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Bisphenol A effects in transthyretin and barrier integrity markers in the choroid plexus: implications in amyloid beta catabolism

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Abstract

The choroid plexus is a multifunctional tissue responsible for a wide range of homeostatic functions crucial to the central nervous system, including secretion of cerebrospinal fluid, synthesis and secretion of important peptides and regulation of the chemical substances exchange between the blood and the cerebrospinal fluid, through the blood-cerebrospinal fluid barrier. Transthyretin, a protein highly expressed and secreted by choroid plexus to the cerebrospinal fluid, is the major amyloid-beta scavenger protein, contributing to its clearance. Sex hormones, as estrogens, upregulate transthyretin expression in choroid plexus, and as a consequence its regulation may be disrupted by substances that interfere with various cellular pathways regulated by endogenous hormones, known as endocrine disruptors chemicals. The human population is exposed to many chemicals with such properties, such as bisphenol A. Therefore, the present study analysed the effects of the endocrine disruptor bisphenol A on transthyretin expression in newborn rats by Whole-Mount fluorescent staining and Western blot, and at the mRNA level by Real time RT-PCR. Moreover, the effects of beta-amyloid on transthyretin expression were also investigated using the same techniques in choroid explants of newborn and young rats. Blood-cerebrospinal fluid barrier plays an important role in the regulation of molecules movement between choroid plexus and cerebrospinal fluid, and its disruption can happen when choroid plexus functions are impaired. Thus, one purpose of this work was to determine the effects of both compounds, beta-amyloid and bisphenol A, in blood-cerebrospinal fluid barrier integrity through the evaluation of some membrane protein levels present in this barrier, namely, occludin, E-cadherin, claudin-1 and zonula occludens-1.

Beta-amyloid treatment in rat choroid plexus seems to trigger transthyretin upregulation, in a dose-response manner. Transthyretin mRNA levels in newborn rat choroid plexus explants increased much more than in young explants. Increased transthyretin expression levels were not correlated with secretion levels. Additionally, beta-amyloid at 1 μ g/mL increased reactive oxygen species production in choroid plexus. Low doses of bisphenol A affected transthyretin expression in rat choroid plexus in a non-monotonic dose response way, accordingly to data previously reported in other studies with bisphenol A. The same response profile was observed in transthyretin protein and mRNA levels measured, with higher transthyretin levels verified at 50nM of bisphenol A. As reported before with beta-amyloid treatment, also bisphenol A led to an increase of transthyretin expression in choroid plexus cells, which was not altered with significance the secretion levels of this protein.

Beta-amyloid and bisphenol A clearly influence transthyretin expression in rat choroid plexus, in a dose-response manner, and in a non-monotonic dose response, respectively. In accordance to previous reports, increasing beta-amyloid levels induced transthyretin upregulation.

Increased transthyretin production by choroid plexus seems to be a protective mechanisms to avoid beta-amyloid fibrillization and consequent toxicity. Bisphenol A interfered with transthyretin expression, in both positive and negative ways. Therefore, bisphenol A levels might lead to up or down of transthyretin regulation, and consequently, leading to impairment of beta-amyloid levels in brain. Blood-cerebrospinal fluid barrier integrity might be compromised by beta-amyloid and bisphenol A injuries, which explains alteration in secretion rates of controls for treated choroid plexus explants. However, further investigation is required to analyse evolution of transthyretin expression by choroid plexus throughout life, and would be also important evaluate bisphenol A effects in blood-cerebrospinal fluid barrier protein levels, to better understand bisphenol A consequences in beta-amyloid clearance.

Keywords

Choroid Plexus, Transthyretin, Amyloid-Beta, Bisphenol A, Blood-cerebrospinal fluid barrier, Tight junctions

Resumo alargado

No sistema ventricular cerebral encontram-se 4 plexos coróides, um em cada ventrículo, os quais desempenham importantes funções, diretamente envolvidas na homeostasia do sistema nervoso central. Destas funções destacam-se a secreção do líquido cefalorraquidiano, a síntese e secreção de inúmeras substâncias bioactivas (proteínas, citocinas, vitaminas), bem como a regulação da passagem de moléculas entre a corrente sanguínea e o líquido cefalorraquidiano, através da barreira sangue-líquido cefalorraquidiano. Esta barreira formada pelas células epiteliais do plexo coróide é composta por inúmeras proteínas membranares denominadas *tight junctions* e *adherens junctions*, que são fundamentais para manter a sua integridade, e assegurar a função secretora do plexo coróide. O plexo coróide é ainda responsável pela síntese e secreção da transtirretina, a principal proteína envolvida no catabolismo do péptido beta amilóide, cuja deposição no cérebro é uma das principais marcas etimológicas da doença de Alzheimer. Quando secretada para o líquido cefalorraquidiano, a transtirretina forma complexos estáveis com o beta-amilóide, evitando a sua agregação e fibrilação, e consequentemente, os efeitos tóxicos inerentes à acumulação destes agregados. A expressão da transtirretina é regulada positivamente pelas hormonas sexuais, nas quais se incluem os estrogénios. Estas hormonas podem ver as suas ações mimetizadas por certos compostos denominados por disruptores endócrinos, cuja crescente difusão no meio ambiente e entre as populações tem levantado várias questões sobre a sua implicação na saúde pública. Um dos disruptores endócrinos mais estudado é o bisfenol A, um xenoestrogénio, altamente difundido no meio ambiente, e ao qual a exposição humana foi comprovada em inúmeros estudos. Posto isto, este trabalho tem como principal objetivo avaliar o efeito do bisfenol A na expressão da transtirretina, em explantes de plexo coróide de rato, e a sua consequente relação no catabolismo do beta-amilóide. Além disto, também se averiguou se a expressão da transtirretina no plexo coróide sofre modificações, em ratos de diferentes idades, e se essa expressão tem alguma relação com o aumento dos níveis de beta-amilóide. Adicionalmente, a expressão de algumas proteínas descritas na barreira sangue-fluido cefalorraquidiano (occludina, e-caderina, claudina-1 e *zonula occludens-1*) foi avaliada nos explantes de plexos coróides após o tratamento com o beta-amilóide e o bisfenol A, de modo a perceber se estes compostos interferem na integridade desta barreira.

A expressão da transtirretina foi analisada *ex vivo*, em explantes de plexos coróides de ratos recém-nascidos e de ratos jovens tratados com beta-amilóide e, em explantes de recém nascidos tratados com bisfenol A. Para tal, recorreu-se a várias técnicas: *Whole mount* (imunohistoquímica por fluorescência), *Real-time PCR* e *Western Blot*. A localização e expressão das proteínas membranares foi estudada através de *Whole mount* e *Western blot*.

Nos explantes de plexo coróide tratados com beta-amilóide houve um aumento na expressão da transtirretina, correlacionado com o aumento da concentração de beta-amilóide, que se verificou em ambas as idades estudadas, de forma semelhante. A maior diferença ocorreu relativamente à expressão de mRNA da transtirretina nos explantes dos animais recém-nascidos, onde os níveis de transtirretina são bastante elevados, mais do que nos explantes dos jovens. Por outro lado e, contrariando o esperado, não houve um aumento na excreção desta proteína, mas sim um ligeiro decréscimo. Foi ainda avaliada a produção de espécies reativas de oxigénio nos explantes de plexo coróide de ratos jovens onde houve um aumento significativo relativamente aos controlos. Nos plexos coróides tratados com bisfenol A, verificaram-se modificações na expressão da transtirretina mesmo com baixas doses do composto, as quais estão de acordo com os níveis referidos em estudos epidemiológicos. Além disto, os níveis de expressão da transtirretina nestes explantes mostraram seguir uma curva com resposta não-monotónica, tal como observado em vários outros estudos, e característico da exposição aos disruptores endócrinos. A expressão da transtirretina nos plexos coróides foi maior para a concentração de 50nM de BPA comparativamente às outras concentrações testadas, tanto para os níveis da proteína como para os de mRNA. Contudo, a secreção da transtirretina não acompanhou o aumento da sua expressão.

Tanto o beta-amilóide como o bisfenol A mostraram-se capazes de interferir na expressão da transtirretina no plexo coróide de rato, de forma dose-dependente e não-monotónica, respetivamente. O aumento da produção de transtirretina pelo plexo coróide quando os níveis de beta amilóide estão aumentados parece tratar-se de um mecanismo de proteção para evitar a agregação do péptido e consequente toxicidade. Relativamente ao bisfenol A, este consegue modular a expressão da transtirretina tanto positivamente como negativamente, o que poderá ter consequências nos níveis de transtirretina produzidos e libertados para o líquido cefalorraquidiano, e assim, interferir nos níveis de beta amilóide no cérebro. Tendo em conta as discrepâncias observadas entre os níveis de expressão da transtirretina e da sua secreção, para ambos os compostos estudados, a integridade da barreira sangue-líquido cefalorraquidiano poderá estar comprometida, e dessa forma, contribuir para o desequilíbrio do beta-amilóide.

Assim, tanto o beta-amilóide como o bisfenol A interferem na produção de transtirretina no plexo coróide de rato. Contudo, é necessário investigar a relação entre a transtirretina e o beta-amilóide ao longo do envelhecimento, para perceber se é a diminuição da transtirretina a responsável pela acumulação do beta-amilóide, nomeadamente na doença de Alzheimer, ou se é o péptido que contribuiu para a disfunção do plexo coróide levando à diminuição da transtirretina. Será ainda importante investigar os níveis das proteínas da barreira sangue-líquido cefalorraquidiano, para melhor entender de que forma o bisfenol A pode levar a um aumento de beta amilóide, ao interferir não só na expressão da transtirretina como também nas funções do plexo coróide.

Palavras-chave

Plexo coróide, transtirretina, beta-amiloide, bisfenol A, barreira sangue-líquido cefalorraquidiano, *tight junctions*

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Abbreviations and Acronymes List

A-Beta	Amyloid-Beta
AD	Alzheimer's Disease
AJ	Adherens Junction
Apo	Apolipoprotein
APP	Amyloid Precursor Protein
BBB	Blood-Brain Barrier
BCSFB	Blood-Cerebrospinal Fluid Barrier
BPA	Bisphenol A
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
Cld	Claudin
CNS	Central Nervous System
CP	Choroid Plexus
CPEC	Choroid Plexus Epithelial Cells
CSF	Cerebrospinal Fluid
DCFHA-DA	2,7-Dichlorodihydrofluorescein Diacetate
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ED	Endocrine Disruptor
EDC	Endocrine Disruptor Chemical
ER	Estrogen Receptor
JAM	Junctional Adhesion Molecule
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl Fluoride
PVDF	Polyvinylidene Difluoride
RBP	Retinol Binding Protein
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
TJ	Tight Junction
TTR	Transtyretin
ZO	Zonula Occludens

I. Introduction

1. Choroid Plexus

Choroid plexuses (CPs) are highly vascularized tissues, with a simple structure, confined to the ventricular system of the brain (Figure 1). This system is divided in four ventricles: two laterals, one third and a fourth, and each one retains the localization of one choroid plexus (CP) (Johansson *et al.*, 2008, Wolburg and Paulus, 2010, Damkier *et al.*, 2013). In the lateral ventricles of the mammalian brain, CPs form a sheet-like structure, whereas in the third and fourth ventricles, branched villus-like structures are observed instead (Damkier *et al.*, 2010).

CP is formed by a single layer of cuboidal epithelial cells that reside on a basement membrane. This epithelial basement membrane is characterized as a network of fenestrated capillaries surrounded by connective tissue composed by fibroblasts and immune cells (e.g., mast cells, macrophages, granulocytes), and a rich extracellular matrix (Miyoshi and Takai, 2005, Wolburg and Paulus, 2010, Terry *et al.*, 2010, Damkier *et al.*, 2013). The choroid plexus epithelial cells (CPEC) are connected by tight junctions (TJs), adherens junctions (AJs) and desmosomes (Damkier *et al.*, 2013). CPEC contain numerous mitochondria, Golgi apparatus, smooth endoplasmic reticulum and lysosome-like vesicles which demonstrate their intense synthetic capacity (Marques *et al.*, 2013). Ependymal cells have numerous microvilli from the ventricle facing (apical side), and extensive infolding at blood facing (basolateral side), thus providing a large surface for contact between epithelium and the cerebrospinal fluid (CSF) the apical side and epithelium and the CP interstitial fluid on the basolateral side (Skipor and Thiery, 2008).

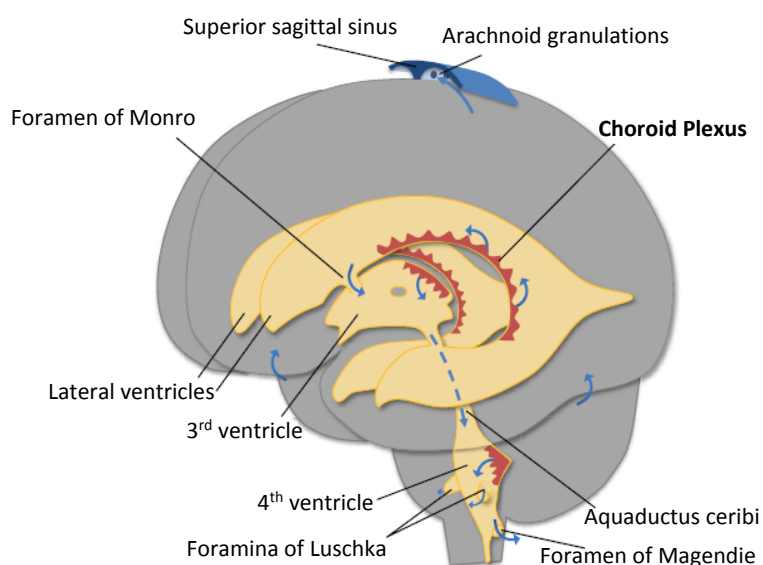


Figure 1 - Ventricular system of brain and CSF circulation. CSF flow forms in lateral CPs, drains via the foramen of Monro to the 3rd ventricle and of this to 4rd ventricle through aqueductus cerebri/Sylvius. CSF leaves ventricular brain through foramina of Luschka and foramen of Magendie. Adapted from (Damkier *et al.*, 2013).

1.1. Functions

CP is a multifunctional tissue responsible for a wide range of homeostatic functions crucial for the central nervous system (CNS), among which stand out: secretion of the CSF, regulation of the exchange of chemical substances from the blood to the CSF through the blood-cerebrospinal fluid barrier (BCSFB), and synthesis and secretion of important biologically active substances as vitamins, growth factors, peptides and hormones (Serot *et al.*, 2000, Johansson *et al.*, 2008, Skipor and Thiery, 2008, Johanson *et al.*, 2011b). These functions will be clarified in the next sections, with more emphasis on the characteristics of BCSFB and synthesis of transthyretin (TTR) due to their role in the present work.

1.1.1. Production and secretion of cerebrospinal fluid

CP is responsible for the constant formation and drainage of CSF, producing about 70-80% of this fluid (Skipor and Thiery, 2008, Johanson, 2008). This allows the formation of a unique circulatory system capable to perform diverse metabolic and signalling functions, which directly affect brain homeostasis (Johanson, 2008). CPs from the lateral ventricles form the flow of the CSF, which drains via the foramen of Munro into the third ventricle, and then by the Aqueduct of Sylvius into the fourth ventricle. At this point, CSF leaves the ventricular system and passes through paired foramina of Luschka and foramen of Magendie into the subarachnoid spaces where it fills the basal cisterns of the brain and the spinal cord (Figure 1). The return of the CSF occurs either directly, via arachnoid villi in the venous sinuses of the brain, or via lymphatic drainage pathways (Segal, 2001, Smith *et al.*, 2004, Skipor and Thiery, 2008, Lehtinen *et al.*, 2013). CSF functions in the CNS include physical protection, intracranial pressure regulation, waste removal and provision of a supportive environment (Serot *et al.*, 2003, Johanson *et al.*, 2008, Erickson and Banks, 2013). The CP is an important source of molecules that circulate in the CSF, contributing with compounds like vitamins, hormones and peptides (Skipor and Thiery, 2008, Spector and Johanson, 2013). Whether CSF plays an important role in controlling and maintaining a proper environment to the CNS, CSF homeostasis depends mainly of CP. Composition of CSF is strictly regulated by the transport systems expressed in CPEC, located at the basolateral and apical membranes, which are essential for the bidirectional movement of various substances across the BCSFB (Redzic *et al.*, 2005, Coisne and Engelhardt, 2011, Erickson and Banks, 2013). As the CSF rapidly and widely disseminates the substances circulating on it that have crossed the BCSFB, throughout the CNS, stability and integrity of this barrier is essential to avoid the access of deleterious substances to brain (Johanson *et al.*, 2011a).

1.1.2. The Blood-Cerebrospinal Fluid Barrier

Three barrier layers limit and regulate molecular exchange at the interfaces between the blood and the neural tissue or its fluid spaces: the blood-brain barrier (BBB), the BCSFB and the arachnoid barrier (Figure 2) (Abbott *et al.*, 2006, Ueno, 2007, Abbott *et al.*, 2010). CNS barriers have an essential role in CNS homeostasis maintenance, establishing a stable and adequate environment that allows normal brain function. Movement of molecules between blood stream and CSF is tightly controlled, to avoid oscillations in ionic composition, by preventing the entrance of macromolecules, neurotransmitters and neurotoxins, by elimination of metabolites, and provision of nutrient support (Skipor and Thiery, 2008, Abbott *et al.*, 2010, Marques *et al.*, 2013).

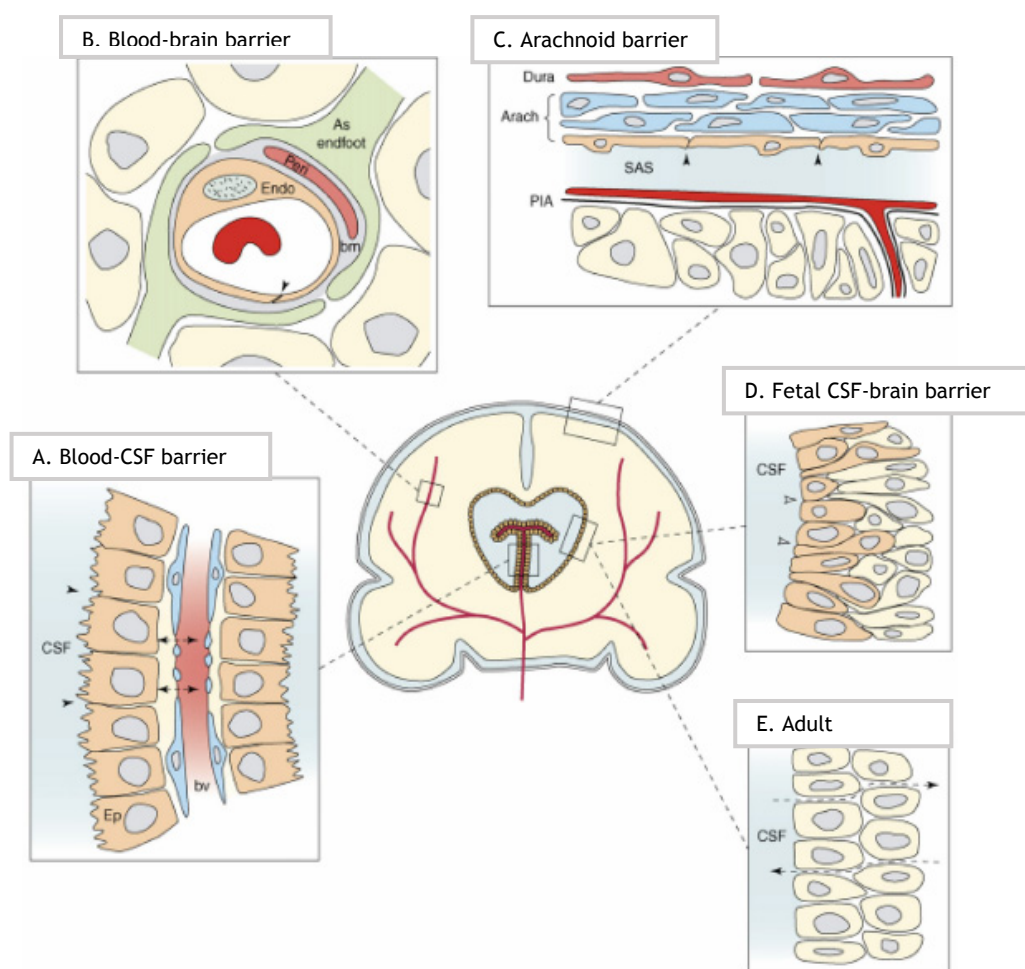


Figure 2 - Barrier interfaces in adult and developing brain (Saunders *et al.*, 2008). **A.** BCSFB is a barrier between CP blood vessels and the CSF. CPEC have apical tight junctions that restricts intercellular passage of molecules; **B.** BBB constitutes a barrier between the lumen of cerebral blood vessels and brain parenchyma; **C.** The arachnoid barrier. The blood vessels of the *dura* are fenestrated providing a weak barrier function; **D.** The fetal CSF-brain barrier has its localization between the CSF and brain parenchyma. It shows properties of a functional barrier at the early developing of brain; **E.** The adult ventricular parenchyma shows fatter cells and loss of strap junctions which are present in the fetal CSF-brain barrier. Consequently, the passage of large molecules as proteins is no longer prevented.

The arachnoid barrier is provided by the avascular arachnoid epithelium, underlying the dura, and completely enclosing the CNS. Due to its avascular nature and relatively small surface area, this barrier layer do not show a conditional role in exchange of molecules between the blood and the CNS (Abbott *et al.*, 2006). BBB, the interface with largest surface area (Neuwelt *et al.*, 2011), is a selective barrier that separates the nervous system from circulating blood, and is formed by the cerebrovascular endothelial cells between blood and brain interstitial fluid (Abbott *et al.*, 2006, Goncalves *et al.*, 2013). BBB and BCSFB constitutes the two main barriers that divide the CNS from the periphery, participating together in CNS homeostasis. The BCSFB is formed by CPEC facing the CSF at their apical membrane (Abbott *et al.*, 2010).

The BCSFB has three general barrier functions. First, the TJs between epithelial cells lining the CP form a physical barrier to facilitate the diffusion of molecules, especially the large and hydrophilic ones, between blood and CSF, and therefore paracellular diffusion does not occur to any great extent. Second, CPEC form an enzymatic barrier involved in uptake and degradation of many substances originated either in the brain or in the blood (e.g., serotonin, noradrenalin and their metabolites). CPEC express a lot of enzymes that degrade peptides including carboxy-, amino-, and endopeptidases. And third, CPEC contain a variety of specific and non-specific transport systems, that both mediate the entry of essential nutrients (e.g., glucose, amino acids) and regulatory substances into the brain, and also facilitate the elimination of xenobiotics and endogenous waste products from the CSF to the circulating blood. The BCSFB restricts and regulates large molecular traffic, which justifies the low amount of protein found in CSF compared with plasma (Skipor and Thiery, 2008). The barrier and secretory functions of CPEC are maintained by the polarized expression of a number of specific transmembrane transport systems that allow for the directed transport of nutrients into the CSF and removal of toxic compounds out of the CSF (Coisne and Engelhardt, 2011). The presence of intercellular TJ between cells that form the interface periphery-CNS is essential for BCSFB carry out all these functions (Saunders *et al.*, 2013).

1.1.2.1. The Epithelial Junctional Complex of the Blood-cerebrospinal fluid barrier

TJs are the most apical component of the epithelial junctional complex, which includes also AJ and desmosomes (Figure 3A) (Tsukita *et al.*, 2001, Balda and Matter, 2008). AJs play an important role, being responsible for generating the polarized distribution of plasma membrane proteins and for establishing other cell-cell junctional complexes, as TJs and desmosomes (Szmydynger-Chodobska *et al.*, 2007). The principal constituents of AJs are the transmembrane proteins of the cadherin family, to which E-cadherin belongs (Miyoshi and Takai, 2005). TJs

assembly is promoted by homotypic interactions between two proteins on the surface of adjacent cells: E-cadherin and nectin (Ca^{2+} independent adhesion molecule and member of Immunoglobulin G superfamily) (Schneeberger and Lynch, 2004). This interaction is established indirectly, Afadin binds to the C-terminal of nectin and also to β -catenin, which in turn binds to C-terminal of E-cadherin (Schneeberger and Lynch, 2004). TJs act as dynamic barriers, regulating the diffusion of water, ions, and other small molecules through the paracellular space between neighbouring cells. They also play a barrier function, maintaining cell polarity, through the restriction of the diffusion of apical and basolateral membrane components. TJs signalling is bi-directional, therefore signals that are transmitted from the cell to TJs regulate its assembly and function, and TJs coordinately receive and transmit information back to the cell, regulating gene expression, which consequently produces cellular responses such as proliferation and differentiation (Terry *et al.*, 2010).

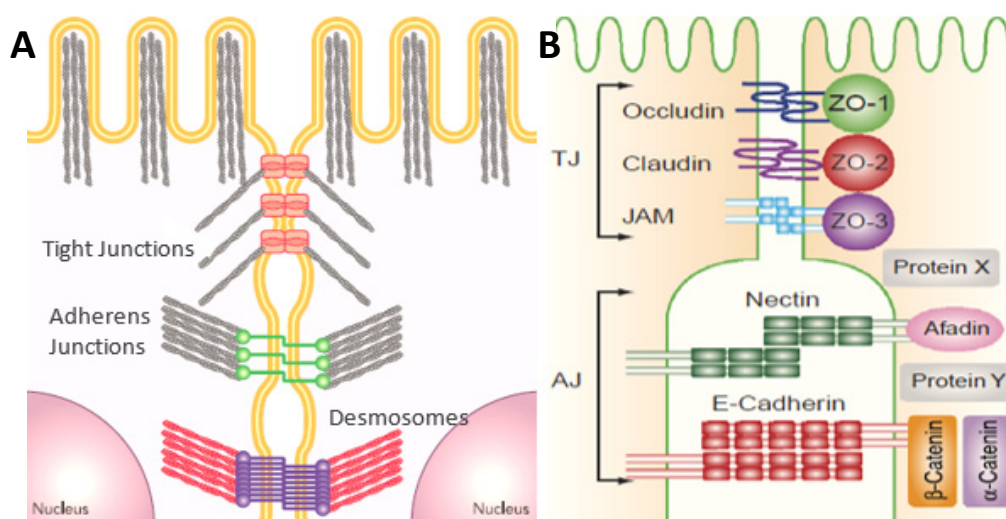


Figure 3 - Epithelial junctional complex of BCSFB. A. Typical composition of epithelial junctional complex with tight and adherens junctions, and desmosomes; B. Some of proteins found at tight junctions (occludin, claudins, JAM and ZO proteins), and at adherens junctions (E-cadherin, catenins, nectin, afadin). Adapted from (Terry *et al.*, 2010) and (Miyoshi and Takai, 2005, Corbett *et al.*, 2012).

Generally, epithelial TJs composition consists in transmembrane and cytoplasmic proteins (Goncalves *et al.*, 2013). The major transmembrane proteins are the tetraspan proteins occludin, claudins and the single-span proteins JAMs (junctional adhesion molecule) (Balda and Matter, 2008). The JAMs (~40 kDa) belongs to the immunoglobulin subfamily, and therefore are associated with the immune system (Hwang *et al.*, 2013). It is possible to find JAMs both at TJs and AJs (Miyoshi and Takai, 2005). These proteins are involved in cell-cell adhesion/junctional assembly of epithelial cells (Tsukita *et al.*, 2001). Transmembrane proteins are intrinsically related with permeability and paracellular transport at TJs (Terry *et al.*, 2010, Hwang *et al.*, 2013). The cytoplasmic plaque proteins of TJs, the zonula occludens (ZO) proteins (ZO-1, 2, 3), are responsible for connecting transmembrane proteins to actin cytoskeleton, and

also contribute to the recruitment of cytosolic molecules which are involved in cell signalling (Schneeberger and Lynch, 2004).

1.1.2.2. Principal adherens and tight junctions proteins of the blood-cerebrospinal fluid barrier

The expression of AJs and TJs proteins in CP was analysed in various studies (Lippoldt *et al.*, 2000a, Lippoldt *et al.*, 2000b, Wolburg *et al.*, 2001), among them are E-cadherin, occludin, claudin-1 (Cld-1) and ZO-1.

E-cadherin is a single-pass transmembrane glycoprotein which exhibits Ca^{2+} dependent homophilic interactions with opposing molecules on neighbouring cells (Szmydynger-Chodobska *et al.*, 2007, Canel *et al.*, 2013), and is important for tissue morphogenesis and polarity (Tunggal *et al.*, 2005). This protein has a major role in the assembly of AJs (Miyoshi and Takai, 2005), by establishing links to the actin cytoskeleton through catenins, and then forming adhesive contacts between cells (Ueno, 2007). The intracellular domain of E-cadherin binds to β -catenin and p120-catenin, which binds to α -catenin, and this in turn interacts with actin. This connection between the catenin-E-cadherin complex and the actin cytoskeleton promotes strong cell-cell adhesion (Tunggal *et al.*, 2005, Szmydynger-Chodobska *et al.*, 2007, Canel *et al.*, 2013). Occludin (60-65 kDa) was the first transmembrane protein of TJs identified (Furuse *et al.*, 1993) and is expressed in several tissues with similar patterns, including the brain (Hwang *et al.*, 2013). This protein seals neighbouring cells (Miyoshi and Takai, 2005) and, together with claudins (Clds), participates in TJs formation (Overgaard *et al.*, 2011). Claudins, more exactly Cld-1 and -2, were found to belong to TJs, some years after occludin (Furuse *et al.*, 1998). These proteins belong to a family of small proteins (20-34 kDa) (Van Itallie *et al.*, 2004) with 27 members known in mice and humans (Goncalves *et al.*, 2013). Of all transmembrane proteins in TJs, Clds are those that demonstrate a major important role in specific paracellular barrier properties that characterize the epithelial barriers as BCSFB (Overgaard *et al.*, 2011). Claudins, and thus Cld-1, are pointed as the basis of the selective size, charge, and conductance properties of the paracellular pathway (Van Itallie and Anderson, 2004, Tunggal *et al.*, 2005). Another protein expressed in CP, ZO-1 (220 kDa) is a protein member of the membrane-associated guanylate kinase family of proteins (Miyoshi and Takai, 2005, Szmydynger-Chodobska *et al.*, 2007, Balda and Matter, 2008). ZO-1 provides structural support to the epithelial cells, connecting actin skeleton to several proteins of TJs (occludin, clds, ZO-2 and -3) and indirectly to E-cadherin, contributing to the control of TJ assembly (Miyoshi and Takai, 2005, Ueno, 2007, Balda and Matter, 2008).

As mentioned, the expression of AJs and TJs proteins in BCSFB has extensively been studied. Both AJs proteins, cadherins and catenins, were identified in the epithelial cells of this barrier

(Wolburg *et al.*, 2001), and the expression of diverse Clds (Cld-1, 2, 3) was reported in CP (Kratzer *et al.*, 2012) and CPEC (Coisne and Engelhardt, 2011). Kratzer *et al.*, 2012 analysed the expression of 14 members of Clds family in CP. Of all tested, Cld-1, -2, and -3 showed the highest levels of expression, and of those Cld-1 stands out as the most expressed. Cld-6, -9, -10, -12, -19 and -22 expression was also observed in CP. Additionally, the expression of those TJs proteins during the various phases of development was analysed. During development, the expression of Cld-2, -9 and -22 increased whereas Cld-3 and -6 decreased. Therefore expression of TJs proteins in CP starts early development, indicating an active barrier function. Moreover, occludin and ZO-1 are also expressed in CP. The immortalized epithelial cell line derived from rat CP (Z310 cell line) also expresses occludin, ZO-1, E-cadherin and β -catenin making it a suitable model for barrier studies, since alterations in their expression contributes to barrier breakdown (Vargas *et al.*, 2010, Goncalves *et al.*, 2013).

1.1.3. Protein Synthesis

CP has the ability to regulate chemicals on brain by limiting the access of substances from the blood stream to the CNS, and also by serving as unique source of essential molecules to the cerebral compartment. Several studies demonstrated the presence of protein and/or mRNA of cytokines, growth factors and hormones in the CP. Example of those are interleukin-1 β , interleukin-6, tumour necrosis factor- α , Insulin-like growth factor 2, nerve growth factor, transforming growth factor- β , vascular endothelial growth factor, transferrin, TTR, vasopressin, retinol acid and leptin (Redzic *et al.*, 2005, Skipor and Thiery, 2008). CP also transport folate, vitamin B6, vitamin B12, vitamin C, and probably vitamin E. TTR is the most abundant protein, representing 25% of the newly proteins synthesized by CP and 50% of CP secreted proteins to CSF (Serot *et al.*, 1997, Segal, 2001, Abate-Shen and Shen, 2002, Serot *et al.*, 2003)).

1.2. Transthyretin

Transthyretin, originally named “prealbumin”, is a protein with 55 kDa found in human serum and CSF, mainly produced in liver and CP (Soprano *et al.*, 1985). This protein is composed by four identical subunits (\approx 14 kDa each) (Du and Murphy, 2010). Its gene is expressed in liver, pancreas, kidney, CP, retinol epithelium and leptomenigeal epithelium (Richardson, 2009, Buxbaum and Reixach, 2009, Li and Buxbaum, 2011). In brain, TTR production is restricted to CP and meninges, with the first being the major site of its production (Sousa *et al.*, 2007). Prealbumin was renamed to TTR, due to its function in *transport of thyroid* hormones and *retinol* (vitamin A) (Sousa *et al.*, 2007). This CP protein carries up about 80% of thyroxine (T₄),

hormone required for cell cycle regulation in the CNS, and is the major plasma retinol transporter through binding to retinol binding protein (RBP) (Blay et al., 1993, Southwell et al., 1993, Richardson, 2009, Li and Buxbaum, 2011, Du et al., 2012). Binding of TTR to RBP prevents filtration of retinol by kidneys, and it is also indirectly involved in the delivery of retinol to target cells. To date, apolipoprotein A-I, neuropeptide Y and amyloid-beta (A-beta) peptide have been identified as natural TTR substrates (Liz et al., 2012). TTR acts as an endogenous detoxifier of protein oligomers with potential neuroprotective effects, through the inhibition of amyloid fibril formation (Casella et al., 2013). For this reason, much attention has been given to this protein and its role in reducing A-beta oligomers, and consequently, by protecting against Alzheimer's disease (AD) (Buxbaum and Reixach, 2009).

1.2.1. Transthyretin role in Amyloid Beta clearance

TTR was the third CSF protein found to interact with A-Beta, after apolipoprotein E (ApoE) and Apolipoprotein J (Apo J) (or clusterin), and is the major A-Beta scavenger protein in human CSF (Schwarzman *et al.*, 1994). The ability of TTR to form stable complexes with A-Beta through its monomers, avoiding amyloid formation (Schwarzman *et al.*, 1994), and the decreased TTR levels in CSF of AD patients (Riisoe, 1988, Serot *et al.*, 1997, Velayudhan *et al.*, 2012), point to an important role of this protein in AD. Additionally, lower TTR levels in plasma were correlated with severe cognitive decline, making this protein a serious candidate as an AD biomarker (Velayudhan *et al.*, 2012).

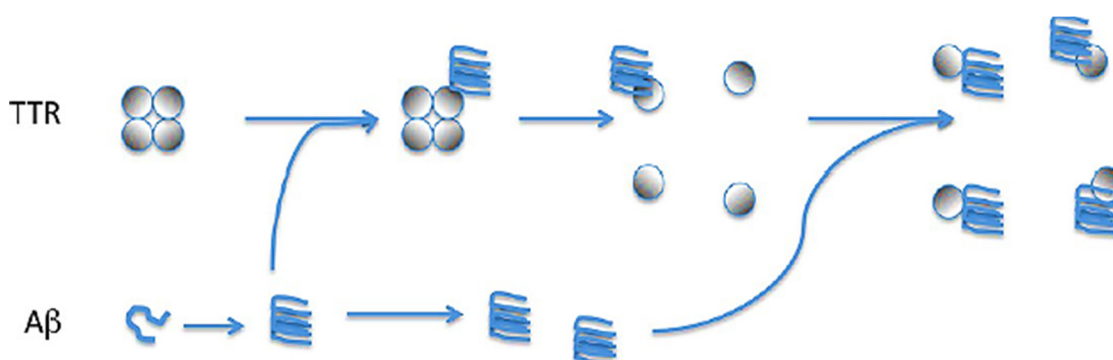


Figure 3 - Scavenger function of TTR. TTR in A-Beta presence change its tetrameric conformation to monomeric, which interact with A-Beta peptide and avoiding its aggregation (Yang *et al.*, 2013).

TTR can bind, in both intra and extracellular environment, due a chaperone-like manner (Buxbaum *et al.*, 2008), to all A-Beta forms (monomers, oligomers and fibrils). However it binds with more affinity to soluble fibrillar A-Beta oligomers, which are believed to be the most toxic forms of A-Beta (Yang *et al.*, 2013, Casella *et al.*, 2013). Usually, TTR tetramers are very stable with this conformation being essential to the transport function of this protein. However,

TTR monomers bind more to A-Beta than tetramers (Du and Murphy, 2010), indicating that conformational molecular changes are important to the binding (Du *et al.*, 2012) and TTR monomers are required to sequester A-Beta (Yang *et al.*, 2013). It was, recently, reported that the presence of A-Beta triggers the destabilization of TTR tetramers, allowing TTR monomers to bind A-Beta, preventing its aggregation, deposition and interaction with cells (Figure 5) (Yang *et al.*, 2013). These results together with the upregulation of TTR in transgenic mice overexpressing mutant amyloid precursor protein (APP), resulting in slow disease progression and lack of neurodegeneration (Stein and Johnson, 2002), and accelerated A-Beta deposition in the absence of TTR (Choi *et al.*, 2007), supports the natural protective role assigned to TTR in AD (22).

Moreover, TTR inhibits the influx of Ca^{2+} caused by the oligomers, further preventing other consequences of A-Beta, such as oxidative stress, membrane leakage and apoptosis. Overall, the molecular mechanism by which TTR protects cells against the deleterious effects of protein aggregation seems to involve two different levels of intervention: (1) inhibition of protein aggregation and fibril formation, as previously demonstrated; and (2) neutralization of protein oligomer toxicity once the oligomers are formed (Casella *et al.*, 2013). Proteolysis has been proposed as a possible mechanism by which TTR process A-Beta, cleaving this in smaller and less amyloidogenic peptides, which can be eliminated by cells (Costa *et al.*, 2008a).

1.2.2. Transthyretin regulation by Sex Hormones

Several epidemiological and neuropathological studies have provided evidence for gender related differences in AD, with women being more susceptible to this disease than men (Ruitenber *et al.*, 2001, Oliveira *et al.*, 2011). A possible explanation relates the loss of estrogens and progesterone that occur during menopause, which is much more abrupt than testosterone loss by men during aging (Yue *et al.*, 2005, Oliveira *et al.*, 2011). TTR probably regulates brain A-Beta levels in a gender-associated manner, under the control of testosterone and estrogen levels (Oliveira *et al.*, 2011).

Both 17 β -estradiol and dehydrotestosterone (DHT) up-regulated TTR expression in liver (Goncalves *et al.*, 2008) and CP (Quintela *et al.*, 2008, Quintela *et al.*, 2009) of mice and rat. Furthermore, progesterone also enhanced TTR expression (Quintela *et al.*, 2009, Quintela *et al.*, 2011). Estrogens are indicated by the majority of studies as neuroprotective, reducing A-Beta formation, and protecting against neuronal toxicity, apoptosis and oxidative stress (Wilson *et al.*, 2000, Greenfield *et al.*, 2002, Amtul *et al.*, 2010). Moreover, 17 β -estradiol, by increasing TTR levels in brain, leads to inhibition of A-Beta aggregation (Amtul *et al.*, 2010), which highlight the importance of this sex hormone in AD, and also supports the relevant role of TTR in AD onset and progression.

2. Implications of Choroid Plexus Senescence

Generally, aging has an impact in all systems of human body, and CP is not an exception. CP morphology alterations comprise epithelial atrophy, basement membrane thickening, fibrosis and calcifications of stroma, and increased number of cells presenting Biondi bodies (Serot *et al.*, 2000, Serot *et al.*, 2012), that intensifies with aging. BCSFB permeability increases while CP protein synthesis and CSF turnover are reduced (Johanson *et al.*, 2011b). The CSF volume increases, following augmented resistance to CSF drainage, probably as a result of a combination of calcification of the arachnoid villi, thickening of the arachnoid membrane, and central vascular hypertension (Redzic *et al.*, 2005, Erickson and Banks, 2013). The decrease of CSF antioxidant properties might be explained by reduction in vitamin E and ascorbic levels which are two principal scavengers of the free radicals of CSF. A decrease in turnover leads to an increasing contact-time between proteins and glucose which promotes the glycation of proteins, including TTR, and also increases oxidative stress (Shuvaev *et al.*, 2001, Vicente Miranda and Outeiro, 2010, Serot *et al.*, 2012). Further, A-Beta retention time in brain increases, contributing to its accumulation in CP, and other processes as apoptosis, oxidative stress, BCSFB disruption, cell death and probably further decreases CSF turnover (Erickson and Banks, 2013).

CP is involved in the most basic aspects of neural function, which means that even modest changes in CPs may trigger some important effects (Vargas *et al.*, 2010). Moreover, the age-related alterations in CP, described above, also take place in AD, but are much more accentuated (Serot *et al.*, 2000, Serot *et al.*, 2012). Therefore, this ventricular structure has received particular attention in the last years due to its importance and role in the CNS pathologies, particularly in AD (Krzyzanowska and Carro, 2012).

2.1. Amyloid Beta Metabolism

A-Beta, a 4 kDa amino acid metalloprotein, is a consequence of APP metabolic processing, that occurs when APP is cleaved by the enzymes β -secretase (BACE-I) and γ -secretase (Figure 5) (Shirwany *et al.*, 2007, Rowe and Villemagne, 2013). APP point of cleavage mainly result in three forms of A-Beta with 38, 40 and 42 amino acid residues. This is particularly important because A-Beta₄₂, the longer form, is far more prone to oligomerize and form fibrils than another A-Beta form (Walsh and Selkoe, 2007). It is likely that A-Beta form aggregates, forming toxic species as dimers, oligomers and fibrils. It is still unknown which of these species presents more toxicity (Ittner and Gotz, 2011), although some studies points to be smaller species of A-Beta (oligomers) (Walsh *et al.*, 2002, Gong *et al.*, 2003, Li *et al.*, 2011). The secreted A-Beta may have several fates, which include reabsorption in capillaries, degradation by proteases and drainage into the CSF (Shirwany *et al.*, 2007, Serot *et al.*, 2012).

Currently, A-Beta is seen as the best marker of AD pathology (Rembach *et al.*, 2013). Typically, 90% of healthy individuals present the A-Beta₄₀ form predominantly, and only 10% present A-Beta₄₂. In contrast, in AD, this ratio changes dramatically, with A-Beta₄₂, the principal compound of amyloid plaques, increasing to approximately 50% (Shirwany *et al.*, 2007).

2.2. Choroid plexus dysfunction and Amyloid Beta accumulation in brain

CP dysfunction normally means that its functions, as secretion, synthesis and transport are compromised (Krzyzanowska and Carro, 2012). Consequently occurs a decrease in CSF turnover, TTR levels, and increases sequestering of proteins synthesized by CP, and oligomerization of A-Beta₄₂. TTR as a scavenger of A-Beta, plays an important role in its clearance, whereby changes in TTR gene expression in the CP and decreased TTR levels in CSF, as consequence of CP age-related modifications, might relate to AD (Serot *et al.*, 2000, Sousa *et al.*, 2007). Concordantly, deposition of A-Beta isoforms, including A-Beta₄₂, in AD patients, is mainly due to failures in its clearance rather than to overproduction (Mawuenyega *et al.*, 2010, Pascale *et al.*, 2011), and levels of TTR in the CSF of AD patients are inversely correlated with A-Beta plaque burden (Merched *et al.*, 1998).

Thus, it is expected that A-Beta accumulates in CP, as already observed in the CP of AD patients (Vargas *et al.*, 2010, Krzyzanowska and Carro, 2012). Moreover, impairment of CP functions as consequence of A-Beta deposition, mainly of A-Beta₄₂, was shown in AD patients CP, by increasing nitric oxide production and interfering with BBB integrity, due to disrupted expression of TJs proteins as ZO-1 (Vargas *et al.*, 2010). In other words, CP dysfunction with aging initiates a cascade that leads to accumulation of A-Beta in brain, which consequently enhances modifications in CP performance (Figure 4). As already mentioned, estrogens have an important role in the regulation of TTR expression, and decreased levels of this hormone are verified with aging. Consequently, this reduction in estrogen production might block the natural protective response, where TTR expression increases, and so contributing to A-Beta accumulation.

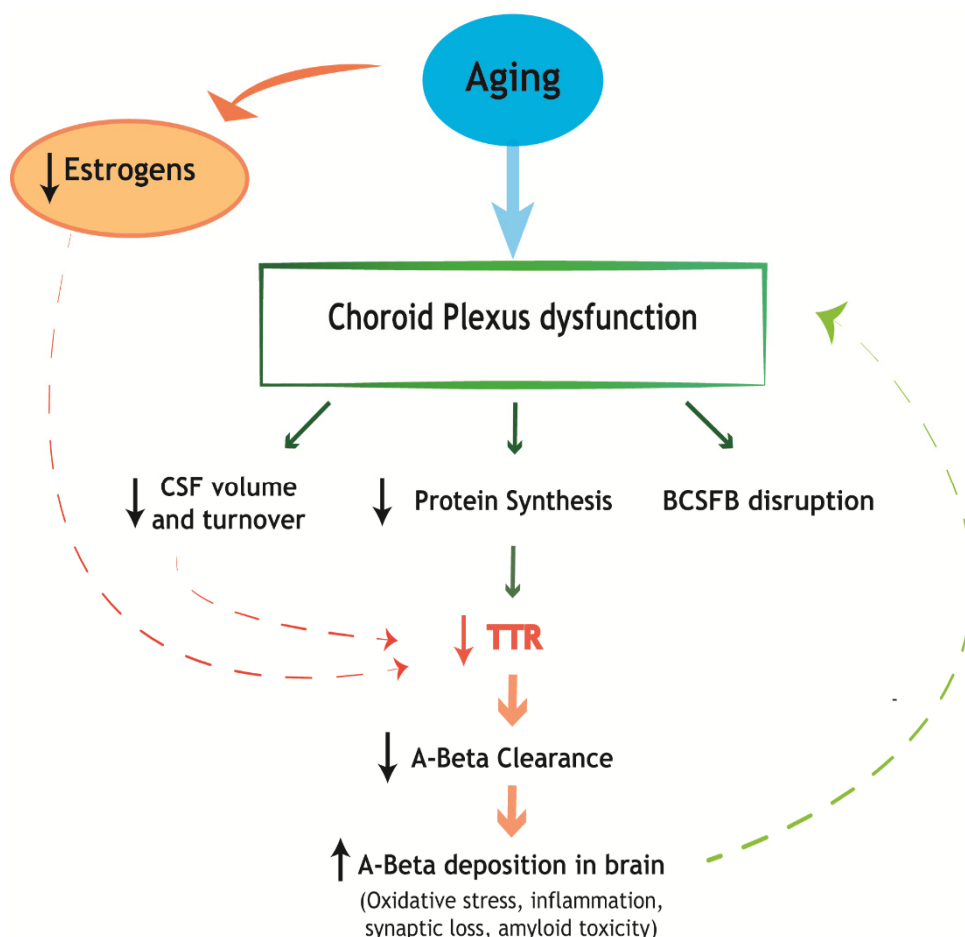


Figure 4 - Correlation between CP dysfunction and accumulation of A-Beta in brain. Aging contributes to impaired CP functions, which in turn leads to decreased TTR levels. Additionally, estrogens levels decrease with aging, contributing to TTR decrease. Crucial to A-Beta clearance, compromised TTR expression might lead to A-Beta deposition in brain.

3. Endocrine Disruptors

There is an increasing concern about the effects of human and wildlife exposition to compounds that have the ability to interfere with endogenous hormones, the so called endocrine disruptors (EDs). These are exogenous compounds that possess the potential to interfere with hormonal regulation and the normal endocrine system, through several mechanisms including blocking, mimicking and displacing endogenous hormones. Consequently, production, release, metabolism, and elimination of natural hormones can be modified (Wetherill *et al.*, 2007, Casals-Casas and Desvergne, 2011, Weiss, 2012). Although there are many sources of endocrine disruptors, the major concern is attributed to synthetic chemicals with endocrine-disrupting properties (EDCs), due to its large and continuous production in western societies (Casals-Casas and Desvergne, 2011).

A major mechanism of EDC-mediated metabolic disruption is through EDC interaction with nuclear receptors, including sex steroid hormone receptors, receptors acting as xenobiotic sensors, and receptors specialized in metabolic regulations (Casals-Casas and Desvergne, 2011). EDCs include biocides, industrial compounds, surfactants, and plasticizers. Bisphenol A (BPA), probably the most studied EDC (Flint *et al.*, 2012, Rogers *et al.*, 2013), belongs to this last category.

3.1. Bisphenol A

BPA is a nonsteroidal xenoestrogen that exhibits approximately 10^{-4} the activity of estradiol (Flint *et al.*, 2012). Heat resistance and elasticity are two of BPA characteristics that lead to its increased use and crescent production worldwide, with more than 10 million of tons produced every year (Fenichel *et al.*, 2013). This chemical is mainly used as intermediate to produce resins and polymers. It is possible to found BPA in a wide range of materials and products: bottles, coatings, pipes, dental sealants, printing store receipts, canned food, nail polishes and flame-retardant materials (Weiss, 2012, Boas *et al.*, 2012, Asimakopoulos *et al.*, 2012). Human exposure to BPA is extensive, as many of these products are easily handled by humans, and moreover, BPA can leach from those (high temperatures, acidic or basic environments) (Weiss, 2012, Boas *et al.*, 2012, Asimakopoulos *et al.*, 2012, Fenichel *et al.*, 2013). Furthermore, BPA has been detected in samples of water, sewage leachates, indoor and outdoor air and dust (Vandenberg *et al.*, 2010). Although, the oral via is the most likely route of human exposure to BPA, transdermal and inhalation should also be considered, as these can avoid most of the first-pass hepatic metabolism, contrarily to oral exposure. The dermal contact may exist with air, dust and water contaminated with BPA (Vandenberg *et al.*, 2007).

3.1.2. Bisphenol A metabolism and molecular mechanism of action

After oral absorption, BPA is rapidly metabolized in the liver into bisphenol A-glucuronide, a highly soluble metabolite, without hormonal activity, that is then eliminated through urine (Mileva *et al.*, 2014). The half-life of BPA estimated is about 6h (Volkel *et al.*, 2002, Calafat *et al.*, 2008). Although, BPA is mostly metabolized in liver and intestines, BPA can originate others metabolites, which may present more risk to physiological functions than BPA itself (Mileva *et al.*, 2014). Unconjugated BPA has been frequently detected in urine and blood samples in many different studies. This indicates that an internal exposure to the parent compound exist, which is estrogenically active (Vandenberg *et al.*, 2010).

Estrogenic properties of BPA were described for the first time in 1936, and since then various experiments were performed that confirmed these properties (Casals-Casas and Desvergne,

2011). BPA binds differently to both estrogen receptors (ERs) - ER α and ER β - displaying a 10-fold higher affinity for ER β (Gould *et al.*, 1998, Kuiper *et al.*, 1998). Initially, BPA was thought to have a very weak estrogenic function due to be much less potent than estradiol. More recently, it was observed that BPA promotes cellular responses through many different pathways, and at very low doses, with changes in cell function from picomolar doses, below the levels expected for classical nuclear ERs binding (Wozniak *et al.*, 2005, Welshons *et al.*, 2006). BPA has also been shown to bind a membrane-associated ER and produce non-genomic steroid actions with the same efficacy and potential than estradiol. BPA can cause effects in animal models at doses in the range of human exposures, indicating that it can act at lower doses than predicted from some *in vitro* and *in vivo* assays (Vandenberg *et al.*, 2010). In addition to affect estradiol hormone function, BPA can also antagonize thyroid hormone and androgen actions (Welshons *et al.*, 2006, Flint *et al.*, 2012). Yang *et al.*, 2009, reported a possible relation between BPA exposure and increased oxidative stress and inflammation, and that postmenopausal women present more predisposition related with estrogen levels and receptor occupancy. BPA exerts cellular and tissue-type specific effects and non-monotonic dose-responses at cellular and intracellular levels at low physiologically relevant concentrations (Wetherill *et al.*, 2007). In the same tissue, different cell types present unique estrogen-stimulated gene expression, and BPA activity may alternate between agonist and antagonist (Welshons *et al.*, 2006).

BPA may elicit cellular responses through several mechanisms. Briefly, BPA may disturb the proper estrogen nuclear hormone receptors activity, and consequently, interfere with endogenous estrogens activity; also can affect the androgen system, disrupt the thyroid hormone function, and affect development, differentiation and function of the CNS and of the immune system (Moriyama *et al.*, 2002, Wetherill *et al.*, 2007).

3.1.3. Bisphenol A levels in human tissues and fluid

With the aim of evaluate if BPA represents a threat to the human population, several studies have been performed for this purpose recently. Unconjugated BPA, the form that has estrogenic activity, has been measured repeatedly in various types of human samples including urine, plasma, saliva, breast milk, among others, using various analytical techniques (Welshons *et al.*, 2006, Vandenberg *et al.*, 2007, Calafat *et al.*, 2008, Gentilcore *et al.*, 2013).

For reference, in a study where urine samples were collected and analysed, 92.6% of people accused the presence of BPA (Calafat *et al.*, 2008). Vandenberg *et al.* (2007) reviewed the published data of more than 80 studies about human exposure to BPA, and reported that the average urine BPA levels had a range of 1-3 ng/mL, and moreover, the concentration of BPA in serum were between 0.3-4.4 ng/ml, corresponding to 1-19.4 nM. Furthermore, the exposure of human population to BPA is widespread, with BPA levels detected in most children and adults

(Vandenberg *et al.*, 2007). This data suggested a continuous exposure to BPA (Calafat *et al.*, 2008) and this amount of BPA to which human are exposed may cause adverse effects in health (Vandenberg *et al.*, 2010).

All endogenous estrogens, androgens, and thyroid hormones decline with aging, and play a role in brain plasticity. Moreover, all three endocrine systems are vulnerable to EDCs (Weiss, 2012). In addition, BPA showed to be able to cross BBB (Sun *et al.*, 2002), which demonstrates that the brain is exposed to this compound.

3.1.1. Metabolic effective dose

BPA has become the chemical model for examples of non-monotonic dose-response functions, due to its dose-response curve called U-shaped or inverted U-shaped. Effects are observed at very low doses and high doses, but no effects are observed at intermediary doses. This contradicts the conventional idea that the lack of an adverse effect at certain dose implies that to lower doses the absence of an effect will maintain. Thus, to non-monotonic dose-response curves, the effects of low doses cannot be predicted by the effects observed at high doses. These U-shaped curves suggest the existence of two independent mechanisms for low and high doses (De Coster and van Larebeke, 2012), probably ER-dependent and ER-independent mechanisms, although far from being completely understood. Thus, BPA might not act as estradiol, which increases the importance of its study and how it affects the various human systems, including the brain.

II. Aim

Aim

The important role of TTR, the principal protein synthesized in choroid plexus, in A-Beta clearance has been supported by many studies, and here its interaction will be assessed in animals with different ages. For other side, it is well documented the importance of sex hormones in TTR regulation and production. For this reason, this work intends to investigate if an endocrine disruptor, Bisphenol A, well known for the widespread exposure to human population, is able to interfere with TTR expression in rat CPs, and consequently if it may affects A-Beta clearance. Additionally, the effect of Bisphenol A, and also A-Beta, in the blood-cerebrospinal barrier integrity will also be subject of analysis, through the measurement of expression of certain membrane proteins found at this barrier.

III. Materials and Methods

1. Animals and Z310 cell line

In this work, several assays with CP explants collected from *Wistar Han* rats (newborn and young) were carried out. All animals were handled in compliance 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/EV). The explants were treated with BPA and A-Beta₄₂ and the effects of these two compounds were evaluated using diverse techniques. In brief, the experiments scheme carried out with these explants are represented in figure 5. Additionally to CP explants, an immortalized rat choroidal epithelium cell line, called Z310, with potential for application in BCSFB research was also used. The major role of Z310 cells in this work relates with optimization processes, specifically Western blot, allowing to avoid a considerer number of animals that would be required to the different optimization steps of this technique, as amount of total protein loaded, transfer time, antibodies dilutions and incubation times.

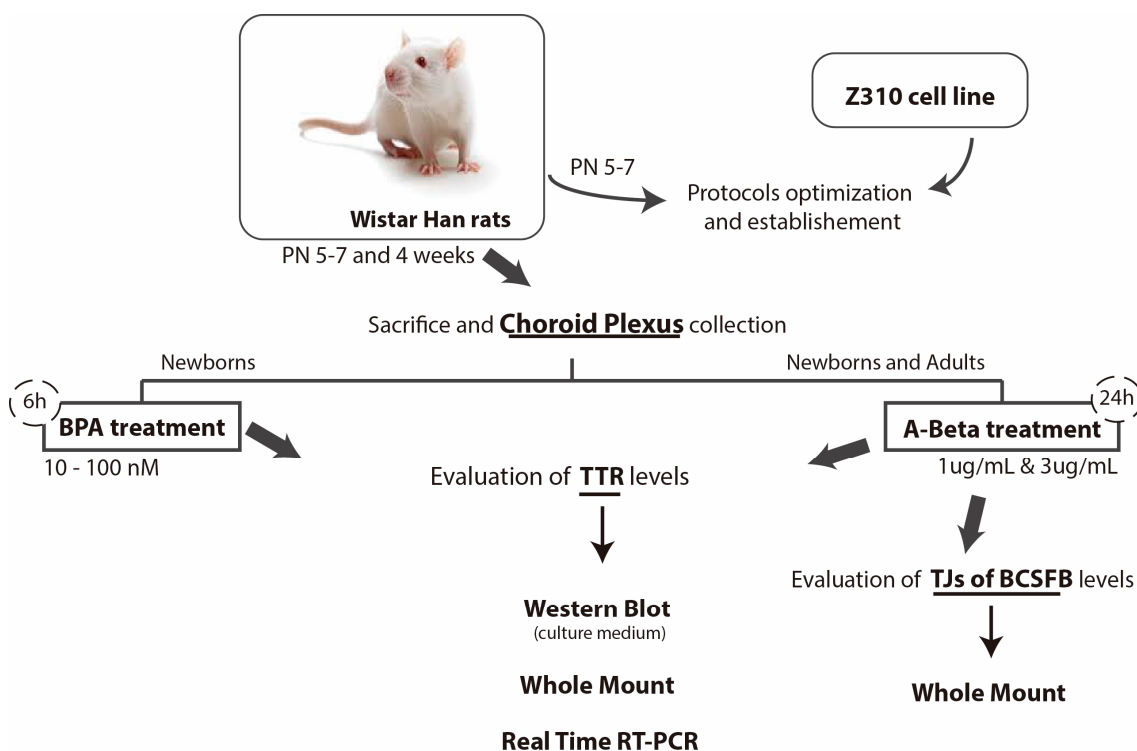


Figure 5 - Scheme of experimental studies about BPA and A-Beta effects in TTR and TJs BCSFB proteins levels, in rat choroid plexus explants. Evaluation of BPA effects was carried out in CPs from newborn *Wistar Han* rats, while A-Beta effects were evaluated in newborn and young rats. CPs were subjected to various techniques, such as Western blot, Whole mount immunohistochemistry and Real-Time PCR. PN- post natal; BPA-Bisphenol A; A-Beta- Beta amyloid; TTR- transthyretin; TJs- tight junctions; BCSFB- Blood cerebrospinal fluid barrier; CP- Choroid plexus.

1.1. Cell Culture

Cell line Z310 was supplemented with Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich), 10% fetal bovine serum (FBS, Biochrom AG, Berlin) and 1% penicillin/streptomycin (Sigma, Saint Louis) in a t-flask, and was kept in AutoFlow DHD CO₂ Air-Jacketed Incubator (NuAire, USA) at 37°C, in a 5% CO₂ atmosphere.

1.1.1. Cells Passage

Always that cellular confluence achieved about 90% it was proceeded to cellular passage, decreasing cellular density to secure the continuous expansion of Z310 cell line. These cells are adherents. Culture medium was removed and cells were washed with phosphate buffered saline (PBS) 1x. PBS was removed and replaced by trypsin-EDTA 0.25% in a volume that ensure cells totally cover, and were incubated for 3 to 5 minutes at 37°C. When most of the cells have detached, culture medium at an equivalent volume of used to trypsin was added. Resuspended cells were collected to a falcon and were centrifuged for 5 minutes, 1300 rpm. Supernatant resulted from centrifuging was discarded and pellet containing cells was resuspended in PBS 1x, and again centrifuged for 5 minutes, 1300 rpm. Supernatant was removed and cells were resuspended in 1 mL of medium culture and placed in the incubator (37°C, 5% CO₂).

1.1.2. Cell Counting

For cell counting, 20 uL were taken from cellular suspension, and equal volume of trypan bue was added, followed by homogenization. Of these preparation, 20 uL were transferred to a Neubauer chamber, for viable cells counting. After counting the cells at the different quadrants, the cells number/mL and the total number of cells in t-flask were estimated, following these formulas:

$$dilution\ factor = \frac{total\ volume}{volume\ of\ sample}$$

$$\frac{number\ cells}{mL} = \frac{number\ of\ cells}{number\ of\ squares} \times dilution\ factor \times 10^4$$

1.1.3. Cells Freezing and Thawing

This process was performed to ensure the perpetuation of the cell line. With this purpose cells were trypsinized, as described before. Cells were diluted in DMEM with 10% of DMSO (Dimethyl sulfoxide), to prevent the formation of crystals that could cause cell lysis. Cells were stored, at first, at -20°C, after at -80°C, and finally, in liquid nitrogen to permanent storage. To thawing cells, cryopreserved cells were resuspended in DMEM with 10% of FBS and 1% of penicillin/streptomycin, after previous thawing at 37°C. Then, resuspension was centrifuged for 5 minutes, 1300 rpm, supernatant rejected and cells washed again with culture medium. After a final centrifuge it was proceeded to cellular culture as previously described.

2. Whole Mount fluorescent staining

Whole mount fluorescent staining is a technique that allows to determinate the cellular localization and distribution of certain proteins in whole tissue, using specific antibodies. In this work, location and expression of TTR (cytoplasmic protein), and occludin, E-cadherin, Cld-1 and ZO-1 (membrane proteins) were assessed by this technique. Further, tissues were subjected to treatment with A-Beta and BPA at nanomolar (nM) concentrations, with the aim of analysing its effect in CP and BCSFB integrity.

2.1. Whole Mount - TTR, Occludin, E-cadherin, Cld-1 and ZO-1

Briefly, lateral CPs were collected from *Wistar Han* rats with 5-7 days and placed into a 48 wells microplate (one CP per well) with paraformaldehyde (PFA) 4% for 45 min at room temperature. CPs were then incubated with a solution of sucrose 30% until deposit in the bottom, and next with a blocking solution 2,5% bovine serum albumin (BSA) 0,2% Triton X-100, overnight at 4°C. In CPs intended to incubate with membrane proteins antibodies, Triton X-100 wasn't used in blocking solution, to avoid compromising cellular membranes. CPs were washed once with PBS-Tween 0.01% (PBS-T) and incubated overnight at 4°C, with respective antibodies diluted in block solutions: rabbit anti-mouse TTR antibody (1:200, Dako), rabbit anti-occludin and anti-E-cadherin (1:50, Santa Cruz Biotechnology) and goat anti-Cld-1 and anti-ZO-1 (1:50, Santa Cruz Biotechnology). After this, CPs were washed several times with PBS-T and then incubated with secondary antibodies, diluted in blocking solutions, Alexa 488 goat anti-rabbit (1:2000, Molecular Probes) and donkey anti-goat 488 (1:1000, Santa Cruz Biotechnology) for 3 hours on dark at room temperature. From the incubation with secondary antibody all next procedures were performed in dark. CPs were again washed with PBS-T before incubation with Hoechst 33342 dye (Molecular Probes, EUA) diluted 1:1000. Finally, CPs were mounted with Entellan and visualized on a confocal microscope LSM 710 (Zeiss, Germany).

The protocol described above was followed to evaluate A-Beta and BPA effects in CP proteins studied in this work. Thus, CPs collected from *Wistar Han* rats with 5-7 days were placed into a microplate (one CP per well) with complete DMEM, and incubated for 6 or 24h at 37°C, in a 5% CO₂ atmosphere, with or without BPA treatment (control). BPA (Sigma Aldrich, Saint Louis) was dissolved in DMSO and diluted in this culture medium at 10, 25, 50, 75, 100 nM and 10 µM. The final concentration of DMSO in each well was less than 0,01%. incubated with or without BPA. Also, CPs collected from *Wistar Han* rats with 5-7 days and 30 days were incubated in complete DMEM for 24h in a 5% CO₂ atmosphere, with or without A-Beta₄₂ (AnaSpec), at 1µg/mL or 3µL/mL. After respective incubation times, the culture medium was removed and CP tissues were washed once with PBS 1x. Then, normal protocol was followed and CPs were incubated with PFA 4%.

2.2. Confocal microscopy images analysis

Confocal microscope allowed the acquisition of various Z-stacks of each CP, at different localizations, after the whole fluorescent staining procedure. These Z-series collects a sequence of several different images/sections of the same region of the tissue. Then, using Blue Edition from Zen 2011 software (Zeiss), mean fluorescence intensity of each stack corresponding to proteins staining was obtained. This staining represents proteins expression in CPs, and then was used to quantify and evaluate the expression levels of proteins in CP explants after treatments.

3. Western Blot

Western blot was carried on with total protein extracted from CPs and Z310 cell line, and with culture medium collected after CP explants incubation with A-Beta₄₂ and BPA.

3.1. Protein Extraction

3.1.1. CP explants

To extract CPs protein, CPs were digested with buffer lysis (5mM HEPES (pH 7,5), 250 mM Sucrose, 10mM NaNO₃, 0.2 mM PMSF, 25 µg/mL leupeptin, 1mM orthovanadate, 10 mM sodium phosphate (pH 7,4), 150 mM NaCl, 2% Triton X-100, 2% deoxycholate, 0.2% SDS and 0.2 mM PMSF) by manual homogenization with a pestle, followed by centrifugation at 10,000 g, for 10 min. After centrifugation, the supernatant, containing protein, was collected. Total protein content in samples was measured using the Bio-Rad Protein Assay according with manufacturer's protocol (Bio-Rad, Hercules, USA).

3.1.2. Z310 cells

Z310 cells resuspension obtained after trypsinization, and twice centrifuged for 5 minutes, 1300 rpm. Then, cells were washed with PBS 1x, centrifuged for 7 minutes, 11000 rpm, 4°C, and supernatant rejected. This process was repeated twice, and at final, cells were resuspended in lysis buffer (same used to CP protein extraction). Total protein content of samples was measured as described before.

3.2. Western Blot - TTR, Occludin, E-cadherin and Cld-1

Total protein 50 µg was separated by SDS-PAGE using 8% and 12,5% gels, after boiled at 95°C for 10 min, and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS). After 1h30 in blocking solution, membranes were washed 10 min with washing solution, TBS containing 0,1% Tween (TBS-T), and then incubated respectively, overnight, at 4°C with anti-TTR (1:250, Dako), anti-Occludin, anti-E-cadherin, anti-Cld-1 and anti-ZO-1 (1:200, Santa Cruz Biotechnology) antibodies diluted in TBS-T. Membranes were washed for 45 minutes in TBS-T, at room temperature, with replacement of washing solution each 15 minutes. Then incubation with HRP-conjugated anti-rabbit and anti-goat secondary antibodies (1:20000) was done. The washing process was repeated as described before, and antibody binding was detected using the ECF substrate (ECF Western Blotting Reagent Packs, GE Healthcare) according to the manufacturer's instructions. Images of blots were captured with the Molecular Imager FX Pro Plus Multilmager system, and densitometry of bands was carried out using the software Quantity One™ (Bio-Rad).

4. Extraction of total RNA

TTR expression was analyzed by Real Time Polymerase Chain Reaction (Real Time PCR) to evaluate the A-Beta and BPA effect on its expression. Before proceeding to Real Time PCR, total RNA was extracted from rat CPs, previously collected, frozen in liquid nitrogen and stored at -80°C. RNA integrity was checked through visualization in 1% agarose gel. Total RNA was quantified in a nanophotometer.

4.1. Extraction

For CP RNA extraction 300 µL of TRIzol Reagent were added to each tube containing a CP followed by manual homogenization with a pestle in order to allow dissolution of cellular

components. After an incubation of 5 minutes at room temperature, 60 μL of chloroform (200 μL chloroform/1mL TRIzol) were added and samples were homogenized by inversion. Samples were incubated at room temperature for 10 minutes and then centrifuged at 4°C for 15 minutes and 12000 g. After this step, the solution split up in three different phases: at the bottom the organic phase (pink) containing proteins and residues of phenol and chloroform, in the intermediary phase there was genomic DNA and in the aqueous phase (transparent) the total RNA. Therefore, the aqueous phase was transferred to a new centrifuge tube in which were isopropyl alcohol (500 μL isopropyl alcohol/1mL TRIzol) added 150 μL followed by mixing for inversion with the purpose to recover total RNA by precipitation. After 10 minutes of incubation at room temperature, and 10 minutes of centrifugation at 4°C, 12000 g, supernatant was rejected and RNA was obtained in a pellet. The pellet was washed once with 500 μL of ethanol 70% in DEPC (diethylpyrocarbonate) water (-20°C), and centrifuged for 5 minutes, 4°C, 7500 g, and the supernatant was rejected again. Finally, ethanol in excess was removed and precipitated RNA was rehydrated with 10 μL of DEPC water and stored at -80°C.

4.2. Determination of total RNA integrity

Integrity of total RNA was analysed by electrophoresis of total RNA on a 1% agarose gel stained with GreenSafe (NZYTech, Portugal). Quality of RNA was confirmed through the presence of 2 bands of rRNA, 18S and 28S, with the strip of 28S with twice the intensity of the 18S strip. Samples were prepared with 2 μL of sample (~300 ng of total RNA), 8 μL of sterile water and 2 μL of loading buffer 10x. The gel was visualized on an UVITEC transilluminator (UVitec Cambridge, United Kingdom).

4.3. Quantification of total RNA

To quantify total RNA a Nanophotometer™ (Implen, Germany), which gives directly total RNA concentration ($\mu\text{g}/\mu\text{L}$) and the ratio A260/A280 (to assess RNA purity) was used. The ratio A260/A280 should be between 1,8 and 2,1.

5. cDNA synthesis

Complementary DNA (cDNA) is synthesized by reverse transcription of mRNA, having as result an identical copy of expressed genes without introns. In this work the NZY M-MuLV Reverse Transcriptase (NZYTech, Portugal) was used according to fabricant recommendations. A MIX, denominated, MIX1 (n+1 reactions) composed by 2 μL of Random Primers and 1 μL of DNTPs was prepared in an eppendorf, for each sample. PCR tubes were prepared with 3 μL of MIX1, ~500

ng of total RNA extracted of each sample and sterile water, performing a final volume of 17 μ L. Then, PCR tubes were placed in TProfessional Basic Gradient thermocycler (Biometra) at 65°C for 5 minutes, and immediately transferred to ice. Meanwhile, a MIX2 was prepared in a new eppendorf, where 2 μ L of RT buffer (5x buffer) and 1 μ L of MMLV were added, for each sample. To each of the previous PCR tubes 3 μ L of MIX2 were added, and then these were incubated at 25°C for 10 minutes, followed by 50 minutes at 37°C. To stop the reaction, a final incubation at 70°C for 15 minutes was carried out. The cDNA was then stored at -20°C until use.

6. PCR

Through conventional PCR it was possible to confirm the expression of the TTR gene in rat CP. In this procedure Taq Polymerase (NZYtech, Portugal) was used to amplify DNA fragments, in accordance with manufacturer's recommendations. To each sample tested was prepared a MIX with 2.5 μ L 10x Buffer, 1.5 μ L MgCl₂ (50 mM), 1 μ L dNTPS (10mM), 0.2 Taq Polymerase, 0.3 μ L Forward primer (25 μ mol) and 0.3 μ L Reverse primer (25 μ mol), completed with sterile water to a volume of 24 μ L. The oligonucleotides initiators (primers) were chosen through Primer-BLAST-NCBI-NIH program. To each reaction was added 1 μ L of cDNA, with the exception of control, which was added 1 μ L of sterile water. To proceed to gene amplification was used the TProfessional Basic Gradient thermocycler (Biometra). For begin, samples were placed at 95 °C for 5 minutes, which was followed by 40 cycles of 95°C for 30 seconds, next for 45 seconds at optimal annealing temperature for each primer, 30 seconds at 72 °C and, at last, 5 minutes at 72 °C. PCR products were run out on an agarose gel 1,5% in the presence of GreenSafe to stain DNA. For this, 8 μ L of each PCR product and 2 μ L of loading buffer 10x were deposited in the agarose gel. In the first well was deposited 5 μ L of HipperLadder II (Bioline).

Gene/ acession no.	Fragment size (bp)	Temperature (°C)	Primer Forward	Primer Reverse
TTR NM_012681.2	119	56	GGACTGATATTTGCGTC TGAAGC	ACTTTCACGGCCACATCG AC
CycA NM_017101.1	163	58	CAAGACTGAGTGGCTG GATGG	GCCCGCAAGTCAAAGAAA TTAGAG

7. Real-Time RT-PCR

Real-time RT-PCR allowed quantification of the relative expression of TTR gene in *Wistar Han* rats (5-7 days), after treatment of CPs with BPA and A-Beta. First, the procedure was optimized through testing primers efficiency to TTR gene with 4 different dilutions of cDNA (1:1 (stock), 1:2, 1:4 and 1:8). After each cycle, the emission of SYBR®Green I (Fermentas) fluorescence was determined, and the relative quantity of each mRNA was quantified by the iCycler software. The profiles, generated at the end of each assay, of the melting curve confirmed the transcription of one specific transcript. Further, the resulting melting curves of real-time allowed evaluate the dimers primers formation and purity of the amplified product. To normalize the levels of TTR gene expression the cyclophilin A gene was used as endogenous control (*housekeeping gene*). For each assay, to perform a volume of 19 μ L, was added 10 μ L of SYBR®Green I dye, 0.8 μ L of Fw primer and 0.8 of Rv primer diluted 1:20, 1 μ L of cDNA dilutions mentioned before, and sterile water (Fermentas). The amplification conditions used were 95°C for 3 minutes, 40 cycles of 95°C for 15 seconds, 56°C for 30 seconds and 72°C for 30 seconds in a thermocycler iQ™5 (Bio-Rad, EUA). Data treatment was carried out by the Pfaffl method (Pfaffl, 2001), and statistically, data was treated with One-Way ANOVA.

8. Reactive Oxygen Species Assay

The effect of A-Beta in reactive oxygen species (ROS) production was evaluated in young rat CPs, using a fluorescent probe, DCFH-DA (2,7-Dichlorodihydrofluorescein Diacetate, Sigma). DCFH-DA is able to diffuse through cell membranes, and when hydrolysed by intracellular esterases form DCFH (non-fluorescent), which is rapidly oxidized to highly fluorescent dichlorofluorescein (DCF) in the presence of ROS. Whole mount fluorescent staining protocol, already described before was used to assess ROS levels, but with some alterations. In brief, CPs of *Wistar Han* rats (~4 weeks) were collected to a microplate, and incubated in DMEM with 10% FBS and 1% penicillin/streptomycin, for 24h at 37°C, in a 5% CO₂ atmosphere with or without treatment of 1 μ g/mL of A-Beta₄₂ (AnaSpec). After 24h, culture medium was removed from wells and PFA 4% was added for 50 minutes. PFA was replaced for a solution of sucrose 30% until CPs deposit in the bottom. Then, CPs were incubated overnight at 4°C with blocking solution (2.5% BSA, 0.2% Triton X-100). After, CPs were incubated with DCFH-DA at 10 μ M, for 20 minutes at 37°C in dark, washed with PBS 1x, incubated with Hoechst 33342 (1:1000) and washed with PBS. Finally, CPs were observed on a confocal microscope LSM 710. Fluorescent intensity in CP explants was measured using the Blue Edition of Zen 2011 (Zeiss) software.

9. Statistical analysis

Statistical analysis of TTR and BCSFB protein levels in rat CP explants was carried out with GraphPad Prism (Version 5). Data was compared and expressed as mean \pm SEM. Comparisons of means between two groups was performed using Student's *t* test, and between three or more groups by one-way ANOVA followed by Dunnett's test. Results were considered statistically significant when $p < 0.05$. Data collected from real-time PCR experiments were analysed using the mathematical model proposed by Pfaffl (Pfaffl, 2001), which allows the determination of differences in expression between control and treated CP explants, taking into account reaction efficiency, and reference gene normalization. All comparisons between three or more groups were compared by means of one-way ANOVA followed by Dunnett's test. Results were statistical significant when $p < 0.05$.

IV. Results

1. Protocols optimization and establishment

1.1. Expression and localization of TTR in rat CP

1.1.1. Whole Mount fluorescent staining

To visualize the cytoplasmic expression of TTR protein, CP explants were incubated with primary (anti-TTR) and secondary antibody (Alexa Fluor 488). Additionally, tissues were treated with Hoechst to nuclei stain. Afterwards, tissues were observed by confocal microscopy. As can be seen at green in figure 6, it was possible to visualize a cytoplasmic localization of TTR protein, in rat CP.

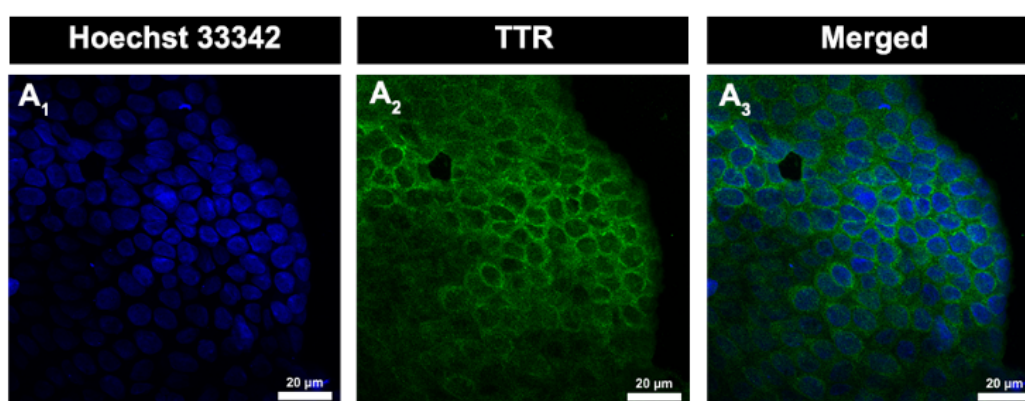


Figure 6 - Expression and localization of TTR in rat CP by confocal microscopy (A1-A3). A1. Staining nucleus with Hoerscht 33342 (blue). A2. Expression of TTR in cytoplasm of CP tissue (green stain). A3. Merged A1 and A2. Magnification: 63x.

1.1.2. Western Blot

In order to minimize the number of animals necessary, we optimized Western blot protocol to TTR protein in Z310 cell line. A band detection corresponding to the monomer isoform (~15 kDa) of TTR, can be observed in figure 7. After protocol optimization and correct visualization of TTR expression in Z310 cell line, the protocol was initially applied to protein samples extracted from CP explants without any treatment, for protocol validation purposes. The method transposition success, from Z310 cells to CP explants, is showed in figure 7. Where is also observed a band around ~15 kDa corresponding to TTR monomer isoform.

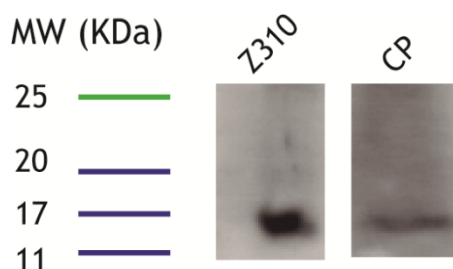


Figure 7 - Western blot optimization protocol of TTR protein, in Z310 (left) and CP (right). Both figures show a band of ~15 kDa, corresponding to monomer form of TTR.

As mentioned before, TTR is the main protein synthesized and secreted by CP to CSF, where it forms stable complexes with A-Beta. For this reason, the BPA or A-Beta effects on TTR expression in CP explants was assessed through the establishment of Western blot method for CPs culture medium, collected after CPs treatment with the mentioned compounds. Furthermore, was also carried out a Western blot analysis to culture medium samples from CP explants without any kind of treatment. Figure 8, shows the resulting blot, where TTR migrated to the 15 kDa region, identified as TTR protein monomer isoform, which is known to present more affinity to bind to A-Beta.

Culture medium used to incubate CP explants has itself some TTR protein content. Therefore to ensure that TTR protein detected is from CPs explants and not from the culture medium, we also carried out a Western blot analysis in samples of culture medium without CP incubation. The results clearly show that for the same medium volume loaded (30 μ L) and under the same conditions, CPs are the main cause for TTR protein presence and detection in culture medium (Figure 8). In order to ensure that the medium protein content contribution to the observed band in all performed Western blot analysis was identical, the same volume (30 μ L) of culture medium of each sample was loaded in SDS-page-gel.

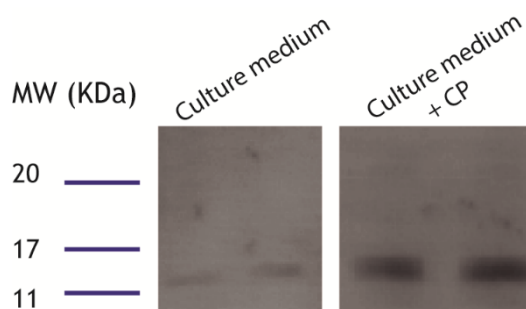


Figure 8 - TTR Western blot performed with culture medium samples (30 μ L) after incubation for 6h with and without CP. Although both blots show TTR detection at ~15 kDa, culture medium samples where CP incubates (right) shows a much stronger bands.

1.1.3. RT-PCR

RT-PCR results confirm TTR mRNA expression in rat CP explants, through specific amplification of the nucleotides sequences of TTR gene. The amplified products were run out in a 1.5% agarose gel stained with GreenSafe, and it was confirmed that the size of fragments obtained (119pb) corresponded to TTR gene, by comparison with HipperLadder II. Three different temperatures (53°C, 56°C and 57°C) were tested to establish the optimal annealing temperature of TTR primers, which was revealed to be 56°C. Further, in control/negative no amplification was verified, indicating absence of contamination (Figure 9).

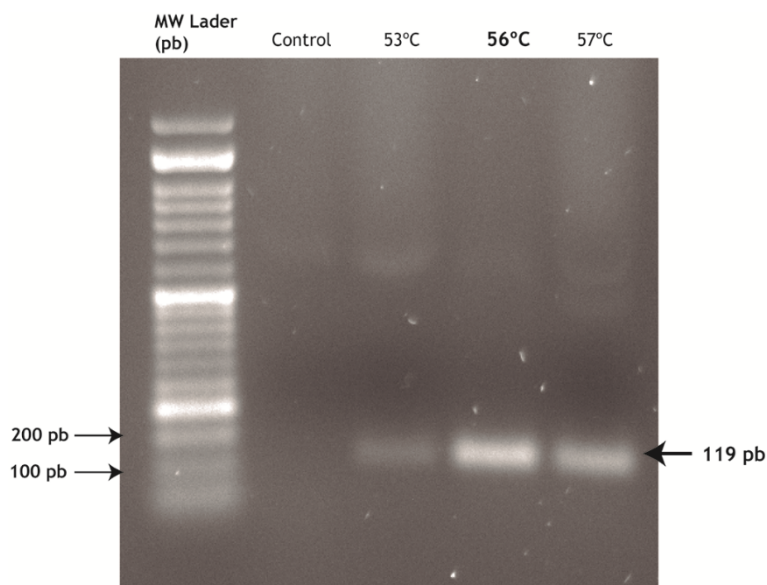


Figure 9 - Electrophoresis in 1.5% agarose gel stained with GreenSafe of cDNA PCR products of TTR gene. From left to right it is represented the molecular weight ladder, the control (C-) and the fragment obtained to temperature of 53°C, 56°C and 57°C.

1.2. Localization and expression of tight and adherens junctions proteins at the blood-cerebrospinal fluid barrier of rat CP

1.2.1. Whole-Mount fluorescent staining

The localization and expression of some tight and adherens junctions proteins (occludin, E-cadherin, Cld-1 and ZO-1) expressed at BCSFB, was confirmed in newborn rat CP explants. CP explants were exposed to appropriated antibodies and visualized in a confocal microscope. Representative images of CP explants to each protein and respective membrane staining are showed in next figures (Figure 10).

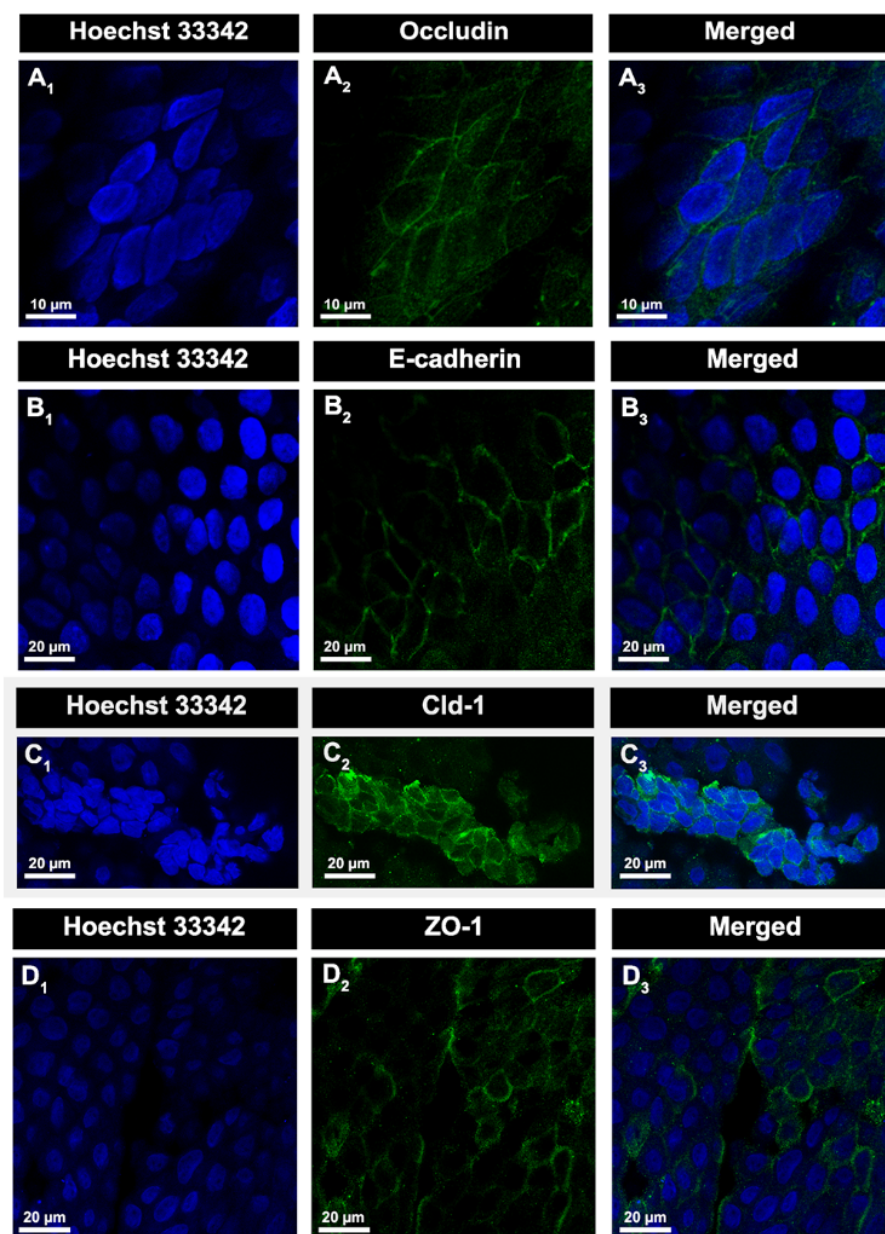


Figure 10 - Localization and expression of some TJs and AJs proteins of BCSFB, in rat CP explants, by confocal microscopy. (A1-3) Occludin; (B1-3) E-cadherin; (C1-3) Claudin-1; (D1-3) ZO-1.

Figure 10 shows the fluorescent staining of occludin, E-cadherin, Cld-1 and ZO-1 in CP explants without any treatment. The confocal images present some differences in the staining of the various proteins, and in the intensity of their staining with for example, Cld-1 showing a more intense staining. Additionally, since is the whole tissue that is stained, its normal observe overlay of cells, which difficult obtaining a full plan of cells, even using Z-stacks of the tissue as we do.

The establishment of Whole-Mount protocol to BCSFB proteins, in CP explants, allowed to pursuit to the evaluation of BCSFB proteins expression in CP explants treated with A-Beta₄₂,

through comparison of fluorescent intensity of each BCSFB protein, at various treatment conditions.

1.2.2. Western Blot

Western blot technique was performed to confirm the expression of occludin, E-cadherin, Cld-1 and ZO-1 proteins in rat CP, already seen by whole mount fluorescent staining. To date ZO-1 protein detection it is still in optimization.

Although, the main goal was analyse the expression of these membrane proteins in rat CP, Z310 cell line was used to the optimization of Western blot (buffer lysis, amount of loaded protein, transfer time, primary antibody dilution and incubation time). Thus, the expression of these proteins was first verified in Z310 cell line and, posterior, after optimization in rat CP explants (Figure 11). Images were captured with the Molecular Imager FX Pro Plus Multimager system.

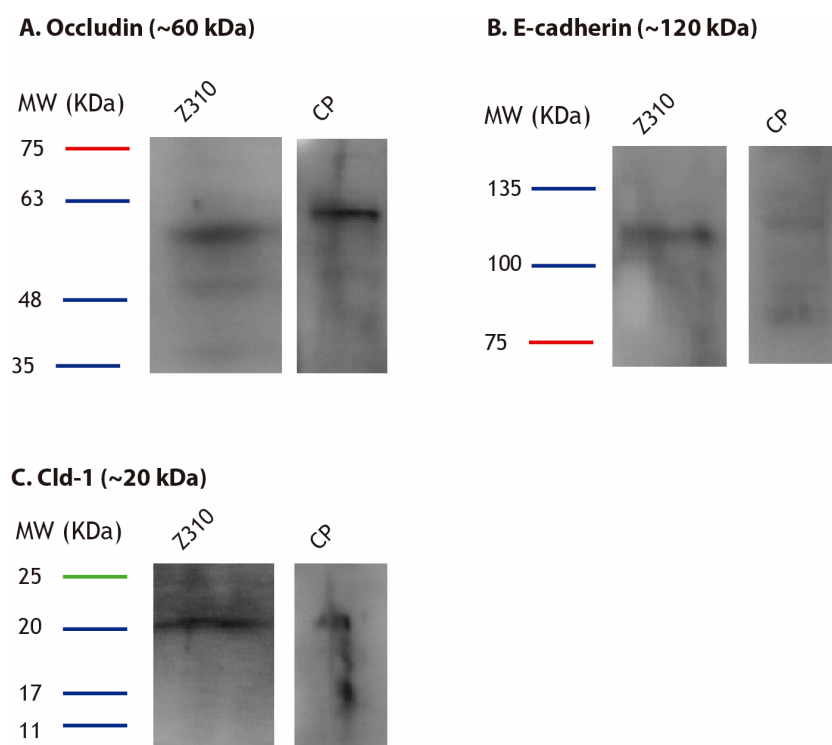


Figure 11 - Western blot optimization and establishment to membrane protein of BCSFB: occludin (A.), E-cadherin (B.), Cld-1 (C.).

Figure 11 shows BCSFB proteins bands in both Z310 cell line and in CP explants, with slightly differences among them. Occludin in Z310 migrates with about 60 kDa, while in CP explants appears with more intensity slightly above, with 63 kDa. E-cadherin was detected in Z310 and in CP explants with approximately the same weight (120 kDa), although in Z310 the band show

more intensity. Cld-1 also migrates with the same molecular weight in both Z310 and CP explants, approximately 22 kDa.

2. TTR expression in rat Choroid Plexus in response to A-Beta insult

2.1. Evaluation of TTR levels after treatment with A-Beta₄₂ for 24 hours

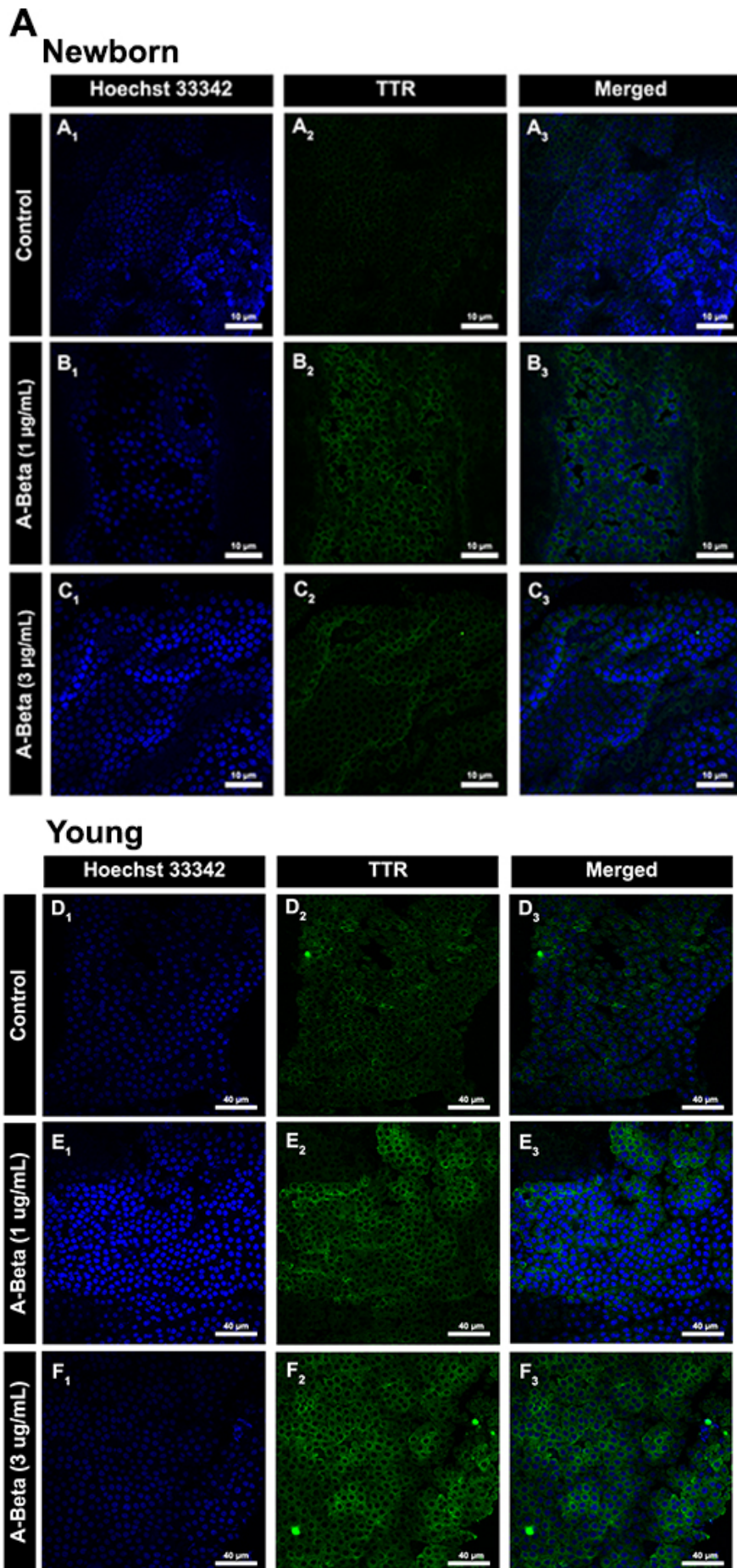
In order to investigate possible alterations in TTR expression in CP during aging, rat CP explants from animals at different stages (5-7 days and 30 days) were treated with A-Beta₄₂ for 24 hours. To evaluate TTR expression levels in rat CP explants in response to A-Beta₄₂ insult three techniques were used: Whole Mount fluorescent staining, Western blot and Real time RT-PCR techniques.

2.1.1. Whole-Mount fluorescent staining

This technique allowed to quantify the green fluorescence intensity corresponding to TTR expression in CP explants, and thus compare TTR expression levels. Figure 12 shows representative images obtained by confocal microscopy of CP explants from newborn and young *Wistar Han* rats, treated with 1 μ g/mL or 3 μ g/mL of A-Beta₄₂ for 24h and controls (non-treated).

Confocal microscopy images (Figure 12A) representing rat CP explants treated with A-Beta₄₂ show an increase in green fluorescence when compared with controls, in both newborn and young rat CP explants.

In newborn rat CP explants, it was observed a significant increase in TTR levels of treated CPs with 1 μ g/mL or 3 μ g/mL, when comparing to controls ($p < 0.001$). Although, no differences were observed between the two tested A-Beta₄₂ concentrations. Meanwhile, in young rat CP explants, TTR expression increased proportionally to A-Beta₄₂ concentration.



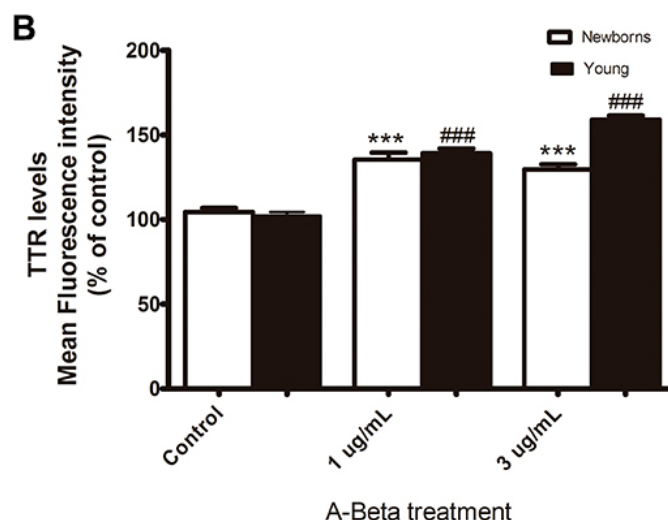


Figure 12 - A-Beta₄₂ effect in TTR expression in rat CP explants at different ages. (A) Representative confocal images of TTR expression (green fluorescence) in CP explants from newborn (A1-C3) and young rats (D1-F3), treated with A-Beta₄₂ for 24h. (A1-A3) and (D1-D3) - Control/non-treated; (B1-B3) and (E1-E3) - Treated with 1ug/mL of A-Beta₄₂; (C1-C3) and (F1-F3) - Treated with 3ug/mL of A-Beta₄₂. Magnification: 63x. (B) Comparison of TTR levels through measurement of mean fluorescence intensity, with different A-Beta₄₂ treatments (1ug/mL and 3ug/mL) in CP explants from newborn (white bars) and young (black bars) rats. Bar graphs represents means \pm SEM, N=4 (**p<0.001; ###p<0.001 vs. control).

2.1.2. Real Time RT-PCR

mRNA TTR levels in newborn and young rat CP explants treated with 1ug/mL or 3ug/mL of A-Beta₄₂, and controls were assessed by Real time RT-PCR. Normalization of TTR gene expression was ensured using Cyclophilin A gene as housekeeping gene.

In figure 14 it is observed, not only a great alteration between treated and non-treated rat CP explants, as also a different response related to animals age. In newborn rat CP explants, TTR expression levels extremely increased in a dose dependent manner, with higher levels achieved in rat CP explants treated with 3ug/mL. Similarly, in young rat CP explants the highest TTR levels were obtained in explants treated with 3ug/mL. However, the observed values were far behind the levels obtained in newborn rat CP. Moreover, compared to controls, TTR levels do not presented significant changes in young rat CP explants treated with 1ug/mL.

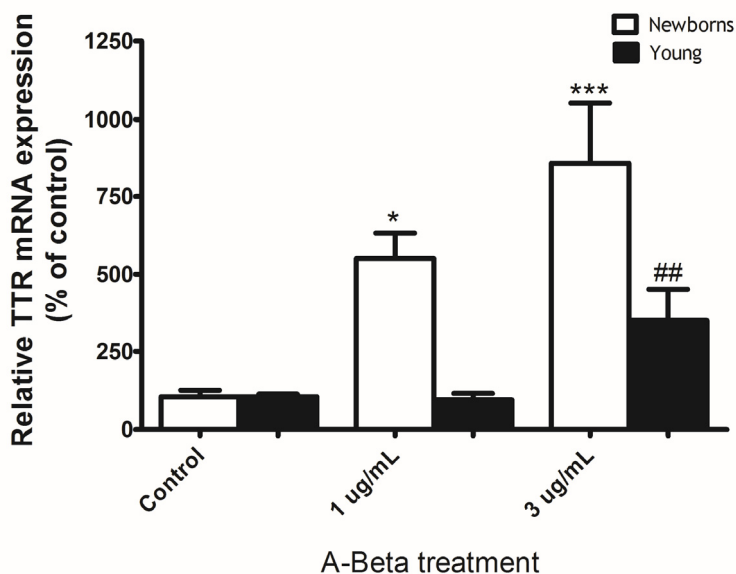


Figure 13 - Comparison of TTR mRNA levels by Real time RT-PCR in newborn and young rat CP explants treated with A-Beta₄₂ for 24h. CP explants treated with A-Beta₄₂ show increased TTR levels comparative to controls, with exception of young CP explants treated with 1ug/mL. Bar graphs represents means ± SEM, N=5 (*p<0.05, ***p<0.001, ##p<0.01 vs. control).

2.1.3. Western Blot

Increased TTR levels observed in CP explants by Whole-Mount and Real time RT-PCR seems to be an effect of A-Beta₄₂ exposure. Thus, it is expected that the amount of TTR protein secreted by CP explants also increase. To determine whether this occurs, samples of culture medium from newborn and young rat CPs explants incubated with A-Beta₄₂ for 24h were carried out in Western blot. Resulting blots (Figure 13) were analysed by densitometry, and results can be observed in the graphic of Figure 13B.

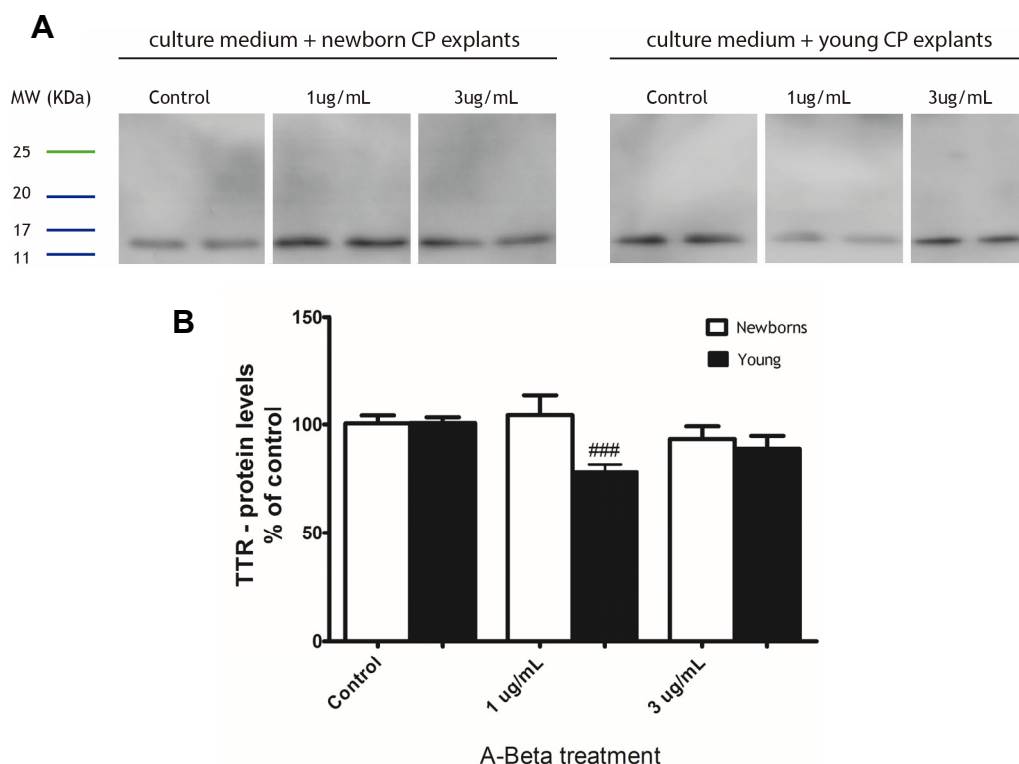


Figure 14 - Evaluation of A-Beta₄₂ effect in TTR secretion by Western blot. Western blot was performed with culture medium collected from newborn and young rat CP explants treated with 1ug/mL or 3ug/mL of A-Beta₄₂ for 24h and control/non-treated. **(A)** Representative blots resulted from TTR Western blot of culture medium samples. **(B)** Comparison of TTR levels in culture medium after blots analysis by densitometry. Images were captured with the Molecular Imager FX Pro Plus Multilimager system. Values are expressed as a percentage of non-treated samples, which were considered 100%. Bar graphs represents means ± SEM, N=7 (###p<0.001 vs. control).

TTR levels in culture medium from rat CP explants treated with A-Beta₄₂ do not show alterations comparative to controls, with the exception of culture medium samples of young rat CP explants treated with 1ug/mL. Young rat CP explants treated with A-Beta₄₂ (1ug/mL) secreted less TTR to culture medium than controls (p<0.001). Moreover, in rat CP explants exposed to A-Beta₄₂, it was not observed a correlation between TTR expression levels in Whole-Mount and Real time RT-PCR and the TTR secretion levels in Western blot of culture medium samples.

2.2. Blood-Cerebrospinal fluid barrier membrane proteins expression in response to A-Beta insult

2.2.1. Whole-Mount fluorescent staining

BCSFB integrity of newborn rat CP explants, after treatment with A-Beta, was accessed by Whole-Mount fluorescent staining through the measurement of expression levels of some membrane proteins, found at TJs and AJs of CPEC (Figure 15-18).

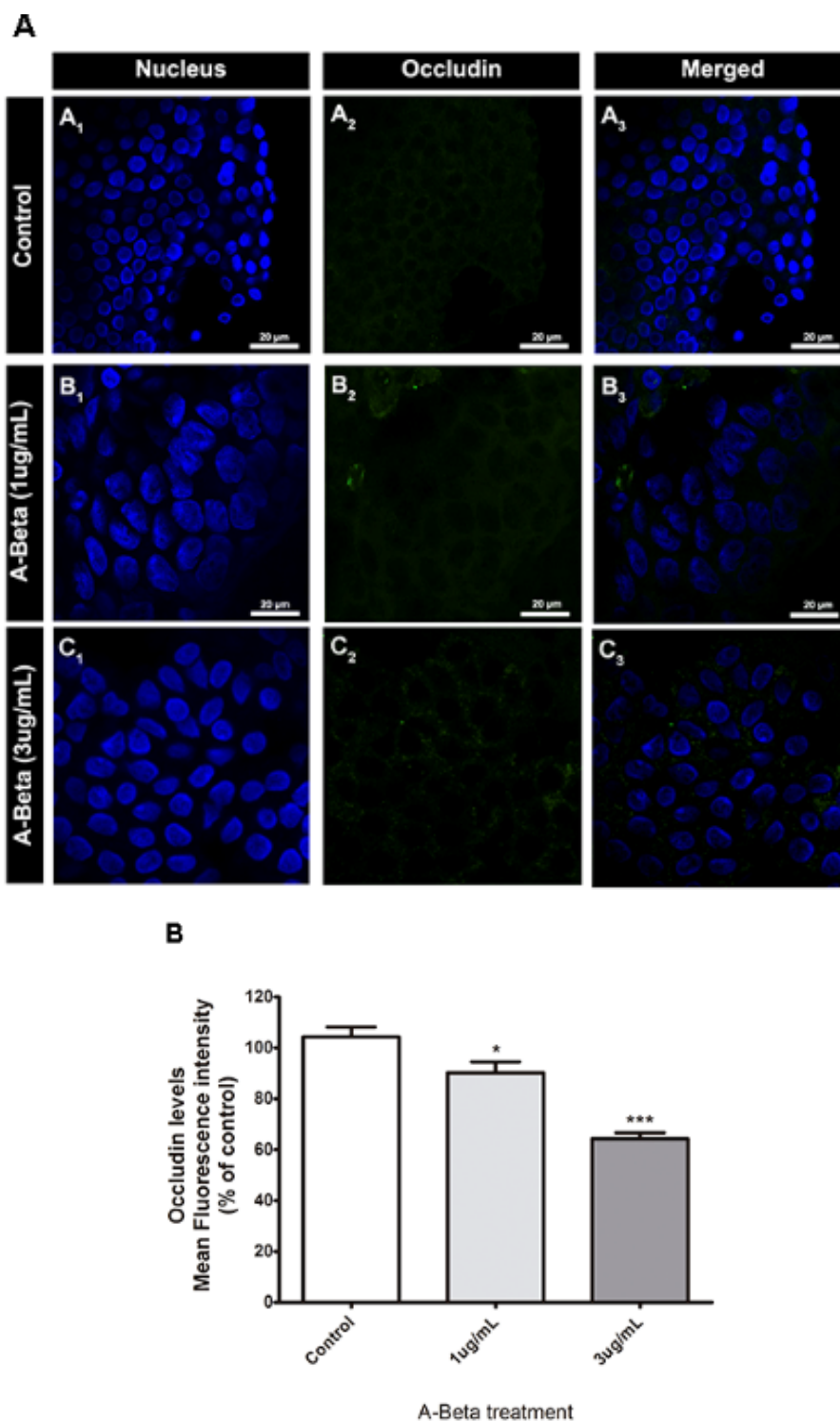


Figure 15 - A-Beta₄₂ effect in occludin protein expression in rat CP explants. (A) Representative confocal microscopic images of BCSFB occludin protein expression, in newborn rat CP explants treated with A-Beta₄₂ for 24h. (A1-A3) - Control/non-treated; (B1-B3) - Treated with 1ug/mL of A-Beta₄₂; (C1-C3) - Treated with 3ug/mL of A-Beta₄₂. Magnification: 63x. (B) Comparison of occludin protein levels, through measurement of mean fluorescence intensity, with different A-Beta₄₂ treatments (1ug/mL and 3ug/mL). Bar graphs represents means ± SEM, N=2 (*p<0.05; ***p<0.001 vs. control).

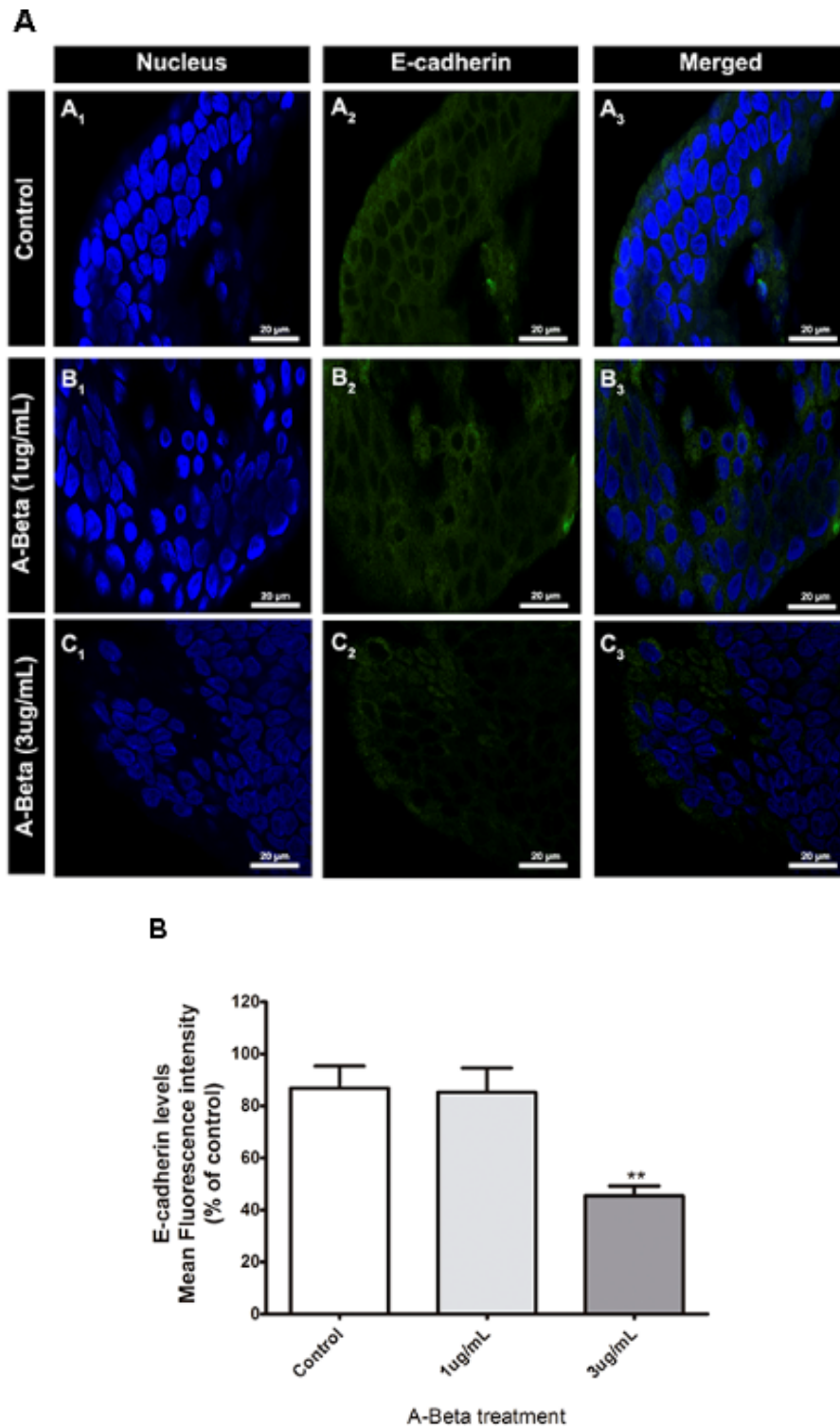


Figure 16 - A-Beta₄₂ effect in E-cadherin protein expression in rat CP explants. (A) Representative confocal microscopic images of BCSFB E-cadherin protein expression, in newborn rat CP explants treated with A-Beta₄₂ for 24h. (A1-A3) - Control/non-treated; (B1-B3) - Treated with 1ug/mL of A-Beta₄₂; (C1-C3) - Treated with 3ug/mL of A-Beta₄₂. Magnification: 63x. (B) Comparison of E-cadherin protein levels, through measurement of mean fluorescence intensity, with different A-Beta₄₂ treatments (1ug/mL and 3ug/mL). Bar graphs represents means ± SEM, N=2 (**p<0.01 vs. control).

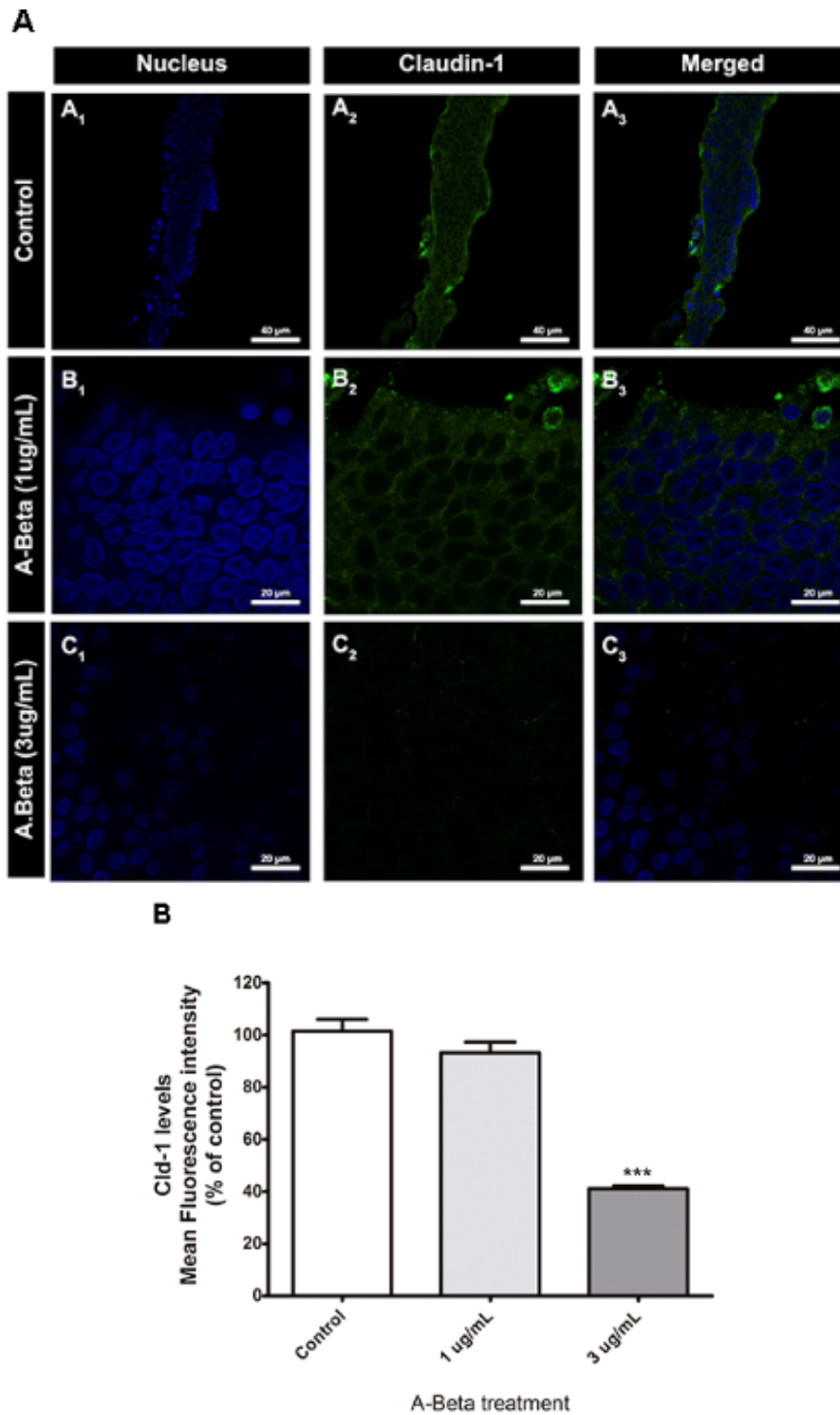


Figure 17 - A-Beta₄₂ effect in Cld-1 protein expression in rat CP explants. (A) Representative confocal microscopic images of BCSFB Cld-1 protein expression, in newborn rat CP explants treated with A-Beta₄₂ for 24h. (A1-A3) - Control/non-treated; (B1-B3) - Treated with 1ug/mL of A-Beta₄₂; (C1-C3) - Treated with 3ug/mL of A-Beta₄₂. Magnification: 63x. **(B)** Comparison of Cld-1 protein levels, through measurement of mean fluorescence intensity, with different A-Beta₄₂ treatments (1ug/mL and 3ug/mL). Bar graphs represents means ± SEM, N=2 (***)p<0.001 vs. control).

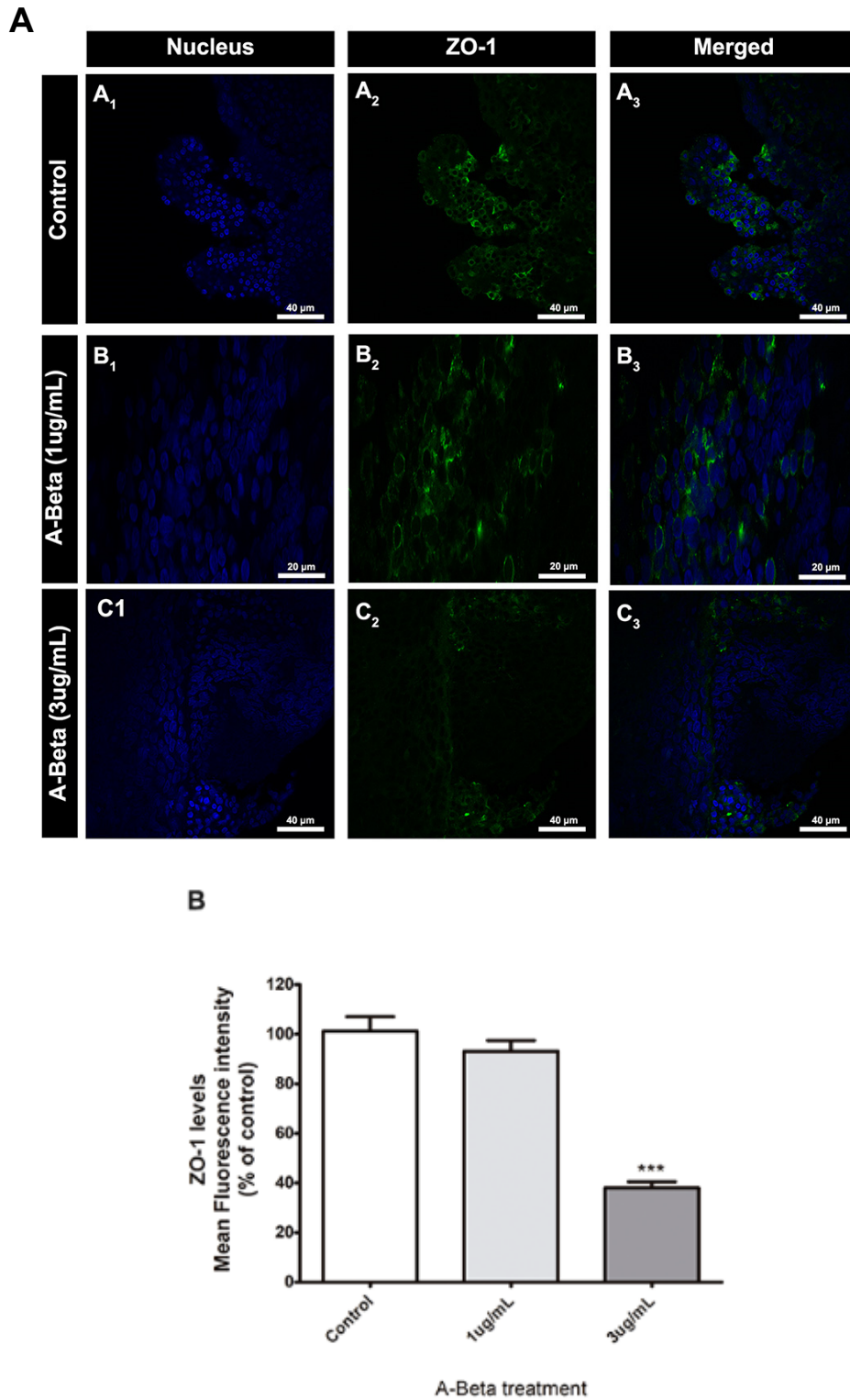


Figure 18 - A-Beta₄₂ effect in ZO-1 protein expression in rat CP explants. (A) Representative confocal microscopic images of BCSFB ZO-1 protein expression, in newborn rat CP explants treated with A-Beta₄₂ for 24h. (A1-A3) - Control/non-treated; (B1-B3) - Treated with 1ug/mL of A-Beta₄₂; (C1-C3) - Treated with 3ug/mL of A-Beta₄₂. Magnification: 63x. (B) Comparison of ZO-1 protein levels, through measurement of mean fluorescence intensity, with different A-Beta₄₂ treatments (1ug/mL and 3ug/mL). Bar graphs represents means ± SEM, N=2 (***)p<0.001 vs. control).

Confocal images of the various BCSFB proteins in study, and respective fluorescence intensity quantification, showed a similar response in rat CP explants treated with A-Beta₄₂. A-Beta₄₂ at 1µg/mL decreased the occludin protein expression, when compared to controls, but no differences were observed in other membrane proteins. Meanwhile, when rat CP explants were treated with a higher A-Beta₄₂ concentration, 3µg/mL, a significant decrease was verified in all the membrane proteins (occludin, E-cadherin, Cld-1 and ZO-1). Thus, indicating a negative effect of A-Beta in BCSFB, probably interfering with its function and consequently in the secretion function of CP. Moreover, E-cadherin, Cld-1 and ZO-1 levels decreased to levels around 40% of control. On the other side, occludin levels had a less pronounced decline, to approximately 60% of control.

2.3. Amyloid Beta oxidative stress in choroid plexus: production of reactive oxygen species

Oxidative stress plays an important role in cellular damage, and A-Beta neurotoxicity seems to mediate this phenomenon, particularly in AD. To determine the possible A-Beta₄₂ effect in ROS generation in CP explants, a fluorescent dye, DCFH-DA was used to measure the ROS production in young rat CP explants

To date, it was only possible to determinate ROS production in young rat CP explants treated to 1µg/mL of A-Beta₄₂, although was also aimed to study the phenomenon with the 3µg/mL concentration, and at the different ages. Representative images relative to ROS production by young rat CP explants treated with 1µg/mL can be visualized in Figure 19A.

The CP explants treatment with 1µg/mL of A-Beta₄₂ lead to an increase in ROS production in young rat CP explants, as can be seen in figure 19 (A₂ and B₂) through the different fluorescence intensity displayed.

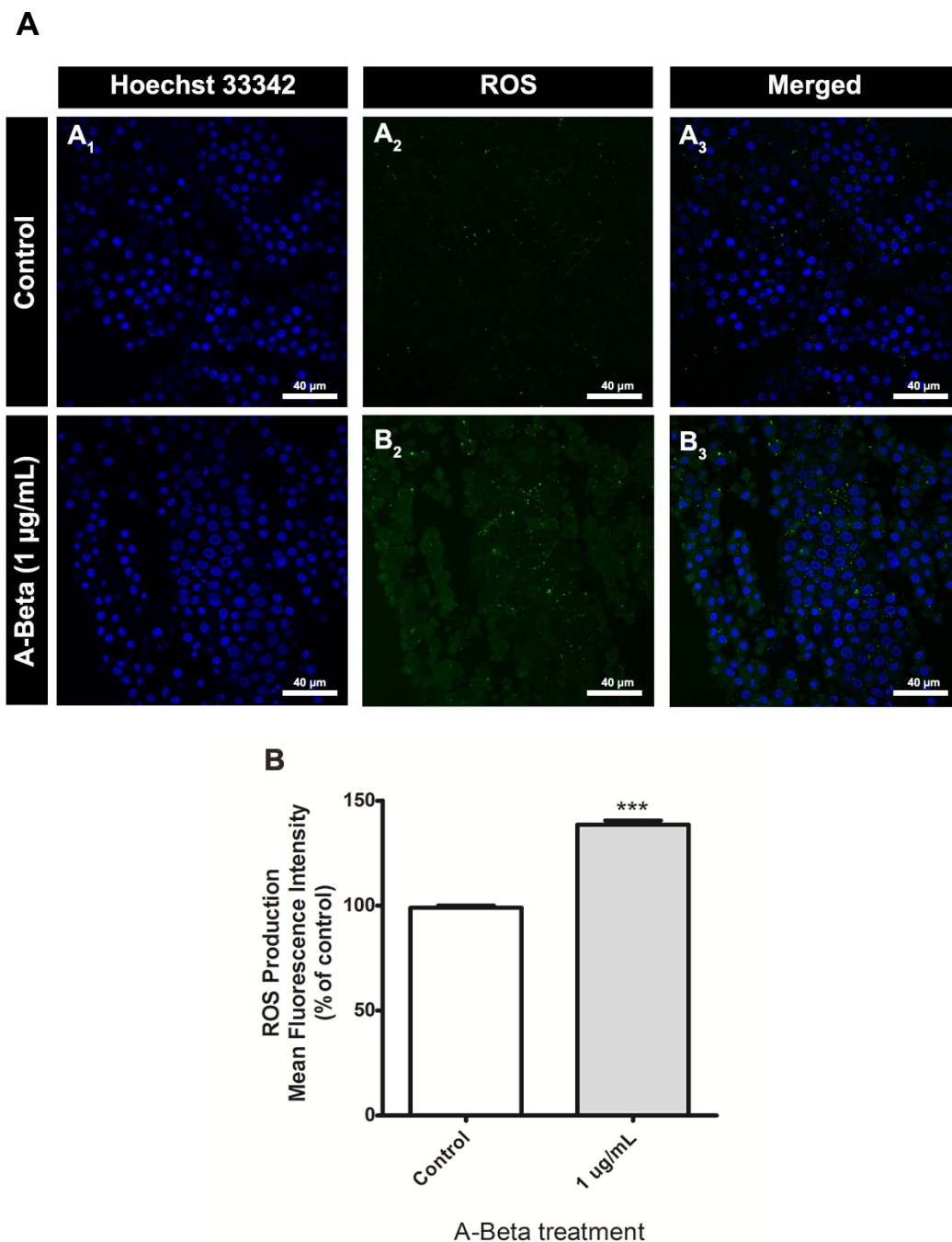


Figure 19 - Ex vivo effects of A-Beta₄₂ on ROS production in young rat CP explants. (A) Representative confocal microscopy images of ROS production (green staining); (A1-A3) control/ non-treated CP explants; (B1-B3) treated CP explants with 1ug/mL of A-Beta₄₂ show a strongest staining (green) corresponding to DCFH-DA. Magnification: 63x. **(B)** Comparison of ROS production in CP explants treated with A-Beta₄₂ and non-treated, through measurement of fluorescence intensity. ROS levels increased in young rat CP explants treated with 1ug/mL of A-Beta₄₂ when compared to control. Bar graphs represents means ± SEM, N=3 (**p<0.001 vs. control).

3. BPA effects in TTR expression in rat Choroid Plexus

3.1. TTR expression after 24 hours of BPA treatment

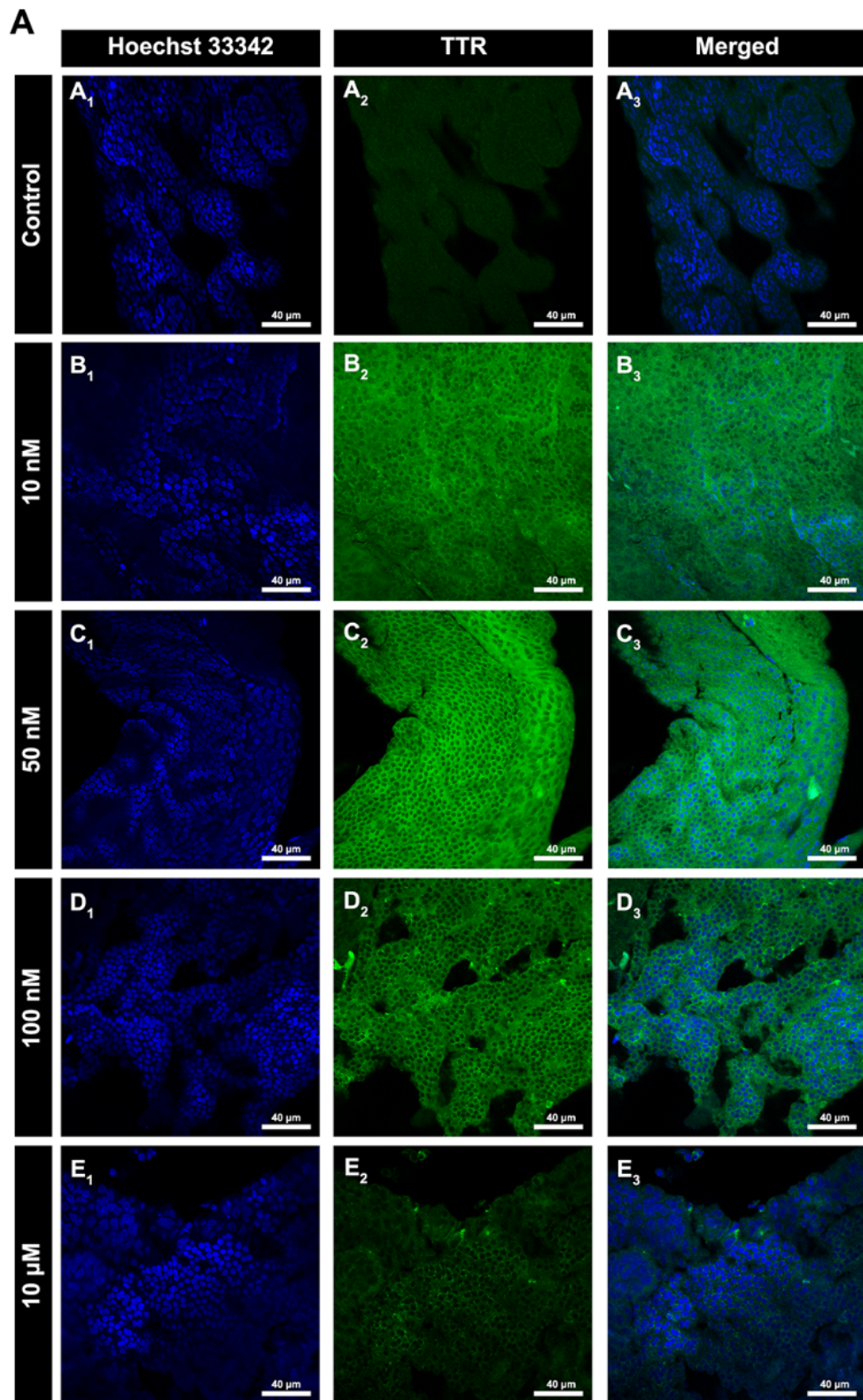
Rat CP explants were incubated with 10, 50, 100 nM and 10 μ M of this EDC for 24h, taking into account BPA levels found in the environment and even conditions used by other BPA studies. TTR levels were assessed by Whole-Mount fluorescent staining. All experiments realized with BPA were performed in CP explants of newborn rats.

3.1.1. Whole-Mount fluorescent staining

Whole-Mount fluorescent staining in rat CP explants treated with BPA allowed to capture various images by confocal microscopy (Figure 20A). Posteriorly, quantification of mean fluorescence intensity (green staining) corresponding to TTR expression was performed and analysed (Figure 20B).

Figure 20A, shows representative confocal images of TTR expression in rat CP explants treated with BPA for 24h. It are easily observed alterations in the intensity of the green staining in all rat CP explants treated with BPA in comparison with the staining in non-treated CP explants. Thus verifying that the CP explants exposure to BPA cause alterations in TTR expression.

Quantification of fluorescence intensity of TTR staining (Figure 20B) show increased TTR levels in all explants treated with BPA comparative to controls, as observed in confocal images. Interestingly, we observed alterations in TTR expression even using the lowest BPA concentration (10nM). Moreover, higher TTR levels were achieved, about 300% more than controls, with 50nM of BPA treatment. Also is important to denote that TTR expression in CP explants incubated with BPA presented a non-monotonic curve, with TTR levels increasing from controls to 50nM of BPA, and decreasing from this concentration to the higher concentration studied (10 μ M).



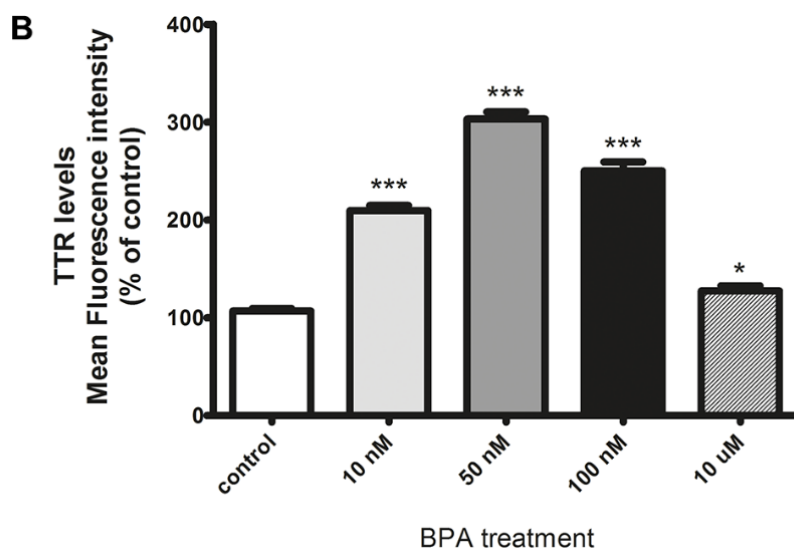


Figure 20 - TTR expression in newborn CP explants after 24h of BPA treatment. (A) Representative confocal microscopy images of TTR expression in CP explants treated with BPA and non-treated. (A1-A3) Control/ non-treated; (B1-B3), (C1-C3), (D1-D3) and (E1-E3) treated with BPA at 10nM, 50nM, 100nM and 10 μM respectively. Magnification: 63x. (B) Comparison of TTR expression levels in rat CP explants treated with different concentrations of BPA through fluorescence intensity measurement. In accordance with the confocal images, green fluorescence intensity increases from controls to 10 nM and even more to 50 nM, but decreases from 50 nM to 100nM and 10 μM of BPA. Bar graphs represents means \pm SEM. N=2 (* p <0.05, *** p <0.001 vs. control).

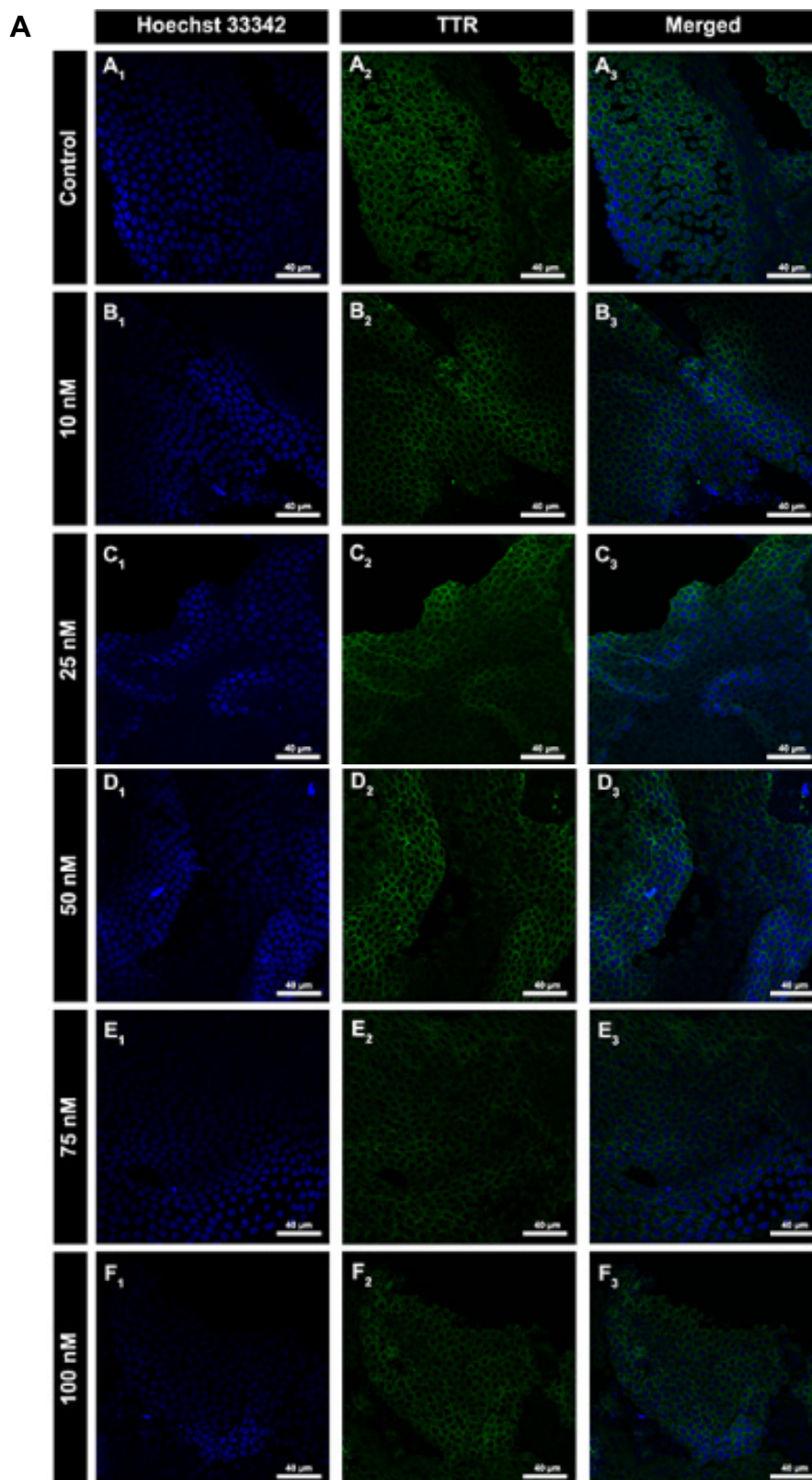
Although, CP explants treated with BPA for 24h showed significant changes in TTR expression levels, BPA metabolism is described in literature as fast, and highly variable between different subjects, and thus presenting a great variety in half-life. Indeed, in most cases after 6 hours, BPA has been totally or mostly metabolized. For this reason, we also assessed BPA effects on TTR expression in CP explants after 6 hours of incubation.

3.2. TTR expression after 6 hours of BPA treatment

CP explants were collected and treated with BPA for 6h and the effects were assessed not only by Whole-Mount fluorescent staining, but also by Western blot and Real-time RT-PCR. To choose the concentrations of BPA used in these assays, the results observed with BPA incubation during 24h were taken into account, with some adjustments. Thus, explants were treated with 10, 25, 50, 75 and 100 nM of BPA. The 10 μM concentration tested before was excluded, once it is far from the reported BPA levels to human exposure.

3.2.1. Whole-Mount fluorescent staining

Figure 21A, shows TTR expression (green staining) in representative confocal images of rat CP explants treated with BPA and controls.



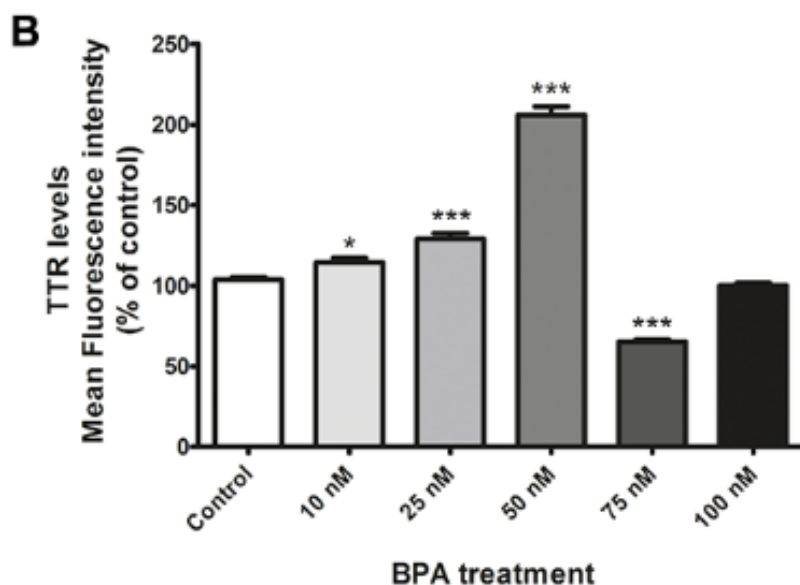


Figure 21 - TTR expression in rat CP explants after 6h of BPA treatment. (A) Representative confocal microscopy images of TTR expression in newborn CP explants treated with BPA and non-treated. (A1-A3) Control/ non-treated; (B1-B3), (C1-C3), (D1-D3), (E1-E3) and (F1-F3) treated with BPA at 10nM, 25nM, 50nM, 75nM and 100nM, respectively. Magnification: 63x. (B) Comparison of TTR expression levels in rat CP explants treated with different concentrations of BPA through fluorescence intensity measurement. Bar graphs represents means \pm SEM. N=5 (* p <0.05, *** p <0.001 vs. control).

BPA effects in TTR expression, after 6 hours of treatment, seems to follow the same tendency observed for 24 hours of treatment. TTR levels in CP explants increased comparative to controls when explants were treated with 10, 25 and 50nM of BPA. The highest levels of TTR were verified for the 50nM concentration. To higher BPA concentrations, as 75nM, TTR levels decreased comparative to controls. In case of 100nM of BPA no alterations were observed, presenting similar levels to controls. Interestingly, TTR levels at 75nM of BPA were lower than at 100nM.

3.2.2. Real-Time RT-PCR

Real-time RT-PCR was performed to compare the expression levels of TTR mRNA gene in CP explants treated with BPA and controls (Figure 18). To normalize TTR gene, Cyclophilin A gene expression was used as endogenous control.

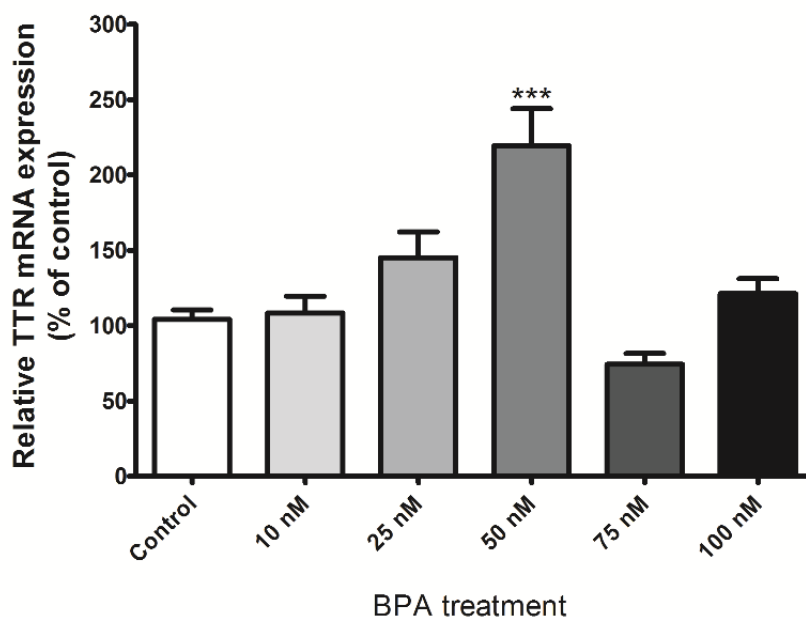


Figure 22 - Comparison of TTR mRNA levels by Real time RT-PCR in CP explants treated for 6h with BPA. The same tendency observed before by whole mount is verified but samples from the same conditions shows some variability. Although, BPA at 50nM clearly and significantly increased TTR mRNA levels. Bar graphs represents means \pm SEM, N=5 (***) $p < 0.001$ vs. control).

Figure 22, shows a similar response in TTR levels to the one verified in Whole-Mount fluorescent staining. The higher TTR mRNA expression level was obtained for BPA concentration of 50nM. Statistically, only this concentration showed significance relatively to controls. Furthermore, is also important to denote again a rise in TTR levels from 75 to 100nM, supporting the results firstly obtained by Whole-Mount analysis.

3.2.3. Western Blot

As previously mentioned, TTR is secreted to culture medium by the CP explants. Thus, the amount of TTR protein secreted to the culture medium by rat CP explants when treated with 10, 25, 50, 75 and 100 nM of BPA was quantified and compared with controls. The resulting blots are presented below (Figure 23A), also as its densitometry analysis (Figure 23B).

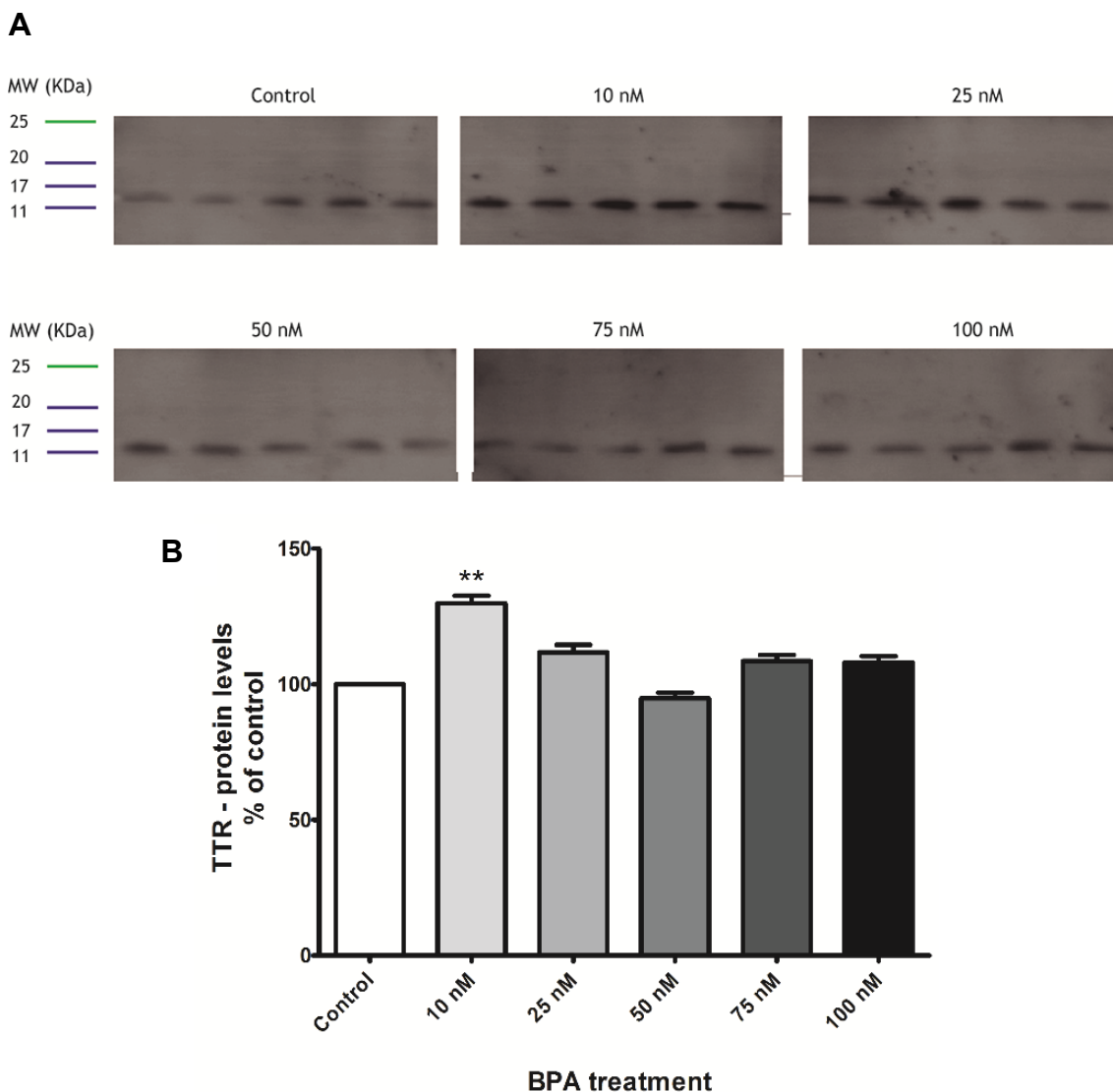


Figure 23 - Evaluation of BPA effect in TTR secretion by Western blot. Western blot was performed with culture medium collected from newborn rat CP explants treated with BPA for 6h. **(A)** Blots resulted from Western blot: control, 10nM, 25nM, 50nM, 75nM, 100nM. **(B)** Comparison of TTR levels in culture medium after analysis of blots by densitometry. Images were captured with the Molecular Imager FX Pro Plus Multilimager system. Values are expressed as a percentage of non-treated samples, which were considered 100%. Bar graphs represents means \pm SEM, N=5 (** $p < 0.01$ vs. control).

Previous results from Whole-Mount and Real Time RT-PCR showed increased TTR expression in rat CP explants treated with BPA over controls, thus being expected an increase in TTR secretion to culture medium during incubation with BPA. Although, Western blot carried out with culture medium samples of rat CP explants treated with BPA, only showed alterations in TTR levels to the concentration of 10nM of BPA ($p < 0.01$). This can be observed in resulting blots in figure 23A and, further densitometry analysis in graphic of figure 23B. For all other BPA concentrations used, no significative alterations in TTR levels in culture medium were registered.

V. Discussion

In the last decades, a great concern about human exposition to BPA has emerged. Several studies indicated that BPA was able to interfere with metabolic and endocrine functions (Masuno *et al.*, 2005, Alonso-Magdalena *et al.*, 2006, Alonso-Magdalena *et al.*, 2010). It was able to produce efficient non genomic steroid actions as well as potential such as estradiol, even at doses normally verified in humans (Vandenberg *et al.*, 2010). Despite the several studies that has been performed with the objective to evaluate BPA risks, BPA effects were never reported in CP to date. CPs are located in the brain ventricular system and participates actively in CNS homeostasis. In brain, CP is the unique local of TTR production (Sousa *et al.*, 2007). TTR has been widely studied due to its neuroprotective potential, since it was found to be the major A-Beta scavenger in CSF (Schwarzman *et al.*, 1994). TTR inhibits A-Beta aggregation and fibrils formation (Giunta *et al.*, 2005), and can participate directly in its degradation, by cleaving it in smaller units, which can be easier eliminated by the cells (Costa *et al.*, 2008a), or indirectly, through A-Beta transport from CNS into serum (Li and Buxbaum, 2011). Thus, due to CP importance for CNS homeostasis, the countless evidences of TTR role to balance A-Beta levels in brain, together with observation of TTR upregulation in CP by exposition to 17- β -estradiol (Quintela *et al.*, 2009), it was purposed to investigate the effects of BPA in TTR expression in CP. Furthermore, the BPA and A-Beta effect in BCSFB integrity, and TTR expression during aging due to A-Beta insult was also investigated.

CP explants treated with A-Beta₄₂ for 24h displayed a TTR upregulation, in a dose-dependent manner. Moreover, the results showed that TTR levels were slightly higher in treated CPs of young rats than in newborns. However, when analysing TTR mRNA levels, newborn rat CPs demonstrated an increased expression relatively to the controls, and even when compared to young rat CPs. These results are in accordance with previous reports supporting TTR production as a physiological response to block A-Beta imbalance, and further prevent its aggregation and toxicity (Stein and Johnson, 2002, Choi *et al.*, 2007, Costa *et al.*, 2007). Furthermore, decrease in A-Beta aggregation rate was linked to TTR, in a strong concentration-dependent manner (Liu and Murphy, 2006).

In literature it was already supported that A-Beta might induce toxicity to neuronal cells through oxidative stress (Costa *et al.*, 2008b, Crouch *et al.*, 2008, Vargas *et al.*, 2010). In this work, analysis performed in young rat CP explants showed that A-Beta significantly increased ROS production which can lead to cytotoxicity (Vargas *et al.*, 2010). Moreover, as already demonstrated by Vargas and co-workers CPEC exposition to A-Beta₄₂ (5 μ g/mL) decreased the ZO-1 protein expression, which can impair CP and BCSFB function originating nefarious effects (Vargas *et al.*, 2010). Therefore, in this work, a broader expression analysis of BCSFB proteins was performed (occludin, E-cadherin, Cld-1 and ZO-1) treating CP explants with A-Beta₄₂, but at a lower concentration values of 1 μ g/mL and 3 μ g/mL. The CP explants exposition to A-Beta₄₂ at a concentration of 3 μ g/mL decreased the proteins expression, which can indicate the protein degradation and, consequently, the possible BCSFB disruption. On the other side, the treatment

with A-Beta₄₂ (1 μ g/mL) showed no alterations on BCSFB proteins expression, in CP explants, indicating a threshold from which A-Beta₄₂ is toxic to BCSFB in CP.

CP explants were also used to investigate BPA effects in TTR expression. BPA at environmental relevant doses induced alteration in both protein and mRNA TTR levels, following a non-monotonic dose response. Effects in TTR expression were observed even with the lowest BPA concentration (10 nM), although the concentration of 50nM of BPA showed the higher impact in TTR levels, significantly increasing its expression. Increased TTR levels suppose that its secretion levels to CSF also increase. However, this was not entirely verified, it was observed that only BPA at 10nM increased TTR secretion, and thus raising the hypothesis of side effects of this EDC in CP.

The risks of BPA to brain functions, since this is a lipophilic compound capable of cross blood-brain barrier (Sun *et al.*, 2002), were investigated in several studies. For example, the adverse effects of BPA in CNS are the decrease of proliferation in neuronal cells, increased ROS production (Kim *et al.*, 2007), inhibition of estradiol-induced hippocampal synaptogenesis (MacLusky *et al.*, 2005), increased Ca²⁺ response to dopamine in both neurones and astrocytes, and induction of caspase-3 activation (Miyatake *et al.*, 2006). BPA action mechanisms remain a controversial question. Although BPA binds to both ERs receptors (α and β) with an affinity several orders of magnitude lower, BPA was shown to exert estrogenic actions at relative low concentrations (Singleton *et al.*, 2004). Some explanations for this fact were proposed as the possibility of BPA bind differently within the ligand-binding domain of Era or ERB and recruits dissimilar coregulators (Safe *et al.*, 2002). BPA might elicit rapid responses by binding to membrane-anchored ERs (Watson *et al.*, 2005) or also BPA binding to estrogen-related receptor Y, an orphan nuclear receptor belonging to the ERR family of receptors that do not directly bind E2 (Ariazi and Jordan, 2006). BPA was recently reported to bind at high affinity with ERRY (Okada *et al.*, 2008). For other side, U-shaped or inverted U-shaped dose-response curves could occur if a ligand, such as BPA, activated two separated pathways with differing threshold sensitivities, but which impinge on a similar downstream pathway (Sharpe, 2010).

Therefore, TTR regulation observed in CP explants by BPA, might be linked, in part, with BPA ability to mimic 17- β -estradiol actions in CP, resulting in TTR increasing levels, and for other side, activation of different pathways by BPA, that no interfere with TTR expression, or interfere at a different manner. Yet, BPA showed promote oxidative stress and inflammation (Chitra *et al.*, 2003, Yang *et al.*, 2009, Hassan *et al.*, 2012), mechanisms known by lead to deleterious effects in CP (Vargas *et al.*, 2010). Thus, is possible that BPA has a toxic effect in CP, and consequently lead to modifications on the functions of this tissue, as alteration in BCSFB proteins expression, in BCSFB integrity, and further in protein secretion rate, as showed before. Although evaluation of BCSFB proteins levels in CP explants treated with BPA was one of the goals of this work, it was not possible to quantify its levels until now, and so, loss of BCSFB integrity could not be proved.

TTR has a major role in keep brain A-Beta levels under control, and thus any interference on TTR production might lead to A-Beta imbalance. In this work, BPA effects in TTR regulation in CP raises the hypothesis that BPA indirectly might contribute to A-Beta imbalance and, therefore to the noxious effects of A-Beta in CP and in brain, as oxidative stress and BCSFB disruption.

VI. Conclusion & Future Perspectives

The increasing exposition to EDCs, especially to BPA, has becoming a public concern. Although much attention has been received in the last years, it is far from being completely understood.

CP is involved in the most basic aspects of neural function, meaning that even modest changes in CP can have far-reaching effects. It synthesizes TTR, the major protein responsible for A-Beta clearance in brain, and accumulation of this peptide in brain is the principal hallmark of AD. TTR expression increased in newborn and young rat CP explants treated with A-Beta₄₂, in a dose dependent-manner. BPA effects on TTR expression in CP were assessed here for the first time, clearly show BPA ability to change TTR expression in rat CP explants, at nanomolar doses. Furthermore, synthesis levels of TTR measured were not correlated with its secretion levels, indicating possible CP impairment. Importantly, TTR roles in A-Beta clearance depends of its synthesis by CP but also of its secretion to CSF, where TTR form complexes with A-Beta avoiding its accumulation and toxicity. For this reason, BPA might has an active role in A-Beta accumulation in brain, by modulate TTR expression and its secretion, modifying A-Beta levels, and therefore, possibly contributing to some neurodegenerative diseases.

BCSFB integrity might be compromised by A-Beta and BPA injuries, which explains alteration in secretion rates of controls when compared to treated CP explants. Several BCSFB proteins levels in CP explants decreased due to A-Beta exposition, supporting this hypothesis.

In future, alterations of TTR expression by CP throughout life should be investigated, possibly using the same techniques optimized in this work, but with older animals at different ages (3, 6 and 9 months) to verify if TTR expression impairment is age-dependent.

This work shows BPA interfering on TTR expression in CP explants, but the mechanisms under this event are uncertain. Taken into account reported TTR upregulation in CP by 17- β -estradiol is possible that one of the mechanisms by which BPA induced TTR production in CP explants is ER-dependent. However, this is a hypothesis that should be verified in the future. In this way, an inhibitor of ERs could be used in CP explants before BPA treatment to assess if ER pathway is linked with BPA action in CP. Another interesting aspect that could be investigated is the chronic effects of BPA on TTR expression in CP, because in this work we only evaluate acute effects in CP explants, and usually, human exposure to BPA it is believed to be continuous.

Additionally, would be important to evaluate the BCSFB proteins levels in CP in response to BPA treatment, in order to verify BPA effects in the integrity of this barrier, and ultimately, in A-Beta clearance.

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