of MT-3 (*46*) at low concentrations. At high concentrations, the opposite effect is observed supporting the initial findings of Uchida and Ihara (*64*), who identified the N-terminal portion of MT-3 as the region responsible for the growth inhibiting effect of the protein.

Another model of neuronal damage (MCAO) has been analyzed in MT-3 KO mice. No significant differences in cerebral infarction after 24-H permanent MCAO were detected between WT and MT-3 KO mice. But, after 2-H MCAO and 22-H reperfusion, cerebral infarction in the MT-3 KO mice was aggravated compared with WT mice. Furthermore, fatal rate of MT-3 KO mice increased from 3 days after MCAO, and neurological deficits at 5 and 7 days after MCAO of MT-3 KO mice were worse than those of WT. These findings indicate that neuronal damage was aggravated by reperfusion injury in the MT-3 KO mice compared with the WT mice, suggesting that MT-3 plays neuroprotective roles in transient cerebral ischemia (*29*).

Further research on the effects of MT-3 on the apoptosis of neurons in the hippocampal CA1 region of senescence-accelerated mouse/PRONE8 (SAMP8) showed that MT-3 increased zinc concentration in the hippocampus of these mice, and also significantly decreased apoptosis in these neurons, dose-dependently. Therefore, MT-3 could attenuate apoptotic neuron death in the hippocampus of SAMP8 mice, suggesting that the protein may lessen the development of neurodegeneration (*40*).

MTs and AD

There are several reports associating MTs with AD. In the early stages of AD, an increase in MT-1/2 has been reported. As this increase had no spatial relationship with amyloid beta (Aβ) plaques or neurofibrillary pathology, the authors proposed that it was a likely result of oxidative stress, inflammation or of a deficient metabolism of heavy metals (*65*). The expression of MT-1 has also been evaluated in three different transgenic mice models of AD: Tg2576 (carrying the β-amyloid precursor protein-APP, harboring the Swedish K670N/M671L mutations), TgCRND8 (Swedish and the Indiana V717F mutations), and Tg-SwDI (Swedish and Dutch/Iowa E693Q/D694N mutations). MT-1 mRNA levels were increased in all transgenic lines studied, in astrocytes and microglia/macrophages surrounding the plaques (*66*). In contrast to MT-1, MT-3 mRNA expression was not significantly altered in any of the models examined probably because MT-3 is insensitive to inflammation (*30*).

There is strong evidence that MTs may have an important role in AD pathogenesis, most probably due to its capacity of zinc storage. AD is characterized by extracellular Zn^{2+} accumulation within amyloid plaques as well as by intraneuronal pro-oxidant Fe^{2+} elevation. It was demonstrated that MT-2A is capable of preventing the *in vitro* copper-mediated aggregation of Aβ40 and Aβ42 and associated neurotoxicity, apparently through the metal-swap between Zn_7MT-2A and Cu^{2+}-Aβ (*67*). Similarly, a metal swap between Zn_7MT-3 and soluble and aggregated Cu^{2+}-Aβ40 abolishes ROS production and related cellular toxicity (*68*). Moreover, Tg2576 mice injected with Zn_7MT-2A show reversal of the AD phenotype, although without a decrease in the amyloid load (*69*). In addition, it was recently described that APP retains ferroxidase activity, preventing oxidative stress by oxidizing Fe^{2+} to Fe^{3+}, and this APP activity is inhibited specifically by Zn^{2+} (*70*). The same authors observed that iron accumulation and APP ferroxidase activity is inhibited by endogenous Zn^{2+}, originated from Zn^{2+}-amyloid aggregates, in AD postmortem neocortices. Therefore, abnormal exchange of cortical zinc that may be correlated with the observed altered levels of MTs, may link amyloid pathology with neuronal iron accumulation in AD (*70*).

MT-2 diminishes the binding of transthyretin (TTR) protein to Aβ (*15*). Considering TTR as an Aβ scavenger (*71*), a less efficient removal of Aβ would be expected when MT-2 levels are increased, and this appears to be the case in AD, as stated above. If so, the up-regulation of MT-1/2 observed in animal models of AD and in AD patients may have detrimental consequences in Aβ clearance.

MT-3 was purified and characterized as a growth inhibitory factor abundantly expressed in the normal human brain, but greatly reduced in AD brains, particularly in astrocytes which inhibited survival and neurite formation of cortical neurons *in vitro* (*60*). Several studies performed subsequently were consistent with these initial findings in AD patients (*72*), and in animal models of disease (*73*). Others generated controversial results. For example, Carrasco et al. (*20*) reported increased MT-3 protein and mRNA levels in AD brains, when compared with similarly aged control brains. Another study by Erickson et al. (*47*) analyzed MT-3 expression in frontal cortices from eight AD and five control brains showing that, neither MT-3 mRNA nor MT-3 protein levels were significantly decreased in the AD group.

There is also evidence that MT-3 protects cerebral cortical neurons in culture from the toxic effect of Aβ peptides. These protective effects seem to result from the diminished capacity of Aβ to form SDS-resistant aggregates, highly neurotoxic, in the presence of MT-3, whereas those formed in its presence are mostly SDS-soluble. These effects are not shared by MT-1/2 (*74*). Only WT MT-3 inhibited the formation of SDS-resistant Aβ aggregates and protected cortical neurons from the toxic effect of Aβ which is different from its neuronal growth inhibitory activity (*46*). Other possible mechanisms underlying the protection of MT-3 from Aβ toxicity may be related to its interaction with TTR by improving its Aβ degrading capacity (*15*).

CONCLUSIONS

Over the last two decades, MT-1/2 and MT-3 have been gaining recognition for their role in neuroprotection and neuroregeneration and therefore are starting to be considered as potential therapeutic agents for several neurological and neurodegenerative disorders. Zinc binding, ROS scavenging, and promotion of neuronal survival are common features of both proteins with a strong impact in neuroprotection and neuroregeneration. The zinc binding and antioxidant properties of MT-1/2 and MT-3 are consensual, have been extensively and consistently demonstrated, and explain most of the neuroprotective actions reported for these two proteins. The capacity to promote neuronal survival is consensual for MT-1/2 but has raised controversial results for MT-3, which may be a consequence from the concentrations of the protein used in several different studies. At low concentrations, the protein induces neuronal survival whereas at higher concentrations it has the opposite effect (*29, 46*). Unquestionable anti-inflammatory properties have been attributed to MT-1/2, but there is a lack of reports on anti-inflammatory properties of MT-3. Moreover, some studies suggest it is irrelevant on the inflammatory process (*30, 34*) what is surprising, as due to their antioxidative properties, MT-3 would be expected to play an important role in the inflammatory response like MT-1/2. The antiapoptotic properties of MT-1/2 may also be shared by MT-3 (*40*) but have not been thoroughly analyzed.

The recent elucidation of receptor-mediated actions of MT-1/2 makes it difficult to discriminate between receptor-mediated actions of MT-1/2 and those arising from its action as a modulator of zinc intracellular levels or as a potent scavenger of ROS. The best demonstrated receptor-mediated actions of MT-1/2 are neurite outgrowth and neuronal survival, but apoptosis and brain inflammation may also be regulated, to some extent, by receptor-mediated actions of MTs (*37, 38, 51, 52*). However, the combination between zinc binding and the antioxidant properties of MTs are likely to play an important role on these two features of MT-1/2. In fact, both zinc and ROS are important modulators of apoptosis. Increased levels of free zinc in astrocytes and neurons are considered one of the major causes of death in ischemic and traumatic incidents. Zinc induces cell death in the CNS via apoptotic and necrotic pathways and often encompasses both mechanisms. Elevated zinc concentrations promote the release of proapoptotic proteins such as cytochrome-c and apoptosis-inducing factor from neuronal mitochondria (*9, 75*). The neuroinflammatory response characterized by microglia activation and astrogliosis, involving recruitment of leukocytes and production of inflammatory mediators as proinflammatory cytokines and TNF-α has a consequent increase in oxygen free radicals and ROS production followed by oxidation and/or nitration of lipids, proteins, DNA, and carbohydrates (*76*). Therefore, chronically activated glia can kill adjacent neurons by the release of these highly toxic products (*77*). Oxidative stress induces the release of zinc from MTs via NO, promoting the activity and expression of antioxidant enzymes, including MT-1/2 itself, thus reducing the oxidative damage and the consequences of the injurious stimulus. On the other hand, zinc itself may be a strong inducer of oxidative stress by promoting mitochondrial and extra-mitochondrial production of ROS and its concentration is influenced by proinflammatory cytokines and by MTs homeostasis, which are in turn affected by proinflammatory cytokines (*77*). Therefore, the anti-inflammatory properties attributed to MT-1/2 are also the likely result from the combination of its regulation of intracellular zinc levels as well as of its antioxidant properties. This is also the property attributed to MT-1/2 for which receptor mediation is not so clear.

Altogether, the neuroprotective and neuroregenerative effects of MT-1/2 most likely result from a combination of its intrinsic features as a potent scavenger of ROS and as zinc binding proteins and its LDLR receptor-mediated actions. The analysis of the intracellular events occurring on binding of MT-1/2 or peptides derived from its domains, strongly suggests that neurite outgrowth and promotion of neuronal survival are mediated by

one or more LDLR. Therefore, MT-1/2 seems to be capable of triggering intracellular pathways associated with neuronal growth, survival and apoptosis, and perhaps even inflammation. It remains elusive, however, if the role of these receptors is restricted to endocytosis of MT-1/2, and downstream signaling events are consequences of the zinc binding and antioxidant properties of MT-1/2, or if MT-1/2 activates the receptors and these trigger the signaling cascades observed, as reported earlier for other ligands (*78*).

Receptor-mediated actions of MT-3 have not been reported so far, but the conservation of the regions known to be involved in receptor binding in MT-1/2 is suggestive that they may also exist. Beyond any doubt, the available data support the potential of MT-1/2 and MT-3 and of peptides derived from MT-1/2 domains (Emtins) capable of crossing the blood-brain barrier, as strong therapeutic candidates for tackling neuronal insults, further mitigating neuronal damage and improving recovery.

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Hormones

1. Introduction

The endocrine system is one of the main systems involved in metabolism. It cooperates with the nervous and reproductive systems, kidneys, gut, liver, pancreas and adipose tissue in the control, communication and maintenance of the body homeostasis, constituting its major controlling system. The endocrine system involves numerous glands and organs that produce, store and secrete hormones within the body. The major endocrine glands/organs are: hypothalamus, pituitary gland, pineal gland, thyroid and parathyroid glands, adrenal gland, pancreas and gonads (ovaries and testis). Additionally, others as brain, lungs, liver, heart, skin, adipose tissue, thymus, kidneys, bones and placenta (Fukumoto and Martin 2009; Gardner 2007; Kershaw and Flier 2004) also produce and release hormones.

Hormones are chemical messengers, produced by glands, or cells individually, which are released to the bloodstream to exert their regulatory functions in target cells. Major functions include: the regulation of the development of male and female sexual characteristics, fertility, growth, digestion, glucose consumption, stress response, mineral and water balances and maintenance of a proper blood pressure. These molecules induce different effects on distinct cells (or tissues) in the body. Once a specific hormone arrives to its target cells, it first interacts with specific high-affinity receptors, present in the cell surface or inside cells, and then, the receptor activation initiates a cascade of associated biochemical reactions within the cell, generating specific responses. Hormones are included in three major groups: the hormones derived from tyrosine (epinephrine, norepinephrine, dopamine and THs – T4 and T3), the steroid hormones (P, aldosterone, cortisol, testosterone – T; and E2) and the peptide and protein hormones (thyrotrophin-releasing hormone - TRH, corticotrophin release hormone - CRH, parathyroid hormone, oxytocin, vasopressin, insulin, follicle-stimulating hormone - FSH and, luteinizing hormone - LH) (Brook 2001; Gardner 2007).

To exert their functions, hormones require: hormonal glands working properly; good blood supply that ensure the correct hormone delivery to target cells; unoccupied receptors in the target cells; and, a regulatory system that controls their own synthesis. Endocrine abnormalities usually result in an excess or a deficit of a specific hormone. Endocrine glands dysfunctions may be due to a problem with the gland itself, a problem in the feedback system and/or to a deficient response by target cells. Decreases in the production of hormones may be related to trauma, disease, infection, crowding of the hormone-producing cells or an inherited gene mutation that affects the quantity, quality and/or structure of the hormone. Furthermore, decreased production of hormones may also be due to a failure in the endocrine organ to produce and release enough hormones to stimulate the target cells. On the other hand, increased production of a specific hormone may be related to a feedback system imbalance, hyperplasia or a tumor in the hormone-producing cells, lack of tissue response, medication use, or an inherited condition. However, the inherited conditions are rare and usually related to deficient or dysfunctional

production of a single hormone or to the hormone production in a particular gland (Brook 2001; Gardner 2007).

2. Steroid Hormones

SHs are lipophilic hormones, derived from cholesterol, and mainly produced in the adrenal gland, gonads, placenta and nervous system and, in lower quantities, in adipocytes. In mammals, the SHs include SSHs and corticosteroids (glucocorticoids and mineralocorticoids). SHs play important roles in reproductive functions (SSHs), carbohydrate regulation (glucocorticoids) and mineral/water balance (mineralocorticoids). They also have important actions in inflammatory and stress responses, bone metabolism, behavior and cognition, mood and cardiovascular system (Brook 2001; Gardner 2007). Once synthesized, SHs are released into the peripheral circulation to act on their target sites, such as female or male reproductive tracts, CNS, bones, vascular system, digestive system, immune system, lungs, skin and kidneys (Wierman 2007). SHs are not stored at high levels in endocrine organs because they are mainly regulated at the synthesis level by negative feedbacks through expression of particular steroidogenic enzymes. After synthesis and release, SHs undergo several metabolic modifications that convert these hormones in inactive water-soluble forms or active ones (Brook 2001; Gardner 2007). SHs act as chemical messengers in the target cells to produce both genomic responses (slow) and non-genomic responses (rapid) (Norman et al. 2004). It is clear that the genomic responses to SHs are mediated by the formation of a complex between the hormone and its cognate steroid-hormone nuclear receptor (genomic pathway). However, several recent reports have described rapid responses mediated by a variety of receptors associated with the plasma membrane or its caveolae components (non-genomic pathway), including a membrane-associated nuclear receptor, which affect subsequent intracellular signaling cascades, both *in vitro* and *in vivo* (Groeneweg et al. 2011; Norman et al. 2004; Yau and Seckl 2012). However, until now, it is uncertain if these non-genomic responses have relevant physiological and clinical impacts.

2.1 Biosynthesis

All SHs are synthesized from a common steroid precursor, cholesterol, by a series of enzymes both P450s and non-P450s. Generally, cholesterol (C27) contains three cyclohexanes (A, B and C) and one cyclopentane (D) rings, with a carbon side chain attached at the 17 position of the D ring. The partial loss of the carbon side chain results in a series of compounds which includes P and corticosteroids, while, the complete loss of the side chain originates androgens, of which T is the primary product and, estrogens, after the aromatization of its A ring.

The adrenal cortex produces three main types of hormones: glucocorticoids (cortisol), mineralocorticoids (aldosterone) and adrenal androgens. As represented in figure 8, the adrenal cortex is divided into three distinct zones: *zona glomerulosa* (outer), *zona fasciculata*

(middle), and *zona reticularis* (inner). The synthesis of cortisol occurs in the *zona fasciculata*, while aldosterone is produced in the *zona glomerulosa* and adrenal androgens in the *zona reticularis*, which also produces cortisol (Lavoie et al. 2009). The first step of adrenal steroidogenesis is the transport of the free cholesterol from the cytoplasm into the mitochondria by the steroidogenic acute regulatory protein (StAR), which is expressed in response to agents that stimulated steroid synthesis (Stocco 2001). In the inner mitochondrial membrane, cholesterol is catalyzed by the side chain cleavage enzyme (P450scc), that cuts the cholesterol carbon side chain between carbon atoms C20 and C22, generating pregnenolone (C21) (Rone et al. 2009), which serves as a substrate for subsequent downstream steroid formation. Pregnenolone is then released into the cytoplasm and, by 3ß-hydroxysteroid dehydrogenase 2 (3ß-HSD2), P is formed. After, 21-hydroxilase (P450c21), converts P in 11-deoxycorticosterone (DOC) (mineralocorticoid pathway) and 17α-hydroxyprogesterone (P450c17) in 11-deoxycortisol (glucocorticoid pathway). These intermediate molecules are converted to the biologically active hormones aldosterone and cortisol through aldosterone synthase and 11ß-hydroxylase (P450c11), respectively. Also, 17α-hydroxylase/17, 20-lyase (P450c17α), in the presence of P450 oxireductase (POR), uses pregnenolone and P as substrate to produce 17α-hydroxysteroids which are, respectively, converted into dehydroepiandrosterone (DHEA) and androstenedione (Reincke et al. 1998). In testis, synthesis of testosterone (T) and other molecules (Sasano et al. 1989) are induced *via* cytochrome P450 enzymes and dehydrogenases: 17ß-hydroxysteroid dehydrogenase (17ß-HSD) converts androstenedione in T (Mindnich et al. 2004); steroid 5α-reductase mediates the production of DHT; and, aromatase converts a part of T into E2. In the ovaries: androstenedione and T are converted to E2 through P450 aromatase and 17ß-HSD actions; and, P450scc and 3ß-hydroxysteroid dehydrogenase-/Δ5-Δ4 isomerase (3ß-HSD) mediates P synthesis (Fig. 8) (Mindnich et al. 2004; Sasano et al. 1988).

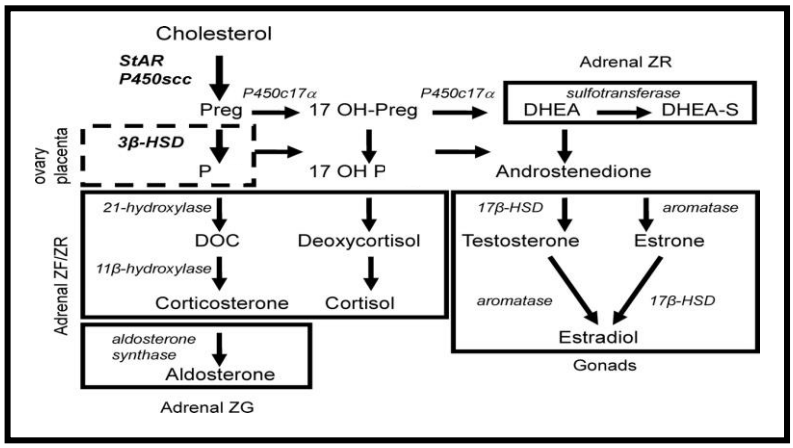

Figure 8: Overview of steroidogenesis. Cholesterol provides the substrate for *de novo* steroidogenesis. StAR (steroidogenic acute regulatory protein) mediates cholesterol delivery to the inner mitochondrial membrane and P450scc (cholesterol side-chain cleavage enzyme) in the adrenal glands, gonads and rodent placenta. The enzymes that mediate all reactions are indicated in italics and the major tissues for each reaction are boxed. Abbreviations include Preg (pregnenolone), P (progesterone), DOC (deoxycorticosterone), ZF (*zona fasciculata*), ZR (*zona reticularis*), ZG (*zona gomerulosa*), 3ß-HSD (3ß-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase), P450c17α (17α-hydroxylase/17,20-lyase), DHEA (dehydroepiandrosterone) and 17ß-HSD (17ß-hydroxysteroid dehydrogenase). Adapted from Lavoie and King 2009.

2.2 Sex Steroids

SSHs include estrogens, androgens and progestins and have been consistently considered for their major roles in reproductive function. In fact, the metabolism of SSHs has been widely studied.

As mentioned, SSHs are also synthesized from cholesterol and have high lipid solubility, which facilitate their cross through cell membranes (Bear 2007). SSHs circulate in bloodstream bound to plasma proteins (hormone-binding globulins and albumin) and, similarly to the other SHs, only when in a free form, they are biologically active.

Concerning SSHs expression in brain, several aspects must be attended because enzymes that mediate SSHs production are expressed in distinct cell types and their distribution may not coincide. In fact, the enzymatic activity, and consequent molecules synthesized, depends on the cell-type: oligodendrocytes have the higher P450scc activity, comparing to neurons and astrocytes; oligodendrocytes, neurons and astrocytes produce pregnenolone; P450c17α is expressed in neurons and astrocytes, but not in oligodendrocytes; and, P450 aromatase is expressed by astrocytes and neurons (Zwain and Yen 1999).

In the past few years, several studies highlighted the neuroprotective effects of SSHs, particularly in neurodegenerative contexts (Vest and Pike 2012; Yaffe 2003). Previous studies well established that in addition to the high quantities of SSHs produced in gonads and adrenal glands, the brain synthesizes *de novo* steroids (neurosteroids) (Mellon and Vaudry 2001), which act on brain, through their receptors, in modulation of intracellular signaling pathways, channels and transcription of genes (Micevych and Sinchak 2008).

2.3 Corticosteroids

Corticosteroids belong to the SHs group and are produced in the cortex of the adrenal gland. Several functions have been attributed to these hormones as they regulate a wide range of physiologic processes, as immune and stress responses and inflammation, behaviour, blood electrolyte/water contents and carbohydrate metabolism. Corticosteroids include two major sub-types of hormones: glucocorticoids and mineralocorticoids. Glucocorticoids (cortisol, cortisone and corticosterone) are involved in the regulation of carbohydrate, protein and fat metabolisms, prevention of inflammation and mediation of stress responses. Mineralocorticoids (aldosterone) are mainly responsible for the control of electrolyte and water balances by through Na^+ reabsorption and K^+ excretion in target tissues (Brook 2001; Gardner 2007).

To date, two extensively investigated sub-types of corticosteroid receptors were identified: the high affinity mineralocorticoid receptor (MR; Type I) and the lower affinity glucocorticoid receptor (GR; Type II) (de Kloet et al. 1998; de Kloet et al. 2005; Mc Ewen et al. 1986).

- ***Glucocorticoids and Cortisol***

Glucocorticoids are the primary end products of the hypothalamic-pituitary-adrenal (HPA) axis, the main neuroendocrine circuit related to the stress response (Charil et al. 2010). They have the capacity to generate adequate responses to physical and emotional stressors (Hellhammer et al. 2009), to regulate carbohydrate (Rooney et al. 1993), protein and lipid metabolisms

(Xu et al. 1993). Also, glucocorticoids have cell type-dependent effects on various immune responses (Amsterdam et al. 2002) mediated by T and B lymphocytes and on the effector functions of monocytes and neutrophils (Amsterdam et al. 2002; Crabtree et al. 1979; Franchimont 2004; Ehrchen et al. 2007). The major glucocorticoid in primates is cortisol and, in rodents corticosterone. It is produced in the *zona fasciculata* of the adrenal gland and is almost ubiquitous in the body. Once synthesized, cortisol circulates in bloodstream almost completely bound with high affinity to corticosteroid-binding globulin (CBG) which protects it against degradation (Brien 1981). It has a marked circadian diary cycle thus, influencing its levels during the daytime: high levels are observed early in the morning, decrease during the day and, are minimal at night, when animals return to sleep. Cortisol (active form) can be converted in cortisone (inactive form) by 11ß-HSD2 while 11ß-HSD1 converts cortisone in cortisol (Lakshmi and Monder 1988) and, the overall ratio of cortisol and cortisone in the bloodstream is the result of the equilibrium between the activities of the two isoenzymes. Cortisol can also be converted in other metabolites through action of other enzymes.

- ***Receptors and Mechanisms of Action***

GR and MR are encoded by NR3C1 (5q31) and NR3C2 (4q31) genes, respectively (Fan et al. 1989; Francke and Foellmer 1989; Hollenberg et al. 1985). Both receptors derived from an ancestral corticosteroid receptor and are ligand activated transcription factors belonging to the nuclear receptors superfamily. MR and GR are tissue- and cell-type specific and, although sharing some complementarity, they are genetically and functionally distinct. In brain, while GRs are widely expressed in the hypothalamus, paraventricular nucleus and brainstem, amygdala, pituitary gland, CP and hippocampus (Herman et al. 1992; Kitraki et al. 1996), the expression of MRs is more restricted and reduced to the hippocampus, septum (Groeneweg et al. 2011; Reul et al. 2000) and CP (Gomez-Sanchez et al. 2009; Lathe 2001). GRs have lower affinity for corticosteroids and only become more activated when hormone levels increase after a stress situation. Contrariwise, MRs have higher affinity for corticosteroids and are extensively occupied under basal conditions (Mc Ewen et al. 1986; Reul and de Kloet 1985). The MR mediated actions are more relevant during the initial response of HPA axis to stress and, GR is the major contributor to the negative feedback of the HPA axis, terminating the stress response and promoting the adaptation and recovering (de Kloet et al. 1998; de Kloet et al. 2005). Thus, the balance between GRs and MRs are essential for maintaining/recovering the homeostasis (Oitzl et al. 2010).

After cortisol (corticosterone in rodents) cross the cell membrane and binds to GR or MR, an homo/heterodimer complex is formed and translocated to the nucleus where it binds to GREs present in target genes. Then, co-activators and/or co-repressors are recruited to activate or repress gene transcription. GRs and MRs can also interact directly with other transcription factors as monomers, thereby modulating gene expression of target genes. Until now, it is more accepted that the classical pathway of GRs/MRs action, through their binding as a dimer to GREs,

is primarily responsible for transactivation of target genes, while the monomeric interaction with other transcription factors seems to represent the major pathway for transrepression (Reichardt et al. 2001). Still, membrane-forms of MRs (Karst et al. 2005) and GRs (Wiegert et al. 2006) and a non identified membrane-localized receptor had also been recently reported as mediators of the rapid actions of glucocorticoids signalling in several brain regions (Groeneweg et al. 2011).

- ***Regulation***

Adrenal glucocorticoid secretion is regulated by the HPA axis. The hypothalamus is a brain structure that establishes the link between the nervous and the endocrine systems and acts as the neuroendocrine control centre in the CNS. It controls the secretion of hormones from anterior and posterior pituitary gland, regulating a number of homeostatic processes (Brook 2001).

The hypothalamus releases CRH into the hypothalamo-hypophyseal portal vein and, then, into pituitary gland, where it stimulates the adrenocorticotrophic hormone (ACTH) from the corticotroph cells located in the anterior lobe of the pituitary gland. Then, ACTH stimulates the adrenal cells, in the *zona fasciculata* and *zona reticularis,* to synthesize cortisol that once released, exerts a negative feedback on CRH and ACTH production and release in the hypothalamus and pituitary gland, respectively (Keller-Wood and Dallman 1984). This mechanism is regulated by several stimuli, including: food intake and nutritional status, mood, circulating cytokines and physical and/or psychological stress. Under physiological conditions, the HPA axis also follows a circadian rhythm activity (Brook 2001; Gardner 2007; Tsigos 2002). Furthermore, basal and induced levels of corticosteroids and reactivity of the HPA axis are also gender-dependent (Critchlow et al. 1963; Kirscbaum et al. 1992; Uhart et al. 2006).

- ***Stress Response: Neurological Roles***

Glucocorticoids have been widely associated with several physiologic processes, including the stress response. Exposure to stressors, activate the HPA system resulting in increased secretion of corticosteroids which are released from the adrenal cortex to the blood, in an effort to re-establish the homeostasis in the body and to re-adaptate to the new stressful conditions (Matousek et al. 2010). The release of corticosteroid hormones upon a stress situation, occurs mainly through cortisol, that is one of the largest endocrine stress inducers in human organisms, the principal hormone related to stress and the final hormone of the HPA system (Dedovic et al. 2009).

Stress is defined as a pathophysiological state associated to specific physiological conditions induced by physical or physiological stimuli (Gardner 2007; Selye 1956). It is related with the pituitary gland and ACTH secretion and, consequent increased production of glucocorticoids, in the adrenal cortex, with effects on metabolism, especially on the nervous and endocrine systems. In the neuroendocrine system, stress affects the synthesis of numerous pituitary

hormones, as ACTH, LH, prolactin, oxytoxin, vasopressin and growth hormone (Armario 1984; Armario et al. 1986). As mentioned, ACTH, once in the blood, is driven to the adrenal cortex where it binds to receptors and promotes the conversion of cholesterol into cortisol (corticosterone in rodents) as a final product (Hellhammer et al. 2009). Previous studies showed that there are gender differences in the HPA axis and, the initial ACTH secretion in response to stress is significantly higher in males than in females (Roca et al. 2005; ter Horst et al. 2012) because in males is observed a higher increase of CRH levels that lead to a consequent increased release of cortisol (Bao et al. 2008a).

Chronic ACTH secretion, promoted by physical and psychosocial stressors, induces constant high levels of glucocorticoids that increase the HPA axis activity. Thus, a continuous increased HPA activity could promote deleterious effects on the structure and function of several brain regions and trigger multiple neuropsychiatric or neurological disorders (Sapolsky 2000), as depression, psychological and physiological stress, dementia, deficits in learning and memory functions and AD (Abel 2005; Hellhammer et al. 2009). Previous reports showed that there are specific areas in the brain affected by glucocorticoids and hippocampus is one of those areas (Beltramini et al. 2004). In this brain structure, glucocorticoids lead to activation or repression of target genes through nuclear receptors (Arriza et al. 1988), mediating the adaptation to stress and regulating the stress response through intrinsic and extrinsic negative feedbacks in the HPA axis (Adzic et al. 2009).

Several studies have associated stress with AD (Bao et al. 2008b; Dong and Csernansky 2009; Souza-Talarico et al. 2008) that is caused by complex interactions between various genetic influences and environmental factors, including stress. AD patients showed high basal cortisol levels and treatment with corticosteroids intensified their cognitive deficit (Alkadhi et al. 2010). Others observed that chronic stress induced glucocorticoid and excitatory amino acid levels that cause hippocampal atrophy, neuronal death and premature aging of hippocampal neurons and, concluded that with the advancing age, there is an increased likelihood of the concurrence of stress and AD (Landfield et al. 2007; Pedersen et al. 2001). Nevertheless, the adaptative or destructive physiological consequences of stress, and consequently glucocorticoids, are dependent on the intensity, type and duration of the stressor and how an organism reacts to restore the equilibrium (Srivareerat et al. 2009; Tran et al. 2011). In line with this, some authors demonstrated that induction of a stress response could prevent some neurodegenerative diseases and may have an anti-inflammatory effect (Schleimer 1993) because in moderately high concentrations of glucocorticoids they can reduce the size of lymph nodes and thymus involution, which, in turn, could increase CNS activity. Several other molecules, including their own receptors, which play roles in various neurological diseases, are also regulated by glucocorticoids.

So, involvement of stress, and concomitant glucocorticoid increases, in several brain diseases is under solid investigation and findings have revealed complex interactions between these hormones and numerous molecules and metabolic brain functions.

Chapter 2: Global Aims

Through the summarized revision of the literature, on chapter 1, it is well defined that CP plays major roles in the maintenance of the brain homeostasis and, disturbances on its structure and/or functions promote CNS imbalances that could trigger the onset and/or progression of various neurodegenerative disorders. In mammals, TTR and MTs (MT-1, MT-2 and MT-3) are expressed by CP cells and mediate several processes within the CNS, including neuroprotection and neuroregeneration. Similarly, various SHs, as glucocorticoids, have been associated with the brain homeostasis, participating on its maintenance/imbalance.

Previous works from our research group showed that some SHs regulate TTR expression and TTR and MT-2 interact. Moreover, we performed *in silico* analyses in TTR and MT-1/2 genes and found GREs in both genes, raising the hypothesis that TTR and MT-1/2 could be regulated by glucocorticoids through these elements.

The global aims of the present research work were to investigate occurrence of a TTR-MT-3 interaction, to clarify the effect of TTR-MT-2 / TTR-MT-3 interaction in TTR-Aß binding and, consequently, in AD. Also, as TTR, MT-1/2, glucocorticoids and CP are related with stress and are involved in brain homeostasis, it was pivotal to study the putative regulation of TTR and MTs (MT-1/2) by glucocorticoids, particularly in the CP.

Chapter 3: Human Metallothioneins 2 and 3 Differentially Affect Amyloid-Beta Binding by Transthyretin

(Paper II)

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Human metallothioneins 2 and 3 differentially affect amyloid-beta binding by transthyretin

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Transthyretin (TTR), an amyloid-beta (Aβ) scavenger protein, and metallothioneins 2 and 3 (MT2 and MT3), low molecular weight metal-binding proteins, have recognized impacts in Aβ metabolism. Because TTR binds MT2, an ubiquitous isoform of the MTs, we investigated whether it also interacts with MT3, an isoform of the MTs predominantly expressed in the brain, and studied the role of MT2 and MT3 in human TTR–Aβ binding. The TTR–MT3 interaction was characterized by yeast two-hybrid assays, saturation-binding assays, co-immunolocalization and co-immunoprecipitation. The effect of MT2 and MT3 on TTR–Aβ binding was assessed by competition-binding assays. The results obtained clearly demonstrate that TTR interacts with MT3 with a K_d of 373.7 ± 60.2 nM. Competition-binding assays demonstrated that MT2 diminishes TTR–Aβ binding, whereas MT3 has the opposite effect. In addition to identifying a novel ligand for TTR that improves human TTR–Aβ binding, the present study highlights the need to clarify whether the effects of MT2 and MT3 in human TTR–Aβ binding observed *in vitro* have a relevant impact on Aβ deposition in animal models of Alzheimer's disease.

Structured digital abstract

- MINT-7905930: *Amyloid beta* (uniprotkb:P05067) *physically interacts* (MI:0915) with *Ttr* (uniprotkb:P02767) by *saturation binding* (MI:0440)
- MINT-7905857: *MT3* (uniprotkb:P25713) *binds* (MI:0407) to *TTR* (uniprotkb:P02766) by *saturation binding* (MI:0440)
- MINT-7905838: *TTR* (uniprotkb:P02766) *physically interacts* (MI:0915) with *MT3* (uniprotkb:P25713) by *two hybrid* (MI:0018)
- MINT-7905914: *Ttr* (uniprotkb:P02766) *physically interacts* (MI:0915) with *Mt3* (uniprotkb:P25713) by *anti tag coimmunoprecipitation* (MI:0007)
- MINT-7905895: *TTR* (uniprotkb:P02767) and *Mt3* (uniprotkb:P37361) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

Introduction

Transthyretin (TTR) is a homotetrameric protein of 55 kDa produced mainly in the liver and in the choroid plexus (CP) of the brain [1], which is known for the transport of thyroid hormones and the indirect transport of retinol [2] via its binding to plasma retinol-binding protein [3]. Within the central nervous

Abbreviations
Aβ, amyloid-beta; AD, Alzheimer's disease; CP, choroid plexus; CPEC, choroid plexus epithelial cell; CSF, cerebrospinal fluid; ER, endoplasmic reticulum; hMT3, human MT3; human TTR, hTTR; MT, metallothionein; RT, room temperature; TTR, transthyretin.

system, TTR is primarily synthesized and secreted into the cerebrospinal fluid (CSF) by the epithelial cells of CP [4]. Recently, TTR has been implicated in behavioural, psychiatric and neurodegenerative disorders, particularly Alzheimer's disease (AD) [5,6].

Previous studies have shown that TTR expression is induced in response to the overproduction of amyloid-β (Aβ) peptides [6] and overexpressed TTR forms stable complexes with Aβ, a key protein on the pathophysiology of AD, sequestering it and preventing its aggregation and/or fibril formation [7]. The physiological relevance of this feature is reinforced by studies showing that, in CSF from AD patients, TTR levels are diminished compared to age-matched controls and that an inverse correlation between TTR levels and senile plaques abundance exists [8–10]. The nature of the TTR–Aβ interaction has been characterized recently; TTR cleaves full-length Aβ, generating smaller peptides with lower amyloidogenic properties, and it is also able to degrade aggregated forms of Aβ peptides [11,12].

Metallothioneins (MTs) are ubiquitous low molecular weight metal-binding proteins (6–7 kDa) involved in the homeostasis of essential trace metals, particularly zinc (Zn^{2+}) and copper [13,14]. There are four distinct MT isoforms: MT1 to MT4. MT1 and MT2 are widely expressed in most tissues, including the central nervous system [15]. MT3 was originally identified in the brain [16], although it is also expressed in the reproductive system, kidney, tongue and CP of rats, whereas MT4 expression is restricted to some stratified squamous epithelia [17,18]. Over the last decade, research on the roles of MTs in brain physiology has demonstrated that MT1 and MT2 are up-regulated in response to injury, protect the brain against neuronal damage, regulate neuronal outgrowth, influence tissue architecture and cognition, and protect against neurotoxic insults and reactive oxygen species [19]. MT3 also protects against brain damage, antagonizes the neurotrophic and neurotoxic effects of Aβ and influences neuronal regeneration, despite having no significant antioxidant role [20–23]. Therefore, MT2 and MT3 are regulated in several neurodegenerative disorders, including AD. Analysis of MT levels in human AD brains and brains of animal models of AD has consistently revealed increased levels of MT1 and MT2 expression [24,25]. MT3 expression, on the other hand, appears to be reduced compared to age-matched controls [16,26,27], although some studies report an opposite trend [28] or no differences in MT3 expression [25,29].

Previously, we have demonstrated that TTR interacts with MT2, either *in vivo* and *in vitro* [30]. Because both TTR and MTs have an impact on Aβ metabolism, we investigated the interaction between TTR and MT3, and characterized the impact of the TTR–MT2 and TTR–MT3 interactions on TTR–Aβ binding.

Results

Analysis of the TTR–MT3 interaction by yeast two-hybrid assays and saturation-binding assays

The existence of an interaction between human TTR (hTTR) and human MT3 (hMT3) was detected by yeast two-hybrid assays. The construct pGBKT7-hTTR, which encodes the full-length hTTR cDNA fused in-frame to the GAL4 DNA binding domain, was used as bait, and the full-length hMT3 cDNA, fused with the GAL4 activation domain, was used as prey in the assay. Positive clones were detected in all of the five experiments carried out, indicating that an interaction between hTTR and hMT3 occurs. Positive and negative controls were run simultaneously, with the expected results being obtained. The hTTR–MT3 interaction was further characterized by saturation-binding assays to determine the K_d of the interaction, which is 373.7 ± 60.2 nM (Fig. 1).

Co-immunolocalization of TTR and MT3

To determine whether TTR and MT3 co-localize *in vivo*, we established CP epithelial cells (CPEC) primary cultures and performed double immunofluorescence staining using antibodies against TTR and MT3. In addition, we used MT3 and endoplasmic reticulum (ER) double immunofluorescence staining to determine whether MT3 is present in the ER. For co-localization, we used the software 25, version 4.4 (Zeiss Imaging Systems, Vertrieb, Germany) and images from MT3 (red channel) and TTR (green channel) or MT3 and ER (green channel) were merged. As shown by the yellow areas in the merged images, TTR and MT3 co-localize in the cytoplasm, particularly in the perinuclear region (Fig. 2A). The co-localization of MT3 and ER (Fig. 2B) suggests that MT3, similar to TTR [30] may also be secreted. Therefore, the TTR–MT3 interaction may occur in this cellular compartment or outside the cell. In preparations where the primary antibodies were omitted, no immunofluorescence was visualized, nor when the MT3 antibody was pre-incubated with MT3.

In vivo co-immunoprecipitation of hTTR and hMT3

More evidence sustaining the hypothesis of the existence of an interaction between hTTR and hMT3 was provided by *in vivo* co-immunoprecipitation

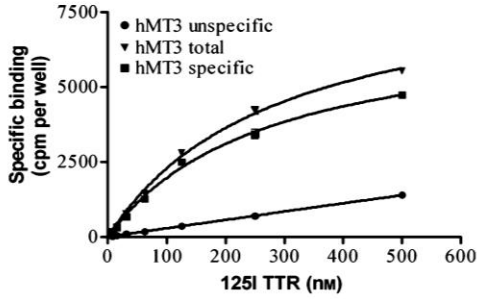

Fig. 1. Saturation-binding assays: binding of [^{125}I]hTTR to hMT3 peptide. Binding of [^{125}I]-hTTR to hMT3 was carried out in 96-well plates coated with 2 μg per well of hMT3. Increasing concentrations of [^{125}I]hTTR were incubated in each well. Unspecific binding was determined by incubating similar amounts of [^{125}I]hTTR in the wells in the presence of a 100-fold molar excess of nonlabelled hTTR. Three replicas of each sample were set up in each experiment. Specific binding was calculated as the difference between total binding and nonspecific binding. Error bars indicate the SEM.

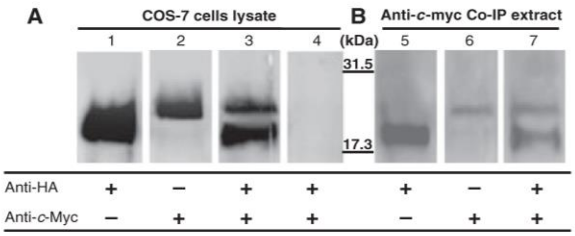

Fig. 3. hTTR and hMT3 expression and interaction. (A) Western blot of COS-7 cells transfected with pCMV-HA-hMT3 (lane 1), pCMV-*c*-Myc-hTTR (lane 2), both constructs (lane 3) or mock transfection (lane 4). The fusion proteins were detected using HA-Tag polyclonal antibody, *c*-Myc monoclonal antibody, or both, according to the scheme shown below. (B) Western blot showing that hMT3 co-immunoprecipitates (Co-IP) with hTTR. Each lane contains 20 μg of immunoprecipitate extract resulting from the immunoprecipitation of the total protein extract with anti-*c*-Myc serum pre-incubated with protein G Plus-Agarose. Lanes 5–7 were incubated with anti-HA, anti-*c*-Myc and both sera, respectively, according to the scheme shown below.

assays. The fusion proteins HA-hMT3 and *c*-Myc-hTTR were expressed in COS-7 cells, transfected with pCMV-HA-hMT3 alone, pCMV-*c*-Myc-hTTR alone or pCMV-*c*-Myc-hTTR + pCMV-HA-hMT3 constructs, as confirmed by western blotting (Fig. 3A). In the co-immunoprecipitation assay, we used protein extracts from cells expressing both fusion proteins (*c*-Myc-hTTR and HA-hMT3). When anti-*c*-Myc was used for immunoprecipitation of *c*-Myc-hTTR, the HA-hMT3 fusion protein was co-precipitated, indicating that both proteins interact in cell extracts, as shown by western blotting (Fig. 3B). As predicted, in

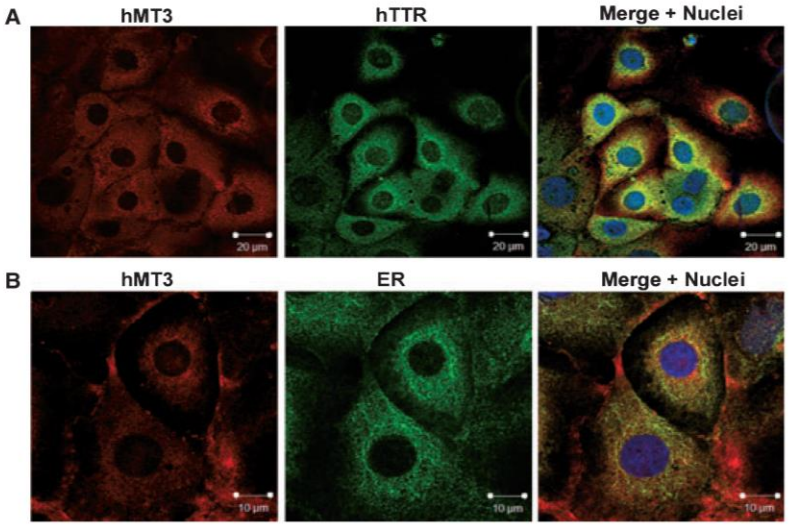

Fig. 2. Confocal microscopy of hMT3 co-localization with TTR and ER in rat CPEC (× 630). (A) Cells were incubated with the primary antibodies, mouse monoclonal anti-hMT3 serum and rabbit polyclonal anti-hTTR serum followed by Alexa Fluor 546 goat anti-(mouse IgG) conjugate (red) and Alexa Fluor 488 goat anti-(rabbit IgG) conjugate (green) (image zoom scan, × 1.0). (B) Cells were stained with a mouse monoclonal anti-hMT3 serum followed by Alexa Fluor 546 goat anti-(mouse IgG) conjugate (red) and a rabbit polyclonal anti-human ATF-6α (ER) followed by Alexa Fluor 488 goat anti-(rabbit IgG) conjugate (green). Co-localization of hMT3/hTTR and hMT3/ER corresponds to the yellow areas in the merged images. The nuclei of cells in (A) and (B) were stained with Hoechst 33342 dye (blue) (image zoom scan, × 2.0).

the western blot set up with protein extracts from cells expressing both fusion proteins, anti-HA and anti-c-Myc, separately and together, were capable of detecting the presence of fusion proteins, confirming that the two proteins interact with each other.

Determination of the effect of MT2 and MT3 in TTR–Aβ binding

The effect of hTTR–MT2 and hTTR–MT3 interactions in TTR/Aβ binding was characterized by competition binding assays using soluble Aβ and recombinant [^{125}I]hTTR (Fig. 4). The inhibition constant (IC_{50}) values calculated in competition binding assays with hTTR alone or with hTTR pre-incubated with hMT2 (Fig. 4A) were 0.409 ± 0.168 and 74.37 ± 0.183, respectively, indicating that pre-incubation of hTTR with hMT2 diminishes the capacity of hTTR to bind Aβ. On the other hand, in an assay identical to that with

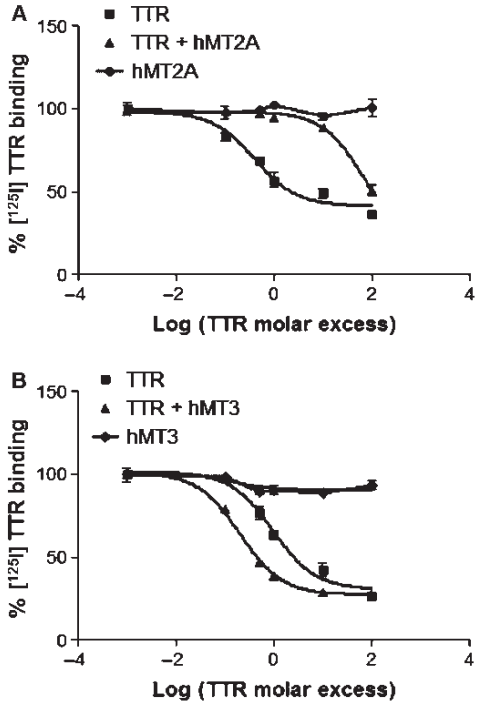

Fig. 4. Binding of [^{125}I]TTR to Aβ in the presence or absence of (A) hMT2 or (B) hMT3. Binding of [^{125}I]TTR to Aβ was carried out in 96-well plates coated with 2 μg per well of soluble $Aβ_{1–42}$. A constant amount of [^{125}I]hTTR was added to each well alone or in the presence of the indicated molar excess of unlabelled competitors (hTTR alone or hTTR pre-incubated with hMT2 or hMT3 peptides at 0, 0.54, 2.7, 5.4, 54 and 540 nM). Specific binding was calculated as that observed with [^{125}I]hTTR alone minus [^{125}I]hTTR in the presence of a 100-fold molar excess of unlabelled protein.

hMT3, the IC_{50} values calculated were 0.987 ± 0.121 for TTR alone and 0.206 ± 0.043 when hTTR was pre-incubated with hMT3, indicating that pre-incubation of hTTR with hMT3 affects hTTR–Aβ binding with a relative affinity of 0.209, strongly suggesting that the capacity of hTTR to bind Aβ is higher in the presence of hMT3 (Fig. 4B).

In both experiments, the presence of hMT2 or hMT3 peptides without previous incubation with hTTR did not affect hTTR–Aβ binding because, in these situations, the relative binding of [^{125}I]hTTR to Aβ was not statistically different.

Discussion

As previously demonstrated, there is an interaction between TTR and MT2, *in vivo* and *in vitro* [30]. Because both TTR and MTs have an impact on Aβ metabolism and deposition, the present study aimed to identify and characterize a putative interaction between hTTR and hMT3 and to determine whether the presence of hMT2 and hMT3 affects hTTR–Aβ binding.

In a first approach, using the yeast two-hybrid technique with hTTR as a bait and hMT3 as a prey, several positive clones were identified, indicating that hTTR and hMT3 interact. However, because this technique often provides false positives [31], we carried out *in vitro* saturation-binding assays and *in vivo* co-immunolocalization and co-immunoprecipitation experiments to further confirm and characterize the interaction.

The K_d calculated for this interaction by *in vitro* saturation-binding assays (373.7 ± 60.24 nM) was in the same order of magnitude as those caculated for other previously reported TTR ligands, such as retinol-binding protein (K_d = 800 nM) [32] or MT2 (K_d = 244.8 nM) [30], indicating that a fairly stable complex occurs.

In vivo studies of co-localization showed that hMT3 and hTTR were both localized in the cytoplasm of CPEC, particularly in the perinuclear region, most likely in the ER, as deduced from the co-localization of hMT3 and ER, and this is also where TTR is present [30]. More consistent evidence of this interaction was provided by *in vivo* co-immunoprecipation studies because when anti-*c*-Myc was used for immunoprecipitation, the HA-hMT3 fusion protein was co-precipitated with *c*-Myc-hTTR. Taken together, the findings of the *in vitro* and *in vivo* experiments support the hypothesis of the existence of an interaction between hTTR and hMT3, which appears to occur in the cytosol of CPEC, most likely in ER.

The next step was to analyze the effect of the hTTR–hMT2 and hTTR–hMT3 interactions on the

capacity of hTTR to bind Aβ. *In vitro* competition binding assays carried out for this purpose indicate that pre-incubation of hTTR with hMT2 reduces hTTR-Aβ binding. On the other hand, when *in vitro* competition binding assays were carried out with hTTR pre-incubated with hMT3, we found that, in contrast to hMT2, pre-incubation of hTTR with hMT3 enhances the hTTR capacity to bind Aβ. Thus, a less efficient removal of Aβ would be expected when hMT3 expression is decreased and hMT2 levels are increased, and this appears to be the case in AD [24,26]. MT3 antagonizes the neurotrophic and neurotoxic effects of Aβ peptides, abolishing the formation of toxic aggregates [23]. This effect may be related to its interaction with TTR, which gains affinity to bind Aβ in the presence of MT3. Therefore, cleavage of full-length Aβ and degradation of aggregated forms of Aβ peptides, which are features that have been attributed to TTR [11,12] should also be enhanced in the presence of MT3.

Despite the differences between hMT2 and hMT3, some consensus amino acid sequences have been conserved and the two proteins share an identity of 70% [27,33]. This includes the CxCAxxCxCxxCxCxxCK sequence that is conserved in all vertebrate metallothioneins [34,35], the existence of two domains, α and β, with a linker between them [36,37], and the total conservation of the 20 cysteines in both molecules [34]. Major differences between hMT2 and hMT3 are the insertion of a threonine in the N-terminal of the β domain (at position 5), the existence of a characteristic motif in the β domain between positions 6 and 9 (CPCP) and an insertion of an octapeptide motif (EAAEAEAE) in the C-terminal of the α domain of hMT3 [15,16,38,39]. Because hTTR interacts with hMT2 and hMT3, it is likely that these interactions occur through the conserved regions of both proteins. The differences between the two MTs may justify their opposing effects on the capacity of TTR to bind Aβ. No differences in the binding of [^{125}I]hTTR to Aβ were found when hMT2 and hMT3 were present in the reaction but had not been pre-incubated with hTTR. This indicates that the effects of MT2 and MT3 in TTR Aβ binding do not result from a competition for TTR between MT2 or MT3 and Aβ, but from the competition of a TTR–MT complex.

The existence of these TTR–MT interactions in CPEC suggests that they may as well, occur *in vivo* in CP, where they may have an important role on Aβ metabolism. The presence of Aβ in brain fluids, including the CSF, is a hallmark of AD, and its accumulation in these fluids increases the severity of the disease. CP has the capacity to remove and degrade Aβ [40,41], contributing to its clearance from the CSF. The mechanisms involved in this process, as well as on overall Aβ homeostasis, are not fully understood, although they appear to require the concerted action of several enzymes involved in Aβ metabolism, such as insulin-degrading enzyme, endothelin-converting enzyme-1, neprysilin and α-secretase, which are all expressed in CP [41]. In addition, TTR, which is also highly expressed in CP and is the most abundant protein in CSF, has gained increasing support as a key protein in Aβ metabolism [11,12]; its capacity to remove Aβ appears to be enhanced by the interplay with MT3 as demonstrated in the present study.

The findings obtained in the present study bring a fresh perspective with respect to the mechanisms implicated in the binding of hTTR to Aβ and highlight the need to clarify whether the apparent effects of MT2 and MT3 in hTTR–Aβ binding have a relevant impact on Aβ deposition in animal models of AD.

Experimental procedures

Analysis of the TTR–MT3 interaction by *in vitro* yeast two-hybrid assays and saturation-binding assays

Yeast two-hybrid system

The full-length hTTR cDNA and the full-length hMT3 cDNA were amplified by PCR using primers hTTRfw and hTTRrv and primers hMT3fw and hMT3rv, respectively (Table 1). Subsequently, the products obtained were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA) and digested with the corresponding endonucleases (Takara Bio Inc., Shiga, Japan), as indicated in Table 1.

The hTTR and hMT3 were cloned in pGBKT7 (Clontech, Shiga, Japan) and pGADT7 (Clontech), respectively. Each plasmid construct was transformed in competent *Escherichia coli* DH5α. Plasmid DNA was extracted from the grown cultures using Wizard® Plus Minipreps DNA Purification System (Promega) and sequenced to confirm the identity of clones.

Each construct was used to transform *Saccharomyces cerevisae* AH109 strain using the Matchmaker GAL4 two-hybrid system 3 (Clontech). The pGBKT7-hTTR construct, which encodes the full-length hTTR cDNA fused in-frame to the GAL4 DNA binding domain, was used as bait and the full-length hMT3 cDNA, fused with the GAL4 activation domain, was used as prey, in accordance with the manufacturer's instructions. Co-transformants were selected on dropout plates (SD base, -Trp-Leu-Ade-His) in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-α-D-galactopyranosidose (Clontech) for 5 8 days at 30 °C.

Table 1. Primer sequences containing adapter sequences to restriction endonucleases designed to amplify full-length hTTR and hMT3. The adapter sequences to restriction sites are shown in bold and underlined in each primer sequence.

Designation	Sequence (5′ to 3′)	Restriction endonuclease
hTTRfw	5′-TTA T**GA ATT C**GG ATG GCT TCT ATCG-3′	*Eco*RI
hTTRrv	5′-TAC A**CT GCA G**TT CCT TGG GAT T-3′	*Pst*I
hMT3fw	5′-TTA T**GA ATT C**AT GCC CGT TCA CCG CCT CCA G-3′	*Eco*RI
hMT3rv	5′-TAC A**GA GCT C**CA CCA GCC ACA CTT CAC CAC A-3′	*Sac*I
hTTRMycrv	5′-TAC A**CT CGA G**TC ATT CCT TGG GAT T 3′	*Xho*I
hMT3HAfw	5′-TTA T**GA ATT C**AT GCC CGT TCA CCG CCT CCA G-3′	*Eco*RI
hMT3HArv	5′-TAC A**CT CGA G**CA CCA GCC ACA CTT CAC CAC A-3′	*Xho*I

Negative controls, in which yeast cells were transformed with one of the constructs alone or without any construct, were included in the experiment. Positive and negative plasmid controls, as provided by the manufacturer, were included in each assay. These experiments were repeated five times.

Saturation-binding assays

hTTR was prepared as described by Almeida *et al.* [42]. For binding studies, hTTR was iodinated with $Na^{125}I$ (Perkin-Elmer, Waltham, MA, USA) using the iodogen method (Sigma-Aldrich, St Louis, MO, USA), in accordance with the manufacturer's instructions. In brief, 1 mCi, 37 MBq of $Na^{125}I$ was added to a reaction tube coated with 100 μg of iodogen, followed by 15 μg of hTTR in NaCl/P_i. The reaction was allowed to proceed on ice for 20 min, and then the iodination mix was desalted in a 5 mL Sephadex G50 column (GE Healthcare, Uppsala, Sweden). Only ^{125}I[TTR] that was more than 95% precipitable in trichloroacetic acid was used in the assays.

For saturation-binding assays, we used the method previously described by Gonçalves *et al.* [30], with minor modifications, and using Zn_7-hMT3 protein (Bestenbalt, Tallinn, Estonia). Briefly, binding of [^{125}I]hTTR to hMT3 was carried out in 96-well plates (Nunc, Maxisorp, Thermofisher, Rochester, NY, USA) coated with 2 μg per well of hMT3 in coating buffer (0.1 M bicarbonate/carbonate buffer, pH 9.6) overnight. Increasing concentrations of [^{125}I]hTTR (as indicated in Fig. 1) in binding buffer (0.1% skimmed milk (Molico; Nestle SA, Vevey, Switzerland) in MEM (Sigma-Aldrich) were incubated in each well for 2 h at 37 °C with gentle shaking. Unoccupied sites were blocked with 5% skimmed dried milk in PBS for 2 h at 37 °C. Three replicas of each sample were set up in each experiment. Binding was determined after five washes with ice-cold PBS with 0.05% Tween 20. Then, 100 μL of elution buffer (NaCl 0.1 M containing 1% Nonidet P40) was added for 5 min at 37 °C, and the content of the wells was aspirated and counted in a gamma counter (Wallac, Wizard; Perkin-Elmer, Waltham, MA, USA). Nonspecific binding was determined by incubating similar amounts of [^{125}I]hTTR in the wells in the presence of a 100-fold molar excess of nonlabelled hTTR. Specific binding was calculated as the difference between total binding and nonspecific binding. Binding data were fit to a one-site model and analyzed by the method described by Klotz and Hunston [43], using nonlinear regression analysis in PRISM software (GraphPad Software Inc., La Jolla, CA, USA), as described by Sousa *et al.* [44]. This assay was repeated three times.

Co-immunolocalization of TTR and MT3

Animals

Wistar rats were housed in appropriate cages at constant room temperature (RT) under a 12 : 12 h light/dark cycle and given standard laboratory chow and water *ad libitum*. Euthanasia was carried after anaesthesia with Clorketam 1000 (50 μL per rat; Vetoquinol SA, Lure, France) and the CP from both the lateral and fourth ventricles of 3 5-day-old rats were dissected under a stereosmicroscope and collected for the establishment of CPEC cultures. All procedures were performed in compliance with the National and European Union regulations for care and handling of laboratory animals (Directive 86/609/EEC).

Primary culture of CP epithelial cells

The method used for the establishment of primary culture of CPEC has been previously described by Gonçalves *et al.* [30]. Briefly, dissected CP were mechanically and enzymatically digested in NaCl/P_i containing 0,2% pronase (Fluka, Ronkonkoma, Germany) at RT for 5 min. Dissociated cells were washed twice in DMEM (Sigma-Aldrich) with 10% fetal bovine serum (Biochrom AG, Berlin, Germany), and 100 units·mL^{-1} of penicillin/streptomycin (Sigma-Aldrich). Cells were seeded into 12 mm poly-D-lysine coated culture wells (approximately two CP per well), and cultured in DMEM supplemented with 100 units·mL^{-1} antibiotics, 10% fetal bovine serum, 10 ng·mL^{-1} epidermal growth factor (Invitrogen, Carlsbad, CA, USA), 5 μg·mL^{-1} insulin (Sigma-Aldrich) and 20 μM cytosine arabinoside (Sigma-Aldrich) in a humidified incubator in 95% air/5% CO_2 at 37 °C. The medium was replaced 24 h after seeding and every 2 days thereafter.

Confluent monolayers of cells were obtained 3 4 days after seeding.

Immunofluorescence

Confluent monolayers of CPEC were washed with DMEM and prefixed with DMEM containing a drop of 4% paraformaldehyde, and then fixed with 4% paraformaldehyde for 20 min at RT. Cells were permeabilized with 1% Triton X-100 in PBS/0.1% Tween-20 for 5 min and blocked with 20% fetal bovine serum in PBS with 0.1% Tween-20 for 4 h at RT. Cells were incubated with the primary antibodies, mouse monoclonal anti-hMT3 serum (dilution 1 : 250) (catalogue number: H00004504-M01A; Abnova, Taipei, Taiwan) and rabbit polyclonal anti-hTTR serum (dilution 1 : 200) (catalogue number: A0002; DakoCytomation, Glostrup, Denmark), overnight at 4 °C. The nuclei of cells were stained with Hoechst 33342 dye (2 μM) (catalogue number: H1399; Molecular Probes, Invitrogen, Carlsbad, CA, USA). Subsequently, cells were washed and incubated 1 h, at RT, with Alexa Fluor 546 goat anti-(mouse IgG) conjugate (1 $\mu g \cdot mL^{-1}$) (catalogue number: A11003; Molecular Probes, Invitrogen) and Alexa Fluor 488 goat anti-(rabbit IgG) conjugate (1 $\mu g \cdot mL^{-1}$) (catalogue number: A11008; Molecular Probes, Invitrogen).

To determine the intracellular localization of MT3, cells were incubated with mouse monoclonal anti-hMT3 serum (dilution 1 : 250) and rabbit polyclonal anti-human ATF-6α serum (c-22799; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) (an ER-transmembrane protein) (dilution 1 : 100) overnight at 4 °C. After washing, cells were incubated with Alexa Fluor 546 goat anti-(mouse IgG) conjugate (1 $\mu g \cdot mL^{-1}$) (catalogue number: A11003; Molecular Probes, Invitrogen) and Alexa Fluor 488 goat anti-(rabbit IgG) conjugate (1 $\mu g \cdot mL^{-1}$) (catalogue number: A11008; Molecular Probes, Invitrogen) for 1 h at RT.

To assess immunostaining specificity, the primary antibodies for TTR, MT3 and ATF-6α were omitted in some preparations as negative controls. In addition, the MT3 antibody was also pre-incubated with MT3 using the same dilution of the antibody and a ten-fold (by weight) excess of MT3 protein (Bestenbalt) in PBS. This pre-absorption was carried out overnight at 4 °C and yielded negative staining. Fluorescence was observed by confocal microscopy in a Zeiss LSM 510 Meta system (Zeiss Imaging \Systems), using a × 63 objective with an image zoom scan of 1.0 (Fig. 2A) or 2.0 (Fig. 2B).

In vivo co-immunoprecipitation of hTTR and hMT3

Plasmid constructs

Full-length TTR and MT3 cDNAs were amplified by PCR using specific primers (Table 1). Subsequently, the products obtained were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega) and digested with *Eco*RI and *Xho*I. The hTTR was cloned in pCMV-*c*-Myc (BD Biosciences, San Jose, CA, USA) and hMT3 was cloned in pCMV-HA (BD Biosciences). Plasmid constructs were sequenced to confirm that cloning had been successful.

Cell culture and transfection

COS-7 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in 25 cm^2 flasks in DMEM supplemented with 100 $units \cdot mL^{-1}$ antibiotics and 10% fetal bovine serum at 37 °C in a humidified incubator in 95% air/5% CO_2. One or two days before transfection, cells were seeded in six-well cell culture plates (150 000 cells per well) and cultured in DMEM containing 10% fetal bovine serum, without antibiotics. Cells at 90 95% confluence were transfected with pCMV-HA-hMT3 alone, pCMV-*c*-Myc-hTTR alone and with pCMV-HA-hMT3 + pCMV-*c*-Myc-hTTR, using Lipofectamine 2000 (Invitrogen), in accordance with the manufacturer's instructions. Forty-eight hours post-transfection, wells were washed with PBS, scrapped, and cells were ressuspended in 2 mL of cold PBS. Cell suspensions were centrifuged at 5000 ***g*** for 5 min at 4 °C. Pellets were ressuspended in nondenaturing cell lysis solution (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 2 $\mu g \cdot mL^{-1}$ leupeptin, 10 mM dithithreitol), and were mechanically lysated. After 15 min of incubation on ice, extracts were sedimented at 5000 ***g*** for 15 min at 4 °C and the supernatants were immediately used or freezed at −80 °C. Protein concentration in lysates from transfected cells was measured using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturer's instructions.

Co-immunoprecipitation

For co-immunoprecipitation, 3 μg of *c*-Myc monoclonal antibody (catalogue number: S1826; BD Biosciences) were incubated with 40 μL of protein G plus-agarose beads (Oncogene, Calbiochem, Boston, MA, USA), in 500 μL of cold PBS, overnight at 4 °C. After washing and centrifugation, the suspension was incubated with protein extracts of COS-7 cells simultaneously transfected with pCMV-HA-hMT3 and pCMV-*c*-Myc-hTTR constructs at 4 °C for 2 h. This mixture was washed three times, centrifuged and resuspended in denaturing lysis buffer (1% SDS, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 2 $\mu g \cdot mL^{-1}$ leupeptin, 10 mM dithiothreitol). The mixture was denatured at 95 °C for 8 min and spun in an Amicon Ultra-15 Centrifugal Filter Device (10 kDa cut-off) (Millipore, Billerica, MA, USA) at 4 °C to remove protein G plus-agarose beads. The eluted

solution was frozen at 80 °C or used for western blotting. This experiment was performed three times.

Western blotting

Protein extracts from transfected cells (pCMV-HA-hMT3 alone, pCMV-*c*-Myc-hTTR alone and pCMV-HA-hMT3 + pCMV-*c*-Myc-hTTR) and co-immunoprecipitation experiments were loaded on 12.5% SDS/PAGE and separated at 148 mA. Separated proteins were transferred to a 0.22 μm poly(vinylidene difluoride) membrane (Bio-Rad) in a transfer buffer containing 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 10.8), 10% methanol and 2 mM $CaCl_2$ for 1 h at 220 mA. After transfer, membranes were incubated for 1 h in 2.5% gluteraldehyde aqueous solution for protein fixation and blocked with 3% hydrolyzed casein in NaCl/Tris (20 mM Tris, 137 mM NaCl, pH 7.6). Each lane in the membrane was cut and incubated with the corresponding primary antibodies from the Matchmaker co-immunoprecipitation kit (Clontech) at RT for 1 h: lane 1 containing protein extracts of cells transfected with pCMV-HA-hMT3 was incubated with HA-Tag polyclonal antibody (dilution 1 : 100) (BD Biosciences); lane 2 containing protein extracts from transfection with pCMV-*c*-Myc-hTTR alone was incubated with *c*-Myc monoclonal antibody (dilution 1 : 500); and lane 3 containing protein extracts from transfection with both constructs was incubated with both antibodies. Lanes containing protein from co-immunoprecipitation experiments (4 6) were incubated with HA-Tag polyclonal antibody (lane 4), *c*-Myc monoclonal antibody (lane 5) or both (lane 6). Blots incubated with HA-Tag polyclonal antibody were incubated with anti-(rabbit IgG) and those incubated with *c*-Myc monoclonal antibody were incubated with anti-(mouse IgG). Incubation with both secondary antibodies was carried out at a dilution of 1 : 20 000 (GE Healthcare, Uppsala, Sweden) for 1 h. Antibody binding was detected using the ECF substrate (ECF Western Blotting Reagent Packs; GE Healthcare, Little Chalfont, UK) in accordance with the manufacturer's instructions. Images of blots were captured with the Molecular Imager FX Pro Plus MultiImager system (Bio-Rad). This experiment was performed three times.

Evaluation of the effect of MT2 and MT3 in TTR–Aβ binding

The effect of hMT2 or hMT3 in hTTR Aβ binding was studied by competition binding assays. Iodination of hTTR with $Na^{125}I$ (NEN Life Science Products) was carried out as described for the saturation-binding assays. The solubilization of $A\beta_{1\ 42}$ (Calbiochem, La Jolla, CA, USA) peptide and the competition method used has been previously described by Costa *et al.* [12]. Briefly, binding of [^{125}I]hTTR to Aβ was carried out in 96-well plates (Nunc) coated with 2 μg per well of soluble $A\beta_{1\ 42}$ in coating buffer (0.1 M bicarbonate/carbonate buffer, pH 9.6), overnight at 4 °C. Unoccupied sites were blocked with binding buffer (0.1% skimmed milk in MEM) for 2 h at 37 °C with gentle shaking. A constant amount of [^{125}I]hTTR was added to each well alone or in the presence of the indicated molar excess of unlabelled competitors (hTTR, hMT2 or hMT3 alone, or hTTR pre-incubated with hMT2 or hMT3 peptides at 0, 0.54, 2.7, 5.4, 54 and 540 nM). Three replicas of each sample were prepared in each assay. Specific binding was calculated as that observed with [^{125}I]hTTR alone minus [^{125}I]hTTR in the presence of a 100-fold molar excess of unlabelled protein. The content of each well was aspirated and measured in a gamma counter (Wallac, Wizard, Perkin-Elmer). Binding data were collected from a minimum of three independent assays.

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Chapter 4: Stress and Glucocorticoids Increase Transthyretin Expression in Rat Choroid Plexus *via* Mineralocorticoid and Glucocorticoid Receptors

(Paper III)

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Stress and Glucocorticoids Increase Transthyretin Expression in Rat Choroid Plexus via Mineralocorticoid and Glucocorticoid Receptors

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Abstract Transthyretin (TTR) is a carrier for thyroid hormones and retinol binding protein. Several mutated forms of TTR cause familial amyloidotic polyneuropathy, an inheritable lethal disease. On the other hand, wild-type TTR has a protective role against Alzheimer's disease. Despite its overall importance in normal animal physiology and in disease, few studies have focused on its regulation. An in silico analysis of the rat TTR gene revealed a glucocorticoid responsive element in the 3′ region of the first intron. Thus, we hypothesised that TTR could be regulated by glucocorticoid hormones and investigated the regulation of TTR expression in response to hydrocortisone in a rat choroid plexus cell line (RCP) and in primary cultures of choroid plexus epithelial cells (CPEC). In addition, the effect of psychosocial stress on TTR expression was analysed in rat liver, choroid plexus (CP) and cerebrospinal fluid (CSF). In RCP and CPEC cultures hydrocortisone upregulated TTR expression, an effect suppressed by glucocorticoid receptor and mineralocorticoid receptor antagonists. Moreover, induction of psychosocial stress increased TTR expression in liver, CP and CSF of animals subjected to acute and chronic stress conditions. Overall, we conclude that stress upregulates TTR expression in CP.

Keywords Transthyretin · Corticosterone · Choroid plexus · Psychosocial stress · Mineralocorticoid receptors · Glucocorticoid receptors

Introduction

Transthyretin (TTR) is a homotetrameric protein of 127 amino acid subunits. Most TTR is produced and secreted by the liver to the peripheral circulation (Felding and Fex 1982) and by epithelial cells of the choroid plexus (CP) of the brain to the cerebrospinal fluid (CSF) (Soprano et al. 1985). TTR is mainly known as a transporter for thyroxin (T_4) and retinol binding protein complexed to vitamin A (Raz and Goodman 1969; Monaco 2000). Mutated forms of TTR cause familial amyloidotic polyneuropathy, an inheritable lethal disease (Saraiva 2001). On the other hand, there are experimental evidences that wild-type TTR has a protective role against Alzheimer's disease (AD) (Santos et al. 2011). Despite its physiological importance in health and disease, there are only a few studies analysing the regulation of TTR expression in the brain: nicotine (Li et al. 2000) and sexual hormones, such as 5α-dihydrotestosterone, 17β-estradiol and progesterone, increase TTR mRNA levels and protein expression in CP (Tang et al. 2004; Quintela et al. 2008; Quintela et al. 2009; Quintela et al. 2011); TTR expression and secretion is increased after administration of leaf extracts of *Gingko biloba* (Watanabe et al. 2001); TTR mRNA levels increased in CP from aged rats after short-term consumption of fish oil (Puskas et al. 2003). Also, maternal separation stress and restrain stress reduce TTR expression in the brain of rats (Kohda et al. 2006; Joo et al. 2009).

Several studies have associated stress with AD mainly due to the disruption of hypothalamic–pituitary–thyroid (HPT) axis through thyroid hormones imbalances (Lizcano and

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Rodriguez 2011) and to the over activation of hypothalamic–pituitary–adrenal (HPA) axis that could lead to hippocampal damage, which is one of the hallmarks of this neurodegenerative disease (Dong and Csernansky 2009). Stress has also been associated with other depressive and cognitive diseases (Lizcano and Rodriguez 2011). AD patients show high basal cortisol levels, and treatment with corticosteroids seems to intensify their cognitive deficits (Alkadhi et al. 2010). Low levels of free triiodothyroxine (T_3) (Kapaki et al. 2003) and high levels of T_4 have also been found in some AD patients (Kapaki et al. 2003; de Jong et al. 2009) and there is an association between hypo- or hyperthyroidism and AD (Tan and Vasan 2009). The glucocorticoid hormones (cortisol in primates and corticosterone in rats) are the primary end products of the HPA axis, the main neuroendocrine circuit related to stress response (Charil et al. 2010), have the capacity to generate adequate responses to physical and emotional stressors (Hellhammer et al. 2009) and play a role in the HPT axis regulation (Helmreich et al. 2005). Exposure to stressors activate the HPA system resulting in increased secretion of corticosteroid hormones which are released from the adrenal cortex to the blood, in an effort to re-establish homeostasis in the body and readaptation to the new stressful conditions (Matousek et al. 2010).

An in silico analysis of the rat TTR gene carried out using Genomatix (data not shown) revealed a glucocorticoid responsive element in the 3′ region of the first intron (GGTACAnnnTGTTCT). This responsive element, initially identified in mouse (Wakasugi et al. 1986), is conserved in humans (Sasaki et al. 1985), suggesting that TTR expression could be regulated by glucocorticoids. In addition, both glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA and/or protein expression was previously described in the CP (Sousa et al. 1989; Kitraki et al. 1996; Lathe 2001; Cinalli and Sainte-Rose 2004; Amin et al. 2005; Sinclair et al. 2007; Gomez-Sanchez 2010). Therefore, we investigated the regulation of TTR expression in response to hydrocortisone in a rat choroid plexus cell line (RCP) and rat primary choroid plexus epithelial cell (CPEC) cultures, and in the liver, CP and CSF of rats are subjected to acute and chronic stress induction.

Material and Methods

RCP Cell Culture

RCP cells (American Type Culture Collection, Manassas, VA, USA) were cultured in 75 or 150-cm^2 flasks in Dulbecco's modified Eagle medium (DMEM) with 100 U/mL antibiotic and 10% foetal bovine serum at 37°C in a humidified incubator with 95% air⁄5% CO_2. Two days before stimulation, cells were seeded in triplicate in six-well culture plates (100,000 cells per well; Nunc, Apogent, Denmark), in serum-free DMEM containing 100 U/mL antibiotic 24 h before incubation with hormones. In order to verify if the RCP cell line reproduces data obtained in experiments with CPEC cultures, we incubated RCP cells with 17β-estradiol (E2; Sigma-Aldrich, Inc., St. Louis, MO, USA) as described by Quintela and colleagues in CPEC cultures (Quintela et al. 2009). Thus, RCP cells at 70–75% confluence were incubated with E2 previously diluted in a serum-free DMEM containing 100 U/mL antibiotics, for 6, 12, 24 and 36 h with 0, 1, 10, 100 or 1,000 nM E2. Thereafter, RCP cells were incubated with hydrocortisone, the synthetic and injectable form of cortisol (Sigma-Aldrich, Inc.), and diluted in a serum-free DMEM containing 100 U/mL antibiotics, for 6, 12, 18, 24 and 36 h with 0, 10, 100 or 1,000 nM of hydrocortisone. In a second experiment, the cells were incubated with 0 or 100 nM of hydrocortisone for 12 h, in the presence, or absence, of glucocorticoid receptor antagonist (mifepristone—RU486; 1.16 μM; Sigma-Aldrich, Inc.) and/or mineralocorticoid receptor antagonist (spironolactone—Spiro; 1 μM; Sigma-Aldrich, Inc.). Each experiment was repeated three times.

Cellular Viability Assay—MTT

Viability of RCP cells was measured using the MTT assay. Replicates of the experiments described above were incubated with 0.5 mg/mL 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma- Aldrich, Inc.) in Krebs solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose and 10 mM hepes, pH 7.4) for 90–120 min at 37°C. MTT is converted by viable cells to a water-insoluble precipitate that was solubilised with the addition of 40 mM HCl in isopropanol, and after a 20-min incubation in the dark with agitation, the absorbance was measured at 570 nm using a microplate reader (Benchmark, Bio-Rad, Hercules, CA, USA). This experiment was repeated three times.

Primary Cultures of Rat Choroid Plexus Epithelial Cells

Newborn rats used for the establishment of primary cultures of CPEC were euthanized with Clorketam 1000 (50 μL/rat; Vetoquinol, S.A., Lure, France). The method used in the establishment and maintenance of primary cultures of CPEC has been previously described (Quintela et al. 2008; Martinho et al. 2010). CPEC cells were incubated for 6, 12, 18, 24 and 36 h with 0, 10, 100 or 1,000 nM of hydrocortisone. The experiment was repeated three times.

Induction of Psychosocial Stress in Rats

A total of 60 adult Wistar–Han rats (3 months old) of about 178–267 g (males) and 150–230 g (females) were submitted to a psychosocial stress experiment through the increase of

animal density: test groups were composed of nine animals housed in appropriate polypropylene cages of 480×375×210 mm (166 cm^2 floor area/animal) while control groups were composed by three animals in the same cage (500 cm^2 floor area/animal) for 24 h (acute stress) or 9 weeks (chronic stress). A total of four test cages and eight control cages were used in this experiment. Animals were housed at constant room temperature in a 12/12-h light/dark cycle and given standard laboratory chow and water ad libitum. All procedures were performed in compliance with the National and European Union rules for the care and handling of laboratory animals (Directive 2010/63/EU). Tissue sampling was carried out in animals euthanized with carbon dioxide. All experiments were conducted under aseptic conditions, and all efforts were made to minimise animal suffering and to reduce the number of animals used.

At the end of both the acute and the chronic stress experiments, all animals were sacrificed at the same daytime (at 10 a.m.). The CP was collected from the lateral ventricles, and CSF was collected from cisterna magna. Serum was obtained from blood samples by a centrifugation of 20 min at 9,000 rpm. Liver was also dissected and frozen with the other samples at −80°C for subsequent analysis. The corticosterone (CORT) concentration in serum was measured in all animals.

Measurement of Serum Corticosterone

Serum from rats was prepared by collecting trunk blood in Microtainer tubes (BD Biosciences, Erembodegen, Belgium). After 30 min at room temperature (RT), samples were centrifuged for 20 min at 9,000 rpm. The supernatant (serum) from each tube was collected, and CORT concentration (in micrograms per decilitres) was determined by a certified veterinary diagnostic centre (Cedivet, Oporto, Portugal), using the Corticosterone kit (Siemens, Deerfield, IL, USA) and the Siemens Immulite® 1000 Immunoassay Analyser (Siemens).

Total Protein Preparation and Western Blot

Protein extracts were prepared as described previously (Martinho et al. 2010). Briefly, cells or tissues were lysated with 50 μL of a denaturing cell lysis buffer (50 mM Tris–HCl pH 7.4, 5 mM EDTA, 5 mM EGTA, 0.2% Triton X-100, 1 mM PMSF, 2 μg/mL leupeptin and 10 mM DTT) for 15 min on ice. Suspensions were centrifuged at 5,000 × *g* for 15 min at 4°C and the pellets discarded. Total protein in the supernatant of each sample was measured using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol.

Western blots were done as described previously (Martinho et al. 2010). Briefly, protein extracts from tissues (5 μg) or cells (RCP or CPEC; 50 and 30 μg, respectively) were loaded on 12.5% polyacrylamide SDS-PAGE gel and separated at 148 mA. Separated proteins were transferred to a PVDF membrane of 0.22 μm (Bio-Rad) in a transfer buffer containing 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, pH 10.8), 10% methanol and 2 mM $CaCl_2$ for 1 h at 680 mA. After transfer, membranes were incubated for 1 h in 0.25% gluteraldehyde aqueous solution for protein fixation and blocked with 3% hydrolysed casein in TBS (20 mM Tris, 137 mM NaCl, pH 7.6). The membrane was then incubated with the rabbit anti-human prealbumin antibody (1:250; Dako-Cytomation, Glostrup, Denmark) at RT for 1 h. After washing, blots were incubated with anti-rabbit IgG at a 1:20,000 dilution (Amersham, Uppsala, Sweden) for 1 h. Blots were normalised using the mouse anti-β actin antibody (1:10,000; Sigma-Aldrich, Inc.), for 1 h at RT, followed by an incubation with anti-mouse IgG at a 1:20,000 dilution (Amersham) for 1 h. Antibody binding was detected using the ECF substrate (ECF Western Blotting Reagent Packs; GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Images of blots were captured with the Molecular Imager FX Pro Plus MultiImager system (Bio-Rad), and the intensity of bands was quantified by densitometry, using Quantity One software (Bio-Rad).

Total RNA Extraction and Real-Time PCR

Total RNA was extracted from tissues or CPEC using TRI Reagent (Sigma-Aldrich, Inc.), following the manufacturer's instructions. Total RNA of each sample was quantified using UV spectrophotometry at 260 nm (Pharmacia Biotech, Ultrospec3000, Denmark), and its integrity was assessed by ethidium bromide agarose gel (1%) electrophoresis. Total RNA (1 μg) was reverse transcribed for 1 h at 37°C in a 20-μL reaction containing First Strand 5× buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$; Invitrogen, Carlsbad, CA, USA), 10 mM DTT, 0.5 mM of each dNTP (dATP, dCTP, dGTP and dTTP; Amersham), 20 U of RNAse Out (Invitrogen), 25 pmol of random hexamerprimers (Invitrogen) and 200 U of M-MLV reverse transcriptase (Invitrogen).

TTR mRNA levels were analysed by real-time PCR in CPEC incubated with different hydrocortisone concentrations (0, 10, 100 or 1,000 nM) during various time periods (6, 12, 18, 24 and 36 h), and in CP and livers from male and female rats subjected to the stress induction experiments. Beta 2 microglobulin (β2m) and cyclophilin A (CycA) were used as endogenous controls. Sequences of all PCR primers used are indicated in Table 1. To analyse TTR mRNA levels in the liver, reactions were carried out using 1 μL of cDNA (1/10 dilution) in a 20-μL reaction containing 10 μL of SYBR Green supermix (Fermentas, Thermo Fisher Scientific, Ontario, Canada) and 6, 4 or 4 pmol of each pair of primers (rβ2m, rCycA or rTTR, respectively). For analysis of TTR mRNA levels in CP and CPEC, the following conditions were used: 1 μL of cDNA in a 20-μL reaction containing 10 μL of

Table 1 Primer sequences of rat β2 microglobulin (rβ2m), cyclophilin A (rCycA) and transthyretin (rTTR) used in real-time PCR

Designation	Sequence (5′–3′)
rβ2m fw	CCG TGA TCT TTC TGG TGC TTG TC
rβ2m rv	CTA TCT GAG GTG GGT GGA ACT GAG
rCycA fw	CAA GAC TGA GTG GCT GGA TGG
rCycA rv	GCC CGC AAG TCA AAG AAA TTA GAG
rTTR fw	GGA CTG ATA TTT GCG TCT GAA GC
rTTR rv	ACT TTC ACG GCC ACA TCG AC

SYBR Green supermix and 3, 4.2 or 6 pmol of each pair of primers (rβ2m, rCycA or rTTR, respectively). In all cases, amplification conditions were: 95°C for 3 min and 35 cycles at 95°C for 15 s, 57°C for 30 s and 72°C for 30 s. Amplified PCR fragments were checked by melting curves: reactions were heated from 55°C to 95°C with 10-s hold at each temperature (0.05 C/s). All primers were validated by quantitative real-time PCR reactions with decreasing cDNA concentrations (1; 1:10; 1:100; 1:1,000), and the reaction efficiencies were calculated. Real-time PCR was carried out using the iCycler IQ™ System (Bio-Rad), and fluorescence was measured after each cycle. Every reaction was done in triplicate and each experiment was repeated three times.

Statistical Analysis

Data were compared by means of one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism (version 5). Results were considered statistically significant when $p<0.05$.

Data collected from real-time PCR experiments were analysed with the mathematical model proposed by Pfaffl: $2^{-(\Delta\Delta Ct)}$ (Pfaffl 2001), which allows the determination of significant differences between control and treated animals, taking into account reaction efficiency, and reference gene normalisation, when more than one endogenous gene was used. All comparisons between two groups were made by Student's *t* test. Data were compared by means of one-way ANOVA followed by Dunnett's test. Results were statistically significant when $p<0.05$.

Results

Effects of Hydrocortisone on Choroid Plexus Cells

RCP Cellular Viability Was Not Compromised by Hydrocortisone Incubation

To ensure that cellular viability of RCP cells had not been compromised by hormonal incubation, the MTT assay was carried out, upon hydrocortisone incubation. The results showed that cell viability was not compromised ($p>0.05$) with any of the concentrations used, 100 and 1,000 nM, and in all time periods tested (6, 12, 18, 24 and 36 h) (Fig. 1).

E2 Increases TTR Expression in the RCP Cell Line

As no previous expression studies have been reported in this cell line, we began this study by subjecting RCP cells to E2 incubation, as described previously (Quintela et al. 2009). The results obtained showed that E2 increases TTR expression in RCP cells when these are incubated for 12 h with 10 and 100 nM and for 24 h with 1 and 10 nM of E2. The maximal TTR upregulation was observed after 24 h of incubation with 10 nM E2 (Supplemental data).

Hydrocortisone Increases TTR Expression in RCP Cell Line

The effects of hydrocortisone were assessed in RCP cells incubated for 6, 12, 18, 24 and 36 h with different hormone concentrations (0, 10, 100 or 1,000 nM). The incubation of RCP cells with hydrocortisone at 10, 100 or 1000 nM increased TTR expression levels after 12, 18 and 24 h of incubation, but this upregulation was more pronounced after 12-h incubation with 100 nM hydrocortisone (Fig. 2).

To investigate if the corticosteroids affect TTR expression through GR and/or MR receptors, a second experiment was performed. Thus, RCP cells were incubated with 0 or 100 nM of hydrocortisone for 12 h with or without a GR antagonist (mifepristone—RU486) and/or a MR antagonist (spironolactone). The results obtained in this experiment showed that the GR and/or MR antagonists abrogated the hydrocortisone-induced increase in TTR expression in RCP cells (Fig. 3).

Hydrocortisone Increases TTR Expression in CPEC Cells

To ascertain whether the overall response of RCP cells to hydrocortisone was identical in CPEC cells, we set-up similar experiments to those performed with RCP cells in CPEC. Briefly, cultures were incubated for 6, 12, 18, 24 and 36 h with different hydrocortisone concentrations (0, 10, 100 or 1,000 nM). The results showed that hydrocortisone also upregulated TTR protein expression in these cells at 10, 100 and 1,000 nM after 12 and 18 h of incubation. As observed in RCP cells, the maximum increase was observed after 12 h of incubation with 100 nM of hydrocortisone (Fig. 4). TTR mRNA levels attained the highest levels after 6 and 12 h of incubation with 100 nM of hormone (Fig. 5).

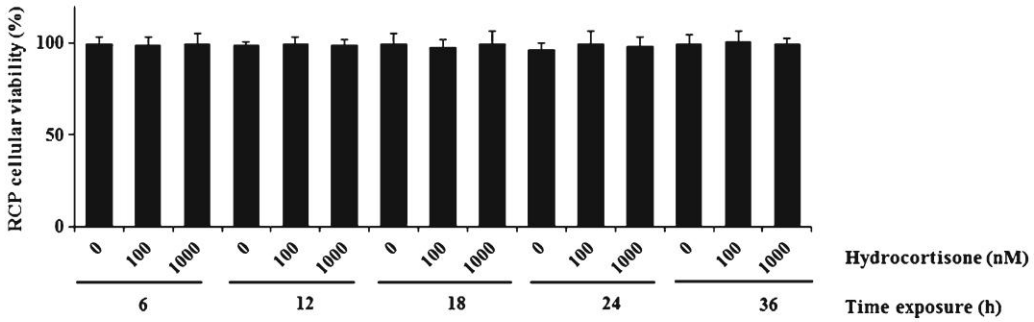

Fig. 1 Comparison of cellular viability of RCP cells (in percent of controls) after incubation with 0, 100 or 1,000 nM of hydrocortisone

Effects of Psychosocial Stress

Psychosocial Stress Increases Corticosterone Levels

To certify that the increased animal density used in the experiments had been effective in inducing stress (acute and chronic), CORT concentration was measured in rat serum, showing that both the acute and chronic stress induction protocols raised the hormone levels in males and in females (Fig. 6) ($p<0.05$). In the acute stress experiment, this increase was more pronounced in males than in females ($p<0.05$). Comparing to chronic stress, acute stress was responsible for the highest increase in CORT concentration ($p<0.01$) in males and in females. The increase in CORT concentration in the chronic stress experiment was higher in females than in males suggesting that males adapted to chronic stress conditions more effectively than females ($p<0.05$). On the other hand, males were more sensitive than females to acute stress, as can be deduced from the higher concentration of CORT in males' serum ($p<0.05$).

TTR Expression Is Increased in Stressed Animals

TTR expression was analysed after the induction of stress in liver and CP and CSF. In these tissues TTR protein expression increased after induction of acute or chronic stress, both in males and in females.

In liver, TTR protein levels increased nearly twofold in animals subjected to acute and chronic stress, either males or females, compared to the controls (Fig. 7). In animals subjected to acute stress, TTR mRNA levels followed a similar trend as TTR protein, but males exhibited higher levels of TTR mRNA than females. On the other hand, males subjected to chronic stress conditions showed lower levels of TTR mRNA than females. In males, acute stress conditions induced

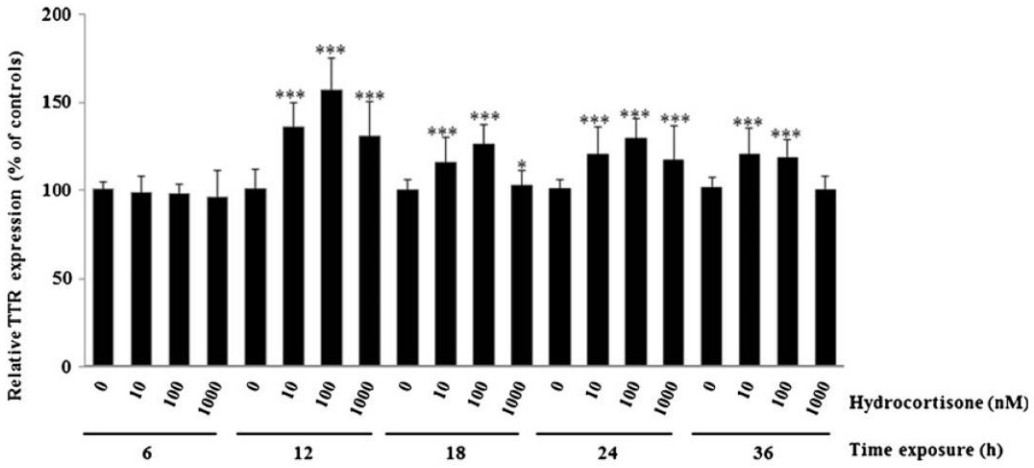

Fig. 2 Comparison of TTR protein levels (in percent of controls) in RCP cell line incubated with hydrocortisone. Cells were incubated with 0 (control), 10, 100 or 1,000 nM of hydrocortisone during 6, 12, 18, 24 or 36 h. Protein extracts (50 μg) were resolved by SDS-PAGE followed by Western blot. Images were captured with the Molecular Imager FX Pro Plus MultiImager system and quantified by densitometry. *Bar graphs* indicate means ± SEM from at least three independent experiments (*$p<0.05$; ***$p<0.001$)

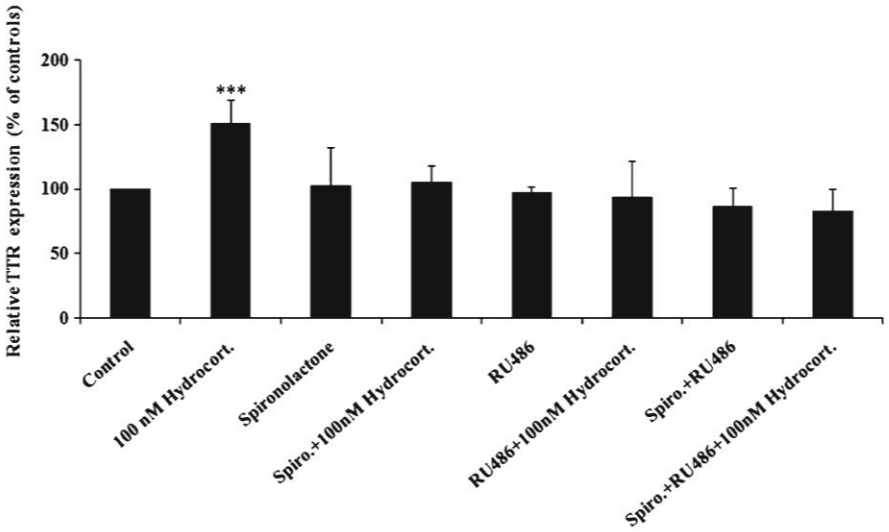

Fig. 3 Effects of glucocorticoid and/or mineralocorticoid receptor antagonists, mifepristone (*RU486*) and spironolactone, respectively, on hydrocortisone (*hydrocort*) induction of TTR protein levels (in percent of controls) in RCP cells. Cells were incubated with 0 (*control*) or 100 nM of hydrocortisone with 500 ng/mL of mifepristone and/or 1 μM of spironolactone. Protein extracts (50 μg) were resolved in SDS-PAGE followed by Western blot. Images were captured with the Molecular Imager FX Pro Plus MultiImager system and quantified by densitometry. *Bar graphs* indicate means ± SEM from at least three independent experiments (***$p<0.001$)

higher levels of TTR mRNA expression than chronic stress. No such differences were seen in females (Fig. 8).

In CP, no significant differences were observed in TTR protein levels between animals subjected to acute and chronic stress (Fig. 9). However, males subjected to acute stress had higher TTR protein levels than females ($p<0.05$). CP TTR mRNA levels, followed the same general pattern as protein. Males subjected to acute stress had higher TTR mRNA levels than females housed in the same conditions, while females subjected to chronic stress had higher TTR mRNA levels than males ($p<0.001$) (Fig. 10). Interestingly, in CSF, the induction of acute stress resulted in a higher increase in TTR protein levels in females than in males, in opposition to what was seen in CP ($p<0.01$) (Fig. 11).

Discussion

Chronic stress induces higher glucocorticoid levels causing hippocampal atrophy, neuronal death and premature ageing

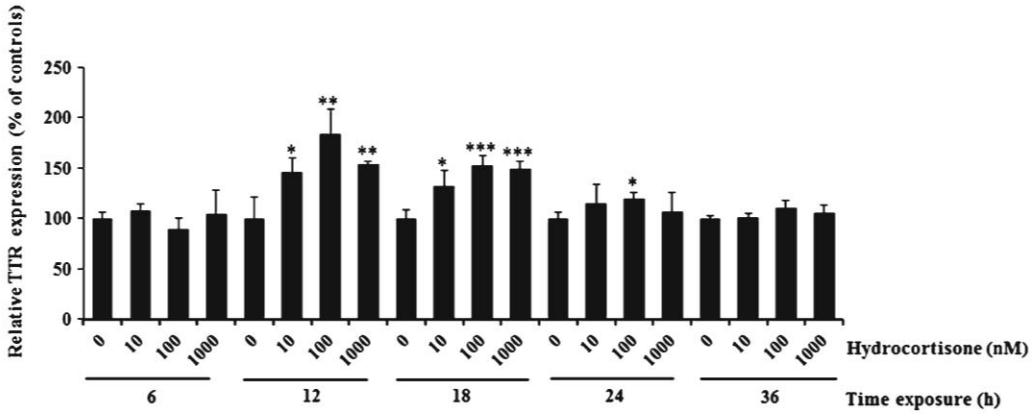

Fig. 4 Comparison of TTR protein levels (in percent of controls) in CPEC cultures incubated with hydrocortisone. Cells were incubated with 0 (control), 10, 100 or 1,000 nM of hydrocortisone for 6, 12, 18, 24 or 36 h. Protein extracts (40 μg) were resolved in SDS-PAGE followed by Western blot. Images were captured with the Molecular Imager FX Pro Plus MultiImager system and quantified by densitometry. *Bar graphs* indicate means ± SEM from at least three independent experiments (*$p<0.05$; **$p<0.01$; ***$p<0.001$)

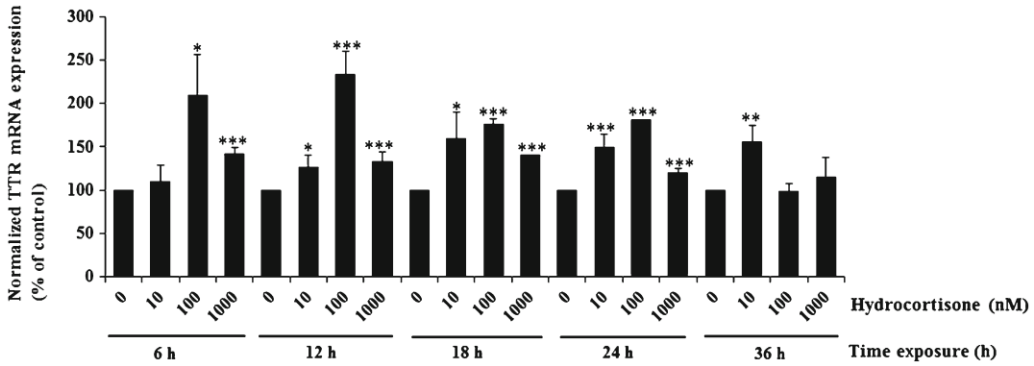

Fig. 5 Fold induction of TTR mRNA expression in CPEC cells incubated with 0 (control), 10, 100 or 1,000 nM of hydrocortisone during 6, 12, 18, 24 or 36 h. TTR mRNA levels were compared by real-time PCR using β2-microglobulin and cyclophilin A as endogenous controls and are represented as normalised expression. Fold differences among groups were calculated using the formula $2^{-(\Delta\Delta Ct)}$. Comparisons between two groups were made by Student's *t* test. Data were compared by means of one-way ANOVA followed by Dunnett's test. Values are expressed as a normalised value of non-treated cells, which were considered 100%. *Bar graphs* indicate means ± SEM from at least three independent experiments, each of them in triplicate. Significant statistical differences are indicated (*$p<0.05$; **$p<0.01$; ***$p<0.001$)

of the hippocampal neurons that may lead to memory impairment, depression and physiological stress (Hellhammer et al. 2009; Herzog et al. 2009) and, with the advancing age, even to AD (Pedersen et al. 2001; Landfield et al. 2007; Dong and Csernansky 2009). In addition, stress also influences the HPT axis and consequently thyroid hormone homeostasis, through the alteration of its production and metabolism (Kuhn et al. 1998; Kelly 2000;) with implications in diseases such as depression and AD (Lizcano and Rodriguez 2011). We have been investigating the regulation of TTR expression in response to endogenous factors that have been associated with AD, and for which we have found responsive elements in the TTR gene (Quintela et al. 2008; Quintela et al. 2009). We identified a responsive element for cortisol (GGTACAnnnTGTTCT) in the 3′ region of the first intron of the TTR gene, and therefore this study focused on the analysis of TTR expression in the RCP cell line and CPEC cultures in response to hydrocortisone and in response to acute and chronic stress induction in the liver, CP and CSF of adult rats. Before testing the response of RCP cells to hydrocortisone, we analysed their response to E2, using a similar procedure as described previously for CPEC cultures (Quintela et al. 2009). Our results showed that E2 upregulates TTR expression in RCP cells resembling the results obtained in CPEC cultures. Therefore, this cell line was considered a suitable model for subsequent in

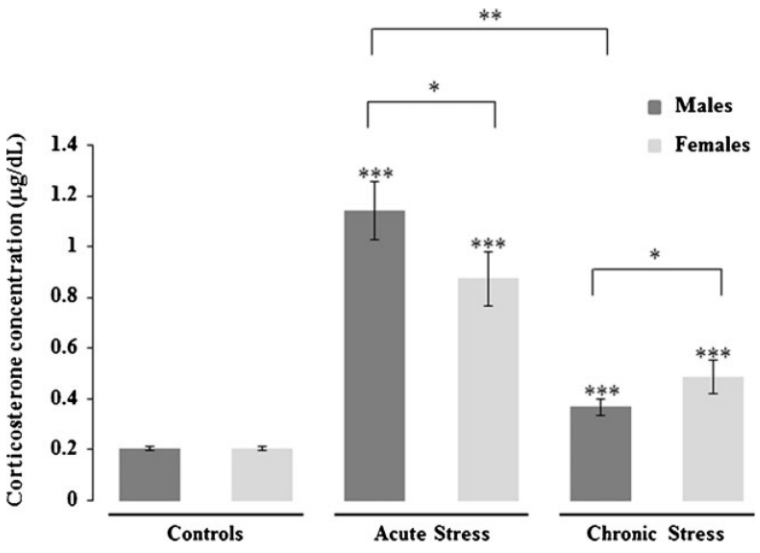

Fig. 6 Corticosterone (CORT) concentration (in micrograms per decilitres) measured in males (*dark grey*) and females (*light grey*) after no stress or acute or chronic stress induction. *Bar graphs* indicate means ± SEM from at least three animals (*$p<0.05$; **$p<0.01$; ***$p<0.001$)

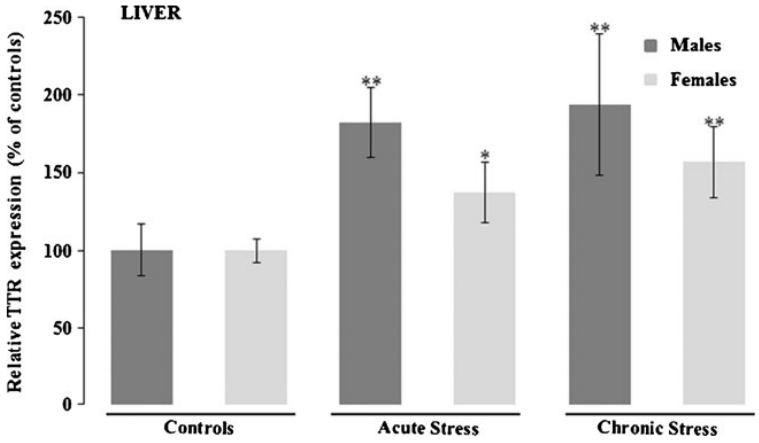

Fig. 7 Comparison of TTR protein levels (in percent) in the liver of rats subjected to acute and chronic stress conditions (males—*dark grey*, females—*light grey*). *Bar graphs* indicate means ± SEM from at least three animals (*$p<0.05$; **$p<0.01$)

vitro studies of CP. Then, we showed that hydrocortisone upregulates TTR expression in these cells via GR and MR receptors as incubation with antagonists for either of these receptors suppressed the hydrocortisone induction of TTR protein expression. In general, under basal conditions (no stress), cortisol binds preferentially to MR forming complexes and triggering a signalling cascade, that ultimately regulates gene transcription. In a stress situation, adrenocorticotrophic hormone (ACTH) is stimulated by corticotropin-releasing hormone, leading to an increased secretion of cortisol into the bloodstream (Bartels et al. 2003). The high cortisol levels saturate MR and gradually occupy GR, forming stable complexes, which are then internalised to the nucleus, regulating their own transcription and the transcription of other genes (Gunnar and Quevedo 2007; Djordjevic et al. 2010; Sterner and Kalynchuk 2010). Previous reports showed that GR and MR could form heterodimers that cooperate and regulate the transcription of certain genes as DNA-bound heterodimers (Liu et al. 1995; Nishi and Kawata 2007; Savory et al. 2001). Both receptors are expressed in CP (Sousa et al. 1989; Kitraki et al. 1996; Lathe 2001; Cinalli and Sainte-Rose 2004; Amin et al. 2005; Sinclair et al. 2007; Gomez-Sanchez 2010), and because induction of TTR expression does not occur when either receptor is blocked, it is likely that both receptors are necessary for TTR upregulation suggesting that formation of heterodimers of MR and GR may be required to exert their function (Mizokami et al. 2004; Brouwer et al. 2005).

Similar experiments were carried out in CPEC cultures, and in spite of minor differences in optimal time periods of

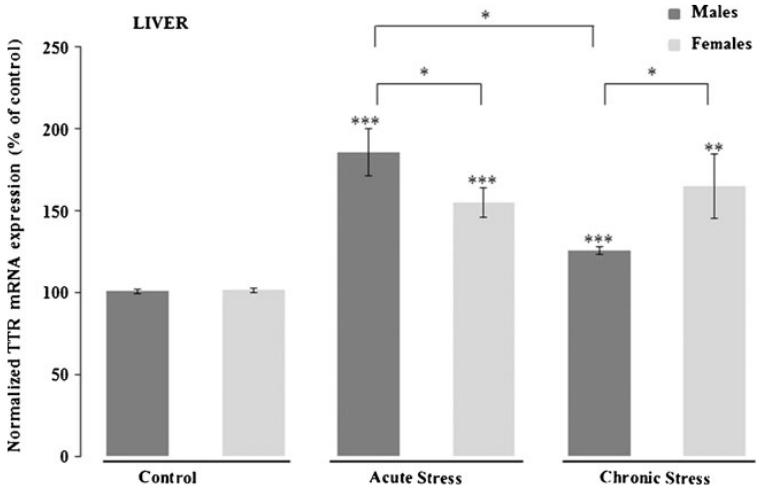

Fig. 8 Fold induction of TTR mRNA expression in rat liver (males—*dark grey*, females—*light grey*) exposed to acute or chronic stress conditions. Values are expressed as a normalised value of non-treated animals, which were considered 100%. Bar graphs indicate means ± SEM from at least three animals. Significant statistical differences are indicated (*$p<0.05$; **$p<0.01$; ***$p<0.001$). Data collected from real-time PCR experiments were analysed using the formula: $2^{(\Delta\Delta Ct)}$. All comparisons between two groups were made by Student's *t* test. Data were compared by means of one-way ANOVA followed by Dunnett's test

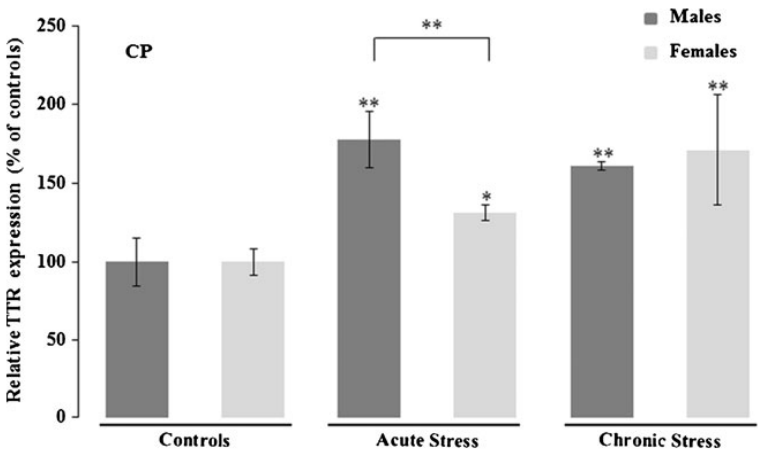

Fig. 9 Comparison of TTR protein levels (in percent) in the CP of rats subjected to acute and chronic stress conditions (males—*dark grey*, females—*light grey*). Bar graphs indicate means ± SEM from at least three animals (*$p<0.05$; **$p<0.01$)

incubation between RCP and CPEC cells, the overall response of TTR expression to hydrocortisone in these cells was similar. Consistent with protein levels, TTR mRNA levels followed the same general trend and also increased registering their maximum after 6 and 12 h of incubation with 100 nM hydrocortisone. As expectable, TTR mRNA increased earlier than protein levels, and a gap of approximately 6 h was registered between the maximum mRNA and protein expression. These time gap between mRNA and protein levels is likely to correspond to the time required for protein synthesis from the increased TTR mRNA transcripts.

In the face of these results, we set-up acute and chronic stress induction experiments in male and in female rats, similar to those described previously (Nagaraja and Jeganathan 2003; Bernatova et al. 2007). The increased animal density in the cages increased CORT concentration in the serum of both males and females subjected to acute or chronic stress protocols. Moreover, in animals subjected to acute stress, CORT concentration was significantly higher than in those subjected to chronic stress and was more pronounced in males than in females. There are several studies in the literature that used increased animal density to promote stress in rodents (Gamallo et al. 1986; Marti et al. 1993; Cook and Wellman 2004; Alkadhi et al. 2005; Marini et al. 2006; Ulrich-Lai et al. 2006; Tran et al. 2010). All reports pointed that after acute stress exposure plasma CORT levels are increased. However, after chronic stress exposure, controversial effects have been reported with some authors reporting decreases or no alterations in hormone levels (Bugajski et al. 1994; Djordjevic et al. 2010), while others report increased hormone levels (Gamallo et al. 1986; Fukuda and Morimoto 2001; Bernatova and Csizmadiova 2006; Puzserova et al. 2006; Lightman 2008; Dong and Csernansky 2009; Vicario et al. 2010). The differences observed by several authors may be explained by

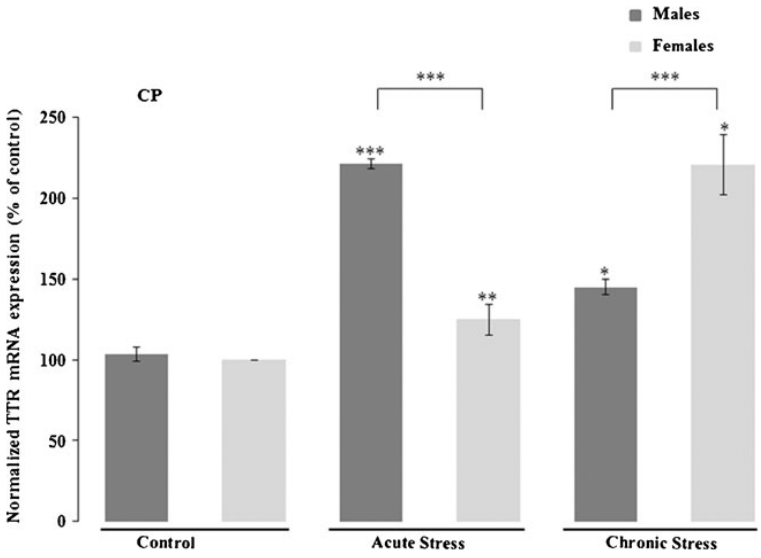

Fig. 10 Fold induction of TTR mRNA expression in rat CP (males—*dark grey*; females—*light grey*) exposed to acute or chronic stress conditions. Values are expressed as a normalised value of non-treated animals, which were considered 100%. *Bar graphs* indicate means ± SEM from at least three animals. Significant statistical differences are indicated (*$p<0.05$; **$p<0.01$; ***$p<0.001$). Data collected from real-time PCR experiments were analysed using the formula: $2^{(-\Delta\Delta Ct)}$. All comparisons between two groups were made by Student's *t* test. Data were compared by means of one-way ANOVA followed by Dunnett's test

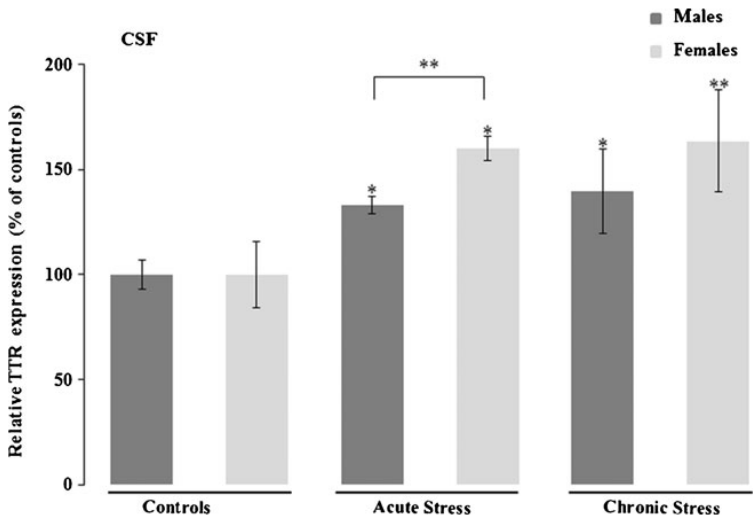

Fig. 11 Comparison of TTR protein levels (in percent of controls) in the CSF of rats subjected to acute and chronic stress conditions (males—*dark grey*; females—*light grey*). *Bar graphs* indicate means ± SEM from at least three animals (*$p<0.05$; **$p<0.01$)

the different capacity of resilience and susceptibility between rats exposed to the same stressor (Sterner and Kalynchuk 2010). Predictability and controllability of stress could lead to habituation and consequent modifications in the response of the HPA axis to stressors (Grissom et al. 2007; Sterner and Kalynchuk 2010). Also, cortisol has a marked circadian diary cycle, and sample collection for CORT measurement should be consistently done at the same daytime (Clow et al. 2010). According to previous results reported by others, the CORT levels in our experiments remained higher after chronic stress induction, but lower than in animals subjected to acute stress, suggesting that although some habituation to the stressful conditions had occurred, the HPA axis remained overactivated until the end of chronic stress exposure.

Cortisol levels are differentially regulated between males and females. In females, the activation of the HPA axis by stress induces differences in the oestrus cycle (Herzog et al. 2009) that could interfere in their stress responses. The vast majority of stress experiments have been made with males, and as such, they may not reflect the reality in females. Stress studies comparing males and females have shown that defeat is more stressful to males than to females, while social instability is more stressful for females than for males (Haller et al. 1999). In addition, social housing can enhance coping with stress in females, whereas in males, housing does not positively influence the stress sensitivity (Westenbroek et al. 2003). Female rats can be kept under chronic stress for weeks without habituation (Herzog et al. 2009). Our results show that chronic stress induced higher levels of CORT in females than in males, contradicting previous studies in which females seem to adapt to stressful conditions more easily than males (Brown and Grunberg 1995; Bao et al. 2008).

Following our in vitro data, we found an increase in TTR mRNA and protein expression, in response to acute and chronic stress in liver, CP and CSF. Nevertheless, our findings contradict some of the few published reports on which the expression of TTR was analysed in response to stress in mice and rats: rats subjected to a chronic restraint stress showed downregulation of TTR expression in liver and in serum (Sun et al. 2010); DNA microarray assays in rat pups subjected to hours-long maternal separation also decreased TTR levels in hippocampus samples, but a single-prolonged stressor upregulated TTR expression (Kohda et al. 2006); decreased TTR mRNA expression has been detected in the cortex of animals subjected to chronic immobilisation stress but not in CP (Joo et al. 2009).

Brain TTR expression sites are not totally clarified: some authors described that CP and meninges are the only brain sites of TTR production (Sousa et al. 2007) while others have also observed TTR expression in the cortex and hippocampus, thus making the subject controversial (Stein and Johnson 2002; Joo et al. 2009). In addition, although the analysis of some other parameters as bodyweight, food intake and behavioural changes has been done, CORT levels have not been measured in the animals involved in these studies and so, these are not comparable with our experiments. Our results showed that TTR protein and mRNA levels were increased in liver, CP and CSF from rats subjected to stress-inducing conditions with raised CORT serum levels, and these experiments corroborated data obtained from in vitro studies.

The upregulation of TTR expression in CP from animals subjected to acute stress was more pronounced in males than in females, following the same trend as CORT, therefore suggesting a positive correlation between CORT levels and TTR expression. Contrarily, the opposite was observed in the CSF after acute stress exposure, on which the increase in TTR levels was higher in females, suggesting a negative correlation between CORT levels and TTR expression. A

possible explanation for the observed differences in TTR expression in CP and CSF between genders after acute stress may be related to the ability of CP to release TTR into the CSF in response to stress induction.

During and after a stress situation, especially a long-term stress exposure, besides the overactivation of HPA axis, the HPT axis is also affected as it suppresses the secretion of pituitary thyrotropin and its response to thyrotropin-releasing hormone is blunted, with concurrent decreased levels of T_3 (Mizokami et al. 2004) and increased amounts of T_4 (Brouwer et al. 2005). Therefore, as TTR is a carrier of T_4 from the bloodstream to the brain, a possible additional effect of the stress mediated increase in TTR expression in the HPT axis is suggested, with a potential occurrence of a feedback and/or interaction between each of the axis and TTR expression.

In the case of AD, given the apparent opposite effects of cortisol and TTR in disease progression, it would be expectable that, contrarily to our results, the high levels of cortisol, induced by psychosocial stress, decreased TTR expression. Nevertheless, AD is a multifactorial disease caused by complex genetic and environmental interactions, and the increase in TTR expression promoted by cortisol may not be sufficient by itself to inhibit the deleterious effects promoted by high levels of cortisol in other brain regions.

Our study does not directly relate cortisol effects and TTR expression with any specific disease. However, as both TTR and cortisol have pivotal roles in various human diseases, especially within the central nervous system, our findings emphasize the importance of TTR regulation by glucocorticoid hormones.

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Chapter 5: Glucocorticoids Regulate Metallothionein-1/2 Expression in Rat Choroid Plexus: Effects on Apoptosis

(Paper IV)

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GLUCOCORTICOIDS REGULATE METALLOTHIONEIN-1/2 EXPRESSION IN RAT CHOROID PLEXUS: EFFECTS ON APOPTOSIS

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Abstract

The choroid plexus (CP) participates in the synthesis, secretion and regulation of the cerebrospinal fluid, in the removal of its toxic compounds, and in the regulation of the availability of essential metal ions to the brain. It expresses and secretes metallothioneins 1/2 (MT-1/2) which are key components in the maintenance of the central nervous system (CNS) metal homeostasis and have anti-apoptotic properties, thereby protecting the brain. Glucocorticoids regulate MT-1/2 expression in several brain regions, but within the choroid plexuses (CPs) it remains unknown. Glucocorticoid levels increase in response to stress with implications in apoptosis. Further, CP expresses glucocorticoid (GR) and mineralocorticoid receptors (MR) turning it into likely glucocorticoid responsive structure. Data prompted us to study the regulation of MT-1/2 expression in response to glucocorticoids in the rat CP, and to investigate its implications in apoptosis. MT-1/2 protein and mRNA expression analysis showed that hydrocortisone up-regulates MT-1/2 expression in rat a choroid plexus (RCP) cell line and in primary cultures of choroid plexus epithelial cells (CPEC) cultures via GR and MR. Also, incubation of RCP cells with hydrocortisone significantly diminished apoptosis, an effect eliminated by the addition of a MT-1/2 antibody. Moreover, induction of psychosocial stress, with concomitant rise of corticosterone levels, increased MT-1/2 expression in liver and in CP of male and female rats, with an exception observed in CP from males subjected to acute stress in which down-regulation in MT-1/2 expression occurred. Altogether, the results obtained demonstrated that stress/glucocorticoids regulate MT-1/2 expression in rat CP, with implications on apoptosis.

Keywords: Metallothioneins; Psychosocial Stress; Glucocorticoids; Choroid Plexus; Mineralocorticoid and Glucocorticoid Receptors; Apoptosis.

1. INTRODUCTION

The choroid plexus (CP) constitutes the blood-cerebrospinal fluid (CSF) barrier being an essential structure for the maintenance of brain homeostasis in development and aging [1]. The choroid plexuses (CPs) participate in the synthesis, secretion and regulation of several biologically active compounds of the CSF [2,3], in the maintenance of brain metal bioavailability, and in the removal of toxic compounds [4, 5] thereby, protecting the brain against neurotoxic insults.

Metallothioneins are non-enzymatic low molecular weight metal-binding proteins (6-7 kDa) and, in mammals, four isoforms have been identified, MT-1 to MT-4. Isoforms 1 and 2 are expressed in all organs, while MT-3 is mostly expressed in the brain [6]. Usually, MT-1 and MT-2 isoforms are referred to MT-1/2 because, in contrast to MT-3, they are very similar and share many structural and functional properties [7], particularly in brain. Expression of MT-1/2 in the central nervous system (CNS) occurs during development and in adulthood, especially in astrocytes, but these proteins are also found in neurons, epithelial cells of choroid plexus (CP) and CSF [6]. MT-3 expression occurs principally in glutamatergic neurons and associated astrocytes [8] and in the ependymal and endothelial cells of CP [7]. Within the CNS, functions of MT-1/2 include maintenance of metal homeostasis, free radical scavenging and antioxidant properties, inhibition of pro-apoptotic mechanisms and enhancement of cell survival and tissue regeneration, highlighting the involvement of MT-1/2 in neuroprotection and neuroregeneration [6]. Stimuli, such as zinc (Zn), copper (Cu) and iron (Fe), cytokines, oxidative agents, and glucocorticoids and a variety of physiological and psychological stressors, which raise endogenous glucocorticoid levels (cortisol in humans and corticosterone in rats) in the bloodstream [9-12], are effective regulators of MT-1/2 expression in the brain. However, nothing is known about MT-1/2 regulation in the CP, where it can hamper metal imbalances that may directly damage its structure, impair specific CP regulatory pathways, and promote metal accumulation itself, leading to

deregulation of oxidative stress, through increased generation of free radicals, and consequent increase of apoptosis [13-15].

Glucocorticoids, which are often used in the treatment of inflammatory conditions, with implications in apoptosis are also potential regulators of MT-1/2 expression in CP as the promoter regions of MT-1/2 genes contain glucocorticoid-responsive elements (GREs) that are responsible for increased MT-1/2 expression following stress induction in other brain regions, [16,17,19]. In addition, CPs express both glucocorticoid (GR) and mineralocorticoid receptors (MR) turning them into a likely glucocorticoid responsive tissue [20-22]. Therefore, we investigated the effect of the glucocorticoid hydrocortisone on MT-1/2 expression *in vitro* in rat choroid plexus cells, and *in vivo*, particularly in the CP of male and female rats subjected to psychosocial stress induction.

2. MATERIAL AND METHODS

2.1 Rat Choroid Plexus cells

The rat choroid plexus (RCP) cell line (American Type Culture Collection, Manassas, VA, USA), was used in a first and preliminary approach to avoid the use of animals as recommended by the 3R's (Directive 2010/63/EU). Cells were cultured in 75 or 150 cm^2 flasks in DMEM with 100 U/mL antibiotic and 10% fetal bovine serum at 37 ºC in a humidified incubator with 95% air / 5% CO2. Two days before stimulation, cells were seeded in triplicate in six-well culture plates (100 000 cells per well; Nunc, Apogent, Denmark), in serum-free DMEM containing 100 U/mL antibiotic 24 h before incubation with hormones. RCP cells at 70-80% confluence were incubated with 0, 10, 100 or 1000 nM of hydrocortisone (Sigma-Aldrich, Inc.), which did not compromised the cellular viability [23], diluted in a serum-free DMEM containing 100 U/mL antibiotics for 6, 12, 18, 24, 36 h. In a second experiment, cells were incubated for 24 h with 0 or

1000 nM of hormone in the presence, or absence, of GR antagonist (mifepristone - RU486; 1.16 µM; Sigma-Aldrich, Inc.) and/or MR antagonist (spironolactone - Spiro; 1µM; Sigma-Aldrich, Inc.). Each experiment was repeated three times.

2.1.1 Immunocytochemistry

The expression of MT-1/2 in RCP cells express was confirmed by immunofluorescence, as previously described [24]. Briefly, confluent monolayers RCP were washed with DMEM and prefixed with DMEM containing a few drops of 4% paraformaldehyde, and then fixed with 4% paraformaldehyde for 20 min at RT. Cells were permeabilized with 1%Triton X-100 in PBS for 5 min and blocked with 20% FBS in PBS with 0.1% Tween-20, for 4 hours at RT. Cells were incubated with the primary antibody, mouse monoclonal anti-MT-1/2 (1:1000; DakoCytomation, Glostrup, Denmark), overnight at 4 ºC. The nuclei of cells were stained with Hoechst 33342 dye (2 µM) (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Subsequently, cells were washed and incubated 1 h, at RT, with Alexa Fluor 546 goat anti-mouse IgG conjugate (1 µg/mL) (Molecular Probes, Invitrogen).

To ensure immunostaining was specific, the primary antibody for MT-1/2 was omitted in a preparation, and the MT-1/2 antibody (1:1000 dilution) was pre-absorbed with a ten-fold (by weight) excess of MT2 (Bestenbalt, Tallinn, Estonia) overnight at 4 ºC. Both yielded negative staining, as expected. Fluorescence was observed by fluorescence microscopy in a Carl Zeiss inverted microscope (Thornwood, NY, USA), using an objective of 63X with an image zoom scan of 1.0.

2.2 Primary cultures of rat choroid plexus epithelial cells

As clear effects in the response of MT-1/2 to hydrocortisone were observed in RCP cells we tested these effects in a most well recognized model of CP, the choroid plexus epithelial cells (CPEC) primary cultures, which have been previously thoroughly characterized by our group [25,26]. So, CPEC cultures were subjected to a similar

experimental setup to confirm the effects on MT-1/2 observed with RCP cells and assess their suitability for further experiments.

Newborn rats used for the establishment of primary cultures of CPEC were euthanized with Clorketam 1000 (50 µL/rat; Vetoquinol, S.A., Lure, France). The method used in the establishment and maintenance of primary cultures of CPEC has been previously described [25]. Briefly, dissected CP were mechanically and enzymatically digested in PBS containing 0,2% pronase (Fluka, Ronkonkoma, Germany) at room temperature (RT) for 5 min. Dissociated cells were washed in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich) with 10% fetal bovine serum (FBS) (Biochrom AG, Germany), and 100 units/mL of penicillin/streptomycin (Sigma-Aldrich). Cells were seeded into 12 mm Poly-D-lysine coated culture wells (2 CP / well, approximately), and cultured in DMEM supplemented with 100 units/mL antibiotics, 10% FBS, 10 ng/mL epidermal growth factor (EGF) (Invitrogen, Carlsbad, CA, USA), 5 µg/mL insulin (Sigma-Aldrich), and 20 µM cytosine arabinoside (Ara-C) (Sigma-Aldrich), in a humidified incubator in 95% air – 5% CO2 at 37 oC. The medium was replaced 24 h after seeding, and every 2 days, thereafter. Confluent monolayers of cells were obtained 3-4 days after seeding. CPEC cells were incubated with hydrocortisone 0, 10, 100 or 1000 nM for 6, 12, 18, 24 and 36 h, as described for RCP cells. This experiment was repeated three times.

2.3 Flow cytometry assessment of apoptosis

As MT-1/2 in RCP cells responded to hydrocortisone incubation in a very similar fashion to CPEC primary cultures, supplementary experiments were carried out in this cell line to reduce the number of animals used in experimental procedures. RCP cells were incubated for 24 h with 0 or 1000 nM of hydrocortisone and/or 0.2 µg/mL of anti-human MT-1/2 antibody (Anti-MT-1/2; DakoCytomation, Glostrup, Denmark). Apoptosis was assessed in these cells by flow cytometry using the Annexin V–FITC and PI kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. A

positive control containing RCP cells incubated for 3 h with 2 µM of staurosporine was included. Cell samples were excited with a 15 mW laser at 488 nm on a BD Biosciences FACSCalibur (BD Biosciences) and acquisition was performed with CellQuestTM Pro Software. Light-scatter and fluorescence signals were acquired logarithmically. Signals from RCP cells corresponding to forward and side scatter (FSC and SSC) and fluorescence from annexin V–FITC/PI labeled cells were accumulated and the resulting fluorescent signals (pulse area measurements) of 2 $\times 10^4$ events were screened by FL-1 (530/30 nm) and FL-2 (585/42) band pass filters. Threshold levels were empirically set on SSC to further reduce electronic and small particle noise. All conditions were done in triplicate and the experiment was repeated three times.

2.4 Induction of Psychosocial Stress in Rats

In vitro data provided experimental evidence that MT-1/2 is regulated by glucocorticoids in CP cells. So, we performed *in vivo* experiments, in order to increase endogenous levels of corticosterone, the main active glucocorticoid in rats, and to assess *in vivo* the resulting MT-1/2 expression in CP, as further described by [23]. Briefly, a total of 60 adult Wistar-Han rats of about 178-267 g (males) and 150-230 g (females) were subjected to a psychosocial stress experiment through the increase of animal number in each cage: test groups were composed of 9 animals housed in polypropylene cages with 166 cm^2 floor area available per animal while control groups were composed by 3 animals in the same cage (500 cm^2 floor area/animal) for 24 h (acute stress) or 9 weeks (chronic stress). Animals were housed at constant room temperature in a 12 h light / 12 h dark cycle and given standard laboratory chow and water *ad libitum*. Moreover, as MT-1/2 expression differs between male and female rats, due to their hormonal background [27,28], the MT-1/2 expression in CP was analyzed in males and females separately. All procedures were performed in compliance with the National and European Union rules for the care and handling of laboratory animals (Directive 2010/63/EU). During the experimental procedures we

made sure that the stress-inducing protocol did not produce fights or injuries both in male and female rats. All experiments were conducted under aseptic conditions and all efforts were made to minimize animal suffering and to reduce the number of animals used. At the end of the acute and the chronic stress experiments, all animals were sacrificed at the same daytime (at 10 a. m.), using carbon dioxide, and tissue sampling was carried out. The CP was collected from the lateral ventricles and CSF was collected from cisterna magna. Serum was obtained from blood samples by a centrifugation of 20 min at 9000 rpm. Liver was also dissected and frozen with the other samples at – 80 ºC for subsequent analysis. As previously addressed by Martinho and co-workers (2012), the corticosterone (CORT) concentration in serum was measured in all animals by a certified veterinary diagnostic centre (Cedivet, Oporto, Portugal), using the Corticosterone kit (Siemens, Deerfield, IL, USA) and the Siemens Immulite® 1000 Immunoassay Analyser (Siemens) [23].

2.5 Total Protein Preparation and Western blot

Protein extracts and Western blots were made as previously described [24]. The total protein concentration in the supernatant of each sample was measured using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Protein extracts from tissues (5 µg) or cells (RCP or CPEC; 50 and 30 µg, respectively), were loaded on 12.5% polyacrylamide SDS-PAGE gel and separated at 148 mA. Separated proteins were transferred to a PVDF membrane of 0.22 µm (Bio-Rad) in a transfer buffer containing 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, pH 10.8), 10% methanol and 2 mM $CaCl_2$ for 1 h at 680 mA. After transfer, membranes were incubated for 1 h in 0.25% gluteraldehyde aqueous solution for protein fixation and blocked with 3% hydrolyzed casein in TBS (20 mM Tris, 137 mM NaCl, pH 7.6). The membrane was then incubated with the mouse anti-MT-1/2 (1:1000; DakoCytomation) at RT for 1 h. After washing, blots were incubated with anti-mouse IgG at a 1:20000 dilution (Amersham, Uppsala, Sweden) for

1 h. Blots were normalized using the mouse anti-β actin antibody (1:10000; Sigma-Aldrich, Inc.), for 1 h at RT, followed by an incubation with anti-mouse IgG at a 1:20000 dilution (Amersham) for 1 h. Antibody binding was detected using the ECF substrate (ECF Western Blotting Reagent Packs; GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol. Images of blots were captured using the Molecular Imager FX Pro Plus MultiImager system (Bio-Rad). MT-1/2 was quantified by densitometry using Quantity One software (Bio-Rad).

2.6 Total RNA extraction and Real-Time PCR

Total RNA was extracted from tissues or CPEC using TRI Reagent (Sigma-Aldrich, Inc.), according to manufacturer's instructions.

Total RNA of each sample was quantified using UV spectrophotometry (260 nm) (Pharmacia Biotech, Ultrospec3000, Denmark), and its integrity were assessed by ethidium bromide agarose gel (1%) electrophoresis.

Total RNA (1 µg) was reverse transcribed (RT) for 1 h at 37°C in a 20 µL reaction containing First Strand 5x buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$; Invitrogen, Carlsbad, CA, USA), 10 mM DTT, 0.5 mM of each dNTP (dATP,dCTP, dGTP, dTTP; Amersham,), 20 U of RNAse Out (Invitrogen), 25 pmol of random hexamerprimers (Invitrogen), and 200 U of M-MLV reverse transcriptase (Invitrogen).

MT-1/2 mRNA levels were analysed by real-time PCR in CPEC incubated with different hydrocortisone concentrations (0, 10, 100 or 1000 nM) during various time periods (6, 12, 18, 24, and 36 h), and in CP and livers from male and female rats subjected to the stress induction experiments. Beta 2 microglobulin (β2m) and cyclophilin A (CycA) were used as endogenous controls. Sequences of all PCR primers used are indicated in table I.

To analyze MT-1/2 mRNA levels in CP, reactions were carried out using 1 µL of cDNA (1/10 dilution) in a 20 µL reaction containing 10 µL of Sybr Green supermix

(Fermentas, Thermo Fisher Scientific, Ontario, Canada), and 3, 4.2 or 4 pmol of each pair of primers (rβ2m, rCycA or rMT, respectively). For analysis of MT-1/2 mRNA levels in CPEC, 1 µL of cDNA was used in a 20 µL reaction containing 10 µL of Sybr Green supermix and 6, 4 or 5 pmol of each pair of primers (rβ2m, rCycA or rMT, respectively). In all cases, amplification conditions were: 95 ºC for 3 min and 35 cycles at 95 ºC for 15 s, 57 ºC for 30 s and 72 ºC for 30 s. Amplified PCR fragments were checked by melting curves: reactions were heated from 55 to 95 ºC with 10 s hold at each temperature (0.05 ºC/s). All primers were validated by quantitative real-time PCR reactions with decreasing cDNA concentrations (1; 1:10; 1:100; 1:1000), and the reaction efficiencies were calculated. Real-time PCR was carried out using the iCycler IQ™ System (Bio-Rad) and Fluorescence was measured after each cycle. Every reaction was done in triplicate and each experiment was repeated three times.

2.7 Statistical Analysis

Data was compared by means of One-way ANOVA followed by Tukey's Multiple Comparison Test using GraphPad Prism (Version 5). Results were considered statistically significant when $p<0.05$.

Data collected from real time PCR experiments was analyzed using the mathematical model proposed by Pfaffl: $2^{-(\Delta\Delta Ct)}$ [29], which allows the determination of differences in expression between control and treated animals, taking into account reaction efficiency, and reference gene normalization, when more than one endogenous gene was used. All comparisons between two groups were made by *t*-Student test. Data were compared by means of one-way ANOVA followed by Dunnett's test. Results were statistically significant when $p<0.05$.

Flow cytometry dot plots were analyzed in FCS Express version 4 Research Edition (De Novo Software™, LA, USA) and comparison between viable and apoptotic/late apoptotic cells was performed using one-way ANOVA followed by Student's *t* test. Results were considered statistically significant when $p<0.05$.

3. RESULTS

3.1 EFFECTS OF HYDROCORTISONE ON CHOROID PLEXUS CELLS

3.1.1 Hydrocortisone increases MT-1/2 expression in RCP cells

MT-1/2 is expressed in CP and CPEC [25], but its expression has not been reported in any CP cell line. Western blot and immunocytochemistry assays (Fig. 1), using an anti-MT-1/2 antibody (DakoCytomation), showed that the RCP cells express this protein. Once MT-1/2 expression in RCP cells had been confirmed, a time-course experiment of 6, 12, 18, 24 and 36 h incubation with 0, 10, 100 or 1000 nM of hydrocortisone was implemented, showing increased expression of MT-1/2 after 24 h of incubation with 0, 100 and 1000 nM of hydrocortisone, in a dose response manner (Fig. 2a). In order to investigate if this up-regulation occurs *via* GR and/or MR receptors, RCP cells were incubated with 0 or 1000 nM of hydrocortisone for 24 h, with or without a GR antagonist (RU486) and/or a MR antagonist (Spiro). GR and MR antagonists completely abolished the hydrocortisone-induced increase in MT-1/2 expression in RCP cells. When cells were incubated with both corticoid receptor antagonists, MT-1/2 expression decreased (Fig. 2b).

3.1.2 Hydrocortisone increases MT-1/2 expression in CPEC

The experiments described above for RCP cells were repeated in CPEC cultures to ensure that RCP is a suitable model for CP studies using glucocorticoids. In fact, the same pattern of up-regulation MT-1/2 expression after incubation with hydrocortisone observed in RCP cells, was obtained in CPEC, although earlier than in RCP cells, after 12 and 18 h of incubation with 100 and 1000 nM of hormone (Fig. 3a). Real-time PCR analysis of MT-1/2 mRNA expression showed a similar pattern, with higher mRNA

levels after 12, 18, 24 and 36 h of incubation with 1000 nM of hormone (Fig. 3b). The maximal effect was seen after 12 h of hydrocortisone incubation.

3.1.3 Hydrocortisone decreases apoptosis in RCP cells

It is well established that MT-1/2 has anti-apoptotic properties [6]. However, the role of glucocorticoids on apoptosis seems to depend on cell type, with demonstrated pro and anti- apoptotic effects in cells of the hematological and epithelial lineages, respectively [30]. Therefore, we investigated the effect of hydrocortisone on the apoptosis of RCP cells (Fig. 4). After 24 h of incubation with 1000 nM of hydrocortisone the ratio of apoptotic/late apoptotic cells decreased comparing to control cells (0 nM) ($p<0.05$), from 17.8% to 9.7% (Fig. 4a and b). This effect was not observed following addition of an anti-MT-1/2 antibody to cell cultures (Fig. 4c), where the percentage of apoptotic/late apoptotic cells remained similar to controls (17.8 and 16.6%, respectively) (Fig. 4d).

3.2 IN VIVO EFFECTS OF PSYCHOSOCIAL STRESS

3.2.1 MT-1/2 expression is regulated by stress in adult male and female rats

MT-1/2 protein and mRNA levels were analyzed in liver and CP of the animals subjected to acute and chronic stress conditions, which raised corticosterone levels, as previously showed [23]. In liver, both acute and chronic stress increased MT-1/2 protein levels. This increase was more pronounced in males than in females in response to acute stress, but no differences in MT-1/2 protein levels were seen between males and females subjected to chronic stress (Fig. 5a). The MT-1/2 mRNA levels followed the same trend in animals subjected to chronic stress induction, except that no differences were seen between males and females (Fig. 5b).

In CP from female rats, MT-1/2 protein expression increased in response to acute and chronic stress induction and no differences were observed between acute and chronic stress. Interestingly, acute stress induction decreased MT-1/2 protein expression in males to approximately one half of control levels, while chronic stress induction increased MT-1/2 protein expression and no differences were observed in MT-1/2 protein expression between males and females (Fig. 6a). Moreover, after acute stress and chronic stress induction, MT-1/2 mRNA levels in CP followed the overall pattern of protein expression (Fig. 6b).

4. DISCUSSION

CPs are key brain structures that constitute a barrier between the CSF and the bloodstream, produce and secrete the CSF and play main roles in maintaining the CNS homeostasis by conferring physical and neurotoxic protection and facilitating the removal of brain metabolites from the CSF [5]. CPs also intervene in the synthesis, secretion and regulation of several biologically active compounds [2,3] and in neurohumoral brain modulation and neuroimmune interaction [31]. CPs also act as a scavenger of metals from the brain and may undergo apoptosis when the metal buffering capacity is exceeded and metal toxicity arises. MT-1/2 are pivotal metalloproteins for the maintenance of essential metal homeostasis, and prevention of oxidative stress by acting as scavengers of ROS. Previous studies showed that in some brain regions, MT-1/2 are highly inducible by cortisol and other glucocorticoid hormones [18], but no studies have focussed on the regulation of MT-1/2 by glucocorticoids in CPs. Given the importance of CPs and MT-1/2 for the homeostasis of brain metals, and regulation of oxidative stress, we investigated MT-1/2 expression in response to glucocorticoids in CPEC cultures and RCP cells and in the CP of rats subjected to psychosocial stress.

Once, MT-1/2 expression had been confirmed in the RCP cell line, RCP and CPEC cells were incubated with hydrocortisone showing that this glucocorticoid up-regulates MT-1/2 expression with the results obtained in CPEC cultures and in RCP cells mostly reproduced. Further, GR and MR receptor antagonists suppressed the hydrocortisone induction of MT-1/2 expression in RCP cells. Previous studies have already shown that in liver and some brain regions, as hypothalamus, the up-regulation of MT-1/2 expression by cortisol occurs *via* GR receptor [19,32]. Our results indicate that, in CPs, both receptors are necessary to mediate induction MT-1/2 expression in response to hydrocortisone, as blockage of MR also suppressed this effect, and the simultaneous blockage of both receptors slightly reduced the expression of MT-1/2. Indeed, it has been demonstrated that in the absence of a stress condition, cortisol binds preferentially to MR receptors, but in a stress situation, the high levels of circulating hormone [33] saturate MR, and bind to GR, regulating the transcription of genes [34] and, this seems to be the case of MT-1/2 genes. Although, as the GR antagonist used in the experiments is also a progesterone receptor antagonist, subsequent studies with a selective antagonist or performed *in vivo* with adrenalectomized animals are important to confirm and extend the *in vitro* results.

The anti-apoptotic properties of MT-1/2 and glucocorticoids are well documented [6]. So we analyzed the effects of hydrocortisone induction of MT-1/2 in the apoptosis of RCP cells by flow cytometry. These experiments showed that the incubation with hydrocortisone decreased the number of apoptotic/late apoptotic cells, to approximately one half, and a pre-incubation with an anti-MT-1/2 antibody abrogated this effect. These results suggest that the decrease of apoptotic/late apoptotic RCP cells number may be due to the up-regulation of MT-1/2 protein expression in CPs, corroborating the anti-apoptotic properties of MT-1/2 in this structures, and the results obtained by several authors who described anti-apoptotic effects of glucocorticoids in epithelial cells [30]. In fact, apoptosis of CP cells may disrupt the global brain homeostasis, as reported in many brain dysfunctions and pathologies: high amyloid-

beta levels in CP provoke a significant increase in ROS and caspase expression, leading to increased apoptosis and CSF dysfunction [14]; high apoptotic levels in CP cells of AD brains, have been reported compromising CP permeability and CSF clearance [35]; cytokine TNFα treatment, simulating inflammatory conditions, induces apoptosis of CP epithelial cells and loss of its barrier functions [31]. Our results provided clear evidence that up-regulation of MT-1/2 expression after incubation with glucocorticoids diminishes apoptosis in CP cells and in this regard, glucocorticoids may beneficiate CP integrity and become neuroprotective.

As previously reported in a very recent publication by our group, an experiment of stress induction, with observed increases in CORT levels, was assembled subjecting male and female rats to acute or chronic psychosocial stress protocols [23]. As observed in several studies [17-19,32,36], the induction of psychosocial stress (acute and chronic) consistently increased MT-1/2 expression in liver at the protein and mRNA levels, both in males and in females, and it was higher in males, comparing with females. It has been postulated that metals regulate MT-1/2 synthesis in brain differently from that in liver [37,38]. Although, our results showed that glucocorticoids regulate MT-1/2 expression in brain and liver in a similar fashion, suggesting that metals and glucocorticoids regulation of MT-1/2 expression occur *via* distinct pathways. There are specific areas in the brain, as hippocampus, cerebellum, mesencephalon, pons and corpus callosum where MT-1/2 expression is up-regulated by glucocorticoids [18,32]. In line with this, we observed that, in CP, chronic stress induction increased MT-1/2 protein and mRNA levels both in males and in females. Interestingly, acute stress produced an increase in MT-1/2 expression in females and the opposite effect in males, in which MT-1/2 protein and mRNA levels decreased to approximately one half. So, it seems that regulation of MT-1/2 expression by glucocorticoids in CP is distinct between genders, probably due to the differential readjustment and response to stressors by the HPA axis. Several factors, as animal model, tissue type, gender, age and time and intensity of exposure to a stressor could generate different stress

responses in animals [37,39,40] probably because, basal and induced levels of GR and MR are also gender-, age- and tissue-dependent in terms of density and affinity [41-43]. In fact, as pointed by others, male and female rats exposed to the same stressor, due to their physiological differences, may show dissimilar habituation and stress responses. Moreover, in females, the activation of the HPA axis by stress induces differences in the oestrus cycle [44] that could also interfere in their stress responses.

Some authors showed that high MT-1/2 expression levels may become protective in transient stress and destructive in permanent stress, as in ageing, because they lead to low zinc bioavailability [45]. In contrast, low MT-1/2 leads to successful ageing both in humans and mice [46]. Also, the continuous exposure of an organism to a stress condition generates high circulating glucocorticoid levels, which may have detrimental effects within the brain and have been associated with the onset and progression of many pathological processes including apoptosis in some brain regions, as the cortex and hippocampus [47]. In this context, we point that the putative neuroprotective effects of the higher MT-1/2 expression in CP, promoted by high levels of glucocorticoids, may also, in permanent stress conditions, become harmful. Therefore, further studies will be required to clarify the precise crosstalk between MT-1/2, apoptosis and stress/glucocorticoids in the CNS, which may have far-reaching effects in various human diseases.

5. CONCLUSIONS

Here, we first demonstrated that hydrocortisone regulates MT1/2 expression, via GR and MR, in RCP and in CPEC cultures. Further, we showed that when RCP cells were incubated with hydrocortisone the number of apoptotic cells decreased, an effect abrogated by the addition of a MT-1/2 antibody. From the in vivo studies, we found that the induction of a chronic psychosocial stress increased MT-1/2 expression in liver

and in CP from both male and female rats. Surprisingly, after an acute stress psychosocial induction, males and females showed an antagonistic regulation in MT-1/2 expression, as males presented decreased MT-1/2 mRNA and protein levels while females showed the opposite effect.

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FIGURE LEGENDS

Figure 1: RCP cells express MT-1/2: **(a)** CP protein extracts (50 µg; lanes 1, 2 and 3) were resolved by SDS-PAGE (12,5%) followed by Western blot using mouse anti-MT-1/2 antibody, normalized with the mouse anti-β-actin antibody. Images were captured with the Molecular Imager FX Pro Plus MultiImager system. **(b)** RCP cells were stained with a mouse monoclonal anti-rat hMT-1/2 antibody followed by an Alexa Fluor 546 goat anti-mouse IgG conjugated (red). Nuclei were stained with Hoechst 33342 dye (2 µM). As negative controls, the primary antibody for MT-1/2 was omitted in a preparation (upper) and the MT-1/2 antibody was pre-incubated with MT2 protein using the same dilution of the antibody and a ten-fold (by weight) excess of protein (bottom) (630x).

Figure 2: Effects of hydrocortisone incubation on MT-1/2 expression in RCP cells: **(a)** Cells were incubated with 0 (control), 100 or 1000 nM of hydrocortisone during 6, 12, 24 and 36 h. Protein extracts (50 µg) were resolved in SDS-PAGE followed by Western blot. Images were captured with the Molecular Imager FX Pro Plus MultiImager system and quantified by densitometry. Bar graphs indicate means ± SEM (% of controls) from at least three independent experiments (** - $p<0.01$; *** - $p<0.001$). **(b)** Effects of glucocorticid and/or mineralocorticoid receptor antagonists, mifepristone (RU486) and spironolactone (SPIRO), on hydrocortisone (hydrocort) induction of MT-1/2 protein levels. Cells were incubated with 0 (control) or 1000 nM of hydrocortisone with 16 µM of RU486 and/or 1 µM of SPIRO. Protein extracts (50 µg) were resolved in SDS-PAGE followed by Western blot. Images were captured with the Molecular Imager FX Pro Plus MultiImager system and quantified by densitometry. Bar graphs indicate means ± SEM from at least three independent experiments (* - $p<0.05$; *** - $p<0.001$).

Figure 3: Effects of hydrocortisone incubation on MT-1/2 expression in CPEC cultures: **(a)** Cells were incubated with 0 (control), 10, 100 or 1000 nM of hydrocortisone for 6, 12, 18, 24 or 36 h. Protein extracts (30 µg) were resolved in SDS-PAGE followed by Western blot. Images were captured with the Molecular Imager FX Pro Plus MultiImager system and quantified by densitometry. Bar graphs indicate means ± SEM from at least three independent experiments (* - $p<0.05$; ** - $p<0.01$). **(b)** Fold induction of MT-1/2 mRNA expression in cells incubated with 0 (control), 10, 100 or 1000 nM of hydrocortisone during 6, 12, 18, 24 or 36 h. MT-1/2 mRNA levels were analysed by real-time PCR using β2-microglobulin and cyclophilin A as endogenous controls, and are represented as normalized expression. Fold differences among groups were calculated using the formula $2^{-(\Delta\Delta Ct)}$. All comparisons between two groups were made by t-Student test Data were compared by means of one-way ANOVA followed by Dunnett's test. Values are expressed as a normalized value of non-treated animals, which were considered 1. Bar graphs indicate means ± SEM from at least three independent experiments Significant statistical differences are indicated (* - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$).

Figure 4: MT-1/2 blockage reverses the anti-apoptotic effect of hydrocortisone in RCP cells **Upper panel:** Representative dot plots of green fluorescence of cells (Annexin V - FITC, x-axis) plotted against red fluorescence (PI, y-axis) obtained with cell samples taken after a 24h incubation with 0 nM **(a)** or 1000 nM hydrocortisone **(b)** and cells pre-incubated for 1h with an anti-MT-1/2 antibody (0.2 µg/mL) followed by a 24h incubation with 0nM **(c)** or 1000 nM hydrocortisone **(d).** A total of 20 000 events was collected for each analysis. **Bottom panel:** Correspondent graphic representation of the percentage of viable and apoptotic/late apoptotic RCP cells (% of cells) in each of the treatments described above. Significant statistical differences are indicated (* - $p<0.05$).

Figure 5: Comparison of liver MT-1/2 levels in rats subjected to acute and chronic stress conditions: **(a)** – Protein levels (% of controls); **(b) -** Fold induction of MT-1/2 mRNA expression. Values are expressed as a normalized value of non-treated animals, which were considered 1. Data collected from real time PCR experiments was analyzed using the formula: 2-($\Delta\Delta$Ct). All comparisons between two groups were made by *t*-Student test. Data were compared by means of one-way ANOVA followed by Dunnett's test. Bar graphs indicate means ± SEM from at least three animals. Significant statistical differences are indicated (* - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$).

Figure 6: Comparison of CP MT-1/2 levels in rats subjected to acute and chronic stress conditions: **(a) -** Protein levels (% of controls); **(b) -** Fold induction of MT-1/2 mRNA expression. Values are expressed as a normalized value of non-treated animals, which were considered 1. Data collected from real time PCR experiments was analyzed using the formula: 2-($\Delta\Delta$Ct). All comparisons between two groups were made by *t*-Student test. Data were compared by means of one-way ANOVA followed by Dunnett's test. Bar graphs indicate means ± SEM from at least three animals. Significant statistical differences are indicated (* - $p<0.05$; *** - $p<0.001$).

Figure 1:

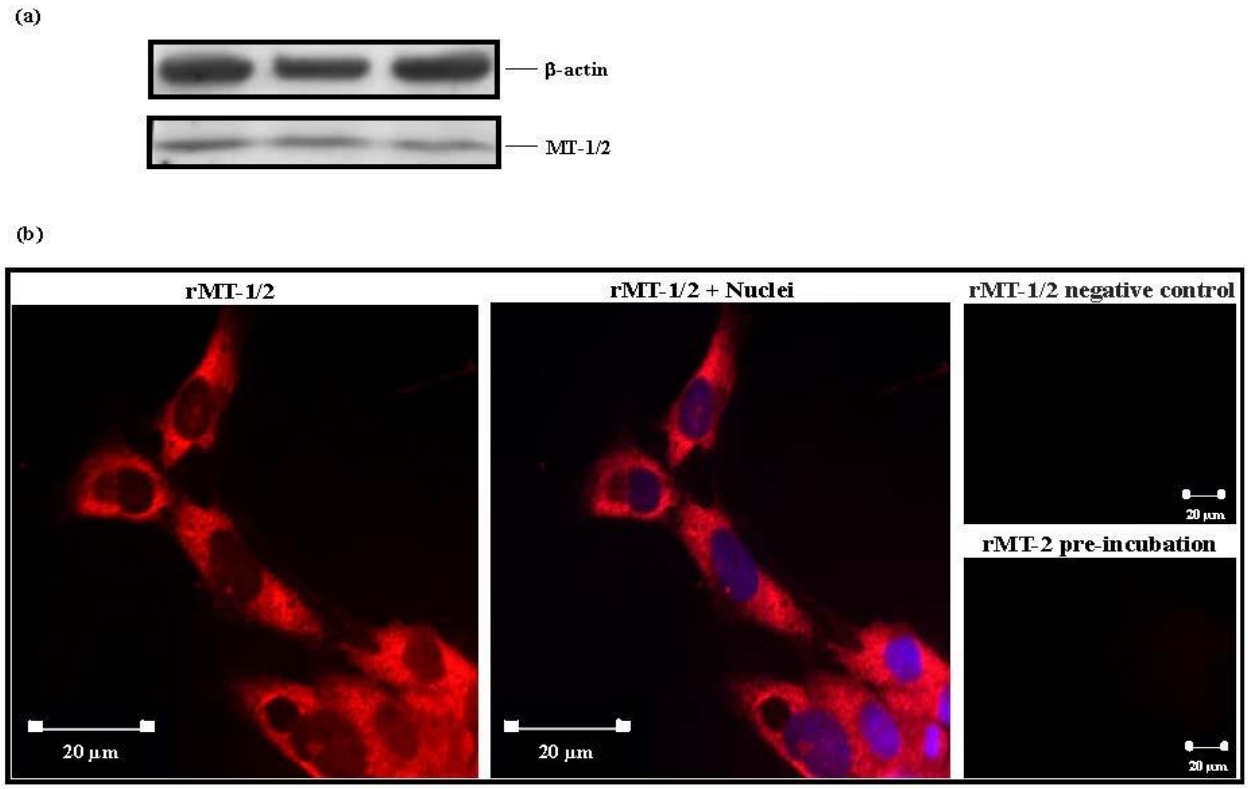
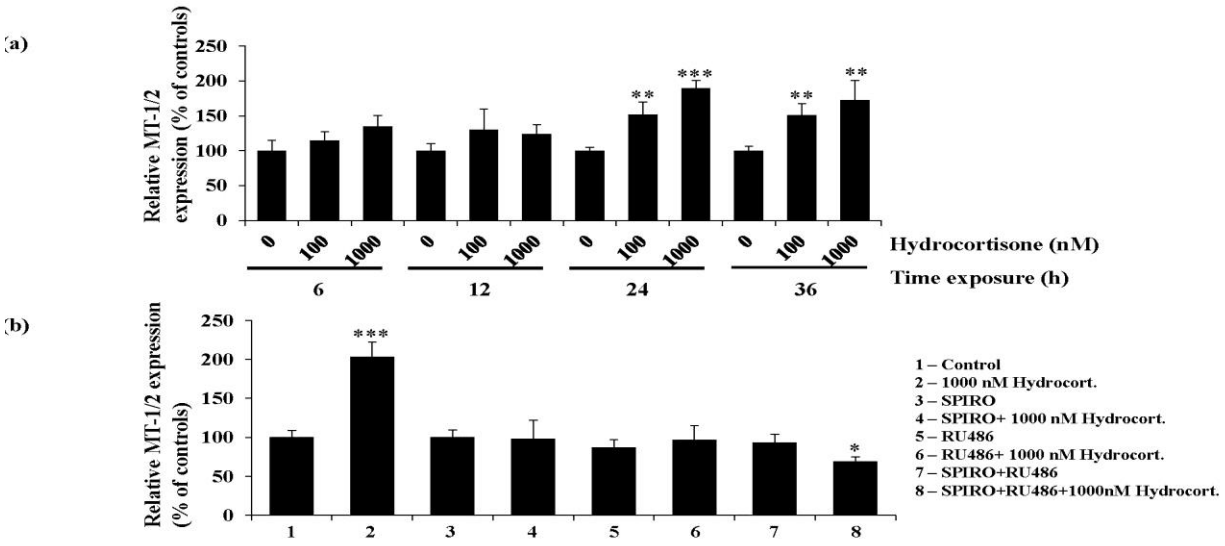


Figure 2:



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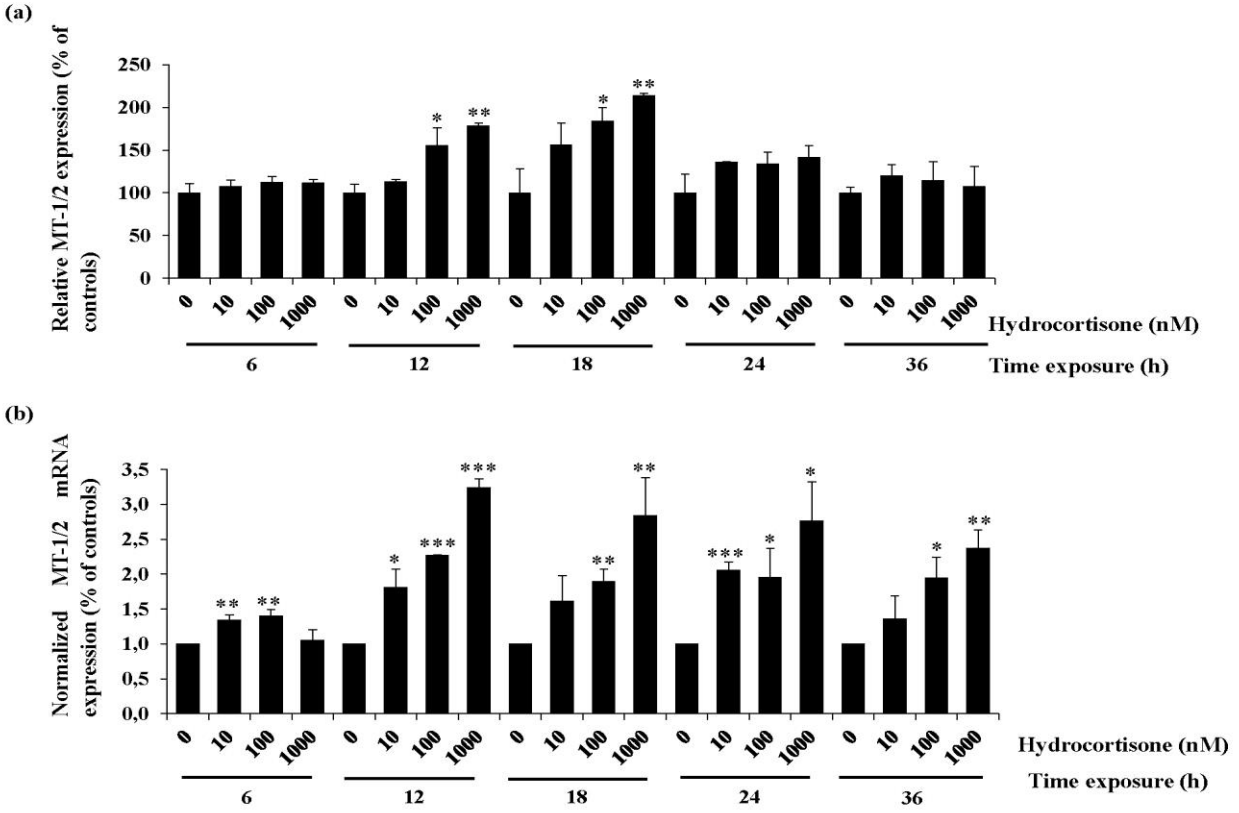

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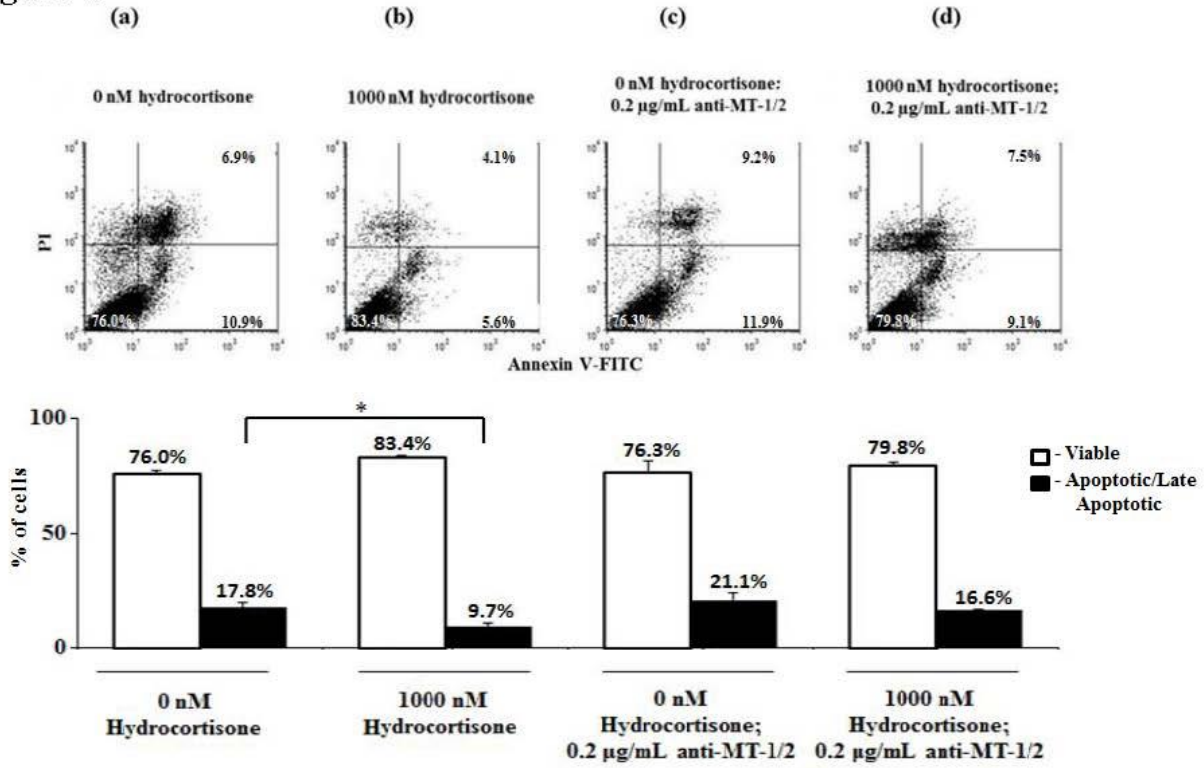

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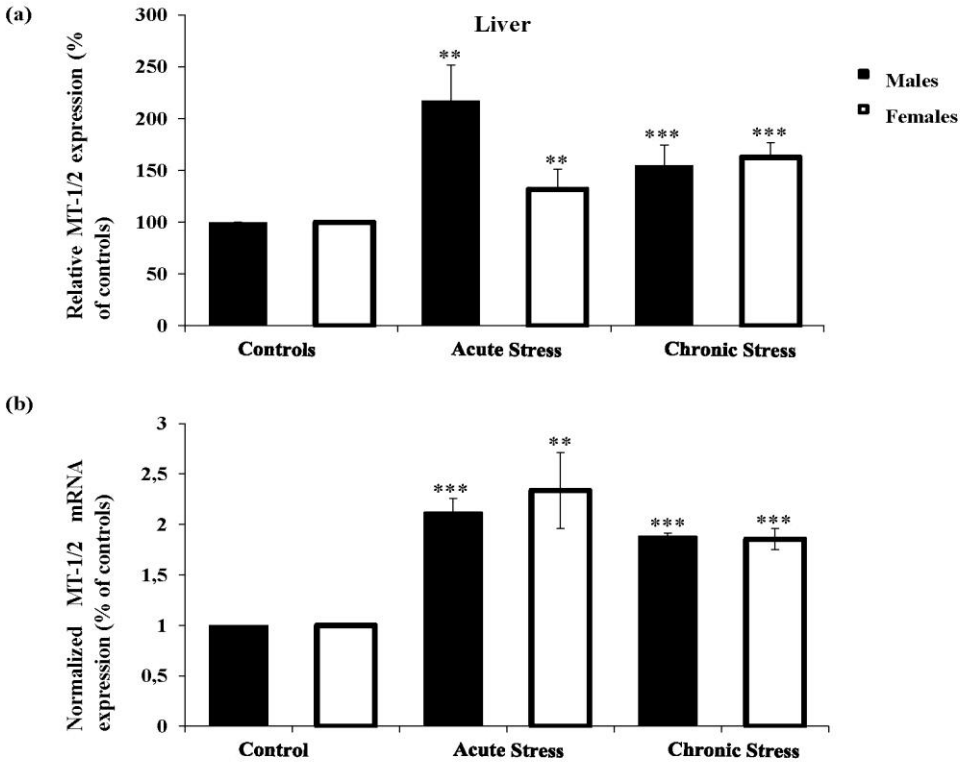

Figure 6:

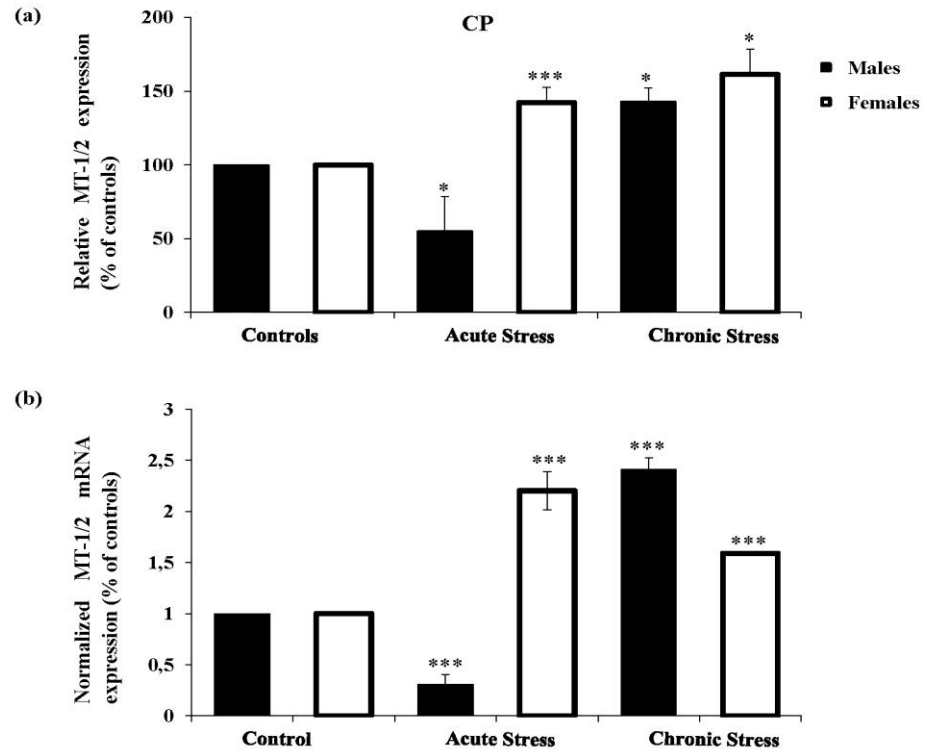

Table I: Primer sequences of rat β2 microglobulin (rβ2m), cyclophilin A (rCycA) and metallothionein (rMT) used in real-time PCR.

Designation	Sequence (5' – 3')
rβ2m fw	CCG TGA TCT TTC TGG TGC TTG TC
rβ2m rv	CTA TCT GAG GTG GGT GGA ACT GAG
rCycA fw	CAA GAC TGA GTG GCT GGA TGG
rCycA rv	GCC CGC AAG TCA AAG AAA TTA GAG
rMT fw	TGT GCC ACA GGA TCC T
rMT rv	GCA GCC CTG GGC ACT T

Chapter 6: General Conclusions and Perspectives

CP is a key brain structure that plays main roles in the maintenance of the CNS homeostasis. As mentioned, it produces, secretes and regulates the CSF and several biologically active compounds, controls the molecular trafficking between the blood and the CSF and is highly sensitive to any alterations in the CSF composition. CP also mediates the relationship between the CNS and the peripheral immune system and participates in the nutrition of the brain, facilitates the removal of brain metabolites, controls the essential metal homeostasis and exerts various functions in neuroprotection and neurorepair processes. Several receptors for growth factors, neurotransmitters and hormones are also present in CP, evidencing that it is a target tissue for numerous molecules. This multiplicity of functions indicates that even small imbalances, they can have extensive effects, thus highlighting the importance of CP, especially in aging and neuropathologic contexts. Among all proteins synthetized by CP cells are TTR and some isoforms of MTs (MT-1, MT-2 and MT-3). TTR is mainly known for the transport of thyroid hormones and retinol through its binding to RBP. Besides, several studies provided clear evidences that TTR is also associated with various neuropathologies, including AD, where it acts as an Aß scavenger, preventing its aggregation and/or deposition and promoting its clearance. Likewise, MTs have various known functions, including metal binding, antioxidant and anti-inflammatory properties and other physiological actions related with neuroprotection and neuroregeneration (Santos et al. 2012).

It was previously demonstrated by our group that TTR binds to MT-2 (Goncalves et al. 2008) with unknown effects on the functions of each protein and on the CNS homeostasis. In order to elucidate if TTR-MTs interactions interfere in Aß metabolism, and consequently in AD progression, we first investigated if TTR binds to MT-3, an isoform predominantly expressed in the brain. The results clearly showed that TTR interacts specifically with MT-3 with a K_d of 373.7 ± 60.24 nM, indicating that a stable complex occurs between these proteins. Also, TTR and MT-3 co-localized with the endoplasmic reticulum of CPECs, particularly in the perinuclear region suggesting that, as described for TTR (Goncalves et al. 2008), MTs are also secreted and the interactions between TTR and MTs may occur inside and outside the CPECs. Furthermore, our results demonstrated that hTTR-hMT2 and hTTR-hMT-3 interactions affect the capacity of hTTR to bind to Aß: the pre-incubation of hTTR with hMT-2 reduced hTTR-Aß binding while with hMT-3 had the opposite effect, enhancing it. So, a less efficient removal/clearance of Aß is suggested when hMT3 expression is decreased and/or hMT-2 levels are increased, as already observed in some animal models of AD and in AD patients (Adlard et al. 1998; Yu et al. 2001).

Some authors observed that MT-3 abolished the formation of toxic aggregates, antagonising the neurotrophic and neurotoxic effects of Aß peptides (Irie and Keung 2001) which, according to our results, is due to the enhanced TTR-Aß binding in the presence of MT-3.

The presence of TTR-MT-2 and TTR-MT-3 interactions in CPECs suggests that they may, as well, occur *in vivo* in CP, where Aß is also processed (Crossgrove et al. 2005; Crossgrove et al. 2007) and cleared from the CSF. However, the precise mechanisms involved on the overall Aß metabolism remain unknown. Still, we propose that cleavage of full-length Aß and

clearance of Aß aggregates, features that have been attributed to TTR (Costa et al. 2008a; Costa et al. 2008b), are improved in the presence of MT-3, and disfavoured in the presence of MT-2.
Our findings brought new perspectives on clarifying the specific mechanisms implicated in the disturbance of hTTR-Aß binding by the presence of MT-2 and/or MT-3. Very recently, Manso and colleagues (2012) analyzed the role of MT-1/2 and MT-3 on Aß metabolism, using Tg2576 mice, and concluded that both MT-1/2 and MT-3 alone affect Aß metabolism in a complex gender-, region- and severity-dependent manner (Manso et al. 2011; Manso et al. 2012a; Manso et al. 2012b). Therefore, future studies using animal models of AD are required to confirm if the observed *in vitro* effects of TTR-MT-2 and TTR-MT-3 interactions in TTR-Aß binding occur, and have effective impacts on Aß metabolism, *in vivo* and, if so, disclose the precise mechanisms/pathways involved.

In general, neurodegenerative disorders are a consequence of various genetic and environmental factors. As referred in chapter 1, stress is a pivotal etiological factor associated with various neurological disorders, including AD (Bao et al. 2008; Dong and Csernansky 2009; Landfield et al. 2007; Souza-Talarico et al. 2008). Glucocorticoids are closely related with stress because they are end products of the HPA axis and are able to generate appropriate responses to stressors (Adzic et al. 2009; Hellhammer et al. 2009; Helmreich et al. 2005).
Numerous authors argued that chronic high levels of glucocorticoids, caused by a continuous exposure to a stress condition, may affect certain brain structures, or regions, and trigger the onset and/or progression of many pathological processes (Hellhammer et al. 2009; Herzog et al. 2009; Munhoz et al. 2008). However, in intermediate levels, others demonstrated that glucocorticoids/stress could also prevent some neurodegenerative diseases (Santos et al. 2010).
Within the brain, many stressors, as hormones, regulate several molecules, as TTR and MT-1/2, with known actions in various CNS pathologies.
It is well defined that a specific stress-inducing stimulus may promote or disfavour the onset/progression of a CNS disorder depending on many aspects. Several factors, as animal model, age, tissue type, gender, intensity and duration of exposure to a stressor could generate different stress responses to the same stimuli (Ebadi et al. 1995; Young et al. 2001) because, basal and induced levels of GR and MR and glucocorticoids are also age-, gender-, duration- and tissue-dependent (Csaba and Inczefi-Gonda 2001; Endres et al. 1979). As such, studies regarding the contribution of stress and glucocorticoids in the regulation of several molecules, involved in brain disorders, are of utmost importance.
Both TTR and MT-1/2 are related with various neuropathologies and are regulated by stress related hormones in some brain regions. Few studies showed that brain TTR expression is altered after stress conditions (Bernstein et al. 1989; Ingenbleek and Young 1994; Kohda et al. 2006). Regarding MTs expression in brain, generally, it is accepted that, contrarily to MT-3, MT-1/2 are stress-related proteins whose expression is highly inducible, in response to numerous stimuli including metals, glucocorticoids, cytokines, oxidative agents and a variety of other

stressors (Aschner and West 2005; Carrasco et al. 1998; Chung et al. 2008; Ebadi et al. 1995; Gasull et al. 1994; Hidalgo et al. 1990; Hidalgo et al. 1994a; Stankovic et al. 2007; Vasak 2005; West et al. 2008). After a stress condition, MT-1/2, but not MT-3 (Aschner and West 2005), are up-regulated in some brain regions (Beltramini et al. 2004; Hidalgo et al. 1991; Hidalgo et al. 1997; Hidalgo et al. 1994b; Jacob et al. 1999) and, authors argued that this could be neuroprotective in transient stress and detrimental in permanent stress conditions, as in aging (Giacconi et al. 2003). Instead, low MT-1/2 levels contribute to the successful aging both in humans and mice (Mocchegiani et al. 2002). Also, previous studies showed that: in the early stages of AD, MT-1/2 expression is increased, as a result of oxidative stress, inflammation or a deficient metabolism of heavy metals (Adlard et al. 1998); and, MT-1 mRNA levels in astrocytes and microglia/macrophages surrounding the Aß plaques are increased in some transgenic mice models of AD while MT-3 mRNA expression is unaltered (Carrasco et al. 2006).

Despite all studies, nothing is known about the regulation of TTR and MT-1/2 expressions by glucocorticoids/stress in CP.

We performed *in silico* analyses in TTR and MT-1/2 genes and found GREs in both genes, suggesting responsiveness to glucocorticoids. Therefore, we focused on the putative regulation of TTR and MT-1/2 genes by glucocorticoids in CP, both *in vitro* and *in vivo*. The results showed that hydrocortisone up-regulated TTR expression in RCP and CPECs cultures, an effect suppressed by GR and/or MR antagonists, suggesting the involvement of both receptors in this regulation. However, as the GR antagonist used is also a PR antagonist, future studies using a selective antagonist, or performed *in vivo* with adrenalectomized animals, are essential to confirm our results.

In *vivo* studies showed that the induction of acute or chronic psychosocial stress (crowding), with increased glucocorticoid levels, up-regulated TTR expression in liver, CP and CSF, both in males and females. Also, this up-regulation was more pronounced in males after an acute stress exposure, indicating that males respond more readily than females to psychosocial stress conditions.

Regarding the regulation of MT-1/2 expression by glucocorticoids in CP, the overall data obtained from *in vitro* studies followed the same trend as described for TTR. Moreover, flow cytometry assays revealed a decrease in apoptosis in RCP cells, from 17.8% to 9.7%, thus confirming the anti-apoptotic properties of MT-1/2 also in CP. From *in vivo* studies, data obtained revealed that induction of chronic psychosocial stress increased MT-1/2 expression in liver and CP both in male and female rats. In turn, induction of acute stress up-regulated MT-1/2 expression in liver, in males and females, and caused opposite effects between genders in CP as it promoted an up-regulation of MT-1/2 expression in females, and a down-regulation in males. Taken together, we concluded that stress regulates MT-1/2 expression in a gender-, tissue- and time exposure-dependent manner. A possible explanation for this could lie in the fact that, as pointed by others, male and female rats exposed to a stressor, due to their physiological differences, may show dissimilar habitutation to stress and distinct stress responses.

With the exception of males after an acute stress situation, our results showed that glucocorticoids increased TTR and MT-1/2 expressions and decreased apoptosis in CP which may beneficiate its integrity and therefore become neuroprotective.
Generally, the damage versus benefit of a specific molecule or stimulus, on a particular pathology, is always dependent on its balancing and, its overall effect, is the sum of its direct and indirect effects in the disorder. In fact, the up-regulation of TTR and MT-1/2 expressions may also lead to a higher bioavailability of both proteins and occurrence of TTR-MT-2 interactions that negatively affect Aß metabolism and, consequently, AD. So, we suggest that the putative and isolated neuroprotective effects promoted by glucocorticoids in CP could be insufficient *per se* to prevent their deleterious effects in other molecules, other brain structures or in the overall progression of a neuropathology, as AD.

In summary, we demonstrated the occurrence of a TTR-MT-3 interaction *in vitro*, highlighted the impact of the interactions between TTR and MTs (MT-2 and MT-3) in TTR-Aß binding and revealed the importance of glucocorticoids in the regulation of TTR and MT-1/2 expressions, particularly in CP.
Our studies do not, directly, relate the effects of glucocorticoids in TTR or MTs expressions with any particular disease. However, both proteins, as well as glucocorticoids and CP, are relevant players in various brain diseases and TTR and MT-1/2/MT-3 are synthetized in CP cells, interact and affect Aß metabolism, thus emphasizing their relevance in brain research, particularly in AD.
In this context, future studies are required to clarify the detailed crosstalk between MTs, TTR, apoptosis and glucocorticoids in the CNS, particularly within the CP. Also, as various brain disorders, including AD, are gender-dependent, it is important to elucidate if this crosstalk differ, or not, between males and females.
Furthermore, it should be noted that we contemplated only few of the numerous molecules and mechanisms involved in AD-like neuropathology. So, subsequent studies regarding the effects of glucocorticoids in the regulation of other important molecules and mechanisms involved in this brain disorder will be useful to the overall understanding of the role of glucocorticoids in AD.
Also, elucidation of the full outcomes promoted by the increased expressions of TTR, MT-1/2 and/or MT-3, in AD animal models and AD patients, are crucial to determine the global impact of these proteins in this disorder. We propose a set of studies involving stress induction/glucocorticoid administration in animal models of AD, crossed with TTR KO and/or MT-1/2 and/or MT-3 KO and determination of their isolated and combined effects in the overall disease progression. Likewise, the determination of the brain Aß levels and cognitive performance will be fundamental to identify the precise roles of these molecules in Aß metabolism and apoptosis and, therefore, in AD progression.
Finally, as TTR and MTs are involved in many CNS disorders and are, simultaneously regulated, by various hormones, particularly SHs, studies including combinations of glucocorticoids and other SHs that also regulate the expression of TTR and MTs, and determination of the sum of their effects in the global disease progression should be conducted.

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Chapter 7: References

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