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Evaluation of a-beta scavengers expression in rat choroid plexus: effects of circadian rhythm and a-beta levels

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Abstract

The choroid plexus, a highly specialized tissue, is involved in a variety of functions such as production and secretion of the cerebrospinal fluid, synthesis and secretion of several bioactive substances and constituting the blood-cerebrospinal fluid barrier. Among others, the choroid plexus has the ability to produce molecules involved in the metabolism/clearance of amyloid β peptide such as transthyretin, gelsolin, apolipoprotein J, metallothionein 2, angiotensin-converting enzyme and insulin-degrading enzyme. Some of these amyloid β scavengers prevent the formation and deposition of amyloid fibrils.

The circadian clock, present in all mammal tissues, is synchronized by *zeitgebers* (e.g., light/dark) over a period of approximately 24 h. In mammals, the brain is involved in the control and coordination of the circadian rhythm, which in turn, is generated by the suprachiasmatic nucleus. Peripheral and extra-suprachiasmatic nucleus clocks exist in other brain areas which are under the control of the suprachiasmatic nucleus. Among these, stands out the choroid plexus by expressing the core clock genes that show a rhythmic expression in both females and males.

The aim of this project is to analyse whether the amyloid β scavengers are influenced by circadian rhythmicity in both sexes in rat choroid plexus explants by Real-time PCR. Moreover, it was also investigated if the expression of these genes/proteins undergoes changes using different concentrations of amyloid β (1 μg/ml and 3 μg/ml) in rats of different ages. Also, amyloid β scavengers expression was evaluated at different ages. For that, Real-time PCR and Western blot techniques were used.

The results herein presented indicate differential expression of amyloid β scavengers with age, sex and amyloid β concentration. However, more studies are needed to investigate, for instance, the circadian oscillation of these genes *in vitro* with different amyloid β concentrations and *in vivo* in Alzheimer´s Disease patients.

Keywords

Choroid plexus, circadian rhythm, amyloid β, transthyretin, gelsolin, apolipoprotein J, metallothionein 2, angiotensin-converting enzyme, insulin-degrading enzyme

Resumo alargado

O plexo coróide, localizado em cada um dos ventrículos cerebrais, é um tecido altamente especializado que se encontra envolvido numa variedade de funções importantes para a homeostasia do sistema nervoso central. Entre estas destacam-se a produção e secreção do líquido cefalorraquidiano, a síntese e secreção de inúmeras substâncias bioativas como fatores neurotróficos, citocinas, vitaminas e uma variedade de proteínas. Para além destas, as células epiteliais do plexo coróide constituem a barreira sangue-líquido cefalorraquidiano com o objetivo de regular a passagem de moléculas entre a corrente sanguínea e o líquido cefalorraquidiano. Quanto à função de síntese do plexo coróide, este possui a capacidade de produzir moléculas com função de metabolismo/*clearance* do péptido ß amiloide. Entre outras, a transtirretina, gelsolina, apolipoproteína J, metalotioneína 2, enzima conversora de angiotensina e enzima de degradação da insulina, fazem parte deste grupo de proteínas. Depois de sintetizadas, as mesmas são secretadas para o líquido cefalorraquidiano onde formam um complexo estável com o péptido ß amiloide e hidrolisam-no em fragmentos menos neurotóxicos. Desta forma, retardam a formação e deposição de fibrilas amiloides (principal característica no desenvolvimento da Doença de Alzheimer e, por sua vez, inibem os efeitos citotóxicos induzidos pelo péptido ß amiloide.

A alteração dos ciclos luz/escuridão regula atividades metabólicas, comportamentais e fisiológicas dos organismos vivos e, estas atividades variam com a altura do dia. Estes ciclos diários são uma resposta da atividade de relógios endógenos, também denominados de relógios circadianos. Em mamíferos, o cérebro encontra-se envolvido no controlo e coordenação do ritmo circadiano. O ritmo circadiano está presente em todos os tecidos de mamíferos com uma periocidade de aproximadamente 24h sendo esta gerada pelo núcleo supraquiasmático. Para além do núcleo supraquiasmático, existem também osciladores circadianos noutras áreas do cérebro e órgãos designados de relógios periféricos os quais estão sob o controlo do núcleo supraquiasmático. De entre os osciladores extra - núcleo supraquiasmático está incluído o plexo coróide, que expressa genes do relógio regulados ritmicamente tanto nas fêmeas como nos machos.

Neste sentido, o principal objetivo deste trabalho foi analisar se os genes envolvidos no metabolismo/*clerance (amyloid ß scavengers)* mencionados anteriormente são influenciados pelo ritmo circadiano em ambos os sexos em explantes de plexo coróide de ratos Wistar *Han*. Também se investigou se a expressão destes genes/proteínas sofre modificações em resposta a diferentes concentrações de ß amiloide (1 µg/mL e 3 µg/mL) em ratos de diferentes idades.

A expressão e localização de transtirretina, gelsolina, apolipoproteína J, metalotioneína 2, enzima conversora de angiotensina e enzima de degradação da insulina foram averiguadas em explantes de plexos coróides recorrendo ao *PCR* convencional e imunohistoquímica por fluorescência *(whole-mount)*. Após a confirmação, a expressão rítmica de cada um deles foi analisada em plexos de machos e fêmeas com 2 meses de idade recolhidos a diferentes horas. Os efeitos de ß amiloide na expressão dos mesmos foram estudados nos explantes de plexo coróide de animais recém-nascidos, jovens e machos e fêmeas adultos. Para esse efeito, a técnica de *Real-time PCR* permitiu a quantificação da expressão génica a diferentes horas do dia e com diferentes concentrações de ß amiloide. Além disso, o nível de expressão foi também avaliado em ratos de diferentes idades. A técnica de *Western blot* possibilitou verificar alterações de expressão provocadas pelo ß amiloide ao nível proteico.

Na experiência do ritmo circadiano, apenas a apolipoproteína J, metalotioneína 2 e enzima de degradação da insulina apresentaram alterações significativas ao longo do dia nas fêmeas. Tanto a apolipoproteína J como a metalotioneína 2 atingiram o seu pico máximo de expressão às 14h (ZT7) e 2h (ZT19). Ao contrário destes, a expressão da enzima de degradação da insulina diminuiu estatisticamente às 2h (ZT19). Ao comparar a expressão destes genes ao longo da idade, estes mostraram ter perfis característicos: a transtirretina diminuiu com a idade, enquanto a gelsolina tem um comportamento contrário; a apolipoproteína J diminuíu nos animais jovens em comparação com os recém-nascidos e aumentou nos machos adultos em relação às duas primeiras idades; a expressão da enzima de degradação da insulina diminuiu em todas as idades relativamente aos animais recém-nascidos. Para além disso, a expressão de cada um deles, com exceção da enzima degradadora, varia entre géneros (machos e fêmeas adultos). Quando o plexo coróide retirado de animais de diferentes idades foi sujeito a diferentes concentrações de ß amiloide, verificou-se que este estímulo aumentou a expressão de transtirretina, gelsolina e apolipoproteína J. Os níveis de transtirretina e gelsolina aumentaram com o aumento da concentração de ß amiloide enquanto a expressão de apolipoproteína J é significativa apenas com 3 µg/mL de ß amiloide. Nos animais jovens, o plexo coróide responde ao estímulo de ß amiloide aumentando a expressão de transtirretina apenas com 3 µg/mL e diminuíndo a expressão de apolipoproteína J na presença de 1 µg/mL de ß amiloide e da enzima de degradação da insulina com 1 e 3 µg/mL de ß amiloide. Os machos adultos não apresentaram quaisquer alterações na expressão de genes, com exceção da enzima de degradação da insulina. Por último, as fêmeas adultas expressaram menor quantidade de transtirretina e apolipoproteína J na presença de ambas as concentrações. O contrário verifica-se quando analisados os níveis de enzima de degradação da insulina em que a sua expressão aumentou com 1 µg/mL de ß amiloide. Em conclusão, os *Aß scavengers* mostram uma expressão diferencial entre idades, géneros e concentração de ß amiloide. No entanto, mais estudos são necessários para investigar, por exemplo, a oscilação circadiana desses genes *in vitro* com diferentes concentrações de ß amiloide e *in vivo* em pacientes com doença de Alzheimer.

Palavras-chave

Plexo coróide, ritmo circadiano, ß amiloide, transtirretina, gelsolina, apolipoproteína J, metalotioneína 2, enzima conversora de angiotensina, enzima de degradação de insulina

Index

Figures list

Tables list

Abbreviations and acronyms list

ACE	Angiotensin-Converting Enzyme
AD	Alzheimer's Disease
AF	Adult Female
Ag	Silver
AJs	Adherens Junctions
AM	Adult Male
ApoJ	Apolipoprotein J
APP	Amyloid Precursor Protein
AR	Androgen Receptors
ArKO	Aromatase Knockout
ARNTL	Aryl Hydrocarbon Receptor Nuclear Translocator-Like
AT	Annealing Temperature
Aß	Amyloid ß
BBB	Blood-Brain Barrier
BCSFB	Blood– Cerebrospinal Fluid Barrier
BSA	Bovine Serum Albumin
Cd	Cadmium
cDNA	Complementary Deoxyribonucleic Acid
CLOCK	Circadian Locomotor Output Cycles Kaput
CNS	Central Nervous System
CP	Choroid Plexus
CPEC	Choroid Plexus Epithelial Cells
CPs	Choroid Plexuses
CRY1	Cryptochrome 1
CRY2	Cryptochrome 2
CSF	Cerebrospinal Fluid
Cu	Copper
DEPC	Diethylpyrocarbonate
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
DR	Dorsal Raphe
E2	17ß-Estradiol
ECL	Enhanced Chemiluminescent
ERα	Estrogen Receptor α
ERß	Estrogen Receptors ß

FBS	Fetal Bovine Serum
GDX	Gonadectomise
GLS	Gelsolin
H_2O_2	Hydrogen Peroxide
Hg	Mercury
IDE	Insulin-Degrading Enzyme
IGL	Intergeniculate Leaflet
ISF	Interstitial Fluid
MeOH	Methanol
MR	Median Raphe
MT	Metallothionein
MT2	Metallothionein 2
NB	Newborn
NC	Negative Control
NO	Nitric Oxide
OVX	Ovariectomy
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline-Tween
PER1	Period 1
PER2	Period 2
PER3	Period 3
PFA	Paraformaldehyde
PMSF	Phenylmethanesulfonylfuoride
PR	Progesterone Receptor
PVDF	Polyvinylidene Difluoride
qPCR	Real Time PCR
RBP	Retinol Binding Protein
RHT	Retinohypothalamic Tract
RT	Room Temperature
SCN	Suprachiasmatic Nucleus
SHs	Sex Hormones
T4	Thyroxine
TAE	Tris-Acetate-EDTA
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline-Tween
TJs	Tight Junctions
tRNA	Total Ribonucleic Acid
TTR	Transthyretin
WR	Work Reagent
YG	Young

Zn	Zinc
ZO-1	*Zonula Occludens* 1

I. Introduction

1. Choroid Plexus

The choroid plexus (CP) is a highly specialized tissue that may provide a readout of the brain's overall status and supply a variety of biological factors essential for normal brain function of the Central Nervous System (CNS). Therefore, it is involved in the establishment and maintenance of the baseline extracellular milieu throughout the CNS under both normal and pathological conditions (reviewed by Ransohoff (2014) and Emerich et al. (2004)).

1.1. Anatomical location of the choroid plexus

There are four interconnected channels within the brain, called ventricles, which are lined by the ependyma. Four choroid plexuses (CPs) reside inside these ventricular system of the brain: two in the lateral, one in the third and another in the fourth ventricles (Figure 1), where they form an interface that connects the peripheral blood to the cerebrospinal fluid (CSF) of the CNS (reviewed by Emerich et al. (2005)).

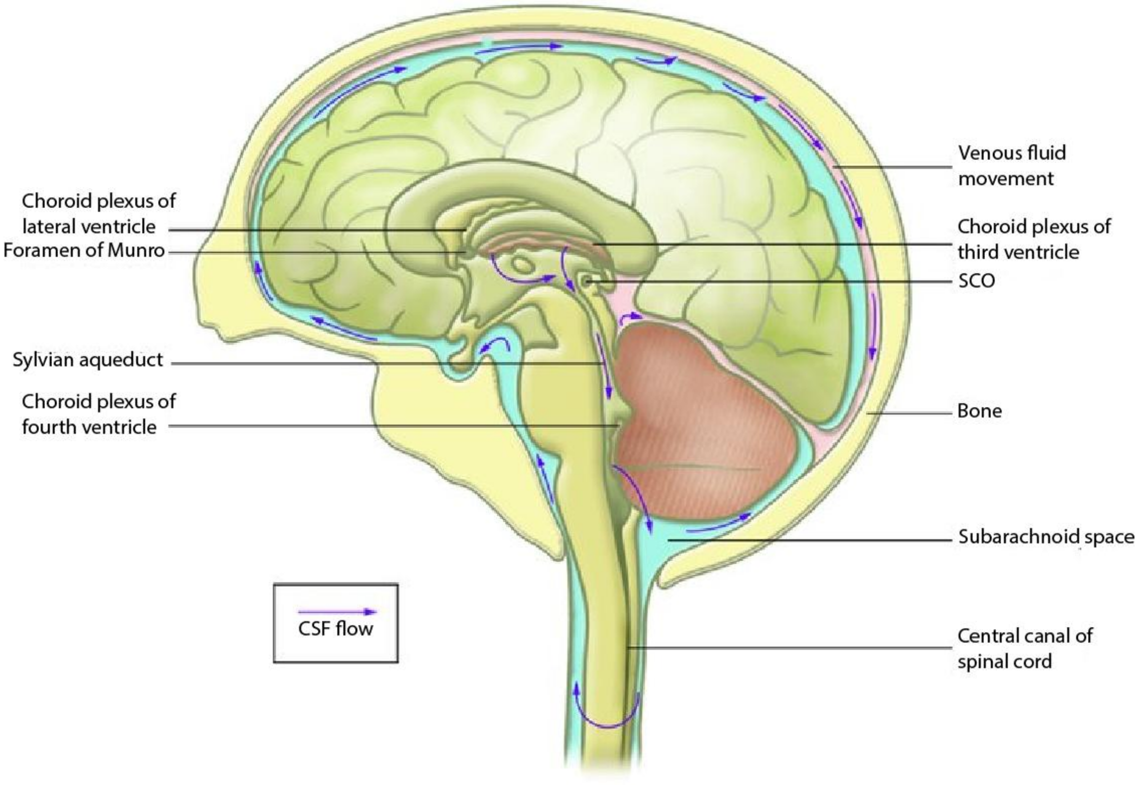


Figure 1 – Anatomical location of the choroid plexus (CP) in brain and cerebrospinal fluid (CSF) flow. The CPs are inserted into the ventricular system and their main function is CSF production and secretion. The CSF is secreted from the CP and travels through the ventricular system in a rostrocaudal direction from the lateral ventricles to the third ventricle via the foramen of Munro, then through the Sylvian aqueduct to the fourth ventricle, and finally into the cisterna magna of the subarachnoid space and the central canal of the spinal cord (adapted from Picketts (2006)).

1.2. Morphology of choroidal tissue

The CP, a highly irrigated structure, consists in epithelial-endothelial vascular convolutions developed from the dorsal roof of the ventricular system. The CP have a relatively simple structure, similar in the lateral, third and fourth ventricles (Figure 2). It is composed of three cellular layers: (i) the apical epithelial cells facing the CSF, (ii) the underlying supporting connective tissue, and (iii) the inner layer of endothelial cells with immediate contact with the blood. Despite its ependymal origin, these cells present characteristics of epithelial cells, being therefore designated by CP epithelial cells (CPEC). Beneath the epithelial layer there is connective tissue, which may contain fibroblasts, mast cells, macrophages, granulocytes or other infiltrates, a rich extracellular matrix, and a vascular network (reviewed by Wolburg and Paulus (2010), Damkier et al. (2013), Emerich et al. (2005) and Mortazavi et al. (2014)).

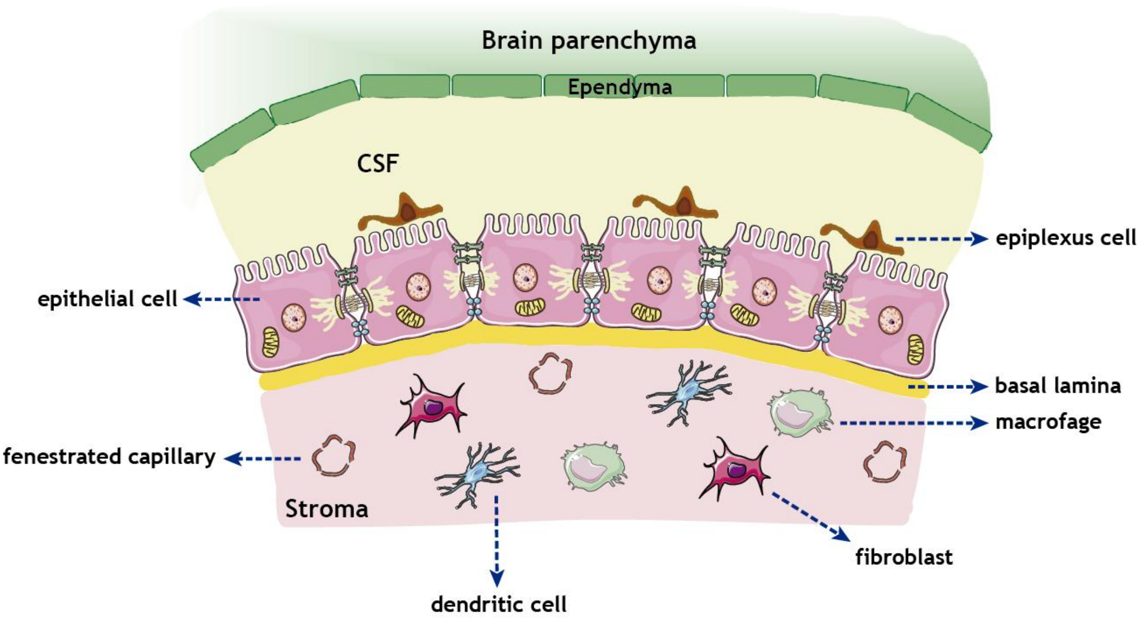


Figure 2 - Morphological illustration of CP and its cellular structure. The CP extends from the ependymal cell layer of the ventricular wall forming a continuous strand of cuboidal epithelial cells resting upon a basal lamina and inner core of connective and highly vascularized tissue. The apical membrane of the epithelial cells faces the CSF where the cells contain numerous infoldings and scattered villi. Adjacent epithelial cells are bound together with the aid of tight junctions (TJs), adherent junctions (AJs) and desmosomes. Globular macrophages, dendritic cells and fibroblasts are found throughout the stroma. The epiplexus cells are located on the apical surface of the epithelial cells.

Tight junctions (TJs), adherens junctions (AJs) and desmosomes in the lateral surface of the CPEC are involved in the linkage of cells. TJs are concentrated in a short length of several complex strands close to the CSF (apical side) and play an important role to physically restrict the movement of hydrophilic molecules to and from the CSF via the paracellular route between the cells, contributing to the blood–CSF barrier (BCSFB). Thus, the movement of polar molecules is limited by the lipid nature of cell walls of the CP and by the TJs. On the other hand, fenestrated capillaries derived from choroidal arteries are surrounded by connective tissue composed by fibroblasts and immune cells. Unlike the capillaries of the cerebral circulation, the capillaries in CP are large and sealed with thin diaphragms, thus providing a little resistance to the movement of small hydrophilic molecules and ions, and allowing the easy passage of other small molecules (e.g., nutrients, vitamins, etc.) into the interstitial fluid (ISF) of the CP (reviewed by Mortazavi et al. (2014), Damkier et al. (2013), Redzic and Segal (2004)). This branched structure is formed by numerous microvilli projected into the ventricles of the brain, providing a large contact surface area between CP epithelium and the CSF on one side, and epithelium and the CP ISF on the other, allowing the exchange of substances between the blood and the CSF (reviewed by Skipor and Thiery (2008) and Redzic (2013)).

CPEC has a large central spherical nucleus with abundant cytoplasm. In addition, there is a large mitochondrial content throughout the cytoplasm, needed to maintain the high cellular respiratory metabolism and energy requirements, a Golgi apparatus located laterally and toward the ventricular lumen, and smooth endoplasmic reticulum and clear vesicles, which are typical features of secretory cells. It was also demonstrated the presence of adrenergic, cholinergic, peptidergic and serotoninergic nerve fibres innervating the blood vessels and the epithelium (reviewed by Emerich et al. (2005), Damkier et al. (2013)).

1.3. Biological functions of the choroid plexus

The CP plays crucial roles in processes that establish, survey and maintain the biochemical and cellular status of the CNS under both normal and pathological conditions. Among these functions, stands out the CSF production and secretion (Wright, 1978), brain defence (Ghersi-Egea and Strazielle, 2001, Engelhardt et al., 2001, Zheng et al., 1991, Gonzalez et al., 2011), and production and secretion of polypeptides (reviewed by Chodobski and Szmydynger-Chodobska (2001)). Regarding the defence function, the BCSFB located at the CP epithelium regulates the paracellular diffusion of hydrophilic solutes and transcellular diffusion of lipophilic compounds from circulating plasma into extracellular fluids of the brain, and exclude xenobiotics. Thus, it provides the controlled environment required for optimal CNS function (reviewed by Redzic (2011)). Besides the BCSFB, CP is capable to monitor the CSF for the presence of noxious compounds, absorb and remove xenobiotics and endogenous waste products from the CSF to the circulating blood (Breen et al., 2002). Additionally, the CP is also involved in the neuro-immune system (Engelhardt et al., 2001), where it promotes a surveillance mechanism defending against blood-borne pathogens. It also appears to assist in recovery processes by secreting neuroprotective compounds (reviewed by Chodobski and Szmydynger-Chodobska (2001)). In addition, there are several transport systems in CP that control the entry of nutrients (Spector and Lorenzo, 1975, Segal, 2001) and also facilitate the elimination of xenobiotics and endogenous waste products from the CSF to the circulating blood (Crossgrove et al., 2005). It has also been described its function in neurogenesis, suggesting an important role in cellular repair and replacement in the CNS (Li et al., 2002).

1.3.1. Production and secretion of polypeptides

Choroidal epithelium is not only a target but also a major source of biologically active compounds in the CNS. mRNA and/or protein for a number of neuropeptides, growth factors, hormones and its receptors, and cytokines (Table 1) have been identified in the CP. Different hormone receptors mediate the action of hormones on the CP, affecting the local metabolism of the tissue and CSF secretion/composition (reviewed by Santos et al. (2011), Chodobski and Szmydynger-Chodobska (2001)).

In addition to synthesize the mentioned polypeptides and other molecules, the CP also secretes them into the CSF fluid to establish and maintain baseline levels of the extracellular milieu throughout the CNS (Johanson et al., 1999). Since the receptors for some of these polypeptides are localized in CP, this organ plays an integral role in autocrine/paracrine signaling. On the other hand, the release of these substances into the CSF allows CP to exert distal/endocrine-like effects on target cells in the brain and spinal cord due to bulk flow of this fluid (reviewed by Chodobski and Szmydynger-Chodobska (2001)).

The CP plays a key role in enzymatic processing and/or degradation of CSF-derived or CP-born polypeptides, through peptidases expressed on the apical surface of epithelial cells, and is also strategically positioned to ensure the CSF clearance of peptide degradation products and presumably noxious polypeptides, such as ß-amyloid protein (Aß). Thus, CP may play an important role in the pathogenesis of Alzheimer's disease (AD) (reviewed by Krzyzanowska and Carro (2012)).

Table 1 - Polypeptides and hormone receptors synthesized by CP (reviewed by Chodobski and Szmydynger-Chodobska (2001))

Adrenomedullin	Interleukin-6
Angiotensin II	Leptin
Angiotensin-converting enzyme	Megalin
Apolipoprotein E	Metallothioneins-2,3
Apolipoprotein J/clusterin	Nerve growth factor
Arginine	Neurotrophin-3,4
ß-Amyloid precursor protein	Prolactin
Brain-derived neurotrophic factor	Prostaglandin D synthase
Cystatin C	Transferrin
Endothelin-1	Transforming growth factor-α
Fibroblast growth factor	Transforming growth factor-ß
GD-15	Transforming growth factor-ß1-3
GDNF	Transthyretin
Gelsolin	Tumor necrosis factor-α
Growth hormone	Vascular endothelial growth factor
Hepatocyte growth factor	Vasopressin
Insulin	Insulin-like growth factor II
Insulin-degrading enzyme	Interleukin-1ß
Insulin-like growth factor binding protein 2–6	

1.4. Age-related changes of choroid plexus and Alzheimer's Disease

The CP tissue is subjected to various external factors and alters through structural and physiological changes during aging and pathological processes such AD. Aging of the CP is characterized by: (i) CPEC flattening, (ii) thicken of the basement membranes of both endothelial and epithelial cells and of the underlying connective tissue, and (iii) increasing of intracellular inclusions of both lipid byproducts and protein tangles. All of these age-related CP abnormalities are also observed in AD, although greatly enhanced (Serot et al., 2000). According to Serot *et al.*, these morphological modifications imply problematic effects on brain functions, in part due to compromised CSF depuration capacities (Serot et al., 2003).

Furthermore, the decreased rate of CSF secretion by CP and reduced drainage, leads to age-dependent increase in CSF/plasma concentration ratios of many compounds normally removed from the brain by CSF drainage (Garton et al., 1991). In literature reviewed by Serot *et al.,* and Kryzanowska *et al.*, these modifications will probably impair CP functions including synthesis, secretion and transport of proteins and other molecules. Therefore, CP dysfunction might be linked to an ineffective metabolism and clearance of AB, which is one of the principal late-onset AD features, since CP synthesizes and secretes several proteins involved in the regulation of AB brain levels, as will be discussed forward (reviewed by Serot et al. (2003), Krzyzanowska and Carro (2012)).

1.5. Metabolism/clearance mechanisms of Aß through the choroid plexus

Amyloid precursor protein (APP) is processed by the sequential action of ß- and γ-secretase, generating the Aß peptide (~4kDa). Depending on the site of y-secretase cleavage, Aß have 38 to 43 amino acids (Figure 3) (Haass et al., 1992). There are two major species: Aß40 (sAß – soluble Aß) and Aß42 (insoluble Aß). Aß40 is the major species produced and corresponds to 90% of the total Aß peptide; Aß42 is the minor species produced but is more prone to aggregation due to two additional hydrophobic amino acids, and it is also the predominant species accumulated in AD brain plaques. Aß is normally produced by cells under physiological conditions (Shirwany et al., 2007) and is present in the CSF and ISF of both normal and AD brains (Tapiola et al., 2000). In soluble form, Aß is known to bind several circulatory proteins like transthyretin (TTR), apolipoprotein J (ApoJ), among others, which have the ability to degrade Aß peptide (Figure 3) (Schwarzman et al., 1994, Bell et al., 2007, Costa et al., 2008a). In AD, Aß is accumulated inside CP epithelia (Gonzalez-Marrero et al., 2015, Vargas et al., 2010b).

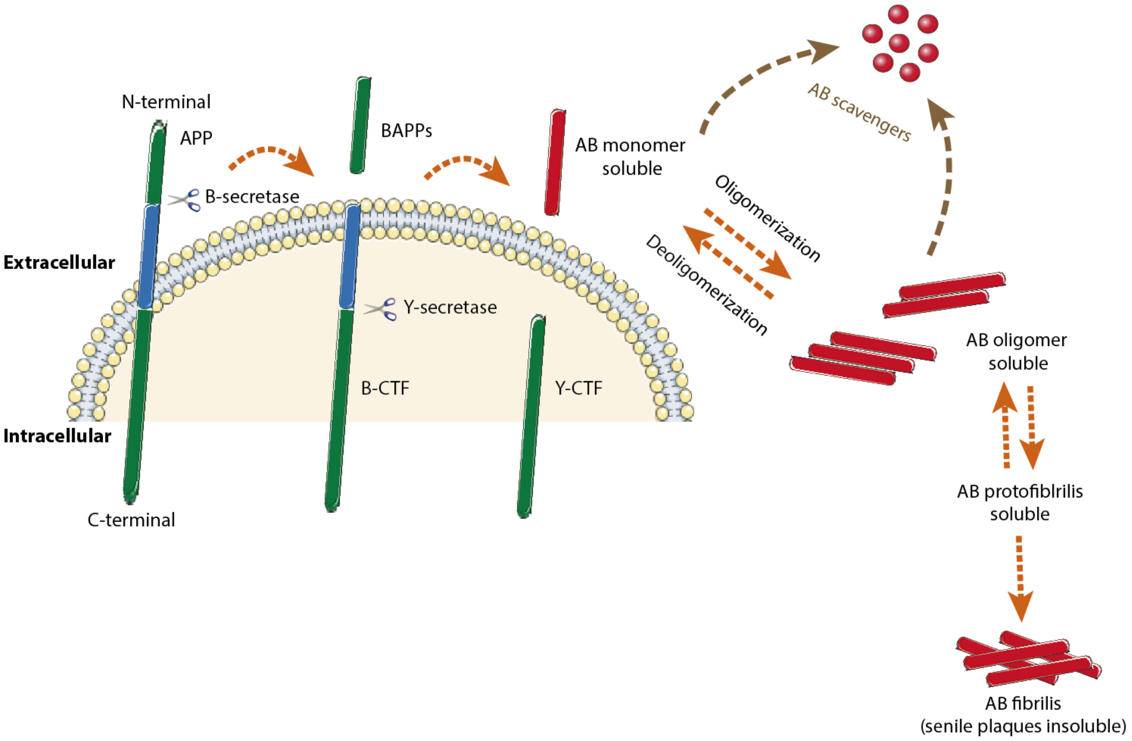


Figure 3 - Illustrative pathway of Aβ formation and metabolism in the brain. Aβ is produced from APP, large integral membrane glycoproteins, by sequential cleavage involving β -secretase and γ-secretase, and released into the extracellular spaces and, in part, to the intravesicular side. The released Aβ undergoes proteolytic degradation by the action of Aβ scavengers, but some Aβ that escapes to degradation, is able to aggregate and form fibrils.

Brain accumulation of Aβ is associated to overproduction, reduction or inadequate metabolic clearance, or an improper balance of Aβ import and export (Mawuenyega et al., 2010, Selkoe, 2000), and it occurs in both normal aging and in AD brains (Silverberg et al., 2010, Selkoe, 2000). Moreover, the correct balance between synthesis and rate of Aβ clearance appears to be directly related to the development of AD, and thus the formation of Aβ plaques would accelerate the onset of the disease symptoms (Bateman et al., 2006). Therefore, degradation of Aβ in CNS could play an important role in its clearance.

The 10-fold Aβ permeability and the greater surface area of CP compared to blood-brain barrier (BBB) allow CP to sequester five times more Aβ than BBB (Strazielle et al., 2000, Crossgrove et al., 2005). In AD brains, Aβ concentration is increased in the CP, and at the same time the expression of Aβ transporters in CP is altered, which consequently decreases Aβ efflux and/or degradation. Crossgrove *et al.*, demonstrates that the CP sequesters Aβ from CSF (Crossgrove et al., 2005). The group showed that Aβ accumulation by BCSFB is because (i) CP takes up the intact Aβ species, (ii) Aβ uptake into CP occurs rapidly and by a non-diffusional uptake process, (iii) CP has a large Aβ storage capacity, (iv) Aβ uptake by the CP does not require several proteins with suggested roles in Aβ binding or transport, and (v) Aβ uptake by CP favors its efflux rather than its influx from blood to the CSF. They also proved that CP may metabolize Aß into smaller fragments following initial uptake, preventing the formation of ß-amyloid plaques. To this, CP has the capacity to remove and degrade Aß, contributing to its clearance from the CSF (Crossgrove et al., 2005) through the expression of various proteins mRNA, some of which mediate cellular clearance of Aß (Rogeberg et al., 2014). Importantly, loss of this Aß clearance route may be a determining factor to the increase of CP Aß seen in AD (Pascale et al., 2011).

1.6. Aß scavengers and choroid plexus

Identification of peptidases involved in Aß metabolism/clearance *in vivo* is important for the development of therapeutic agents for prevention and treatment of AD (Oba et al., 2005). TTR, gelsolin (GLS), ApoJ, metallothionein 2 (MT2), angiotensin-converting enzyme (ACE) and insulin-degrading enzyme (IDE) are some of proteins responsible for degrading Aß peptide.

1.6.1. TTR

TTR, also known as prealbumin, is a homotetrameric protein with 55 kDa (Schreiber et al., 1990). TTR is synthesized by the CP which secretes it to the CSF (Herbert et al., 1986, Dickson et al., 1986), and by the liver that secretes it to blood (Herbert et al., 1986). TTR is the main thyroid hormone carrier protein in the brain, binding about 80% of CSF thyroxine (T4) (Schreiber et al., 1990), and also binds to retinol binding protein (RBP) (Yamamoto et al., 1997) thereby participating in delivery of retinol to target cells in the brain. Within the CNS, TTR is the major protein synthesized by CP (Herbert et al., 1986, Dickson et al., 1986), representing 25% of all CSF proteins secreted by CP.

Although TTR is primarily known as a transporter, there is evidence that TTR can also act as a neuroprotective molecule against AD. The protective effect of TTR against Aß toxicity has been shown in *in vitro* and *in vivo* studies (Schwarzman et al., 1994, Yang et al., 2013, Stein and Johnson, 2002, Costa et al., 2008a, Costa et al., 2008b). Aß overproduction seems to induce TTR expression, with TTR forming stable complexes with Aß *in vitro*, sequestering it, and thus preventing its aggregation and further amyloid formation (Schwarzman et al., 1994, Stein and Johnson, 2002). Also, TTR binds to different Aß species namely soluble, oligomeric and fibrillar Aß (Costa et al., 2008a, Costa et al., 2008b). Moreover, TTR is able to cleave full-length Aß, generating smaller peptides with lower amyloidogenic properties, and also might degrade aggregated forms of Aß peptides (Costa et al., 2008a), thereby inhibiting brain amyloid deposition, and then possibly preventing the onset and/or progression of AD.

The interaction between TTR and Aß occurs through two residues (L82 and L110) identified on TTR (Du et al., 2012). The loss of these binding residues results in reduced TTR ability to bind Aß, which compromises inhibition of Aß aggregation and toxicity (Yang et al., 2013). Additionally, although tetrameric structure is necessary for the protein's transport functions, TTR monomers bind more Aß than tetramers. Actually, it was reported that the presence of soluble toxic Aß oligomers triggers TTR tetramers destabilization, allowing TTR monomers to bind Aß and "scavenge" these toxic oligomers. It has already been reported that TTR concentration decreases in CSF along with age (Zhang et al., 2005, Chen et al., 2005) and in the CSF of AD patients (Serot et al., 1997). In short, inverse correlation between decreased TTR levels in CSF of AD patients and abundance of senile plaques, point to an important role of this protein in AD.

1.6.2. GLS

GLS, with ~90 kDa (Chauhan et al., 1999), is a major actin-binding protein responsible for the limitation of actin filament's growing end, stimulation of its nucleation, and separation of actin filaments. Thus, it plays an important role in normal physiological actin assembly as well as in disease conditions, like cancer, amyloidosis, and AD (Sun et al., 1999). GLS is constitutively expressed in the whole CNS (Tanaka and Sobue, 1994). It is present in all nervous system cell types, including neurons and CP (Matsumoto et al., 2003, Vargas et al., 2010a). GLS can be found as an intrinsic cytoplasmic and secreted protein in plasma and CSF (Paunio et al., 1994). The CP is responsible for the presence of GLS in CSF (Matsumoto et al., 2003).

The secretory form of GLS is known to bind Aß under normal physiological conditions (Chauhan et al., 1999, Qiao et al., 2005, Ji et al., 2008), and moreover, can sequester it and inhibit its fibrillation, and also reduce amyloid load through defibrillation of preformed peptide fibrils (Ray et al., 2000). An *in vitro* study performed by Chauchan *et al.,* showed that GLS inhibited the fibril formation of both Aß40 and Aß42 by more than 90% than observed for Aßs in the absence of GLS (Chauhan et al., 1999). GLS acts as anti-amyloidogenic agent both in plasma and CSF and therefore the failure of Aß sequestration by GLS and imbalance of its levels in CSF/plasma of AD subjects may lead to amyloid formation in AD. GLS also shows the ability to reduce the amyloid burden in a transgenic mouse model of AD (Hirko et al., 2007).

Then GLS might have a protective role in the AD pathology through the regulation of brain Aß levels and consequently avoid its neurotoxic effects. In addition, neuroprotection conferred by GLS against Aß42-induced cytoskeletal alterations, associated to proteolytic degradation of the tight junction-associated protein *zonula occludens* 1 (ZO-1), was showed in CPEC by

(Vargas et al., 2010a). In the same study, a relation between fibrillar Aß42 and CPEC cytoskeletal actin alterations was found, as reflected by an increase in the F-actin content. The F-actin, structure characterized by aggregation of polymerized actin is found in AD brains and its levels are increased in hippocampal neurons treated with Aß (Mendoza-Naranjo et al., 2007). Once again, GLS protected CPEC against AB42 effects. Thus, GLS contributes to the maintenance of the CP monolayer and the blood-CSF barrier integrity, preventing and diminishing Aß toxic effects.

Besides Aß degrading, GLS also plays a role against its oxidative damage. In an experimental study (Ji et al., 2008) where PC12 cells were treated with hydrogen peroxide (H_2O_2), an increase in lipid peroxidation and in oxidative stress was observed. In those cells, GLS expression was increased in a concentration-dependent manner in response to oxidative stress, revealing that GLS may have antioxidant properties.

Qiao *et al.* reported that cytoplasmic GLS can effectively inhibit Aß-induced cell death at a point concomitant with, or upstream to, the mitochondrial events (Qiao et al., 2005). Cytoplasmic form of GLS is associated to the mitochondria, where it can inhibit Aß-induced loss of mitochondrial membrane potential, cytochrome c releasing, and regulates voltage-dependent channels, resulting in the lack of activation of caspases-3, -8, and -9 (Koya et al., 2000). Aß accumulation in CP, the existence of a link between Aß-induced CP cell death, increased production of nitric oxide (NO), and mitochondrial dysfunction in the CP of patients with AD and APP/PS1 mice (Vargas et al., 2010b) has been described. Using CP epithelial cells, it was demonstrated an alteration of the enzyme activity of the respiratory chain complex IV induced by Aß (Vargas et al., 2010a). In this experimental study, GLS reduced brain Aß burden in the APP/Ps1mice, accompanied by inhibition of NO production and cell death, in CP. The group also showed that GLS prevents Aß-induced cell death and NO production in CP cell cultures.

1.6.3. ApoJ

ApoJ or clusterin (encoded by the CLU gene), one of the major CNS apolipoproteins, is a component of plasma and CSF lipoprotein particles (Holtzman et al., 1999). It is a 75-85 kDa glycoprotein that circulates as a disulphide linked heterodimer component of lipid poor in HDL and VLDL, which serves both as a lipid-transport protein with the ability to induce cholesterol efflux, and a molecular chaperone in the cellular stress response. Lower mass versions of ApoJ (~49–60 kDa) are present in the cytosol and nucleus, particularly during cellular stress (reviewed by Elliott et al. (2010)).

By binding to specific receptors, ApoJ controls the availability and clearance of lipids contained in lipoprotein particles by various cells and tissues (Kounnas et al., 1995, Reddy et al., 1996). Besides CP tissue, ApoJ in brain is expressed primarily by astrocytes but also by pyramidal neurons of the hippocampus and Purkinje neurons in the cerebellum (Pasinetti et al., 1994).

ApoJ is a carrier protein for Aß in biological fluids (CSF and blood) (Bell et al., 2007, Hammad et al., 1997), and plays an anti-amyloidogenic role by binding to Aß, thus influencing its clearance across BBB, metabolism and aggregation (Oda et al., 1995). Moreover, ApoJ has been shown to retard the formation of Aß aggregates and may therefore act to maintain Aß in a soluble form and prevent it from forming pathological fibrils (Oda et al., 1994). Furthermore, the ApoJ/Aß interaction has a cytoprotective effect. Relatively to its expression in aging and AD, ApoJ levels were found increased (Ishikawa et al., 2006, Lidstrom et al., 1998).

It has been reported that megalin/LRP2/pg330 is an endocytic receptor for ApoJ (Kounnas et al., 1995) expressed by cells in contact with CSF (CP and ependymal cells) (Kounnas et al., 1994), where it participates in transport processes between the vascular system, CSF and brain tissue. According to Ammar and Closset, ApoJ provides an example of LRP2 regulation by its own ligand, thus increasing LRP2 mRNA and protein expression (Ammar and Closset, 2008).

Megalin, an endocytic receptor for ApoJ, have been implicated in efflux of ApoJ out of the CNS, and Aß binding to ApoJ may enhance clearance of highly pathogenic Aß42 (Hammad et al., 1997, Bell et al., 2007). Megalin-mediated endocytosis of ApoJ/Aß leads to Aß lysosomal degradation (Oda et al., 1995). Hammad *et al.* also indicated that LRP2 is required for ApoJ efflux at BBB (Hammad et al., 1997). According the group, Aß42 binding to ApoJ accelerates BBB Aß42 clearance rate by 83%, which again requires megalin.

1.6.4. MT2

Metallothionein (MT) is a generic name for small (6–7 kDa), heterogeneous, and non-enzymatic proteins, that are rich in cysteine (up to 30%), giving them a high capacity to bind heavy metal ions such as zinc (Zn), cadmium (Cd), copper (Cu), silver (Ag), and mercury (Hg), among others, in biological systems. Presently, fourth MT isoforms have been described: MT1, MT2, MT3, MT4 (reviewed by Santos et al. (2012)). Particularly in brain, MT1 and MT2 isoforms are referred to MT1/MT2 because they are very similar and share many structural and functional properties (Thirumoorthy et al., 2011).

The MT2 (encoded by MT2-A gene) is widely expressed in all organs, including the CNS. It has been found in neurons, epithelial cells of CP and CSF (Goncalves et al., 2008, Chung et al., 2004). Within the CNS, it has been reported that MT1/MT2 is involved in neuroprotection and neurodegeneration, pro-apoptotic mechanisms inhibition, cell survival enhancement, tissue regeneration, maintenance of metal homeostasis, and free radical scavenging (reviewed by Carpene et al. (2007)). MT overexpression has a neuroprotective role during CNS pathological conditions (Penkowa et al., 2002).

Taking into account that CP cells synthesise MT, the MT2/TTR and MT3/TTR interactions were detected *in vivo* and *in vitro* (Goncalves et al., 2008, Martinho et al., 2010). According to Martinho *et al.*, when TTR interact with MT2 its capacity to bind Aß is diminished while MT3/TTR interaction increases the ability of TTR to interact with Aß peptide (Martinho et al., 2010). In AD and old brains, the MT2 expression is increased (Zambenedetti et al., 1998, Hidalgo et al., 2006) which results in a less efficient Aß clearance, since the interaction MT2/TTR decreases TTR/Aß interaction rate.

1.6.5. ACE

ACE, expressed in blood vessels throughout the body, is a carboxyterminal dipeptidyl peptidase (Del Vecchio and Smith, 1981). It is an acidic glycoprotein (~150 kDa) (Wright and Harding, 1992), that catalyses the conversion of angiotensin I to the potent vasoconstrictor angiotensin II (de Lannoy et al., 2001). Thus, ACE is really important for the regulation of fluid homeostasis and blood pressure (Reid, 1992). This enzyme is a widely distributed peptidase, and within the CNS is already been found expressed in CP (Arregui and Iversen, 1978). The ACE active site is located in the extracellular space, and its unbound form circulates in biological fluids, such as plasma and CSF (Zubenko et al., 1985).

ACE has large homologous active domains: N-domain and C-domain (Soubrier et al., 1988). Oba *et al.,* demonstrated that N-domain inhibits Aß aggregation and cytotoxicity *in vitro* by the conversion of Aß42 to Aß40, whereas the angiotensin-converting activity is found predominantly in the C-domain (Oba et al., 2005). ACE has been associated with AD, since CSF of patients with moderate degrees of senile dementia exhibited about half of the ACE activity compared with age and sex-matched control individuals (Konings et al., 1993). *In vitro,* this protein was found to significantly degrade Aß, to retard Aß fibril formation and deposition, and to inhibit cytotoxicity induced by Aß (Zou et al., 2007, Hu et al., 2001), through Aß40 cleavage (Hu et al., 2001). Chronic inhibition of ACE enhances predominant Aß42 deposition *in vivo* (Zou et al., *2007).* In this way, ACE affects susceptibility to AD by degrading Aß and preventing the accumulation of amyloid plaques in brains of AD patients (Hu et al., 2001).

1.6.6. IDE

IDE or insulysin is a ~110 kDa zinc-containing metalloendopeptidase (Qiu et al., 1998) known by its extensively participation on the clearance of Aß (Crossgrove et al., 2007, Behl et al., 2009, McDermott and Gibson, 1997, Qiu et al., 1998), glucagon (Duckworth and Kitabchi, 1974), insulin-like growth factor 1 and 2 (Roth et al., 1984), atrial natriuretic peptide (Ralat et al., 2011), bradykinin (Malito et al., 2008), endorphin (Safavi et al., 1996), insulin (Duckworth and Kitabchi, 1974), and amylin (Bennett et al., 2000). In this manner, the common features of IDE substrates include hydrophobicity and substrate's ability to aggregate and form fibrils (amyloidogenic). This metalloendopeptidase is expressed by cortical and subcortical neurons, and has been detected in the cytoplasm of endothelial cells, pericytes smooth muscle cells (Gao et al., 2004, Dorfman et al., 2010) and in human CSF of both normal and Alzheimer's patients. In addition, IDE is also expressed in the CP apical surface (Behl et al., 2009).

IDE plays an important role in regulating extracellular Aß levels, by hydrolysing several peptide bonds of both Aß40 and Aß42 into less neurotoxic fragments (Bora and Prabhakar, 2010). Diminished activity and levels of IDE in AD brains and aging (Miners et al., 2009) has been giving support to the hypothesis that IDE reduced activity and levels compromise Aß degradation in brain. On the other hand, IDE overexpression reduces Aß levels and retards, or completely prevents, brain amyloid plaque formation (Leissring et al., 2003, Farris et al., 2004). In IDE-/- animals, Farris et al. showed elevated cerebral Aß, which validates the IDE role in AB proteolysis *in vivo* (Farris et al., 2003).

1.7. Regulation of CP functions

The choroidal tissue's functions are subject to neurogenic and endocrine regulatory mechanisms. The neurogenic regulatory mechanism comprises the sympathetic, cholinergic and peptidergic innervations while endocrine system consists of 5-hydroxytryptamine, melatonin, atrial natriuretic peptide, vasopressin, insulin and insulin-like growth factors, glucocorticoid hormones, thyroid and sex hormones, all occurring in CP. Both mechanisms regulate blood flow, and CSF production and the secretory functions of CP (reviewed by Nilsson et al. (1992)).

In this work, special attention is given to sex hormones (SHs).

1.7.1. Sex hormones

SHs, potent regulators of neural function from normal development to neural injury and aging, play a major role in the maintenance of CNS balance and function during life, affecting and modulating several actions. The biosynthesis of these hormones is not restricted to adrenal and gonadal glands but also occurs in nervous system, called neurosteroidogenesis, where act through specific receptors throughout various brain regions (Corpechot et al., 1981).

Aging and AD are often associated with SHs levels decline. The loss or depletion of SHs, with particular impact in women, whose have a rapid and accentuated decrease in both estrogens and progesterone levels. Men are also affected although in a gradual manner. For example, men dehydroepiandrosterone (DHEA) levels by the age of 80 are about 20% of those at age 20 (reviewed by Barron and Pike (2012), Bates et al. (2005)). Clinically, the increased risk of AD in women may be associated with the precipitous loss of estrogens and progesterone at menopause, which is much more abrupt than testosterone loss by men during aging (Yue et al., 2005, Ruitenberg et al., 2001). It is important to highlight that testosterone protection acts through both estrogen (via aromatase conversion) and androgen (either directly or after 5α-reductase conversion to dihydrotestosterone (DHT) pathways (reviewed by Aloisi and Bonifazi (2006), Garcia-Segura et al. (2001)).

In literature review Barron *et al.*, showed that SHs have many beneficial and protective actions in brain, with potential relevance to AD. Among the neuroprotective effects stands out the increase of spine density that favours synaptic plasticity and improve select aspects of cognition and neuron viability that protects neurons against a range of toxic insults including those implicated in AD (Barron and Pike, 2012).

1.7.1.1. The choroid plexus as a target for sex hormones

In addition to peptides synthetized by CP, as previously mentioned, the choroidal tissue also express sex hormone receptors such progesterone (PR) (Quadros et al., 2007), estrogen receptors α and β (ERα and ERβ) (Hong-Goka and Chang, 2004), and androgen receptors (AR) (Alves et al., 2009) which suggest that CP is a target tissue for SH. The expression of both ERα and ERβ in the CP of female and male AD patients when compared to non-AD population, are significantly lower (Hong-Goka and Chang, 2004).

In a study, many mRNA/proteins are differentially expressed in CP in response to SHs. The same experimental study demonstrated that the CP collected from gonadectomized (GDX) female and male rats suffered changes in the expression of genes associated with several pathways, and it is more pronounced in females than in males. Most of these CP expression changes occurred in genes implicated in chemical sensing, metabolism, steroid hormone biosynthesis and circadian rhythm pathways (Quintela et al., 2013).

1.7.1.1.1. Regulation of Aß scavengers by sex hormones

Taken into account the epidemic problematic of AD worldwide, its intrinsic association with imbalanced brain Aß levels, and further uncountable neuroprotective effects of SHs, the regulation of Aß scavengers by these is presented in table 2.

SHs show the ability to increase of Aß scavengers expression is different tissues, namely in brain, such as CP and hippocampus. Therefore, SHs seem to benefit amyloid load reduction, by increasing Aß scavenger's levels, although some contradictory data were reported about ACE.

Table 2 - Aß regulation by SH

Aß scavenger	SHs	Tissue/cell studied	Regulation	Refs
TTR	17ß-estradiol (E2)	Liver, CP	Up regulated	(Goncalves et al., 2008, Quintela et al., 2008, Quintela et al., 2011)
	DHT			
	Progesterone	CP	Up regulate	
GLS	DHT	Adipose tissue	Up regulated	(Bolduc et al., 2004)
ApoJ	Estrogen and progesterone	Endometrial cancer cell lines: KLE and ECC-1	Up regulated	(Won et al., 2012)
MT2	No data available			
ACE	E2	Cerebrocortical cultures *in vivo*	Down regulated	(Jayaraman et al., 2012, Seltzer et al., 1992)
	Progesterone	Cerebrocortical cultures *in vivo*	Up regulated	(Jayaraman et al., 2012)
	E2-only and E2-Progestogen hormone therapy	Serum samples (postmenopausal women)	Down regulated	(Proudler et al., 2003)
IDE	E2	Hippocampal and primary neuron culture	Up regulated	(Zhao et al., 2011)
		Cerebrocortical cultures *in vivo*	Up-regulated	(Jayaraman et al., 2012)
	Progesterone	Cerebrocortical cultures *in vivo*	Up-regulated	

1.7.2. Circadian rhythm

Organisms have developed internal timing systems to adapt to the external day and night cycles. All organisms possess endogenous daily clocks. The periods inherent of clocks, which are not exactly 24h, will then manifest themselves. This is why endogenous daily clocks are also called circadian clocks (Latin: *circa diem*, ‘‘approximately one day’’). *Zeitgebers* (e.g., light) synchronize the circadian clocks to a period of exactly 24h in the natural environment. Indeed, the circadian clock is closely related to the everyday human lives. The principal stimulus to circadian clocks is the cycle of light and darkness. Briefly, the contact of a free-running individual to light, around the time of expected nightfall and dawn, can delay and advance the clock, respectively, whereas light presented during the expected day is unsuccessful. In the case of animals, they are frequently nocturnal either diurnal in their habits (reviewed by Hastings and Maywood (2000), Kwon et al. (2011) and Helfrich-Forster (2004)).

1.7.2.1. Concept of circadian rhythm: location and components

In mammals, the brain is involved in the control and coordination of circadian rhythms. Circadian rhythms, present in almost all mammal tissues, are generated by an endogenous biological clock located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Figure 4). The SCN (“central clock”) is the core of the circadian system and peripheral oscillators, and contains approximately 10 000 neurons in mice and about 50 000 neurons in humans. In addition to controlling daily rhythms, the SCN is equally essential for the synchronization of an organism’s internal circadian timing to the external environmental world, integrating internal and external signals. Nuclei are strategically located for receiving visual input for light-dark entrainment through both direct and indirect retina-to-SCN pathways. The major light input pathway to the SCN is the retinohypothalamic tract (RHT), which arises from a widely distributed population of retinal ganglion cells. Circadian oscillators also exist in various areas of the brain outside the SCN such as the pineal gland, pituitary and arcuate nucleus (reviewed by Videnovic et al. (2014), Morin and Allen (2006), Reppert and Weaver (2001), Kwon et al. (2011)). More recently, the study carried out by Quintela *et al.* showed that CP has the capacity to express clock genes that are subjected to rhythmic expression in female and male rats (Quintela et al., 2014).

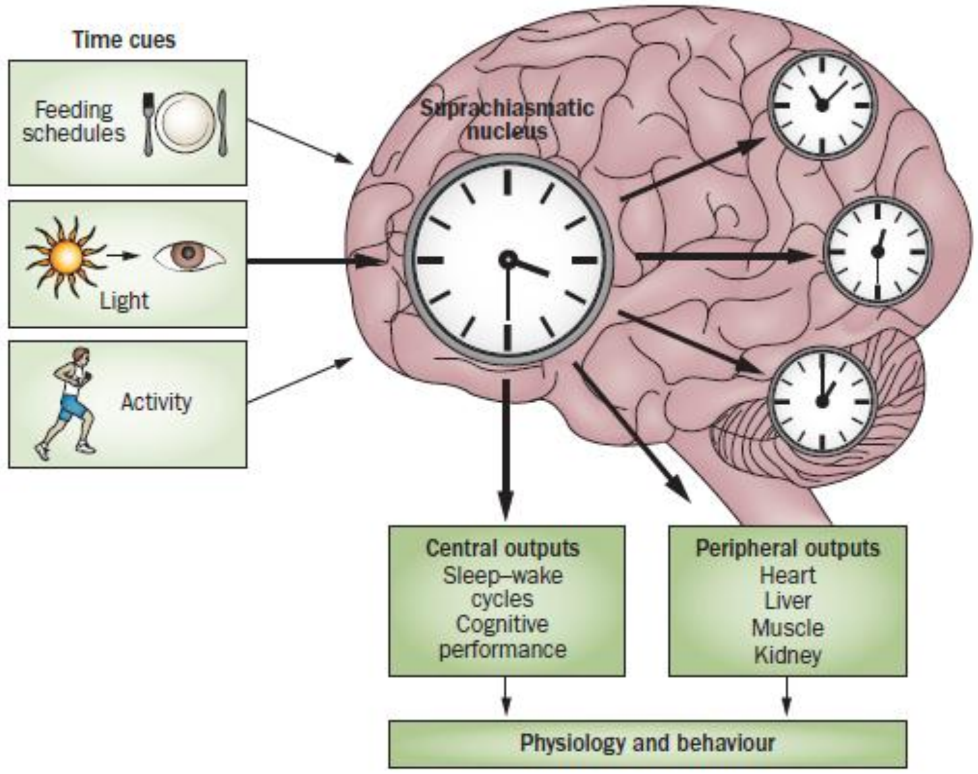


Figure 4 – Illustration of the circadian system. The timing of human biological rhythms is synchronized to the rotation of the Earth, and influenced by numerous external and internal time cues. These stimuli are known as "*zeitgebers*" (germen for 'time giver'). Light is the most important and potent *zeitgeber*. In addition to light, activity, feeding schedules, and the hormone melatonin also influence circadian timing. This synchronization can become disrupted, which eventually leads to misalignment or internal desynchronization. This loss of coordination of circadian rhythms can have negative consequences for sleep–wake cycles and numerous other biological functions (adapted from Videnovic et al. (2014)).

SCN neurons generate rhythmic electrical activity and produce synchronizing signals that control the phases of the oscillations called "peripheral clocks" which are present in liver, heart, lung, kidney, muscle or adipose tissue, skin and eyes. Unlike the central pacemaker, these peripheral oscillators are not directly entrained by light. Peripheral tissues produce rhythmic physiological outputs which are orchestrated by SCN and synchronized with the environment, thus providing optimal activity or response to an organism's needs at the specific time of the day (reviewed by Green et al. (2008), Helfrich-Forster (2004)).

The mechanisms underlying circadian rhythms involve circadian oscillations in gene expression, protein modifications and secretion. These oscillations are controlled by the products of the core circadian clock genes. Despite the differences between central and peripheral clocks, both share the same molecular architecture and capacity to generate sustained circadian rhythms. Nevertheless, one key difference between central and peripheral clocks lies in the degree of their intercellular coupling (reviewed by Partch et al. (2014), Ko and Takahashi (2006)).

1.7.2.2. Molecular basis

Circadian rhythms are regulated by a set of genes, known as core "clock" genes, whose products interact to generate and maintain the rhythms (Figure 5), expressed in many peripheral tissues and in the circadian master clock. Those genes are: *Period* (PER1, PER2 and PER3), circadian locomotor output cycles kaput (CLOCK), aryl hydrocarbon receptor nuclear translocator-like (ARNTL, also known as BMAL1), and plant *Cryptochrome* gene homologues (CRY1 and CRY2). The rhythm is generated by two interconnected molecular feedback loops. In mouse main loop, PER and CRY are described as clock proteins which inhibit the transcription of their own genes after a time delay. Transcription inhibition is accomplished by blocking their transcriptional activators CLOCK and BMAL1. This negative feedback loop leads to rhythmic gene expression and rhythmic protein accumulation, as part of the pre-requisite of rhythmic behaviour. The second feedback loop reinforces the main oscillatory loop through the control of BMAL1 expression in a rhythmic manner. The entrainment of the feedback loops by light, induces transcription of mPER1 and mPER2 genes in mice. So, this *zeitgeber* resets the molecular clocks, leading to its synchronization with the environment. Many metabolic enzymes and metabolism-regulating growth factors and hormones are under the direct control of the circadian clock. When the master clock is injured, the circadian gene expression change in the peripheral tissues, by desynchronization of the peripheral oscillators (reviewed by Videnovic et al. (2014), Helfrich-Forster (2004), Kondratova and Kondratov (2012)).

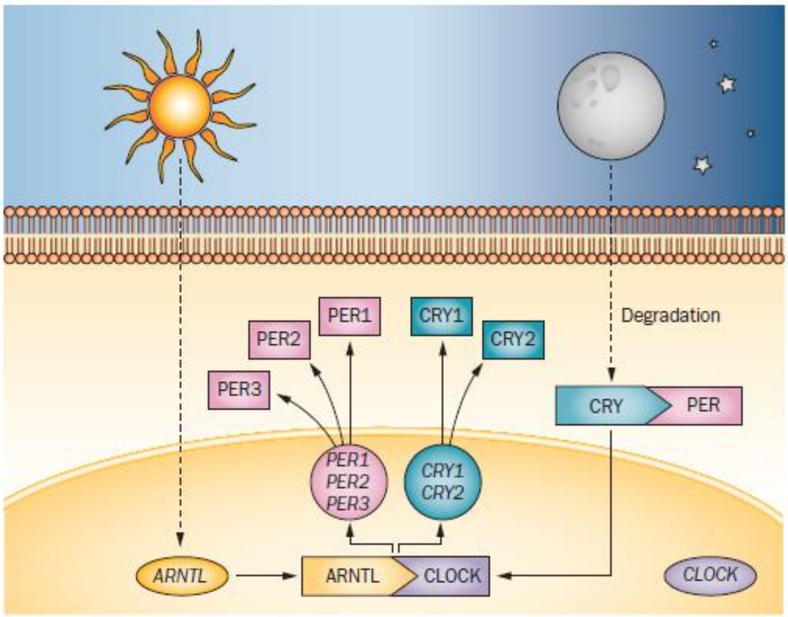


Figure 5 – Molecular organization of the circadian system. PER, CLOCK, ARNTL (also known as BMAL1) and CRY proteins interact to create a self-sustaining negative transcription–translation feedback loop. The CLOCK intracellular level remains steady throughout the 24h period. The ARNTL high level at the

beginning of the day promotes the formation of ARNTL–CLOCK heterodimers, which in turn activate PER and CRY transcription. Since PER accumulates in the cytoplasm, it becomes phosphorylated and degraded by ubiquitylation. CRY accumulates in the cytoplasm late in the subjective day, and translocates to the nucleus to inhibit ARNTL–CLOCK-mediated transcription. At night, the PER–CRY complex is degraded, and the cycle starts again. This feedback loop ensures a high level of ARNTL and low levels of PER and CRY at the beginning of a new circadian day (adapted from Videnovic et al. (2014)).

1.8. Hormonal control of circadian rhythm

Gonadal steroids modify the phase, amplitude and period of circadian rhythms. Sex differences have been identified in almost every aspect of circadian rhythms including free-running rhythms, light-induced phase shifts, sleep and activity patterns, hormonal fluctuations, and rates of re-entrainment. As mentioned, the gonadal hormones (estrogen, progesterone and androgen) control the circadian activity rhythms, and the opposite is also true (Nakamura et al., 2005, Jechura et al., 2000, Iwahana et al., 2008, Quintela et al., 2013). The presence of gonadal receptors in SCN allowing a direct influence of SHs on SCN functions. It seems that these receptors act through different mechanisms in males and females (Figure 6). The SCN has scarce ERs (ERα & ERß), suggesting that estradiol appears to exert its effects on circadian rhythms via extra-SCN sites projecting to the SCN. Therefore, the role of steroids in females is consequently focused primarily on extra-SCN sites of action that are ER-rich and projected to the SCN (De La Iglesia et al., 1999). In contrast, ARs are prevalent in the SCN. AR expression is highly localized in the central retinorecipient “core”, which receives dense retinal input of the SCN (Karatsoreos et al., 2011). As known, the testosterone is widely aromatized to estradiol in the brain, although androgens act directly in the SCN to modulate circadian rhythms. AR activation within SCN cells is in position to impact fast cellular processes, as well as slower responses involving regulation of gene transcription (Karatsoreos et al., 2007). Hormones which act directly in the SCN may alter neural inputs, modify its structure and synaptic connectivity and modulate the responsiveness of structures that receive projections from the SCN, or some combination of these factors (Brockman et al., 2011).

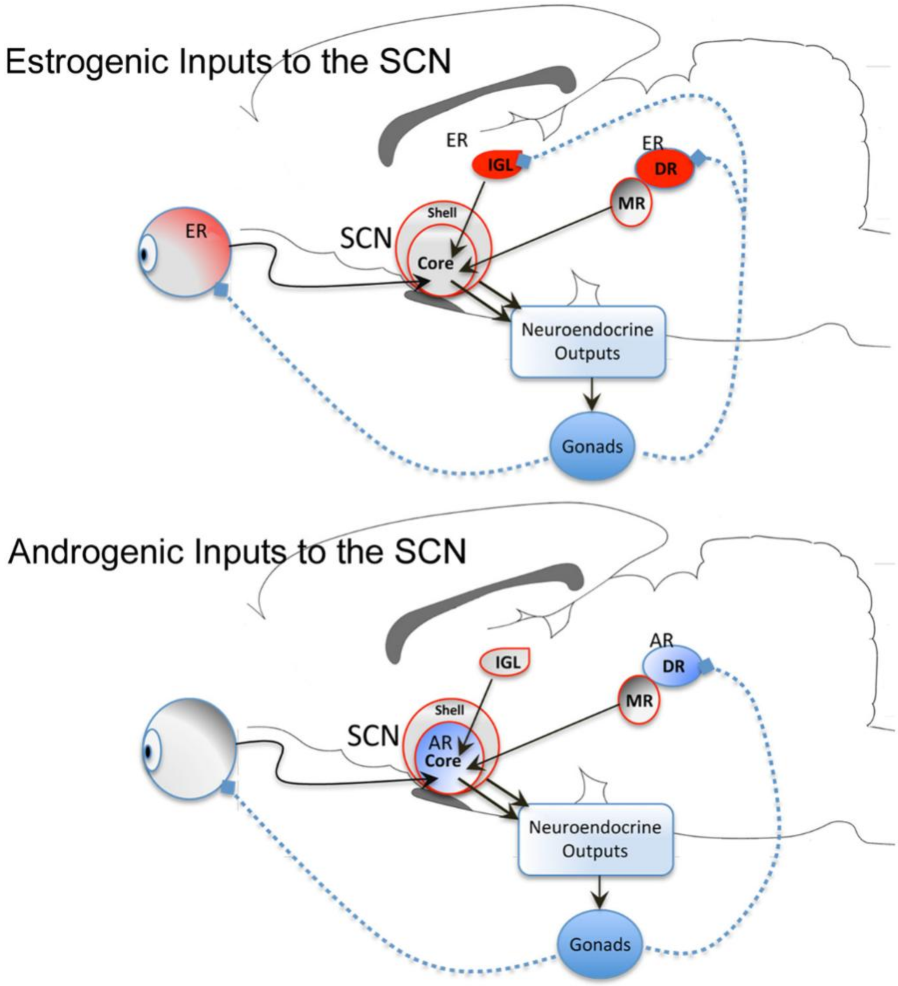


Figure 6 – Differences in action mechanisms of ER and AR in SCN. ER-rich nuclei (red), including the retina, the intergeniculate leaflet (IGL), and the dorsal raphe (DR), via the median raphe (MR), project to the SCN (top). The DR also contains androgen receptors and projects to the SCN. However, unlike the ER, ARs are densely located in the core SCN (bottom). Testosterone can be aromatized into estradiol and thus may have dual androgenic/estrogenic impacts on the system (adapted from Mong et al. (2011)).

In the absence of SHs, males and females show different temporal patterns of activity. The GDX males severely reduced activity at the start of the night, while ovariectomy (OVX) in females had no effect on period or activity (Iwahana et al., 2008). Brockman *et al.* documented that estradiol deficiency during development modulates the expression of circadian and daily rhythms in male and female aromatase knockout mice (Brockman et al., 2011). In this experiment, WT males, but not aromatase knockout (ArKO) males, retained the ability to respond to steroid hormones; the time of activity onset, free running period in constant darkness, and total daily activity were significantly different in GDX compared to intact males. In contrast, gonadectomy did not alter the expression of these variables in ArKO males. ArKO females had a longer free running period in constant darkness compared to WT females regardless of gonadal state. Ovariectomized ArKO females had a significantly late activity onset compared to intact ArKO females and ovariectomized WT females, despite all 3 groups being estrogen deficient. Phase shifts in response to light pulses given at different times of the day shown an interaction between genotype, sex, and circulating steroids. More recently, Butler *et al.* showed that the effects of androgens on circadian clock are attributable to an interaction between hormonal status and circadian parametric responses to light. The effects of androgens are a result of hormonal modulation of photic input and not due to a change in the inherent period of oscillators in the absence of light. Independent of light, androgens alter clock function and the distribution of activity during the night (Butler et al., 2012).

1.9. Circadian rhythm & Alzheimer Disease

Aging of the circadian system contributes to the decline in mental performance of aged brains, but the circadian clock may also be involved in specific age-associated neurodegenerative diseases, such as AD. The disruption of sleep and circadian rhythms is one of the most common and earliest signs of AD; when disease progresses, the abnormalities in the circadian clock and sleep worsen. Deposition of Aß plaques and neurofibrillary tangles in patients with AD means that there is some failure in the systems responsible for the degradation of damaged proteins and other cytoplasmic components. Two major systems responsible for the degradation of cellular proteins are ubiquitin-proteasome-dependent system and autophagy. In patients with AD, the clearance of autophagic Aß-containing vacuoles is defective. The circadian clock is one of the potential systems that have the ability to regulate autophagic activity. Although this process remains unclear, it is suggested a molecular connection between the circadian oscillator and autophagy, once the expression of several genes associated with autophagy rhythmically oscillate in a circadian manner. In the face of all these data, the circadian clock could be a novel treatment of disorders of the aging brain, namely AD (reviewed by Kondratova and Kondratov (2012)).

II. Aim

Aim

It has already been established that several genes of the brain clock machinery are expressed in the CP of female and male rats that may work as a peripheral clock. Since CP synthesizes several peptides and enzymes associated with Aß metabolism/clearance and it is regulated by SHs, the objectives of this project aimed at:

1 - analyse if CP Aß scavengers are influenced by circadian rhythmicity in both female and male Wistar *Han* rats;

2 – evaluate how Aß scavengers respond to an Aß42 stimulus a long day time.

III. Materials and Methods

1. Animals experiments and tissue collection

Animal experiments were carried out in *Wistar Han* rats. Animals were maintained with food and water *ad libitum* under conditions of constant temperature (20 ± 2°C) and 12 hour cycles of artificial light. All were handled in agreement with the National Institute of Health guidelines and the National and European Union rules for the care and handling of laboratory animals (Directive 2010/63/EU).

The figure 7 schematically represents the whole laboratory procedure in CP explants.

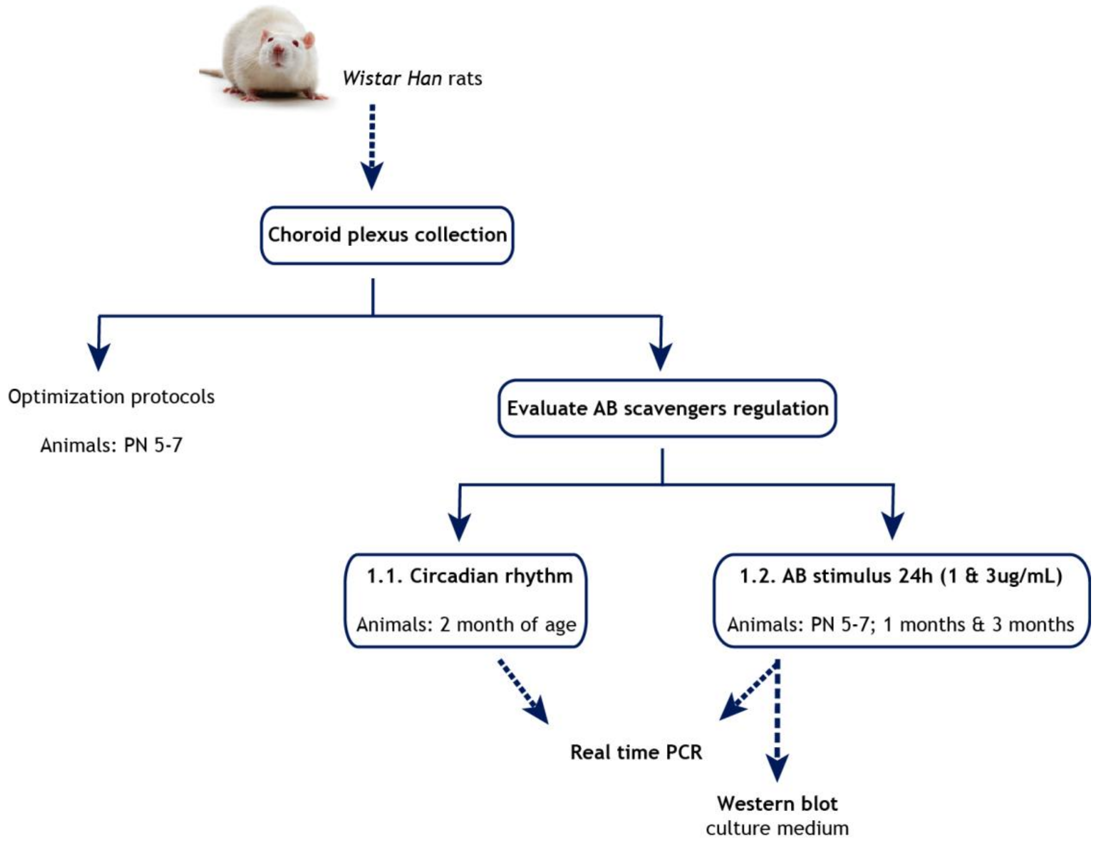


Figure 7 – Schematic illustration of experimental studies performed in order to analyse Aβ scavengers regulation by circadian rhythm and Aβ42 stimulus for 24h in rat choroid plexus. The assessment of the influence of the circadian oscillation in Aβ scavengers expression was carried out in CPs collected from rats with two month of age through real time PCR, while Aβ42 effects in these scavengers were evaluate in CPs from newborns (PN 5-7), young (1 month) and adult male and female rats by real time PCR and Western blot techniques.

In a preliminary approach to assess the expression of Aβ scavengers, CPs were collected from 5 newborn rats (PN 5-7), frozen at -80ºC or fixed in paraformaldehyde (PFA) 4%. The next two experiments were set to evaluate the effect of circadian rhythm and Aß incubations on the expression of several Aß scavengers.

1.1. Circadian rhythm

For assessing the effect of circadian rhythm, the animals (two months-old, 24 females and 24 males; n=6 of both sexes per each hour), were euthanized at 8h, 14h, 20h and 2h corresponding to ZT1, ZT7, ZT13, and ZT19, respectively. Rats euthanized at 20h and 2h were completely deprived of light. CP of lateral ventricles were collected and frozen in liquid nitrogen for future procedures.

1.2. Aß stimulus 24h

Another experiment aimed at evaluating the effect of Aß on the expression of Aß scavengers. For these studies CPs were collected from newborns (PN 5-7), young (1 month), and adult female and male rats (three month of age) and incubated in Dulbecco's Modified Eagle Medium (DMEM) completed with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin for 24h at 37ºC and 5% CO_2 atmosphere, with or without Aß42 (AnaSpec) at 1 or 3 µg/mL (n=6 per each condition). At the end of the stimuli, culture medium and tissues were collected. Culture media was frozen at -20ºC, while CP tissue was frozen in liquid nitrogen.

2. Total RNA extraction

Total RNA (tRNA) was extracted from all CPs collected. All procedures were carried out on ice since RNA is temperature sensitive, and due to the easy enzymatic degradation of RNA it is necessary to use agents which inactivate RNases, like water treated with diethylpyrocarbonate (DEPC).

The extraction of tRNA was carried out by homogenizing each CP in 300 µL TripleXTractor reagent from (TRIzol, Grisp) using a pestle, to allow the separation of the cellular components, followed by an incubation of 5 minutes at room temperature (RT) for complete dissociation of nucleoprotein complexes. Later, 60 µL of chloroform (200 µL chloroform/1 mL TRIzol) was added and tubes were inverted for homogenization. Afterwards, samples were incubated again at RT for 10 minutes and then centrifuged at 4ºC for 15 minutes at 12 000 g.

This centrifugation allowed the solution to separate into three different phases. The aqueous phase (transparent) containing RNA was collected and transferred to a new centrifuge tube and 150 µL of isopropyl (500 µL isopropyl/1 mL TRIzol) was added, followed by homogenization by inversion to enable recovery of RNA by precipitation. Next, samples were incubated for 10 minutes at RT, centrifuged at 4ºC for 10 minutes at 12 000 g, and finally the supernatant was rejected. The RNA, in the form of a white precipitate (pellet), was washed once with 500 µL 75% ethanol in DEPC water (-20°C), centrifuged at 4ºC for 5 minutes 7500 g, and the supernatant was discarded. Finally, excess ethanol was removed and the tRNA pellet was rehydrated in DEPC water and stored at -80°C for later use.

The integrity of tRNA was examined by electrophoresis gel 1% agarose in Tris-acetate-EDTA (TAE) 1x diluted in DEPC water, stained with GreenSafe (NZYTech) for gel visualization on an UVITEC transilluminator (UVitec). The quality of tRNA was confirmed by the presence of two bands — 18S and 28S (the last with the double of the intensity). When RNA is degraded, this proportion might be affected.

In the end, the quantification of tRNA was performed using a nanospectrophotometer Nanophotometer (Implen). Reading from nanospectrophotometer gives directly tRNA concentration (µg/µL) and purity of tRNA by ratio A260/A280. tRNA is considered pure when the ratio is between 1.8 and 2.1. If the ratio is <1.8, tRNA is contaminated with proteins and phenols, while a ratio >2.1 means that tRNA is contaminated with genomic DNA.

3. cDNA synthesis

Complementary DNA (cDNA) synthesis was performed using reverse transcription of mRNA, obtaining an exact copy of expressed genes without introns. The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) was used for this purpose according to fabricant recommendations. For that, in each PCR tube 1 µL of Random Hexamer primer, ~ 500 µg of tRNA extracted previously and sterile water, performing a final volume of 12 µL was added. After incubation at 65ºC for 5 minutes on a thermal cycler (T100 Thermal Cycle, Bio-rad) and immediately cooled on ice, MIX 1 (n+1) of 8 µL per each reaction was prepared with the following components: 4 µL 5x Reaction Buffer, 1 µL RiboLock RNase Inhibitor (20 U/µL), 2 µL 10 mM dNTP and 1 µL RevertAid M-MuLV RT (200 U/µL) and added to the initial mixture. After this step, samples were again incubated on the thermal cycler (T100 Thermal Cycle, Bio-rad) at 25ºC for 5 minutes followed by 42ºC during 50 minutes. The reaction ends at 70ºC for 5 minutes. At the end, cDNA was stored at -20ºC for later use.

4. Conventional PCR

The enzymatic amplification technique of DNA confirmed the expression of TTR, GLS, ApoJ, MT2, ACE and IDE genes in rat CP. For this purpose, Taq Polymerase (NZYtech) was used to amplify DNA fragments, in accordance to the manufacturer's recommendations. After optimization of the PCR reaction the appropriate annealing temperature (AT) for each gene was selected. All PCR reactions were performed in a final volume of 25 µL (23.5 µL "common" MIX (n+1) + 1.5 µL of cDNA). The "common" MIX was composed of components required for the enzymatic amplification and sterile water to a total volume of 23.5 µL. To each reaction 1.5 µL cDNA was added, except for the negative control to which 1.5 µL of sterile water was added. The primer's sequences were selected from studies already performed in rat animals. The sequences were confirmed by the NCBI-BLAST database (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The choice of primers was performed so that the sequences must be in different exons, in order to avoid the risk of amplifying genomic DNA. Amplification was performed on a thermal cycler (T100 Thermal Cycle, Bio-rad). The sequences for each primer, their respective size (bp) and the optimal AT optimized are shown in table 3.

Table 3 - Sequence of primers and its size

Gene	Fragment size (bp)	AT (°C)	Forward primer	Reverse primer
TTR	119	56	GGA CTG ATA TTT GCG TCT GAA GC	ACT TTC ACG GCC ACA TCG AC
GLS	141	58	GGT GCA GAG GCT CTT CCA GG	CTG CCG GAG CCA CAC CAC TG
ApoJ	215	54	CTG ACC CAG CAG TAC AAC GA	AGC TTC ACC ACC ACC TCA GT
MT2	126	57	TGT GCC ACA GAT GGA TCC T	GCA GCC CTG GGA GCA CTT
ACE	97	58	CAG AAT CTA CTC CTC CAC TGG CAA GGT	TCG TGA GGA AGC CAG GAT GT
IDE	125	56	TGG CTG TGG ACG CAC CAA GGA	GGG TGG CGC TTC GGA AAG GT

The amplified products were then visualized on an agarose gel 2% in the presence of GreenSafe (GreenSafe Premium, NzyTech), confirming that each fragment obtained had the expected size by comparison with molecular weight marker GRS Ladder 50bp (Grisp). After visualization on transilluminator, the duly amplified PCR products were sent to the company StabVida (Portugal) for sequencing, and the corresponding sequences of the genes under study were confirmed over again by the NCBI-BLAST database by comparison with the sequences of *Rattus norvegicus* database.

5. TTR, GLS and ApoJ Whole-mount Immunohistochemistry staining

The immunohistochemistry staining method is based on using a primary antibody specific for the protein under study and a secondary antibody linked to a fluorescent molecule. Fluorescence emission occurs when there is interaction between the antibodies.

CPs were collected from Wistar *Han* rats with 5-7 days of age and placed into a 48-well microplate (one CP per well) with PFA 4% for 45 minutes at RT. Subsequently, CPs were incubated overnight with 30% sucrose solution until their deposition on the bottom. After, CPs were incubated in block solution 2.5% bovine serum albumin (BSA) 0.2% Triton X-100 during 4h at RT. CPs were washed in Phosphate buffered saline-Tween 0.01% (PBS-T) and incubated 4h at RT with the primary antibodies diluted in block solution: rabbit anti-TTR (1:200; Dako), rabbit anti-ApoJ (0.5 µg/mL; Abcam) and rabbit anti-GLS (0.5 µg/mL; Abcam). Tissues were washed 6 times in PBS-T and incubated 3h on dark at RT with the secondary antibody Alexa Fluor 488 goat anti-rabbit (1:2000; Invitrogen) also diluted in blocking solution. From this step, all next procedures were performed in the dark. CPs were over again washed with PBS-T before incubation with Hoechst 33342 dye (1:1000; Invitrogen). In the end, tissues were mounted with mounting media (Dako, NZYtech) and visualized on a confocal microscope LSM 710 (Zeiss).

6. Real time PCR

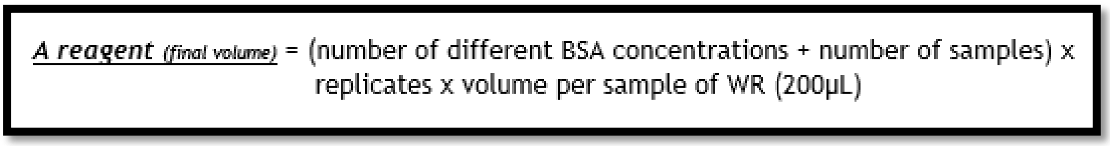
The real time PCR (qPCR) technique allowed the relative quantification of TTR, GLS, ApoJ, MT2, ACE and IDE gene expression, both along the day and in the presence of the stimulus with Aß42. The conditions were optimized to test the efficiency of the reactions for each gene with 4 different dilutions, and the experiments were performed in duplicate. The fluorescence emission of SYBR Green was determined after each cycle, and the relative amount of each mRNA was quantitated using the iCycler software (Bio-Rad). SYBR Green emits fluorescence only when it intercalates in the DNA strain. The emitted fluorescence is proportional to the amount of the product formed. To normalize the levels of gene expression, Cyclophilin A gene was used as an endogenous control. Each reaction had a total volume of 20 µL: MIX and cDNA. MIX was constituted with 10 µL of SYBR Green (Thermo Scientific Maxima SYBR Green/Fluorescein qPCR Master Mix (2x)), 0.8 µL of 5 µmol Forward and Reverse primers, and sterile water. The amplification conditions used were: 95ºC for 3 minutes, 40 cycles of 95ºC for 15 seconds, optimizing annealing temperature of each primer for 30 seconds and 72ºC for 30 seconds in a thermocycler iQ5 (Bio-Rad). The data were processed using the ΔΔCt method (Livak and Schmittgen, 2001).

The primer efficiency tests were: TTR gene was tested with 1 µL cDNA diluted 1:1 (stock), 1:5, 1:25 and 1:125 at a temperature of 56°C, 40 cycles; GLS and ApoJ genes were optimized at 58ºC 40 cycles with 2 µL of cDNA diluted 1:1 (stock), 1:2, 1:4 and 1:8; primer efficiency for MT2 gene was tested with 2 µL of cDNA diluted 1:1 (stock), 1:5, 1:10; 1:25 at 60ºC 40 cycles; ACE primer efficiency was adjusted for 1.5 µL of cDNA diluted 1:1 (stock), 1:5, 1:25; 1:125 40 cycles at 60ºC; finally, IDE primers efficiency was established with 1 µL of cDNA at dilutions 1:1 (stock), 1:5; 1:25; 1:125 40 cycles at 56ºC.

7. Protein extraction and quantification

The CPs were digested with a pestle in RIPA lysis buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10mM PMSF (phenylmethanesulfonylfuoride) and protein koktail (Roche)). Next, the samples were centrifuged at 10 000 g for 10 minutes, and the supernatant containing protein was collected and stored at -20ºC.

The quantification of proteins was performed in a 96-well plate using BCA protein assay kit (Pierce, Thermo Scientific). The first stage of the procedure is to construct a calibration curve using different concentrations of BSA diluted in PBS: 500 mg/mL, 250 mg/mL, 125 mg/mL, 62.5 mg/mL, 31.25 mg/mL, 15.625 mg/mL, 7.8125 mg/mL, 3.9062 5mg/mL and 0 mg/mL. In the later stage, Work Reagent (WR) BCA where the final volume of A reagent is reflected by the following formula, was prepared:



A reagent *(final volume)* = (number of different BSA concentrations + number of samples) x replicates x volume per sample of WR (200µL)

The final volume of B reagent is 50:1 of A reagent. After, 200 µL of WR was added to all wells used. For curve calibration, in addition to WR, 10 µL of different concentration BSA was added to each well; for samples, 10 µL of samples diluted 1:10 in lysis buffer were added to plate. Next, the plate was stirred for 30 seconds and then was incubated during 30 minutes at 37ºC. After incubation, was observed a purple colour. The colour intensity is directly proportional to protein concentration. In the end, absorbance is read at 562nm by spectrophotomer (XMark Microplate Spectrophotomer, Bio-Rad). The readying was analyzed through Microplate Manager Software (Bio-Rad).

8. TTR & GLS Western blot

The Western blot technique is used for detection and/or relative quantification of proteins from extracts of total protein. This technique is based on polyacrylamide gel electrophoresis to separate proteins, denatured beforehand according to their size. Then, under the action of the electric current, the proteins are transferred from the gel to a membrane. To get the antibody epitope, which may not be accessible in the three dimensional conformation of the protein, it is necessary to denature proteins. Once protein is transferred, the membrane is blocked to prevent non-specific binding of the primary and/or secondary antibodies. Then, the membrane is incubated with specific primary antibody of the protein of interest, in order to detect its presence. It is important to wash the membrane to remove nonspecifically bound primary antibody. The secondary antibody contains horseradish peroxidase (HRP) which catalyzes the oxidation of luminol into a reagent which emits light when it decays. When HRP forms a complex with the protein of interest on the membrane, the amount and location of light whose emission is catalyzed by HRP is directly correlated with the location and amount of protein on the membrane. When the membrane is in contact with enhanced chemiluminescent (ECL), is detected the presence of HRP. The image is analyzed by densitometry, allowing thus the protein quantification.

Loading buffer 4x (glycerol, SDS 10%, Tris-HCl ph 6.8, <1% bromophenol blue, 4% ß-mercaptoethanol) was added to both DMEM and CP explants samples. Later, these samples were boiled at 95ºC during 10 minutes. After protein denaturation, 30 µg of CP's total protein and 15 µL of DMEM were separated by SDS-PAGE through stacking (4.7%) and resolving (12.5%) gels. Electrophoresis race held on 120V in electrophoresis solution (100 mL Western buffer 1x pH 8.3 (3 g Tris, 14.4 g glycine, miliQ water to 1 L) and 10% SDS). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes with 0.45 µm pore during 55 minutes at 75V, previously activated with methanol (MeOH) and Tris-buffered saline (TBS) equilibrated in the electrotransfer solution (100 mL Western buffer 1x pH 8.3 and 20% MeOH). Membranes were blocked with 5% nonfat dry milk in TBS for 1h30 at RT. After this period, the membranes were incubated with the primary antibodies rabbit anti-TTR (1:500; Dako) and rabbit anti-GLS (0.5 µg/mL; Abcam), overnight at 4ºC with constant slow agitation. These antibodies were diluted in TBS containing 0.1% Tween (TBS-T). Membranes were washed three times for 15 minutes in TBS-T at RT, followed by incubation with a secondary antibody anti-rabbit (1:50000; Santa Cruz Biotechnology) for 1h at RT also in constant slow agitation. The washing process was repeated. Then membrane was incubated with ECL substract (Bio-Rad) according to the manufacturer's instructions. Protein bands were detected by chemiluminescence, using ChemiDoc MP Imaging system (Bio-Rad) and densitometry of bands was performed using the software ImageLab 5.1 (Bio-Rad).

During the optimization process for Western blot, different conditions for GLS detection were tested, namely total protein amount (40 µg/µL, 35 µg/µL and 30 µg/µL), primary antibody concentration (1 µg/mL, 0.5 µg/mL and 0.25 µg/mL) and incubation time (overnight at 4ºC and 1h at RT).

9. Statistical analysis

Statistical analysis of Aß scavengers in both experiences was carried out using GraphPad Prism (Version 5). All experimental data were compared and expressed as mean ± SEM. Comparisons of means between groups was performed using one-way ANOVA followed by Dunnett's test for Aß 24h experience to compare all treated groups relatively to the control, while Bonferroni Multiple Comparison test was used to compare Aß scavengers expression in different ages and for circadian rhythm experiment. Results were considered statistical significant when $p<0.05$.

IV. Results

1. Expression of Aß scavengers genes in CP

In a preliminary approach to assure the detection of the genes under study and assay the most appropriate annealing temperatures, conventional PCR analysis of the expression of TTR, GLS, ApoJ, MT2, ACE and IDE genes was carried out. The size of the fragments obtained was confirmed by 2% agarose gel by comparison GRP Ladder 50bp (Figure 8).

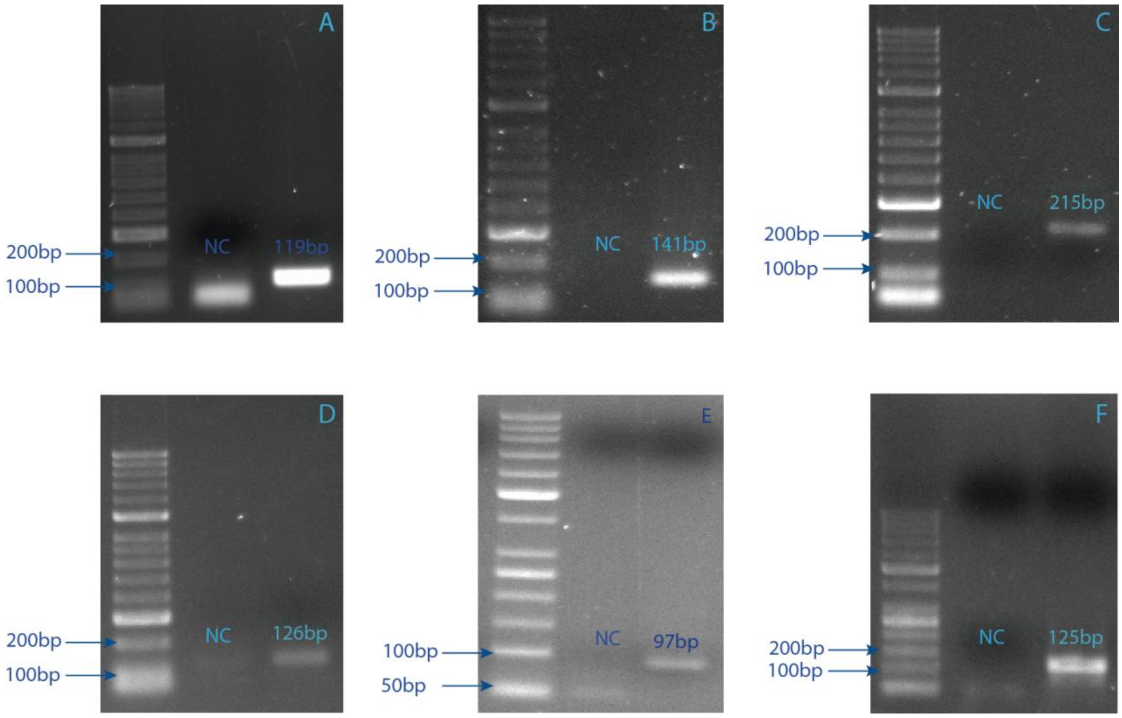


Figure 8 – Aß scavenger's expression in rat CP. Agarose gels 2% show the molecular weight marker (left), the negative control (NC) and respective gene fragment: **A** – TTR (119 bp); **B** – GLS (141 bp); **C** – ApoJ (215 bp); **D** – MT2 (126 bp); **E** – ACE (97 bp); **F** – IDE (125 bp).

The fragment observed in figure 8A refers to the TTR gene with a size of 119 bp; the fragment in figure 8B matches to GLS gene with a molecular weight of 141 bp; the fragment in figure 8C corresponds to ApoJ gene with a size of 215 bp; the fragment in figure 8D corresponds to the MT2 gene with a molecular weight of 126 bp; the fragment of figure 8E indicates ACE gene with a size of 97 bp, and finally, figure 8F shows the fragment which corresponds to IDE gene with a molecular weight of 125 bp.

2. Cellular distribution of Aß scavengers in rat CP

The whole-mount immunohistochemistry staining technique allowed to determine the cellular location of TTR, GLS, and ApoJ in rat CPs. CPs were incubated with the respective primary antibody and then incubated with the secondary antibody conjugated with fluorochrome, which binds to the primary antibody and emits fluorescence in the green wavelength. In addition, nuclei were also stained with Hoechst 33342 dye, which emits blue fluorescence. The fluorescence of tissues was observed in a confocal microscope, showing that all the proteins have a cytoplasmic location. The negative controls do not present any staining (Figure 9).

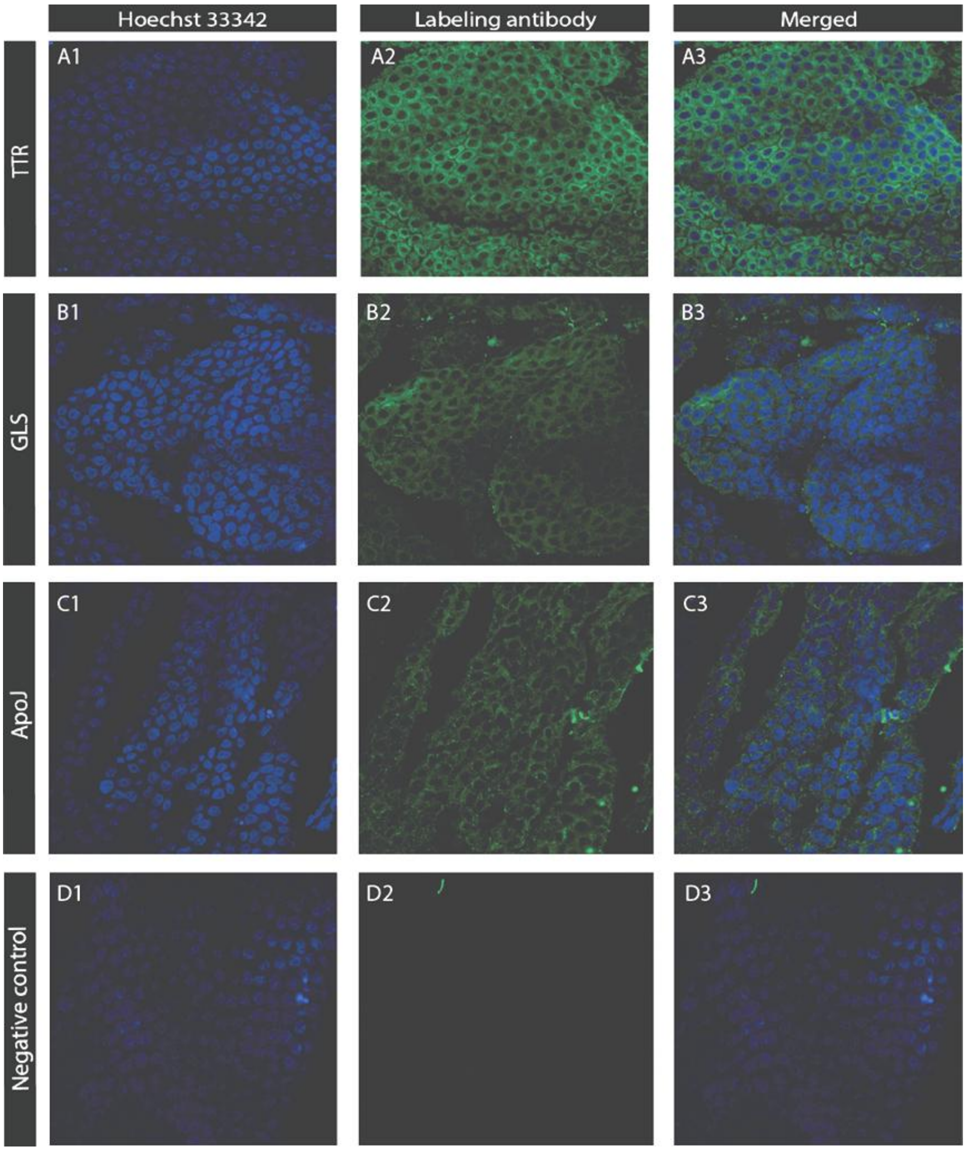


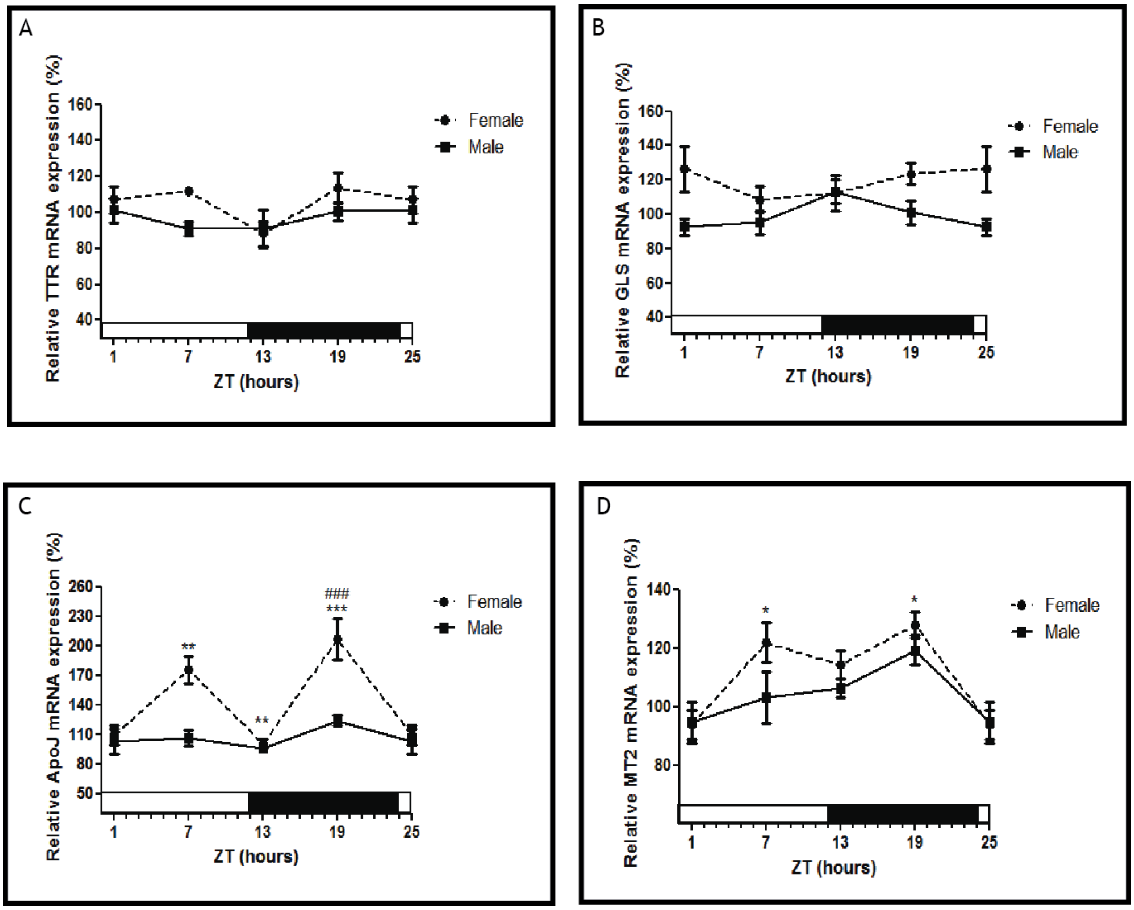
Figure 9 – Cellular localization of Aß scavenger's in rat CP by confocal microscopy (A1-D3). A1, B1, C1 - nucleus staining with Hoechst 33342 (blue). **A2, B2, C2** – Localization of each protein (TTR, GLS

and ApoJ, respectively) in the cytoplasm of CP tissue (green). **A3, B3, C3** – Merged of A1-A2, B1-B2, C1-C2. **D** – Negative control. All figures were obtained in a magnification of 63x.

3. Regulation of Aß scavengers by the circadian rhythm

3.1. Real time PCR

The qPCR technique allowed us to analyze the differences in mRNA expression levels of TTR, GLS, APOJ, MT2, ACE and IDE genes in male and female two months old rats for 24h. To normalize the expression levels of these genes, cyclophilin A was used as an endogenous control. Figure 10 represents the variation in gene expression during 24h.



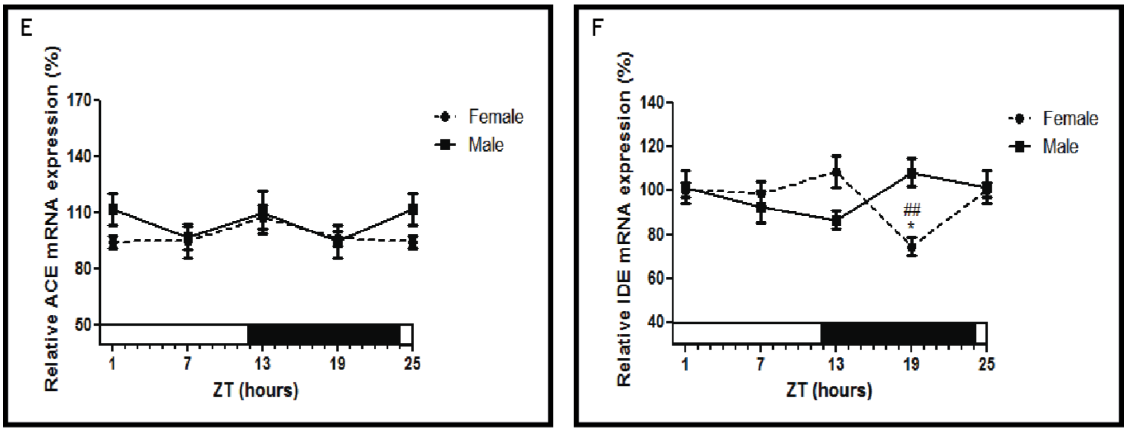


Figure 10 – Relative Aβ scavenger's expression in rat CP at different hours of the day. The open and filled bars on the x-axis of graphs a-e represent 12 h of daytime and 12 h of darkness, respectively. Data from ZT1 and ZT25 are double plotted in panel. Full lines represent the males and the dotted lines represent females. Results are expressed as mean ± SEM; n=3-6 (* p< 0.05; **, ## p< 0.01; ***, ### p< 0.001).

TTR (Figure 10A), GLS (Figure 10B) and ACE (Figure 10E) did not show significant variations in its expression at any time of the day either in female or in male animals. ApoJ expression revealed a characteristic profile: in Figure 10C, the expression of ApoJ in female animals increases statistically reaching its peak twice a day, at 14h (ZT7) and 2h (ZT19), with statistically significant differences between ZT1 vs ZT7 (** p< 0.01), ZT1 vs ZT19 (*** p< 0.001) and ZT13 vs ZT19 (### p< 0.001). After reaching its peak at ZT7, ApoJ mRNA expression fell by ZT13 (p<0.01), increasing again at ZT19. Unlike females, males did not exhibit alterations in ApoJ expression at each sampling point. MT2 expression (figure 10D) was statistically rhythmic only in females, ZT1 vs ZT7 (* p< 0.05) and ZT1 vs ZT19 (* p< 0.05). Like ApoJ and MT2, also IDE (Figure 10F) revealed variations in its expression levels only in females. IDE mRNA expression showed a maximum at ZT13, and declined during the dark phase, ZT1 vs ZT19 (* p< 0.05) and ZT13 to ZT19 (** p< 0.01) ZT1 vs ZT19 and ZT13 vs ZT19.

4. Regulation of Aß scavengers through Aß42 stimulus during 24h

4.1. Real time PCR

qPCR also enabled the comparison of the expression of Aß scavenger genes in CP explants collected from newborn, young (1 month) and adult male and female (3 months) rats, with or without being subjected to Aß42 treatment for 24h, using three different concentrations.

The comparison of the relative mRNA expression of TTR, GLS, ApoJ and IDE between these age groups are presented in figure 11.

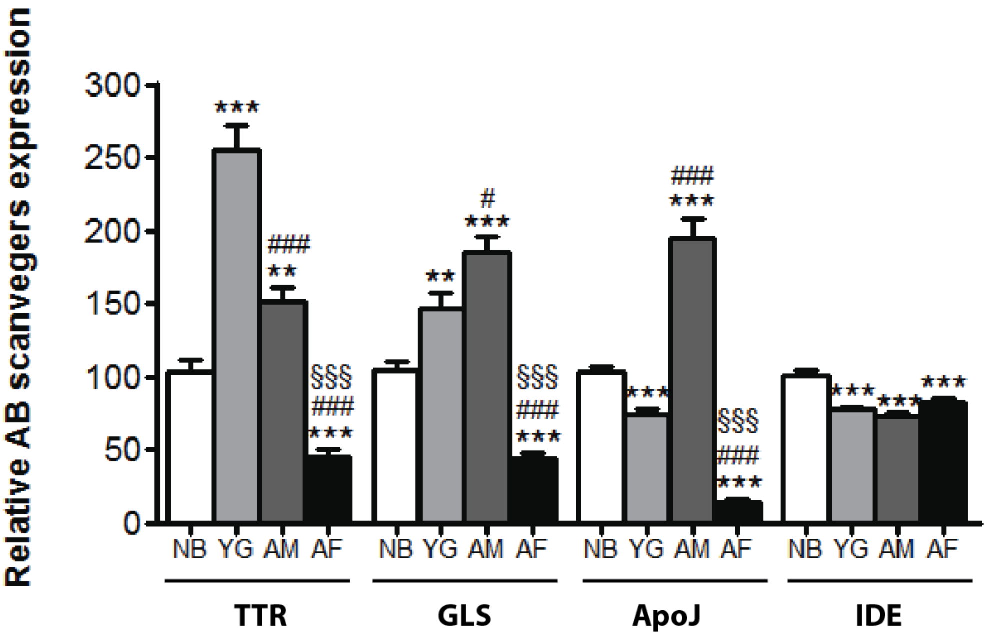
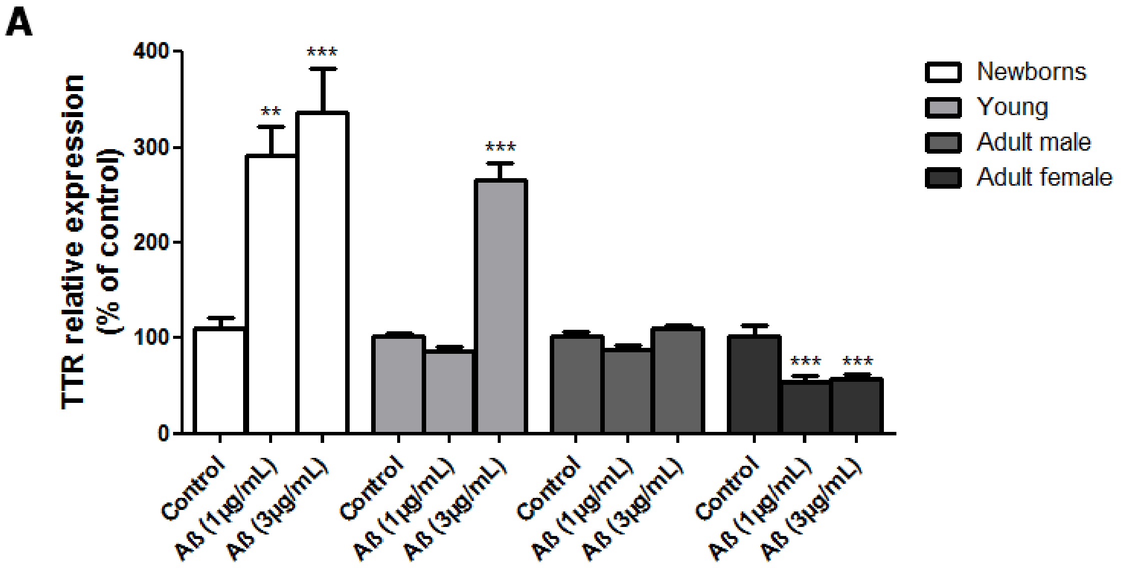
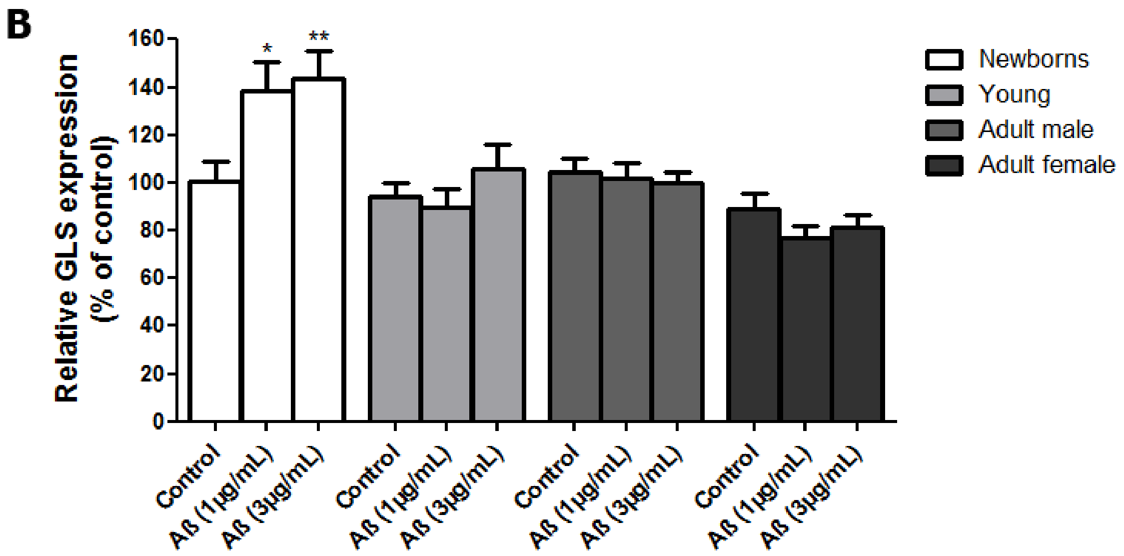


Figure 11 – Comparison of Aß scavenger's expression between young (YG) and adult male (AM) and female (AF) rats considering as controls (100% expression) the newborn (NB) explants. Results are expressed as mean ± SEM; n = 4-6 (# $p<0.05$; ** $p<0.01$; ***, ###, §§§ $p<0.001$).

Figure 11 shows that TTR, GLS, ApoJ and IDE expression vary between age groups with each of them presenting a different pattern of expression. Differences in the expression patterns were also seen between adult males and females, except in IDE expression. TTR reaches its higher expression when animals are with 1month of age. In adult males (3 months), TTR expression still augmented in comparison to newborn rats but decreases in relation to animals with 1 month. Regarding to GLS expression, it also increases in CP young rats. However, in opposite to TTR, the adult male presented the higher GLS expression compared to newborn and young CP rats. The ApoJ mRNA expression, contrarily to TTR and GLS, decreases in young animals. Like GLS, ApoJ expression increases in adult male. Interestingly, in adult female, TTR, GLS and ApoJ show low expression with advancing age. Even comparing animals of the same age, the expression of TTR, GLS and ApoJ decreases considerably in females. IDE has totally different expression pattern relative the others genes. Its expression seems to decreases in young and adult male animals while increasing in adult female.

To evaluate how the CP explants respond to an Aß42 stimulus, the CP tissue was incubated for 24h with two different concentrations: 0 µg/mL (control), 1 µg/mL and 3 µg/mL of Aß42. Figure 12 shows the CP expression of different Aß scavengers in newborn, young (1 month) and adult males and females (3 months) under Aß42 treatment.




B
Relative GLS expression
(% of control)
160
140
120
100
80
60
40
20
0
*
**
Control
Aß (1µg/mL)
Aß (3µg/mL)
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Aß (1µg/mL)
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Control
Aß (1µg/mL)
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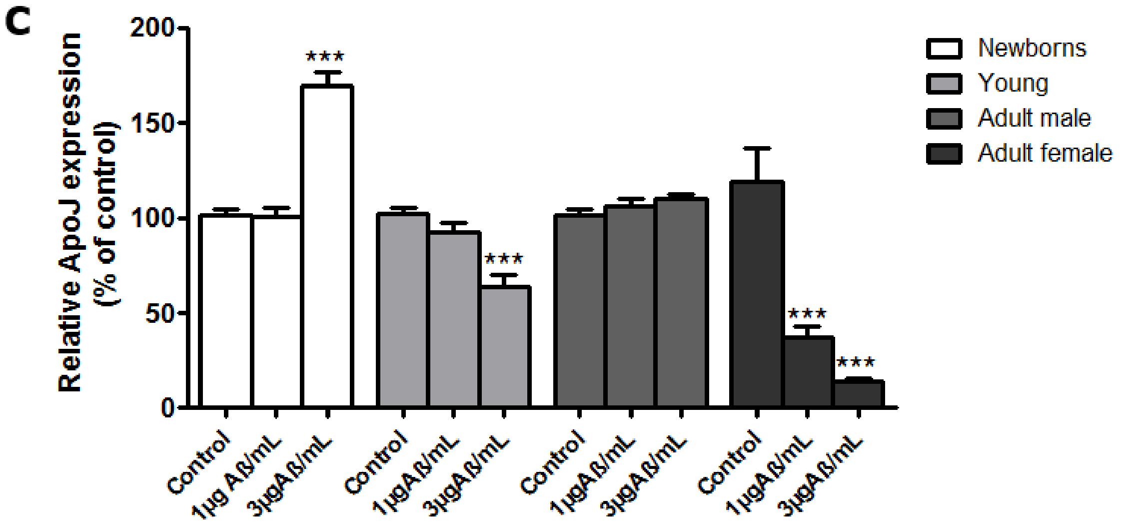
Control
Aß (1µg/mL)
Aß (3µg/mL)
Newborns
Young
Adult male
Adult female


C
Relative ApoJ expression
(% of control)
200
150
100
50
0
***
***
***
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Control
1µg Aß/mL
3µgAß/mL
Control
1µgAß/mL
3µgAß/mL
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3µgAß/mL
Control
1µgAß/mL
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Newborns
Young
Adult male
Adult female

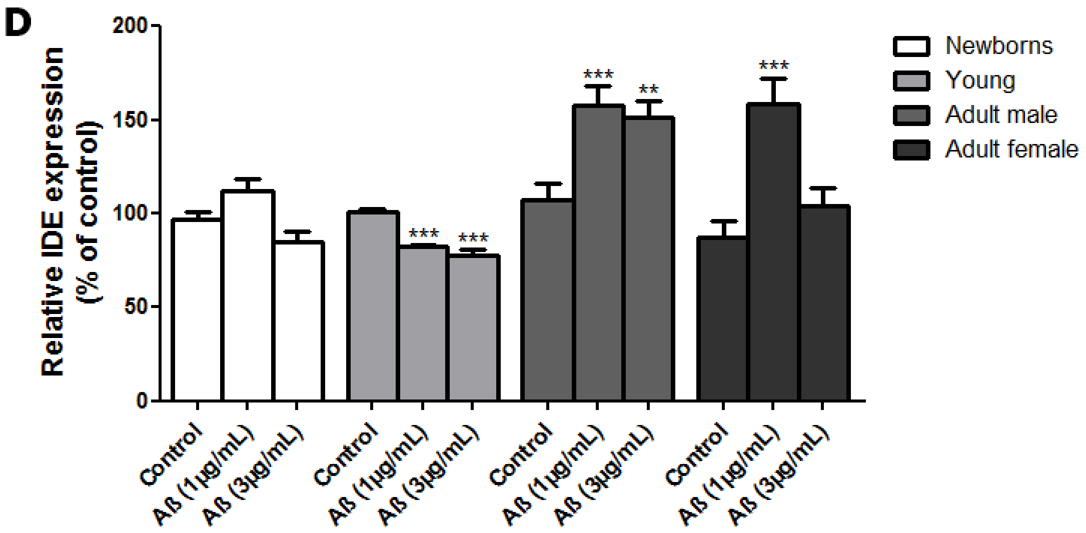

Figure 12 - Relative Aß scavenger's expression in newborn, young (1 month) and adult male and female (3 months) rats CP in presence of Aß42 stimulus for 24h. Results are expressed as mean ± SEM; n = 4-6 (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Figure 12A shows that TTR expression increases 3 times in response to both concentrations of Aß42 insults in the CP of newborn rats but these effects tends to decrease along aging. In young rat CPs there was still an increase in TTR expression when CP tissue was treated with 3 µg/mL. There were no significant changes in TTR expression in young rat CP explants treated with 1 µg/mL compared to control. Interestingly, while in adult males TTR expression remained constant in response to different Aß42 concentrations a decrease in TTR expression was seen in adult females with both concentrations.

Only CP explants from newborn rats had increased expression of GLS, in response to incubation with Aß42 (Figure 12B). No significant differences were observed in the other age groups.

Regarding ApoJ (Figure 12C) only in newborn CPs, 3 µg/mL of Aß42 increased mRNA ApoJ. In CPs collected from older animals, there were decreased expression of ApoJ, except in males where no changes were observed.

IDE mRNA expression (Figure 12D) displays a different pattern in rat CP compared with the other Aß scavengers. In newborns rat CPs there was no response to Aß42, while in young animals a slight decrease in IDE expression was observed. In adult animals IDE expression increased with 1 and 3 µg/mL of Aß42 males, while in adult females only 1 µg/mL of Aß42 increased the expression of this Aß scavenger.

4.2. Western blot

Since changes in gene expression levels are observed in Aβ scavenger's using the qPCR technique, variations at protein expression levels are also expected. Since the choroidal tissue has the ability to secrete the proteins and, these in turn, form stable complexes with Aβ, the Western blot technique was performed to evaluate the levels of TTR and GLS in the culture medium.

Figures 13A and 14A represent blot from culture medium of TTR and GLS, respectively. The same volume (15 µL) of culture medium of each sample was loaded in SDS-PAGE.

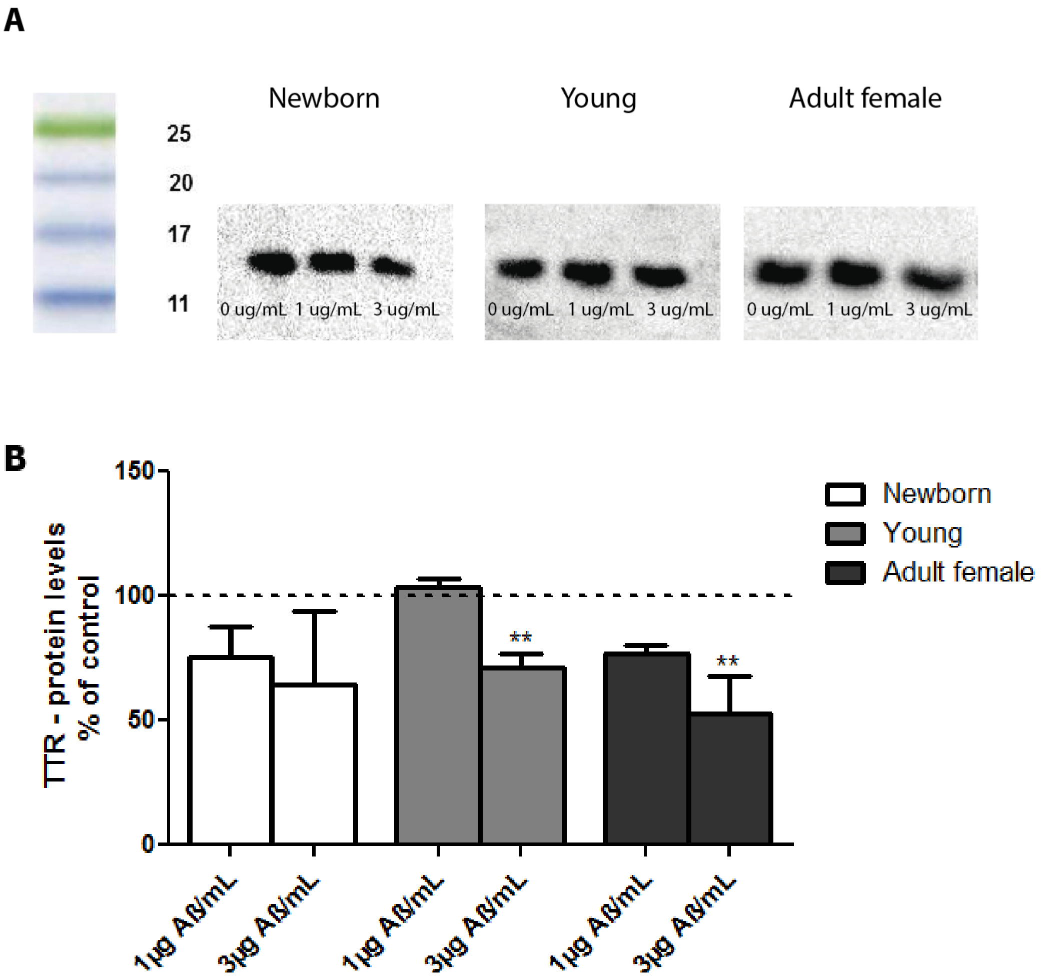

Figure 13 - Evaluation of Aβ42 levels effect in TTR secretion by Western blot. A - representative blot of culture medium collected from newborn, young and adult female rat CP tissue treated with 0 µg/mL (control), 1 µg/mL and 3 µg/mL during 24h. A band was detected corresponding to the monomer isoform (~15 kDa) of TTR. **B** – Comparison of TTR levels in culture medium normalized for the total amount of protein was analysed by the software ImageLab 5.1 (Bio-Rad). Results are expressed as mean ± SEM; n = 4.

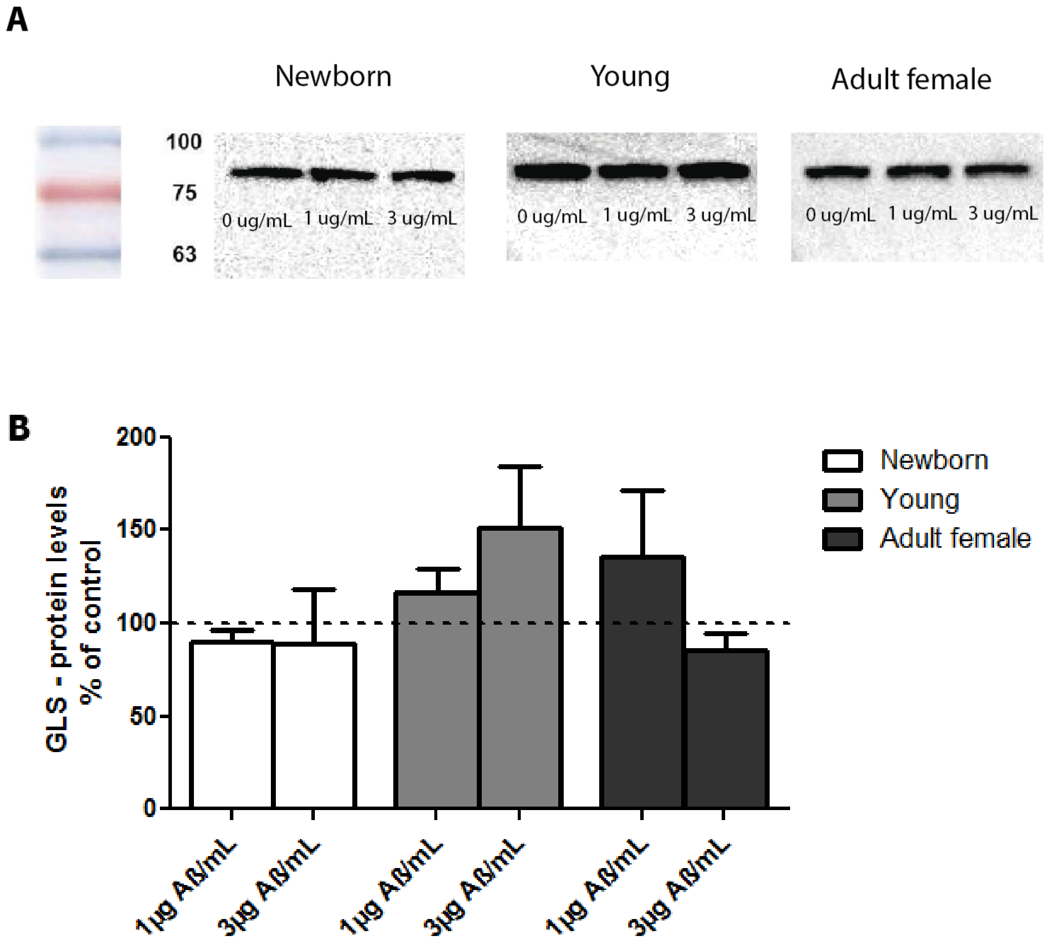

Figure 14 – Evaluation of Aβ42 levels effect in GLS secretion by Western blot. A – representative blot of culture medium collected from newborn, young and adult female rat CP tissue treated with 0 μg/mL (control), 1 μg/mL and 3 μg/mL during 24h. A band was detected corresponding to the ~86 kDa of GLS. **B** – Comparison of TTR levels in culture medium normalized for the total amount of protein was analysed by the software ImageLab 5.1 (Bio-Rad). Results are expressed as mean ± SEM; n = 4.

The TTR (~15 kDa) protein levels (Figure 13B) in culture medium samples, collected before Aβ42 incubation, decreases when CP tissue was treated with 3 μg/mL in both young and adult female rats. Conversely, GLS (~86 kDa) (Figure 14B) protein levels did not show any significant alteration compared to controls in all age groups under study.

V. Discussion

The impaired clearance of Aß peptide leads to the development of AD pathology, therefore the importance to reduce Aß production and, in turn, its load. Currently, more attention is given to choroidal tissue and its neuroprotective functions in neurodegenerative disorders (Borlongan et al., 2008, Emerich et al., 2006), namely AD (Vargas et al., 2010b, Carro et al., 2005). By expressing peptidases, CP plays a key role in enzymatic processing and/or degradation of several peptides such as Aß protein (Crossgrove et al., 2005). Morphological alterations of choroidal epithelium cause impaired synthesis, secretion and transport of protein functions, resulting in the increase of amyloid load (reviewed by Serot et al. (2003) and Krzyzanowska and Carro (2012)). There are many studies demonstrating the expression of TTR, GLS, ApoJ, MT2, ACE and IDE in choroidal epithelial cells and their neuroprotective effects against Aß toxicity (Yang et al., 2013, Ji et al., 2010, Bell et al., 2007, Martinho et al., 2010, Oba et al., 2005, Behl et al., 2009). All of these Aß scavengers possess the ability to bind Aß peptide, to hydrolyse it into less neurotoxic fragments, to retard Aß fibril formation and deposition, or to inhibit the cytotoxicity induced by Aß (Costa et al., 2008a, Costa et al., 2008b, Bora and Prabhakar, 2010, Zou et al., 2007, Oda et al., 1995, Ray et al., 2000).

All organisms have developed endogenous daily clocks, present in almost all mammalian tissues, to adapt to the external day and night cycles. This endogenous daily clock, called circadian clocks (approximately one day, ~24h), is crucial for the synchronization of an organism's internal circadian timing to the external environment, integrating both signals (reviewed by Videnovic et al. (2014), Hastings and Maywood (2000)).

The transcriptome analyses from animal samples like blood, brain, liver, kidney, skeletal muscle, and heart, have revealed that many genes outside the core clock genes undergo daily variations in expression levels (Yang et al., 2007, Panda et al., 2002, Akhtar et al., 2002, Nakamura et al., 2005, Karatsoreos et al., 2011). More recently, convincing evidence that there is a rhythmic rise and fall in the transcriptional activity of hundreds of genes in the six different regions (dorsolateral prefrontal cortex, amygdala, cerebellum, nucleus accumbens, anterior cingulate cortex and hippocampus) of healthy brains was provided (Li et al., 2013). Moreover, CP has the capacity to express clock genes (Quintela et al., 2014). However, no data is available about the expression of the scavenger genes here studied in choroidal tissue along the day. Our data show that each of the Aß scavenger's analysed displays different circadian patterns. Even more, each gene shows gender-related differences in its mRNA expression. This differences between both sexes might be due to the CP response to SHs (Quadros et al., 2007, Hong-Goka and Chang, 2004), which in turn may influence gene expression (Quintela et al., 2013). In addition, circadian rhythm is also under sex hormone control (Nakamura et al., 2005, Jechura et al., 2000, Iwahana et al., 2008). In a study performed by Quintela *et al.*, the clock genes expressed in choroid epithelium are subjected to rhythmic expression in females and males (Quintela et al., 2014). The results presented here provide a strong evidence that SHs regulate TTR, GLS, ApoJ, MT2, ACE and IDE in CP.

Only ApoJ, MT2 and IDE demonstrate different expression in female rats during both day light and night. In other words, the genes seem to be influenced by the CP biological and by of SHs.

The expression of Aß scavenger's expression along life, was studied in rats within three different age groups. Expression differences were observed on TTR, GLS, ApoJ and IDE levels in newborn (PN 5-7), young (1 month) and adult males and females (3 months) rats. TTR mRNA begun to decrease when animals reached 1 month of age. This is in concordance with other studies which report an age-dependent expression of TTR in serum (Buxbaum et al., 2008) and CSF (Zhang et al., 2005, Chen et al., 2005), and loss of the ability of CP tissue to synthesize, secrete and transport proteins (reviewed by Krzyzanowska and Carro (2012)). Importantly, as it is shown herein, SHs levels also might play their own role. As early demonstrated, TTR serum levels were significantly higher in males when compared with females (Buxbaum et al., 2008, Han et al., 2011). Evidences of SHs influencing the expression of Aß scavengers has also been reported, namely in liver and CP, were SHs up-regulate TTR expression (Goncalves et al., 2008, Quintela et al., 2008, Quintela et al., 2011). Moreover, it is well known that SHs are subjected to age-related changes in their levels, which are more pronounced in women than man (Yue et al., 2005, Ruitenberg et al., 2001), what may explain the differences observed in gene expression between adult males and females in choroidal tissue. The expression pattern of GLS and ApoJ appears to be identical, and contrarily to TTR, GLS and ApoJ expression increased in adults. Increase in GLS levels with age might be associated with previous observations of augmented GLS expression in response to oxidative stress (Ji et al., 2008, Ji et al., 2009), which in turn is related with the aging process (reviewed by Cui et al. (2012)). The increase of ApoJ levels with age presented here are also supported by other studies in human pituitary gland (Ishikawa et al., 2006). This ApoJ mRNA increase may be explained by the rise of cell damage and stress during aging (Ishikawa et al., 2006). Similarly to TTR, it was also showed here differences in expression between males and females in both GLS and ApoJ (Kwekel et al., 2013), suggesting also the regulation of these Aß scavenger's by SHs in CP. In a study by Won *et al.,* the estrogen and progesterone increase ApoJ expression in endometrial cancer cell lines KLE and ECC-1 (Won et al., 2012). In case of GLS, DHT up-regulate its expression in adipose tissue (Bolduc et al., 2004). Otherwise, IDE expression shows different expression patterns compared to other Aß scavenger's. IDE expression shows a decrease in an age-related manner compared to newborn rats, which is in accordance with an experimental study that show diminished IDE levels and activity in aging brains (Miners et al., 2009). Although here no differences in expression between males and females were detected, others show E2 and progesterone up-regulate IDE expression in hippocampal and cerebrocortical cultures, and *in vivo* (Zhao et al., 2011, Jayaraman et al., 2012).

The analysis of the expression of Aß scavenger's in CP subjected to Aß42 treatment for 24h, showed that all suffered differential expression. TTR and GLS mRNA transcript up-regulation was observed in an Aß42 dose-dependent manner in newborn rats but TTR showed statistical decrease at the protein secretion level. The increase of TTR mRNA expression is in agreement with previous reports suggesting that Aß overproduction seems to induce TTR expression (Stein and Johnson, 2002). However, the 3 µg/mL of Aß42 has a negative effect on TTR secretion to the culture medium in young and adult females which is not concordant with the increase of TTR mRNA in young animals and is suggestive of post-translational regulation events or may be related to decrease CP's secretory function. Comparing TTR expression of young and adult females to newborn animals treated with Aß42, its expression seem to be decreased which is consistent with the previous results which show diminished TTR expression with age. Regarding GLS mRNA expression in newborn CP rats, it seems to be up-regulated in a concentration-dependent manner (Ji et al., 2009) induced by Aß peptide (Crouch et al., 2008). On the other hand, GLS expression in CP from animals with 1 month of age and older, was unaltered in the presence of Aß42 stimulus. About ApoJ mRNA expression, it behaves in a similar manner to TTR, and its expression was higher when CP was treated with higher Aß42 concentration (3 µg/mL). This is consistent with (Thambisetty et al., 2010) that suggested that increased plasma concentrations of ApoJ in response to greater Aß burden in the medial temporal lobe will prevent, thus, the cell damage and cellular stress by the Aß peptide. Likewise TTR, the ApoJ expression in young and adult females is decreased, which is in agreement with previous results showing that ApoJ expression is higher in young rats in comparison to adult females. Like in TTR expression, also in the case of ApoJ, Aß42 seems to impair peptide synthesis in CP, hence the decreased levels of expression in young and adult females. Additionally, in the presence of Aß42, IDE has a particular pattern of expression. In opposite to other Aß scavengers, no evident variations in IDE expression were observed in newborn rats. While in young animals IDE expression is decreased, in adult male and females it is increased. Interestingly, only IDE showed increased expression in CP treated with Aß42, when compared to controls, in adult female animals.

In summary, with the exception of IDE, the CP of newborn and young animals showed a clear enhanced capacity to protect against Aß insults by higher expression of Aß scavengers, supporting the relevant neuroprotective role of CP in age-dependent processes.

VI. Conclusions and future perspective

The main feature of AD is the accumulation of Aß which causes the toxic damage in the brain. From the early past, it was already known the function of certain proteins involved in metabolism/clearance of the Aß peptide. However, only recently the function of synthesis and secretion of proteins responsible for Aß levels decrease has received more attention in CP tissue. TTR, GLS, ApoJ, MT2, ACE and IDE are some of the proteins expressed in CP that have the additional function of reducing amyloid load by binding to Aß , hydrolyse it into less neurotoxic fragments, retard Aß fibril formation and deposition, and inhibit cytotoxicity induced by Aß.

The circadian rhythm (approximately ~24h), crucial for the synchronization of an organism's internal circadian timing to the external world, and its influence in each Aß scavengers such as regulation of these same scavengers by Aß42 in rat's CP explants was assessed herein for the first time. We found that expression of the Aß scavengers studied vary along the day, with age, sex and Aß42 concentration. The results here presented highlight the SHs effect in circadian oscillation of each gene in CP.

Importantly, roles of Aß scavengers depend on its synthesis by CP but also on its secretion to the CSF, where these form complexes with Aß avoiding its accumulation and toxicity. These study will benefit from the complementary analysis of aging animals (older than 8 month), in addition to newborns, young and adult male and females. Additionally, the regulation of GLS, ApoJ, MT2, ACE and IDE by female and male hormones in CP tissue should be investigated for those where the differential expression between females and males is evident. This may be evaluated through CP stimulation by females and males SHs. Another interesting aspect that could be investigated is circadian oscillation of these genes expression *in vitro* with different Aß42 concentrations and their assessment *in vivo* in AD patients.

VII. Bibliography

AKHTAR, R. A., REDDY, A. B., MAYWOOD, E. S., CLAYTON, J. D., KING, V. M., SMITH, A. G., GANT, T. W., HASTINGS, M. H. & KYRIACOU, C. P. 2002. Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Curr Biol,* 12, 540-50.

ALOISI, A. M. & BONIFAZI, M. 2006. Sex hormones, central nervous system and pain. *Horm Behav,* 50, 1-7.

ALVES, C. H., GONCALVES, I., SOCORRO, S., BALTAZAR, G., QUINTELA, T. & SANTOS, C. R. 2009. Androgen receptor is expressed in murine choroid plexus and downregulated by 5alpha-dihydrotestosterone in male and female mice. *J Mol Neurosci,* 38, 41-9.

AMMAR, H. & CLOSSET, J. L. 2008. Clusterin activates survival through the phosphatidylinositol 3-kinase/Akt pathway. *J Biol Chem,* 283, 12851-61.

ARREGUI, A. & IVERSEN, L. L. 1978. Angiotensin-converting enzyme: presence of high activity in choroid plexus of mammalian brain. *Eur J Pharmacol,* 52, 147-50.

BARRON, A. M. & PIKE, C. J. 2012. Sex hormones, aging, and Alzheimer's disease. *Front Biosci (Elite Ed),* 4, 976-97.

BATEMAN, R. J., MUNSELL, L. Y., MORRIS, J. C., SWARM, R., YARASHESKI, K. E. & HOLTZMAN, D. M. 2006. Human amyloid-beta synthesis and clearance rates as measured in cerebrospinal fluid in vivo. *Nat Med,* 12, 856-61.

BATES, K. A., HARVEY, A. R., CARRUTHERS, M. & MARTINS, R. N. 2005. Androgens, andropause and neurodegeneration: exploring the link between steroidogenesis, androgens and Alzheimer's disease. *Cell Mol Life Sci,* 62, 281-92.

BEHL, M., ZHANG, Y. & ZHENG, W. 2009. Involvement of insulin-degrading enzyme in the clearance of beta-amyloid at the blood-CSF barrier: Consequences of lead exposure. *Cerebrospinal Fluid Res,* 6, 11.

BELL, R. D., SAGARE, A. P., FRIEDMAN, A. E., BEDI, G. S., HOLTZMAN, D. M., DEANE, R. & ZLOKOVIC, B. V. 2007. Transport pathways for clearance of human Alzheimer's amyloid beta-peptide and apolipoproteins E and J in the mouse central nervous system. *J Cereb Blood Flow Metab,* 27, 909-18.

BENNETT, R. G., DUCKWORTH, W. C. & HAMEL, F. G. 2000. Degradation of amylin by insulin-degrading enzyme. *J Biol Chem,* 275, 36621-5.

BOLDUC, C., LAROSE, M., YOSHIOKA, M., YE, P., BELLEAU, P., LABRIE, C., MORISSETTE, J., RAYMOND, V., LABRIE, F. & ST-AMAND, J. 2004. Effects of dihydrotestosterone on adipose tissue measured by serial analysis of gene expression. *J Mol Endocrinol,* 33, 429-44.

BORA, R. P. & PRABHAKAR, R. 2010. Elucidation of interactions of Alzheimer amyloid beta peptides (Abeta40 and Abeta42) with insulin degrading enzyme: a molecular dynamics study. *Biochemistry,* 49, 3947-56.

BORLONGAN, C. V., THANOS, C. G., SKINNER, S. J., GEANEY, M. & EMERICH, D. F. 2008. Transplants of encapsulated rat choroid plexus cells exert neuroprotection in a rodent model of Huntington's disease. *Cell Transplant,* 16, 987-92.

BREEN, C. M., SYKES, D. B., FRICKER, G. & MILLER, D. S. 2002. Confocal imaging of organic anion transport in intact rat choroid plexus. *Am J Physiol Renal Physiol,* 282, F877-85.

BROCKMAN, R., BUNICK, D. & MAHONEY, M. M. 2011. Estradiol deficiency during development modulates the expression of circadian and daily rhythms in male and female aromatase knockout mice. *Horm Behav,* 60, 439-47.

BUTLER, M. P., KARATSOREOS, I. N., LESAUTER, J. & SILVER, R. 2012. Dose-dependent effects of androgens on the circadian timing system and its response to light. *Endocrinology,* 153, 2344-52.

BUXBAUM, J., KOZIOL, J. & CONNORS, L. H. 2008. Serum transthyretin levels in senile systemic amyloidosis: effects of age, gender and ethnicity. *Amyloid,* 15, 255-61.

CARPENE, E., ANDREANI, G. & ISANI, G. 2007. Metallothionein functions and structural characteristics. *J Trace Elem Med Biol,* 21 Suppl 1, 35-9.

CARRO, E., SPUCH, C., TREJO, J. L., ANTEQUERA, D. & TORRES-ALEMAN, I. 2005. Choroid plexus megalin is involved in neuroprotection by serum insulin-like growth factor I. *J Neurosci,* 25, 10884-93.

CHAUHAN, V. P., RAY, I., CHAUHAN, A. & WISNIEWSKI, H. M. 1999. Binding of gelsolin, a secretory protein, to amyloid beta-protein. *Biochem Biophys Res Commun,* 258, 241-6.

CHEN, R. L., ATHAUDA, S. B., KASSEM, N. A., ZHANG, Y., SEGAL, M. B. & PRESTON, J. E. 2005. Decrease of transthyretin synthesis at the blood-cerebrospinal fluid barrier of old sheep. *J Gerontol A Biol Sci Med Sci,* 60, 852-8.

CHODOBSKI, A. & SZMYDYNGER-CHODOBSKA, J. 2001. Choroid plexus: target for polypeptides and site of their synthesis. *Microsc Res Tech,* 52, 65-82.

CHUNG, R. S., ADLARD, P. A., DITTMANN, J., VICKERS, J. C., CHUAH, M. I. & WEST, A. K. 2004. Neuron-glia communication: metallothionein expression is specifically up-regulated by astrocytes in response to neuronal injury. *J Neurochem,* 88, 454-61.

CORPECHOT, C., ROBEL, P., AXELSON, M., SJOVALL, J. & BAULIEU, E. E. 1981. Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. *Proc Natl Acad Sci U S A,* 78, 4704-7.

COSTA, R., FERREIRA-DA-SILVA, F., SARAIVA, M. J. & CARDOSO, I. 2008a. Transthyretin protects against A-beta peptide toxicity by proteolytic cleavage of the peptide: a mechanism sensitive to the Kunitz protease inhibitor. *PLoS One,* 3, e2899.

COSTA, R., GONCALVES, A., SARAIVA, M. J. & CARDOSO, I. 2008b. Transthyretin binding to A-Beta peptide--impact on A-Beta fibrillogenesis and toxicity. *FEBS Lett,* 582, 936-42.

CROSSGROVE, J. S., LI, G. J. & ZHENG, W. 2005. The choroid plexus removes beta-amyloid from brain cerebrospinal fluid. *Exp Biol Med (Maywood),* 230, 771-6.

CROSSGROVE, J. S., SMITH, E. L. & ZHENG, W. 2007. Macromolecules involved in production and metabolism of beta-amyloid at the brain barriers. *Brain Res,* 1138, 187-95.

CROUCH, P. J., HARDING, S. M., WHITE, A. R., CAMAKARIS, J., BUSH, A. I. & MASTERS, C. L. 2008. Mechanisms of A beta mediated neurodegeneration in Alzheimer's disease. *Int J Biochem Cell Biol,* 40, 181-98.

CUI, H., KONG, Y. & ZHANG, H. 2012. Oxidative stress, mitochondrial dysfunction, and aging. *J Signal Transduct,* 2012, 646354.

DAMKIER, H. H., BROWN, P. D. & PRAETORIUS, J. 2013. Cerebrospinal fluid secretion by the choroid plexus. *Physiol Rev,* 93, 1847-92.

DE LA IGLESIA, H. O., BLAUSTEIN, J. D. & BITTMAN, E. L. 1999. Oestrogen receptor-alpha-immunoreactive neurones project to the suprachiasmatic nucleus of the female Syrian hamster. *J Neuroendocrinol,* 11, 481-90.

DE LANNOY, L. M., SCHUIJT, M. P., SAXENA, P. R., SCHALEKAMP, M. A. & DANSER, A. H. 2001. Angiotensin converting enzyme is the main contributor to angiotensin I-II conversion in the interstitium of the isolated perfused rat heart. *J Hypertens,* 19, 959-65.

DEL VECCHIO, P. J. & SMITH, J. R. 1981. Expression of angiotensin-converting enzyme activity in cultured pulmonary artery endothelial cells. *J Cell Physiol,* 108, 337-45.

DICKSON, P. W., ALDRED, A. R., MARLEY, P. D., BANNISTER, D. & SCHREIBER, G. 1986. Rat choroid plexus specializes in the synthesis and the secretion of transthyretin (prealbumin). Regulation of transthyretin synthesis in choroid plexus is independent from that in liver. *J Biol Chem,* 261, 3475-8.

DORFMAN, V. B., PASQUINI, L., RIUDAVETS, M., LOPEZ-COSTA, J. J., VILLEGAS, A., TRONCOSO, J. C., LOPERA, F., CASTANO, E. M. & MORELLI, L. 2010. Differential cerebral deposition of IDE and NEP in sporadic and familial Alzheimer's disease. *Neurobiol Aging,* 31, 1743-57.

DU, J., CHO, P. Y., YANG, D. T. & MURPHY, R. M. 2012. Identification of beta-amyloid-binding sites on transthyretin. *Protein Eng Des Sel,* 25, 337-45.

DUCKWORTH, W. C. & KITABCHI, A. E. 1974. Insulin and glucagon degradation by the same enzyme. *Diabetes,* 23, 536-43.

ELLIOTT, D. A., WEICKERT, C. S. & GARNER, B. 2010. Apolipoproteins in the brain: implications for neurological and psychiatric disorders. *Clin Lipidol,* 51, 555-573.

EMERICH, D. F., SKINNER, S. J., BORLONGAN, C. V., VASCONCELLOS, A. V. & THANOS, C. G. 2005. The choroid plexus in the rise, fall and repair of the brain. *Bioessays,* 27, 262-74.

EMERICH, D. F., THANOS, C. G., GODDARD, M., SKINNER, S. J., GEANY, M. S., BELL, W. J., BINTZ, B., SCHNEIDER, P., CHU, Y., BABU, R. S., BORLONGAN, C. V., BOEKELHEIDE, K., HALL, S., BRYANT, B. & KORDOWER, J. H. 2006. Extensive neuroprotection by choroid plexus transplants in excitotoxin lesioned monkeys. *Neurobiol Dis,* 23, 471-80.

EMERICH, D. F., VASCONCELLOS, A. V., ELLIOTT, R. B., SKINNER, S. J. & BORLONGAN, C. V. 2004. The choroid plexus: function, pathology and therapeutic potential of its transplantation. *Expert Opin Biol Ther,* 4, 1191-201.

ENGELHARDT, B., WOLBURG-BUCHHOLZ, K. & WOLBURG, H. 2001. Involvement of the choroid plexus in central nervous system inflammation. *Microsc Res Tech,* 52, 112-29.

FARRIS, W., MANSOURIAN, S., CHANG, Y., LINDSLEY, L., ECKMAN, E. A., FROSCH, M. P., ECKMAN, C. B., TANZI, R. E., SELKOE, D. J. & GUENETTE, S. 2003. Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. *Proc Natl Acad Sci U S A,* 100, 4162-7.

FARRIS, W., MANSOURIAN, S., LEISSRING, M. A., ECKMAN, E. A., BERTRAM, L., ECKMAN, C. B., TANZI, R. E. & SELKOE, D. J. 2004. Partial loss-of-function mutations in insulin-degrading enzyme that induce diabetes also impair degradation of amyloid beta-protein. *Am J Pathol,* 164, 1425-34.

GAO, W., EISENHAUER, P. B., CONN, K., LYNCH, J. A., WELLS, J. M., ULLMAN, M. D., MCKEE, A., THATTE, H. S. & FINE, R. E. 2004. Insulin degrading enzyme is expressed in the human cerebrovascular endothelium and in cultured human cerebrovascular endothelial cells. *Neurosci Lett,* 371, 6-11.

GARCIA-SEGURA, L. M., AZCOITIA, I. & DONCARLOS, L. L. 2001. Neuroprotection by estradiol. *Prog Neurobiol,* 63, 29-60.

GARTON, M. J., KEIR, G., LAKSHMI, M. V. & THOMPSON, E. J. 1991. Age-related changes in cerebrospinal fluid protein concentrations. *J Neurol Sci,* 104, 74-80.

GHERSI-EGEA, J. F. & STRAZIELLE, N. 2001. Brain drug delivery, drug metabolism, and multidrug resistance at the choroid plexus. *Microsc Res Tech,* 52, 83-8.

GONCALVES, I., QUINTELA, T., BALTAZAR, G., ALMEIDA, M. R., SARAIVA, M. J. & SANTOS, C. R. 2008. Transthyretin interacts with metallothionein 2. *Biochemistry,* 47, 2244-51.

GONZALEZ-MARRERO, I., GIMENEZ-LLORT, L., JOHANSON, C. E., CARMONA-CALERO, E. M., CASTANEYRA-RUIZ, L., BRITO-ARMAS, J. M., CASTANEYRA-PERDOMO, A. & CASTRO-FUENTES, R. 2015. Choroid plexus dysfunction impairs beta-amyloid clearance in a triple transgenic mouse model of Alzheimer's disease. *Front Cell Neurosci,* 9, 17.

GONZALEZ, A. M., LEADBEATER, W. E., BURG, M., SIMS, K., TERASAKI, T., JOHANSON, C. E., STOPA, E. G., ELICEIRI, B. P. & BAIRD, A. 2011. Targeting choroid plexus epithelia and ventricular ependyma for drug delivery to the central nervous system. *BMC Neurosci,* 12, 4.

GREEN, C. B., TAKAHASHI, J. S. & BASS, J. 2008. The meter of metabolism. *Cell,* 134, 728-42.
HAASS, C., SCHLOSSMACHER, M. G., HUNG, A. Y., VIGO-PELFREY, C., MELLON, A., OSTASZEWSKI, B. L., LIEBERBURG, I., KOO, E. H., SCHENK, D., TEPLOW, D. B. & ET AL. 1992.

Amyloid beta-peptide is produced by cultured cells during normal metabolism. *Nature,* 359, 322-5.

HAMMAD, S. M., RANGANATHAN, S., LOUKINOVA, E., TWAL, W. O. & ARGRAVES, W. S. 1997. Interaction of apolipoprotein J-amyloid beta-peptide complex with low density lipoprotein receptor-related protein-2/megalin. A mechanism to prevent pathological accumulation of amyloid beta-peptide. *J Biol Chem,* 272, 18644-9.

HAN, S. H., JUNG, E. S., SOHN, J. H., HONG, H. J., HONG, H. S., KIM, J. W., NA, D. L., KIM, M., KIM, H., HA, H. J., KIM, Y. H., HUH, N., JUNG, M. W. & MOOK-JUNG, I. 2011. Human serum transthyretin levels correlate inversely with Alzheimer's disease. *J Alzheimers Dis,* 25, 77-84.

HASTINGS, M. & MAYWOOD, E. S. 2000. Circadian clocks in the mammalian brain. *Bioessays,* 22, 23-31.

HELFRICH-FORSTER, C. 2004. The circadian clock in the brain: a structural and functional comparison between mammals and insects. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol,* 190, 601-13.

HERBERT, J., WILCOX, J. N., PHAM, K. T., FREMEAU, R. T., JR., ZEVIANI, M., DWORK, A., SOPRANO, D. R., MAKOVER, A., GOODMAN, D. S., ZIMMERMAN, E. A. & ET AL. 1986. Transthyretin: a choroid plexus-specific transport protein in human brain. The 1986 S. Weir Mitchell award. *Neurology,* 36, 900-11.

HIDALGO, J., PENKOWA, M., ESPEJO, C., MARTINEZ-CACERES, E. M., CARRASCO, J., QUINTANA, A., MOLINERO, A., FLORIT, S., GIRALT, M. & ORTEGA-AZNAR, A. 2006. Expression of metallothionein-I, -II, and -III in Alzheimer disease and animal models of neuroinflammation. *Exp Biol Med (Maywood),* 231, 1450-8.

HIRKO, A. C., MEYER, E. M., KING, M. A. & HUGHES, J. A. 2007. Peripheral transgene expression of plasma gelsolin reduces amyloid in transgenic mouse models of Alzheimer's disease. *Mol Ther,* 15, 1623-9.

HOLTZMAN, D. M., LADU, M. J. & FAGAN, A. M. 1999. Clusterin-apoE Lipoprotein Particles. *In:* FINCH, C. E. (ed.) *Clusterin in Normal Brain Functions and During Neurodegeneration.*

HONG-GOKA, B. C. & CHANG, F. L. 2004. Estrogen receptors alpha and beta in choroid plexus epithelial cells in Alzheimer's disease. *Neurosci Lett,* 360, 113-6.

HU, J., IGARASHI, A., KAMATA, M. & NAKAGAWA, H. 2001. Angiotensin-converting enzyme degrades Alzheimer amyloid beta-peptide (A beta); retards A beta aggregation, deposition, fibril formation; and inhibits cytotoxicity. *J Biol Chem,* 276, 47863-8.

ISHIKAWA, T., ZHU, B. L., LI, D. R., ZHAO, D., MICHIUE, T. & MAEDA, H. 2006. Age-dependent increase of clusterin in the human pituitary gland. *Leg Med (Tokyo),* 8, 28-33.

IWAHANA, E., KARATSOREOS, I., SHIBATA, S. & SILVER, R. 2008. Gonadectomy reveals sex differences in circadian rhythms and suprachiasmatic nucleus androgen receptors in mice. *Horm Behav,* 53, 422-30.

JAYARAMAN, A., CARROLL, J. C., MORGAN, T. E., LIN, S., ZHAO, L., ARIMOTO, J. M., MURPHY, M. P., BECKETT, T. L., FINCH, C. E., BRINTON, R. D. & PIKE, C. J. 2012. 17beta-estradiol and progesterone regulate expression of beta-amyloid clearance factors in primary neuron cultures and female rat brain. *Endocrinology,* 153, 5467-79.

JECHURA, T. J., WALSH, J. M. & LEE, T. M. 2000. Testicular hormones modulate circadian rhythms of the diurnal rodent, Octodon degus. *Horm Behav,* 38, 243-9.

JI, L., CHAUHAN, A. & CHAUHAN, V. 2008. Cytoplasmic gelsolin in pheochromocytoma-12 cells forms a complex with amyloid beta-protein. *Neuroreport,* 19, 463-6.

JI, L., CHAUHAN, A. & CHAUHAN, V. 2010. Upregulation of cytoplasmic gelsolin, an amyloid-beta-binding protein, under oxidative stress conditions: involvement of protein kinase C. *J Alzheimers Dis,* 19, 829-38.

JI, L., CHAUHAN, A., MUTHAIYAH, B., WEGIEL, J. & CHAUHAN, V. 2009. Gelsolin levels are increased in the brain as a function of age during normal development in children that are further increased in Down syndrome. *Alzheimer Dis Assoc Disord,* 23, 319-22.

JOHANSON, C. E., SZMYDYNGER-CHODOBSKA, J., CHODOBSKI, A., BAIRD, A., MCMILLAN, P. & STOPA, E. G. 1999. Altered formation and bulk absorption of cerebrospinal fluid in FGF-2-induced hydrocephalus. *Am J Physiol,* 277, R263-71.

KARATSOREOS, I. N., BUTLER, M. P., LESAUTER, J. & SILVER, R. 2011. Androgens modulate structure and function of the suprachiasmatic nucleus brain clock. *Endocrinology,* 152, 1970-8.

KARATSOREOS, I. N., WANG, A., SASANIAN, J. & SILVER, R. 2007. A role for androgens in regulating circadian behavior and the suprachiasmatic nucleus. *Endocrinology,* 148, 5487-95.

KO, C. H. & TAKAHASHI, J. S. 2006. Molecular components of the mammalian circadian clock. *Hum Mol Genet,* 15 Spec No 2, R271-7.

KONDRATOVA, A. A. & KONDRATOV, R. V. 2012. The circadian clock and pathology of the ageing brain. *Nat Rev Neurosci,* 13, 325-35.

KONINGS, C. H., KUIPER, M. A., SCHELTENS, P., GRIJPMA, A. M., VAN PELT, W. & WOLTERS, E. C. 1993. Re-evaluation of cerebrospinal fluid angiotensin-converting enzyme activity in patients with 'probable' Alzheimer's disease. *Eur J Clin Chem Clin Biochem,* 31, 495-7.

KOUNNAS, M. Z., HAUDENSCHILD, C. C., STRICKLAND, D. K. & ARGRAVES, W. S. 1994. Immunological localization of glycoprotein 330, low density lipoprotein receptor related protein and 39 kDa receptor associated protein in embryonic mouse tissues. *In Vivo,* 8, 343-51.

KOUNNAS, M. Z., LOUKINOVA, E. B., STEFANSSON, S., HARMONY, J. A., BREWER, B. H., STRICKLAND, D. K. & ARGRAVES, W. S. 1995. Identification of glycoprotein 330 as an endocytic receptor for apolipoprotein J/clusterin. *J Biol Chem,* 270, 13070-5.

KOYA, R. C., FUJITA, H., SHIMIZU, S., OHTSU, M., TAKIMOTO, M., TSUJIMOTO, Y. & KUZUMAKI, N. 2000. Gelsolin inhibits apoptosis by blocking mitochondrial membrane potential loss and cytochrome c release. *J Biol Chem,* 275, 15343-9.

KRZYZANOWSKA, A. & CARRO, E. 2012. Pathological alteration in the choroid plexus of Alzheimer's disease: implication for new therapy approaches. *Front Pharmacol,* 3, 75.

KWEKEL, J. C., DESAI, V. G., MOLAND, C. L., VIJAY, V. & FUSCOE, J. C. 2013. Sex differences in kidney gene expression during the life cycle of F344 rats. *Biol Sex Differ,* 4, 14.

KWON, I., CHOE, H. K., SON, G. H. & KIM, K. 2011. Mammalian molecular clocks. *Exp Neurobiol,* 20, 18-28.

LEISSRING, M. A., FARRIS, W., CHANG, A. Y., WALSH, D. M., WU, X., SUN, X., FROSCH, M. P. & SELKOE, D. J. 2003. Enhanced proteolysis of beta-amyloid in APP transgenic mice prevents plaque formation, secondary pathology, and premature death. *Neuron,* 40, 1087-93.

LI, J. Z., BUNNEY, B. G., MENG, F., HAGENAUER, M. H., WALSH, D. M., VAWTER, M. P., EVANS, S. J., CHOUDARY, P. V., CARTAGENA, P., BARCHAS, J. D., SCHATZBERG, A. F., JONES, E. G., MYERS, R. M., WATSON, S. J., JR., AKIL, H. & BUNNEY, W. E. 2013. Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. *Proc Natl Acad Sci U S A,* 110, 9950-5.

LI, Y., CHEN, J. & CHOPP, M. 2002. Cell proliferation and differentiation from ependymal, subependymal and choroid plexus cells in response to stroke in rats. *J Neurol Sci,* 193, 137-46.

LIDSTROM, A. M., BOGDANOVIC, N., HESSE, C., VOLKMAN, I., DAVIDSSON, P. & BLENNOW, K. 1998. Clusterin (apolipoprotein J) protein levels are increased in hippocampus and in frontal cortex in Alzheimer's disease. *Exp Neurol,* 154, 511-21.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25, 402-8.

MALITO, E., RALAT, L. A., MANOLOPOULOU, M., TSAY, J. L., WADLINGTON, N. L. & TANG, W. J. 2008. Molecular bases for the recognition of short peptide substrates and cysteine-directed modifications of human insulin-degrading enzyme. *Biochemistry,* 47, 12822-34.

MARTINHO, A., GONCALVES, I., CARDOSO, I., ALMEIDA, M. R., QUINTELA, T., SARAIVA, M. J. & SANTOS, C. R. 2010. Human metallothioneins 2 and 3 differentially affect amyloid-beta binding by transthyretin. *FEBS J,* 277, 3427-36.

MATSUMOTO, N., KITAYAMA, H., KITADA, M., KIMURA, K., NODA, M. & IDE, C. 2003. Isolation of a set of genes expressed in the choroid plexus of the mouse using suppression subtractive hybridization. *Neuroscience,* 117, 405-15.

MAWUENYEGA, K. G., SIGURDSON, W., OVOD, V., MUNSELL, L., KASTEN, T., MORRIS, J. C., YARASHESKI, K. E. & BATEMAN, R. J. 2010. Decreased clearance of CNS beta-amyloid in Alzheimer's disease. *Science,* 330, 1774.

MCDERMOTT, J. R. & GIBSON, A. M. 1997. Degradation of Alzheimer's beta-amyloid protein by human and rat brain peptidases: involvement of insulin-degrading enzyme. *Neurochem Res,* 22, 49-56.

MENDOZA-NARANJO, A., GONZALEZ-BILLAULT, C. & MACCIONI, R. B. 2007. Abeta1-42 stimulates actin polymerization in hippocampal neurons through Rac1 and Cdc42 Rho GTPases. *J Cell Sci,* 120, 279-88.

MINERS, J. S., BAIG, S., TAYLER, H., KEHOE, P. G. & LOVE, S. 2009. Neprilysin and insulin-degrading enzyme levels are increased in Alzheimer disease in relation to disease severity. *J Neuropathol Exp Neurol,* 68, 902-14.

MONG, J. A., BAKER, F. C., MAHONEY, M. M., PAUL, K. N., SCHWARTZ, M. D., SEMBA, K. & SILVER, R. 2011. Sleep, rhythms, and the endocrine brain: influence of sex and gonadal hormones. *J Neurosci,* 31, 16107-16.

MORIN, L. P. & ALLEN, C. N. 2006. The circadian visual system, 2005. *Brain Res Rev,* 51, 1-60.

MORTAZAVI, M. M., GRIESSENAUER, C. J., ADEEB, N., DEEP, A., BAVARSAD SHAHRIPOUR, R., LOUKAS, M., TUBBS, R. I. & TUBBS, R. S. 2014. The choroid plexus: a comprehensive review of its history, anatomy, function, histology, embryology, and surgical considerations. *Childs Nerv Syst,* 30, 205-14.

NAKAMURA, T. J., MORIYA, T., INOUE, S., SHIMAZOE, T., WATANABE, S., EBIHARA, S. & SHINOHARA, K. 2005. Estrogen differentially regulates expression of Per1 and Per2 genes between central and peripheral clocks and between reproductive and nonreproductive tissues in female rats. *J Neurosci Res,* 82, 622-30.

NILSSON, C., LINDVALL-AXELSSON, M. & OWMAN, C. 1992. Neuroendocrine regulatory mechanisms in the choroid plexus-cerebrospinal fluid system. *Brain Res Brain Res Rev,* 17, 109-38.

OBA, R., IGARASHI, A., KAMATA, M., NAGATA, K., TAKANO, S. & NAKAGAWA, H. 2005. The N-terminal active centre of human angiotensin-converting enzyme degrades Alzheimer amyloid beta-peptide. *Eur J Neurosci,* 21, 733-40.

ODA, T., PASINETTI, G. M., OSTERBURG, H. H., ANDERSON, C., JOHNSON, S. A. & FINCH, C. E. 1994. Purification and characterization of brain clusterin. *Biochem Biophys Res Commun,* 204, 1131-6.

ODA, T., WALS, P., OSTERBURG, H. H., JOHNSON, S. A., PASINETTI, G. M., MORGAN, T. E., ROZOVSKY, I., STINE, W. B., SNYDER, S. W., HOLZMAN, T. F. & ET AL. 1995. Clusterin (apoJ) alters the aggregation of amyloid beta-peptide (A beta 1-42) and forms slowly sedimenting A beta complexes that cause oxidative stress. *Exp Neurol,* 136, 22-31.

PANDA, S., ANTOCH, M. P., MILLER, B. H., SU, A. I., SCHOOK, A. B., STRAUME, M., SCHULTZ, P. G., KAY, S. A., TAKAHASHI, J. S. & HOGENESCH, J. B. 2002. Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell,* 109, 307-20.

PARTCH, C. L., GREEN, C. B. & TAKAHASHI, J. S. 2014. Molecular architecture of the mammalian circadian clock. *Trends Cell Biol,* 24, 90-9.

PASCALE, C. L., MILLER, M. C., CHIU, C., BOYLAN, M., CARALOPOULOS, I. N., GONZALEZ, L., JOHANSON, C. E. & SILVERBERG, G. D. 2011. Amyloid-beta transporter expression at the blood-CSF barrier is age-dependent. *Fluids Barriers CNS,* 8, 21.

PASINETTI, G. M., JOHNSON, S. A., ODA, T., ROZOVSKY, I. & FINCH, C. E. 1994. Clusterin (SGP-2): a multifunctional glycoprotein with regional expression in astrocytes and neurons of the adult rat brain. *J Comp Neurol,* 339, 387-400.

PAUNIO, T., KIURU, S., KARONEN, S.-L., PALO, J. & PELTONEN, L. 1994. Quantification of serum and cerebrospinal fluid gelsolin in familial amyloidosis, Finnish type (AGel). *Amyloid,* 1, 80-89.

PENKOWA, M., GIRALT, M., CAMATS, J. & HIDALGO, J. 2002. Metallothionein 1+2 protect the CNS during neuroglial degeneration induced by 6-aminonicotinamide. *J Comp Neurol,* 444, 174-89.

PICKETTS, D. J. 2006. Neuropeptide signaling and hydrocephalus: SCO with the flow. *J Clin Invest,* 116, 1828-32.

PROUDLER, A. J., COOPER, A., WHITEHEAD, M. & STEVENSON, J. C. 2003. Effects of oestrogen-only and oestrogen-progestogen replacement therapy upon circulating angiotensin I-converting enzyme activity in postmenopausal women. *Clin Endocrinol (Oxf),* 58, 30-5.

QIAO, H., KOYA, R. C., NAKAGAWA, K., TANAKA, H., FUJITA, H., TAKIMOTO, M. & KUZUMAKI, N. 2005. Inhibition of Alzheimer's amyloid-beta peptide-induced reduction of mitochondrial membrane potential and neurotoxicity by gelsolin. *Neurobiol Aging,* 26, 849-55.

QIU, W. Q., WALSH, D. M., YE, Z., VEKRELLIS, K., ZHANG, J., PODLISNY, M. B., ROSNER, M. R., SAFAVI, A., HERSH, L. B. & SELKOE, D. J. 1998. Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation. *J Biol Chem,* 273, 32730-8.

QUADROS, P. S., PFAU, J. L. & WAGNER, C. K. 2007. Distribution of progesterone receptor immunoreactivity in the fetal and neonatal rat forebrain. *J Comp Neurol,* 504, 42-56.

QUINTELA, T., ALVES, C. H., GONCALVES, I., BALTAZAR, G., SARAIVA, M. J. & SANTOS, C. R. 2008. 5Alpha-dihydrotestosterone up-regulates transthyretin levels in mice and rat choroid plexus via an androgen receptor independent pathway. *Brain Res,* 1229, 18-26.

QUINTELA, T., GONCALVES, I., CARRETO, L. C., SANTOS, M. A., MARCELINO, H., PATRIARCA, F. M. & SANTOS, C. R. 2013. Analysis of the effects of sex hormone background on the rat choroid plexus transcriptome by cDNA microarrays. *PLoS One,* 8, e60199.

QUINTELA, T., GONCALVES, I., MARTINHO, A., ALVES, C. H., SARAIVA, M. J., ROCHA, P. & SANTOS, C. R. 2011. Progesterone enhances transthyretin expression in the rat choroid plexus in vitro and in vivo via progesterone receptor. *J Mol Neurosci,* 44, 152-8.

QUINTELA, T., SOUSA, C., PATRIARCA, F. M., GONCALVES, I. & SANTOS, C. R. 2014. Gender associated circadian oscillations of the clock genes in rat choroid plexus. *Brain Struct Funct,* 220, 1251-62.

RALAT, L. A., GUO, Q., REN, M., FUNKE, T., DICKEY, D. M., POTTER, L. R. & TANG, W. J. 2011. Insulin-degrading enzyme modulates the natriuretic peptide-mediated signaling response. *J Biol Chem,* 286, 4670-9.

RANSOHOFF, R. M. 2014. Physiology. Good barriers make good neighbors. *Science,* 346, 36-7.

RAY, I., CHAUHAN, A., WEGIEL, J. & CHAUHAN, V. P. 2000. Gelsolin inhibits the fibrillization of amyloid beta-protein, and also defibrillizes its preformed fibrils. *Brain Res,* 853, 344-51.

REDDY, K. B., KARODE, M. C., HARMONY, A. K. & HOWE, P. H. 1996. Interaction of transforming growth factor beta receptors with apolipoprotein J/clusterin. *Biochemistry,* 35, 309-14.

REDZIC, Z. 2011. Molecular biology of the blood-brain and the blood-cerebrospinal fluid barriers: similarities and differences. *Fluids Barriers CNS,* 8, 3.

REDZIC, Z. B. 2013. Studies on the human choroid plexus in vitro. *Fluids Barriers CNS,* 10, 10.

REDZIC, Z. B. & SEGAL, M. B. 2004. The structure of the choroid plexus and the physiology of the choroid plexus epithelium. *Adv Drug Deliv Rev,* 56, 1695-716.

REID, I. A. 1992. Interactions between ANG II, sympathetic nervous system, and baroreceptor reflexes in regulation of blood pressure. *Am J Physiol,* 262, E763-E778.

REPPERT, S. M. & WEAVER, D. R. 2001. Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol,* 63, 647-76.

ROGEBERG, M., FURLUND, C. B., MOE, M. K. & FLADBY, T. 2014. Identification of peptide products from enzymatic degradation of amyloid beta. *Biochimie,* 105, 216-20.

ROTH, R. A., MESIROW, M. L., YOKONO, K. & BABA, S. 1984. Degradation of insulin-like growth factors I and II by a human insulin degrading enzyme. *Endocr Res,* 10, 101-12.

RUITENBERG, A., OTT, A., VAN SWIETEN, J. C., HOFMAN, A. & BRETELER, M. M. 2001. Incidence of dementia: does gender make a difference? *Neurobiol Aging,* 22, 575-80.

SAFAVI, A., MILLER, B. C., COTTAM, L. & HERSH, L. B. 1996. Identification of gamma-endorphin-generating enzyme as insulin-degrading enzyme. *Biochemistry,* 35, 14318-25.

SANTOS, C. R., MARTINHO, A., QUINTELA, T. & GONCALVES, I. 2012. Neuroprotective and neuroregenerative properties of metallothioneins. *IUBMB Life,* 64, 126-35.

SANTOS, C. R. A., CARDOSO, I. & GONÇALVES, I. 2011. Key Enzymes and Proteins in Amyloid-Beta Production and Clearance. *In:* MONTE, S. D. L. (ed.) *Alzheimer's Disease Pathogenesis-Core Concepts, Shifting Paradigms and Therapeutic Targets.* InTech.

SCHREIBER, G., ALDRED, A. R., JAWOROWSKI, A., NILSSON, C., ACHEN, M. G. & SEGAL, M. B. 1990. Thyroxine transport from blood to brain via transthyretin synthesis in choroid plexus. *Am J Physiol,* 258, R338-45.

SCHWARZMAN, A. L., GREGORI, L., VITEK, M. P., LYUBSKI, S., STRITTMATTER, W. J., ENGHILDE, J. J., BHASIN, R., SILVERMAN, J., WEISGRABER, K. H., COYLE, P. K. & ET AL. 1994. Transthyretin sequesters amyloid beta protein and prevents amyloid formation. *Proc Natl Acad Sci U S A,* 91, 8368-72.

SEGAL, M. B. 2001. Transport of nutrients across the choroid plexus. *Microsc Res Tech,* 52, 38-48.

SELKOE, D. J. 2000. Toward a comprehensive theory for Alzheimer's disease. Hypothesis: Alzheimer's disease is caused by the cerebral accumulation and cytotoxicity of amyloid beta-protein. *Ann N Y Acad Sci,* 924, 17-25.

SELTZER, A., PINTO, J. E., VIGLIONE, P. N., CORREA, F. M., LIBERTUN, C., TSUTSUMI, K., STEELE, M. K. & SAAVEDRA, J. M. 1992. Estrogens regulate angiotensin-converting enzyme and angiotensin receptors in female rat anterior pituitary. *Neuroendocrinology,* 55, 460-7.

SEROT, J. M., BENE, M. C. & FAURE, G. C. 2003. Choroid plexus, aging of the brain, and Alzheimer's disease. *Front Biosci,* 8, s515-21.

SEROT, J. M., BENE, M. C., FOLIGUET, B. & FAURE, G. C. 2000. Morphological alterations of the choroid plexus in late-onset Alzheimer's disease. *Acta Neuropathol,* 99, 105-8.

SEROT, J. M., CHRISTMANN, D., DUBOST, T. & COUTURIER, M. 1997. Cerebrospinal fluid transthyretin: aging and late onset Alzheimer's disease. *J Neurol Neurosurg Psychiatry,* 63, 506-8.

SHIRWANY, N. A., PAYETTE, D., XIE, J. & GUO, Q. 2007. The amyloid beta ion channel hypothesis of Alzheimer's disease. *Neuropsychiatr Dis Treat,* 3, 597-612.

SILVERBERG, G. D., MILLER, M. C., MESSIER, A. A., MAJMUDAR, S., MACHAN, J. T., DONAHUE, J. E., STOPA, E. G. & JOHANSON, C. E. 2010. Amyloid deposition and influx transporter expression at the blood-brain barrier increase in normal aging. *J Neuropathol Exp Neurol,* 69, 98-108.

SKIPOR, J. & THIERY, J. C. 2008. The choroid plexus--cerebrospinal fluid system: undervaluated pathway of neuroendocrine signaling into the brain. *Acta Neurobiol Exp (Wars),* 68, 414-28.

SOUBRIER, F., ALHENC-GELAS, F., HUBERT, C., ALLEGRINI, J., JOHN, M., TREGEAR, G. & CORVOL, P. 1988. Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. *Proc Natl Acad Sci U S A,* 85, 9386-90.

SPECTOR, R. & LORENZO, A. V. 1975. Folate transport by the choroid plexus in vitro. *Science,* 187, 540-2.

STEIN, T. D. & JOHNSON, J. A. 2002. Lack of neurodegeneration in transgenic mice overexpressing mutant amyloid precursor protein is associated with increased levels of transthyretin and the activation of cell survival pathways. *J Neurosci,* 22, 7380-8.

STRAZIELLE, N., GHERSI-EGEA, J.-F., GHISO, J., DEHOUCK, M. P., FRANGIONE, B., PATLAK, C., FENSTERMACHER, J. & GOREVIC, P. 2000. In vitro evidence that beta-amyloid peptide 1-40 diffuses across the blood-brain barrier and affects its permeability. *J Neuropathol Exp Neurol,* 59, 29.38.

SUN, H. Q., YAMAMOTO, M., MEJILLANO, M. & YIN, H. L. 1999. Gelsolin, a multifunctional actin regulatory protein. *J Biol Chem,* 274, 33179-82.

TANAKA, J. & SOBUE, K. 1994. Localization and characterization of gelsolin in nervous tissues: gelsolin is specifically enriched in myelin-forming cells. *J Neurosci,* 14, 1038-52.

TAPIOLA, T., PIRTTILA, T., MIKKONEN, M., MEHTA, P. D., ALAFUZOFF, I., KOIVISTO, K. & SOININEN, H. 2000. Three-year follow-up of cerebrospinal fluid tau, beta-amyloid 42 and 40 concentrations in Alzheimer's disease. *Neurosci Lett,* 280, 119-22.

THAMBISETTY, M., SIMMONS, A., VELAYUDHAN, L., HYE, A., CAMPBELL, J., ZHANG, Y., WAHLUND, L. O., WESTMAN, E., KINSEY, A., GUNTERT, A., PROITSI, P., POWELL, J., CAUSEVIC, M., KILLICK, R., LUNNON, K., LYNHAM, S., BROADSTOCK, M., CHOUDHRY, F., HOWLETT, D. R., WILLIAMS, R. J., SHARP, S. I., MITCHELMORE, C., TUNNARD, C., LEUNG, R., FOY, C., O'BRIEN, D., BREEN, G., FURNEY, S. J., WARD, M., KLOSZEWSKA, I., MECOCCI, P., SOININEN, H., TSOLAKI, M., VELLAS, B., HODGES, A., MURPHY, D. G., PARKINS, S., RICHARDSON, J. C., RESNICK, S. M., FERRUCCI, L., WONG, D. F., ZHOU, Y., MUEHLBOECK, S., EVANS, A., FRANCIS, P. T., SPENGER, C. & LOVESTONE, S. 2010. Association of plasma clusterin concentration with severity, pathology, and progression in Alzheimer disease. *Arch Gen Psychiatry,* 67, 739-48.

THIRUMOORTHY, N., SHYAM SUNDER, A., MANISENTHIL KUMAR, K., SENTHIL KUMAR, M., GANESH, G. & CHATTERJEE, M. 2011. A review of metallothionein isoforms and their role in pathophysiology. *World J Surg Oncol,* 9, 54.

VARGAS, T., ANTEQUERA, D., UGALDE, C., SPUCH, C. & CARRO, E. 2010a. Gelsolin restores A beta-induced alterations in choroid plexus epithelium. *J Biomed Biotechnol,* 2010, 805405.

VARGAS, T., UGALDE, C., SPUCH, C., ANTEQUERA, D., MORAN, M. J., MARTIN, M. A., FERRER, I., BERMEJO-PAREJA, F. & CARRO, E. 2010b. Abeta accumulation in choroid plexus is associated with mitochondrial-induced apoptosis. *Neurobiol Aging,* 31, 1569-81.

VIDENOVIC, A., LAZAR, A. S., BARKER, R. A. & OVEREEM, S. 2014. 'The clocks that time us'--circadian rhythms in neurodegenerative disorders. *Nat Rev Neurol,* 10, 683-93.

WOLBURG, H. & PAULUS, W. 2010. Choroid plexus: biology and pathology. *Acta Neuropathol,* 119, 75-88.

WON, Y. S., LEE, S. J., YEO, S. G. & PARK, D. C. 2012. Effects of female sex hormones on clusterin expression and paclitaxel resistance in endometrial cancer cell lines. *Int J Med Sci,* 9, 86-92.

WRIGHT, E. M. 1978. Transport processes in the formation of the cerebrospinal fluid. *Rev Physiol Biochem Pharmacol,* 83, 3-34.

WRIGHT, J. W. & HARDING, J. W. 1992. Regulatory role of brain angiotensins in the control of physiological and behavioral responses. *Brain Res Brain Res Rev,* 17, 227-62.

YAMAMOTO, Y., YOSHIZAWA, T., KAMIO, S., AOKI, O., KAWAMATA, Y., MASUSHIGE, S. & KATO, S. 1997. Interactions of transthyretin (TTR) and retinol-binding protein (RBP) in the uptake of retinol by primary rat hepatocytes. *Exp Cell Res,* 234, 373-8.

YANG, D. T., JOSHI, G., CHO, P. Y., JOHNSON, J. A. & MURPHY, R. M. 2013. Transthyretin as both a sensor and a scavenger of beta-amyloid oligomers. *Biochemistry,* 52, 2849-61.

YANG, S., WANG, K., VALLADARES, O., HANNENHALLI, S. & BUCAN, M. 2007. Genome-wide expression profiling and bioinformatics analysis of diurnally regulated genes in the mouse prefrontal cortex. *Genome Biol,* 8, R247.

YUE, X., LU, M., LANCASTER, T., CAO, P., HONDA, S., STAUFENBIEL, M., HARADA, N., ZHONG, Z., SHEN, Y. & LI, R. 2005. Brain estrogen deficiency accelerates Abeta plaque formation in an Alzheimer's disease animal model. *Proc Natl Acad Sci U S A,* 102, 19198-203.

ZAMBENEDETTI, P., GIORDANO, R. & ZATTA, P. 1998. Metallothioneins are highly expressed in astrocytes and microcapillaries in Alzheimer's disease. *J Chem Neuroanat,* 15, 21-6.

ZHANG, J., GOODLETT, D. R., PESKIND, E. R., QUINN, J. F., ZHOU, Y., WANG, Q., PAN, C., YI, E., ENG, J., AEBERSOLD, R. H. & MONTINE, T. J. 2005. Quantitative proteomic analysis of age-related changes in human cerebrospinal fluid. *Neurobiol Aging,* 26, 207-27.

ZHAO, L., YAO, J., MAO, Z., CHEN, S., WANG, Y. & BRINTON, R. D. 2011. 17beta-Estradiol regulates insulin-degrading enzyme expression via an ERbeta/PI3-K pathway in hippocampus: relevance to Alzheimer's prevention. *Neurobiol Aging,* 32, 1949-63.

ZHENG, W., PERRY, D. F., NELSON, D. L. & APOSHIAN, H. V. 1991. Choroid plexus protects cerebrospinal fluid against toxic metals. *FASEB J,* 5, 2188-93.

ZOU, K., YAMAGUCHI, H., AKATSU, H., SAKAMOTO, T., KO, M., MIZOGUCHI, K., GONG, J. S., YU, W., YAMAMOTO, T., KOSAKA, K., YANAGISAWA, K. & MICHIKAWA, M. 2007. Angiotensin-converting enzyme converts amyloid beta-protein 1-42 (Abeta(1-42)) to Abeta(1-40), and its inhibition enhances brain Abeta deposition. *J Neurosci,* 27, 8628-35.

ZUBENKO, G. S., VOLICER, L., DIRENFELD, L. K., FREEMAN, M., LANGLAIS, P. J. & NIXON, R. A. 1985. Cerebrospinal fluid levels of angiotensin-converting enzyme in Alzheimer's disease, Parkinson's disease and progressive supranuclear palsy. *Brain Res*, 328, 215-21.