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Analysis of the regulation of drug uptake and detoxification systems in the blood-cerebrospinal fluid barrier: the role of sex hormones and circadian rhythm

(Versão final após defesa)

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Resumo Alargado

De forma a se adaptarem às alterações diurnas, os organismos vivos desenvolveram ritmos circadianos, que correspondem a alterações com uma ritmicidade de 24 horas nas funções biológicas. Os ritmos circadianos são conduzidos por relógios moleculares que, nos mamíferos, se encontram organizados de forma hierárquica. O núcleo supraquiasmático do hipotálamo opera como oscilador central, tendo a importante função de receber a informação luminosa e sincronizar os osciladores periféricos, de acordo com a hora do dia. O plexo coroide, para além de englobar um relógio molecular modulado pelo estradiol, é responsável por formar uma barreira entre o sangue e o líquido cefalorraquidiano que, através da presença de transportadores de membrana, enzimas de destoxificação, e de junções de oclusão entre as células epiteliais limita a passagem de moléculas do sangue para o líquido cefalorraquidiano e, consequentemente, para o cérebro. Assim, o objetivo do presente trabalho é avaliar o impacto do ritmo circadiano e das hormonas sexuais em transportadores de membrana presentes no plexo coroide.

Neste estudo, avaliou-se inicialmente se os transportadores de membrana Abca1, Abcc1, Abcc4, Abcg4 e Oat3 apresentam uma expressão circadiana e, por sua vez, se essa ritmicidade varia entre machos e fêmeas. Para isso, através da técnica de PCR em tempo real obteve-se o perfil diário da expressão destes transportadores no plexo coroide de ratos fêmea e macho intactos, de ratos fêmea submetidos a ovariectomia e fêmeas controlo eutanasiados a diferentes horas.

Nas fêmeas intactas, observou-se uma expressão circadiana para o Abcc4 e para o Oat3, enquanto que nos machos apenas se verificou para o Abcg4. Apenas a expressão do Oat3 nas fêmeas controlo (não ovariectomizadas) apresentou ritmicidade.

Para verificar se o metotrexato é transportado de forma circadiana através da barreira entre o sangue e o líquido cefalorraquidiano, foi realizado um ensaio in vitro, onde *transwells* cultivados com células da linha celular do plexo coroide humano (HIBCPP) foram incubados com fluoresceína-metotrexato no lado basal a diferentes horas. As concentrações de fluoresceína-metotrexato foram avaliadas nos 3 compartimentos (apical, basal e intracelular), e todos eles apresentaram ritmicidade, o que significa que o metotrexato é transportado de forma circadiana tanto através da membrana basal como da membrana apical. Sendo o metotrexato um substrato do ABCC4, fomos

verificar se este estaria envolvido na ritmicidade do transporte do metotrexato através da barreira entre o sangue e o líquido cefalorraquidiano. Para isso, através de PCR em tempo real observámos que o ABCC4 é expresso de forma circadiana nas células HIBCPP. Posteriormente, o ensaio in vitro foi repetido, usando um inibidor do ABCC4, em que se verificou ritmicidade nos níveis de fluoresceína-metotrexato apenas nos compartimentos apical e intracelular. Assim, o ABCC4, que se encontra localizado na membrana basolateral das células epiteliais do plexo coroide, é responsável pela ritmicidade do transporte de metotrexato através da membrana basal. Por fim, para verificar se o ABCC4 é o único transportador responsável pela ritmicidade do transporte de metotrexato através da membrana basal, realizou-se um novo ensaio *in vitro* em que se inibiu o ABCG2, um transportador localizado na membrana apical. Os resultados obtidos neste ensaio foram semelhantes ao anterior, verificando-se também apenas ritmicidade nos níveis de fluoresceína-metotrexato para os compartimentos apical e intracelular, indicando que o ABCC4 não é exclusivamente responsável pela ritmicidade do transporte de metotrexato através da membrana basal.

Em conclusão, demonstrámos que o Abcc4, o Abcg4, e o Oat3 apresentam uma expressão circadiana no plexo coroide, influenciada pelas hormonas sexuais. Os nossos resultados mostram também que metotrexato é transportado de forma circadiana através da barreira entre o sangue e o líquido cefalorraquidiano e que o ABCC4 está parcialmente envolvido na ritmicidade desse transporte através da membrana basal.

Palavras-chave

Plexo coroide; Ritmos circadianos; Transportadores de membrana; ABCC4; Hormonas sexuais

Abstract

The choroid plexus (CP), localized in the brain ventricles, forms the blood-cerebrospinal fluid barrier, which, by the presence of thigh junctions, detoxification enzymes, and membrane transporters limit the traffic of molecules into the brain. Sex hormones influence several CP functions, including its activity as a circadian oscillator. This study aims to evaluate the impact of sex hormones and circadian rhythms in the function of CPs´ membrane transporters. First, we compared the diary expression of Abca1, Abcc1, Abcc4, Abcg4, and Oat3 transporters in the CP of male and female rats. We observed a circadian expression of Abcc1 and Oat3 in female rats and Abcg4 in male rats, suggesting that sex hormones influence the rhythmicity in the expression of these transporters in CP. Next, we also compared the 24 hours transcription profile of Abca1, Abcc1, Abcc4, Abcg4, and Oat3 in the CP of sham-operated and ovariectomized female rats. We found that Oat3 circadian expression is dependent on ovarian hormones. Using an *in vitro* model of the human blood-cerebrospinal fluid barrier, we also found that methotrexate (MTX), a very unspecific substrate in terms of transport, is transported in a circadian way across this barrier. Moreover, we demonstrate that Abcc4 is also expressed in a circadian way in the human CP epithelial papilloma cells and is partially responsible for the MTX circadian transport across the basal membrane of choroid plexus epithelial cells

Keywords

Choroid plexus; Circadian rhythms; Membrane transporters; ABCC4; Sex hormones

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Acronyms list

ABC	ATP-Binding Cassette
AhR	Aryl hydrocarbon Receptor
AMPA	α-amino-3-hydroxi-5-methyl-4-isoxazolepropionic acid
AVP	Arginine Vasopressin
BBB	Blood-Brain Barrier
BCRP	Breast Cancer Resistance Protein
BCSFB	Blood-Cerebrospinal Fluid Barrier
BMAL1	Brain and Muscle ARNT-Like 1
BSA	Bovine Serum Albumin
CAR	Constitutive Androstane Receptor
CCG	Clock Controlled Gene
CLOCK	Circadian Locomotor Output Cycles Kaput
CNS	Central Nervous System
CP	Choroid plexus
CPEC	Choroid Plexus Epithelial Cells
CREB	cAMP Response Element Binding Protein
CRY	Cryptochrome
CSF	Cerebrospinal Fluid
CYC	Cyclophilin
CYP450	Cytochrome P450
Dbp	D-box binding protein
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
E2	Estradiol
E-box	Enhancer box
ER	Estrogen Receptor
FBS	Fetal Bovine Serum
FL-MTX	Fluorescein-methotrexate
FSH	Follicle-Stimulating Hormone
GABA	Gamma-aminobutyric acid
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GnRH	Gonadotropin-Releasing Hormone
GRP	Gastrin Releasing Peptide
HIBCPP	Human epithelial CP 228 papilloma
ICI 182,780	Fulvestrant
ipRGC	Intrinsically photosensitive Retinal Ganglion Cells
LH	Luteinizing hormone
MDR1	Multidrug Resistance Protein
MRP	Multidrug Associated resistance Protein
MTX	Methotrexate
NBD	Nucleotide-Binding Domain

NFIL3	Nuclear Factor, Interleukin 3 Regulated
NMDA	N-metil D-Aspartate
OAT1	Organic Anion Transporter 1
OAT3	Organic Anion Transporter 3
OB	Olfactory bulb
OOX	Orchidectomized
OVX	Ovariectomized
P4	Progesterone
PARbzip	Proline and Acidic amino acid-Rich basic leucine zipper
PBS	Phosphate Saline Buffer
PCFT	Proton-Coupled Folate transporter
PEPT1	Polypeptide Transporter 1
PER	Period
PFA	Paraformaldehyde
P-gP	P-glycoprotein
PXR	Pregnane X Receptor
RFC	Reduced Folate Carrier
ROR	Retinoic Acid-Related Orphan Receptor
RT-qPCR	Real-time Quantitative Reverse Transcription PCR
SCN	Suprachiasmatic Nucleus
SH	Sex Hormones
SLC	Solute-Carrier
TEER	Transepithelial Electric Resistance
TMD	Transmembrane Domain
TTFL	Transcriptional-Translational Feedback Loop
VIP	Vasoactive Intestinal Peptide
ZT	Zeitgeber time

Chapter I - Introduction

1.1. Circadian rhythms

All life forms, as a result of the earth's rotation, are exposed to diurnal changes in form of day and night. To adapt to those changes, organisms have developed circadian (from the Latin *circa*, meaning 'about' and *dies*, meaning 'day') rhythms, which by simple definition correspond to rhythmically daily oscillations in biological functions (Bell-Pedersen et al., 2005; Bhadra et al., 2017; Panda et al., 2002). At a cellular level, genetic circuits composed of specific genes whose expression oscillates in a 24 hours pattern, produces and regulates the timing of circadian rhythms (Reppert and Weaver, 2002; Takahashi, 2017). These molecular clocks are found in almost every cell and tissue (Buhr and Takahashi, 2013), and the way of how they function and sense the *zeitgebers* (temporal cues) will be described in the next subsections and, in the next section.

1.1.1. Organization of the circadian system

The circadian system shows a hierarchical organization (Figure 1), where the suprachiasmatic nucleus (SCN) operates as the master circadian pacemaker. The SCN, located in the hypothalamus, receives light (main *zeitgeber*) inputs from the retina and synchronizes peripheral oscillators across the body (Hastings et al., 2018, 2019). In other words, SCN can sense the time of the day and prepare the body for daily environmental changes. Through direct or indirect projections, SCN communicates with various brain areas, which many of them contain extra-SCN oscillators that drive behavioral, neurohormonal, and autonomic rhythms, which are responsible for the synchronization of peripheral molecular clocks (Begemann et al., 2020; Hastings et al., 2018; Logan and McClung, 2019).

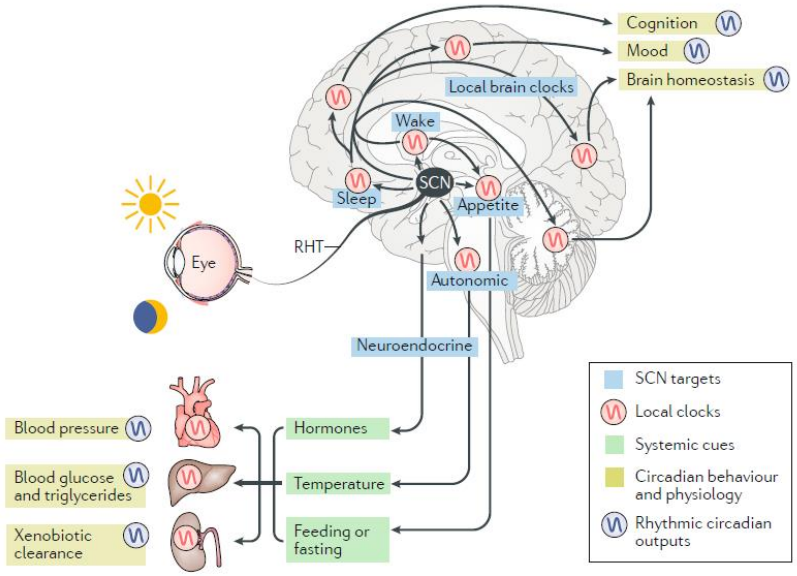

Figure 1. General organization of the circadian system. The suprachiasmatic nucleus (SCN) is the central piece of the circadian system, having the important function of setting the peripheral molecular clocks according to the time of the day. Light entrains the SCN via retinohypothalamic tract and synchronizes the SCN molecular clock. In its turn, SCN communicates with many brain loci that drive behavioral, autonomic, and neuroendocrine rhythms, which are responsible for peripheral molecular clocks synchronization. Adapted from (Hastings et al., 2018).

1.1.2. Molecular mechanism of the mammalian circadian clock

Circadian rhythms are driven by self-sustaining transcriptional-translational feedback loops (TTFLs) (Figure 2). The actual model comprises one core loop and two accessory loops (Cox and Takahashi, 2019; Curtis et al., 2014). In the main loop, the two activator transcription factors: Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-Like 1 (BMAL1) form a heterodimeric complex. The CLOCK-BMAL1 complex binds to enhancer box (E-box) response elements promoting the expression of many clock-controlled genes (CCGs), including the repressor regulators cryptochrome (Cry1 and Cry2) and period (Per1, Per2, and Per3) (Cox and Takahashi, 2019; Mendoza-Viveros et al., 2017; Okamura et al., 2010; Patke et al., 2019; Takahashi, 2017). PER and CRY proteins dimerize and translocate to the nucleus, where interact with CLOCK-BMAL1 complex and repress its own transcription by recruiting chromatin remodeling complexes (Kim et al., 2014; Mendoza-Viveros et al., 2017). PER and CRY proteins are progressively degraded and, after the repressor phase, the CLOCK-BMAL1 complex is available to start a new transcription cycle.

Besides Per and Cry genes, the heterodimeric CLOCK-BMAL1 complex also promotes the transcription of REV-ERβ$_{\alpha}$ and REV-ERβ$_{\beta}$ nuclear receptors, coded by NR1D1 and NR1D2 genes, respectively. REV-ERβ$_{\alpha}$ and REV-ERβ$_{\beta}$ nuclear receptors, compete with retinoic acid-related orphan receptors (RORs), which are positive regulators, for RevDR2 and ROR binding elements (ROREs). Through this pathway, REV-ERβ$_{\alpha}$ and REV-ERβ$_{\beta}$ lead to a repression of BMAL1 transcription (Cox and Takahashi, 2019; Mendoza-Viveros et al., 2017; Takahashi, 2017). The second accessory loop involves the interplay between D-box regulator proteins. The transcription of the D-box binding protein (Dbp) is driven by the CLOCK-BMAL1 complex. DBP binds to D-box response elements and promotes the transcription of many CCGs, including RORa and RORb genes, that together with REV-ERB nuclear receptors are responsible to impose rhythmicity in Bmal1 transcription. In addition to Bmal1, RORs also promote the transcription of the nuclear factor, interleukin 3 regulated (NFIL3), which negatively regulates gene expression through D-box response elements (Cox and Takahashi, 2019; Okamura et al., 2010; Takahashi, 2017).

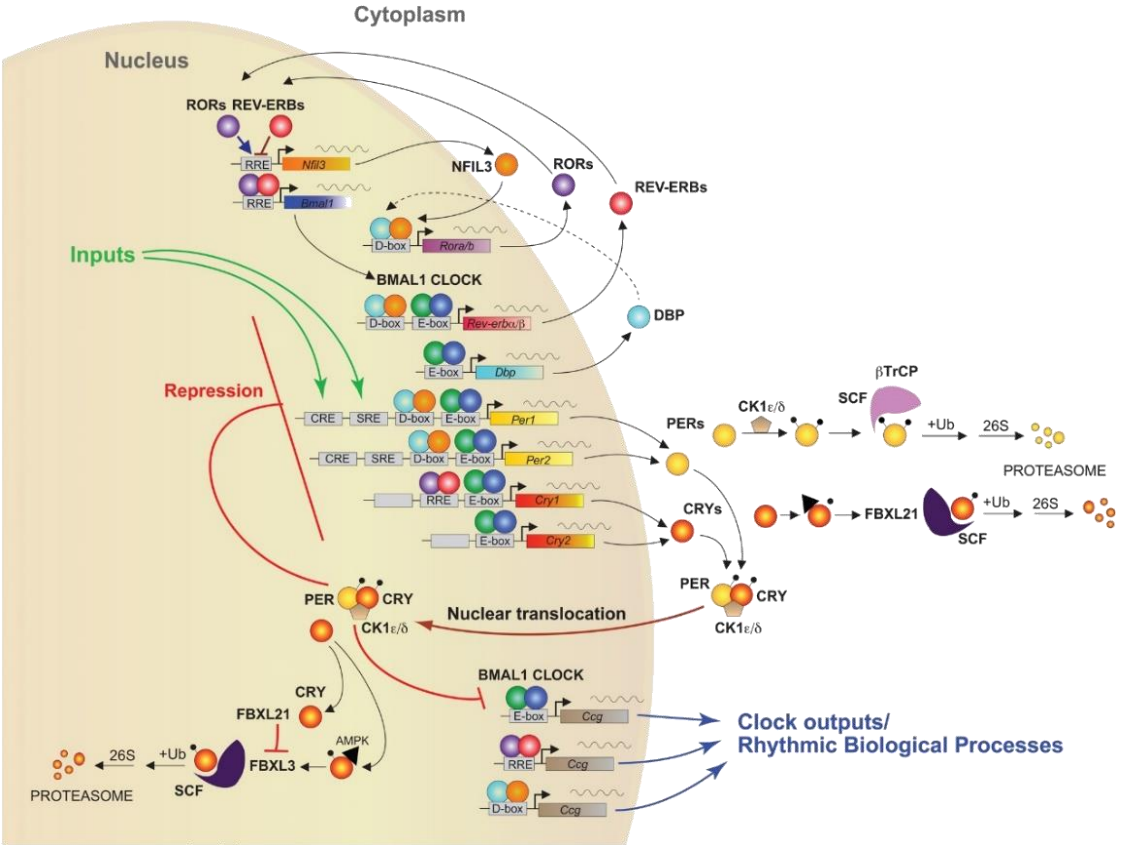

Figure 2. Molecular mechanism of the mammalian circadian clock. In the core feedback loop, the two activator transcription factors, Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-Like 1 (Bmal1) form a heterodimeric complex and bind to E-box response elements, driving the expression of several clock-controlled genes (CCGs), including the repressor regulators Period (Per) and Cryptochrome (Cry). PER and CRY proteins dimerize and translocate to the nucleus, where they repress its own transcription. In the second feedback loop, CLOCK-BMAL1 complex also drives the expression of REV-ERB nuclear receptors, that compete with retinoic acid-related orphan receptors (RORs) for binding

to RevDR2 and ROR binding elements (ROREs) on gene promotors, where negatively regulate the transcription of many CCGs, including Bmal1. In the third feedback loop, the CLOCK-BMAL1 complex drives the expression of the D-box binding protein (DBP), that binds to D-box response elements and promote the expression of many genes, including the ROR genes. RORs are positive regulators and, together with REV-ERBs, impose rhythmicity in Bmal1 transcripts. RORs also drive the expression of the nuclear factor, interleukin 3 regulator (NFIL3), which negatively regulates gene expression through D-box response elements. Adapted from (Cox and Takahashi, 2019).

1.1.3. General characterization of the suprachiasmatic nucleus

The SCN is a hypothalamic brain region, located above the optic chiasm, and is composed of approximately 20.000 neurons (Hastings et al., 2018). The SCN is a GABAergic circuit that, based on neuropeptidergic identity, can be divided into two subregions: core and shell. Core neurons are characterized for receiving light inputs from the retina, and for the expression of the vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP). On the other hand, shell neurons express arginine-vasopressin (AVP) and receive inputs from the retino-recipient core neurons (Fernandez et al., 2016; Mieda, 2019).

The neurons of the SCN exhibit spontaneous activity, that together with membrane potential undergo circadian variations. These properties are ensured by an interplay between the molecular clock and ionic currents. First, TTFLs are responsible for circadian rhythms in ionic conductances, where, during the circadian day, Ca^{2+} and Na^{+} currents are upregulated sustaining a slight membrane depolarization and a higher activity of SCN neurons. Contrarily, at night, K^{+} currents are upregulated, but dorsal astrocytes also contribute to a night-time depolarization and lower electrical activity of SCN neurons (Colwell, 2011; Harvey et al., 2020; Hastings et al., 2018). SCN dorsal astrocytes are responsible for a night peak of extracellular glutamate, which in turn activates the N-methyl-D-aspartate (NMDA) receptors containing NR2C subunits in the presynaptic terminals of dorsal neurons. This activation triggers the night release of gamma-aminobutyric acid (GABA) across the SCN, leading to the hyperpolarization of SCN neurons (Brancaccio et al., 2019; Brancaccio et al., 2017). However, the electrical activity of SCN neurons can entrain the molecular clock, where Ca^{2+} through the cAMP Response Element Binding protein (CREB) leads to Per genes expression. Thus, this interplay is essential for the functions of SCN neurons (Hastings et al., 2018, 2019).

Light is the main *zeitgeber* and entrains the circadian system by the intrinsically photosensitive retinal ganglion cells (ipRGC). ipRGCs project to SCN through the retinohypothalamic tract and release glutamate to SCN core neurons. Glutamate through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA

receptors elevate the intracellular calcium levels which depolarize core neurons and lead to the phosphorylation of CREB by Ca^{2+}/calmodulin-dependent kinase. Phosphorylated CREB can translocate to the nucleus where promote Per genes expression, causing a phase shift in core neurons (Colwell, 2011; Ramkisoensing and Meijer, 2015). Further, core neurons synchronize shell neurons through the release of VIP. VIP act through VIPR receptors in core neurons, leading to the promotion of PER expression in a manner independent of CREB protein (Hamnett et al., 2019). Thus, light is a direct *zeitgeber* capable of depolarize and synchronize SCN neurons, that convey the light information and integrate peripheral clock across the body. However, non-photic *zeitgebers* like physical activity or stress entrain the circadian system through peripheral oscillators (Buttgereit et al., 2015; Tahara et al., 2017).

1.1.4. Extra-SCN brain oscillators

To synchronize peripheral tissue clocks, first, SCN must convey the light information to extra-SNC brain oscillators that drive behavioral, neurohormonal, and autonomic rhythms, which in turn act as synchronizing factors for peripheral tissue oscillators (Begemann et al., 2020; Hastings et al., 2018; Logan and McClung, 2019). SCN downstream clocks can be classified as semiautonomous or slave oscillators. Semiautonomous oscillators are capable of generating autonomous circadian rhythms but require the presence of a master clock for its synchronization. On the other hand, slave oscillators rhythmicity is entirely dependent on inputs from another clock (Guilding and Piggins, 2007). A study performed by *Abe et al.* showed that 14 of 27 brain areas analyzed contain circadian oscillators. The most robust ones were found in the pineal gland, in the pituitary, and in the arcuate nucleus (Abe et al., 2002). These tissues are involved in neuroendocrine functions and all, directly or indirectly receive inputs from the SCN (Chen, 2019; Guilding and Piggins, 2007; Simonneaux and Piet, 2018). Arcuate nucleus together with lateral habenula and dorsal medium hypothalamus are semiautonomous oscillators (Guilding et al., 2009; Salaberry et al., 2019; Sellix et al., 2006).

One of the most intriguing brain areas in chronobiology is the olfactory bulb (OB), known to possess pacemaker-like qualities. First, the OB can drive circadian rhythms independently of SCN. Second, these rhythms are temperature compensated, and third, the OB molecular clock can sense environmental cues, in this case, odorants, and can transmit that information to a downstream oscillator, the piriform cortex (Abraham et al., 2005; Amir et al., 1999; Granados-Fuentes et al., 2004a; Granados-Fuentes et al.,

2004b; Granados-Fuentes et al., 2006). These data from the OB can challenge the classical view of a hierarchical organization of the circadian system, where SCN is alone at the top of the pyramid. Although, more recent studies identified a circadian oscillator in the choroid plexus (CP) more robust than the clock on SCN (Quintela et al., 2018a; Quintela et al., 2015; Yamaguchi et al., 2020).

1.2. Factors Influencing the circadian system

The principal function of the circadian system is preparing the organism for environmental changes, thus, this system must be able to sense and integrate *zeitgebers*. Light (Hastings et al., 2018), temperature (Refinetti, 2010), meals (Wang et al., 2017), stress (Koch et al., 2017), and physical exercise (Gabriel and Zierath, 2019) are examples of external cues that entrain the circadian system, however, the organism needs an internal mediator to transport the information from zeitgebers to the molecular clocks. Hormones can function as internal mediators and, a well-established example are glucocorticoids. Glucocorticoid hormones secretion is under the control of SCN, imposing a circadian rhythm in glucocorticoid levels. In its turn, glucocorticoids can synchronize peripheral clocks across the body (Koch et al., 2017; Oster et al., 2017; Spencer et al., 2018). In addition to zeitgebers, other factors like age (Hood and Amir, 2017; Nakamura et al., 2016) and disease states (Homolak et al., 2018; Tana et al., 2018) can influence the circadian system. For example, in the case of these two factors, melatonin levels and rhythm's amplitude are decreased, which leads to sleep-wake cycles disruption (Hardeland, 2012). So, hormones function as second synchronizers, and disturbances in hormonal signaling impair the biological rhythms. However, despite the well-characterized impact of melatonin and glucocorticoids in circadian rhythms, the role of sex steroid hormones in the circadian system is still scarce.

1.2.1. Sex hormones and circadian rhythms

Sex hormones (SH) like every other hormone show circadian variations (Bao et al., 2003; Diver et al., 2003; Rahman et al., 2019). The release of SH is controlled by the hypothalamic-pituitary-gonadal axis. The hypothalamic Gonadotropin-releasing hormone (GnRH) neurons are the first components of this axis and receive time inputs from the SCN. SCN core neurons project directly to GnRH neurons, whereas shell neurons communicate with them via kisspeptin neurons (Putteeraj et al., 2016; Simonneaux and Piet, 2018). GnRH neurons molecular clock drive the circadian

release of GnRH, which through the hypophisary-portal system act on pituitary cells stimulating the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In its turn, LH and FSH will communicate with ovaries, where regulate the molecular clock controlling the timing of the expression of some genes involved in steroid hormones biosynthesis (Sen and Hoffmann, 2020). In testis, Leydig cells possess a functional molecular clock (Alvarez et al., 2008; Baburski et al., 2019) and, a recent study showed that testosterone rhythms are mainly dependent of LH signaling instead of the molecular clock itself (Baburski et al., 2019). In addition, SH can also affect circadian rhythms. At a central level, SH modulate some SCN functions, but, besides the expression of SH receptors in the SCN, the molecular mechanisms of how SH affect the SCN are poorly understood (Hatcher et al., 2018; Karatsoreos and Silver, 2007). On the other hand, SH can modulate the expression of clock genes, where it´s shown that estrogens drive the expression of Per1 in liver and kidney, Per1 and 2 in the uterus (He et al., 2007; Nakamura et al., 2005; Nakamura et al., 2008), and Per 1,2 and Bmal1 in CP (Quintela et al., 2018a). Progesterone (P4) can drive Per1 expression in the uterus, and testosterone modulates Bmal1 expression in the prostate (Kawamura et al., 2014). So, SH can act as a second synchronizers, that feedback in the SCN and entrain the peripheral clocks.

2. Choroid Plexus

2.1. Choroid Plexus structure

The CPs form the interface between blood and cerebrospinal fluid (CSF). CPs are localized in each of the four brain ventricles: two laterals, one third, and a fourth (Damkier et al., 2013; Lun et al., 2015; Santos et al., 2017). The structure of the CPs is relatively simple (Figure 3). Each CP consists of a monolayer of cuboidal epithelial cells (CPEC; choroid plexus epithelial cells) that lay in a basement membrane, which below resides a network of fenestrated capillaries surrounded by connective tissue rich in fibroblasts and immune system cells (Ghersi-Egea et al., 2018; Liddelow, 2015; Santos et al., 2017; Wolburg and Paulus, 2010).

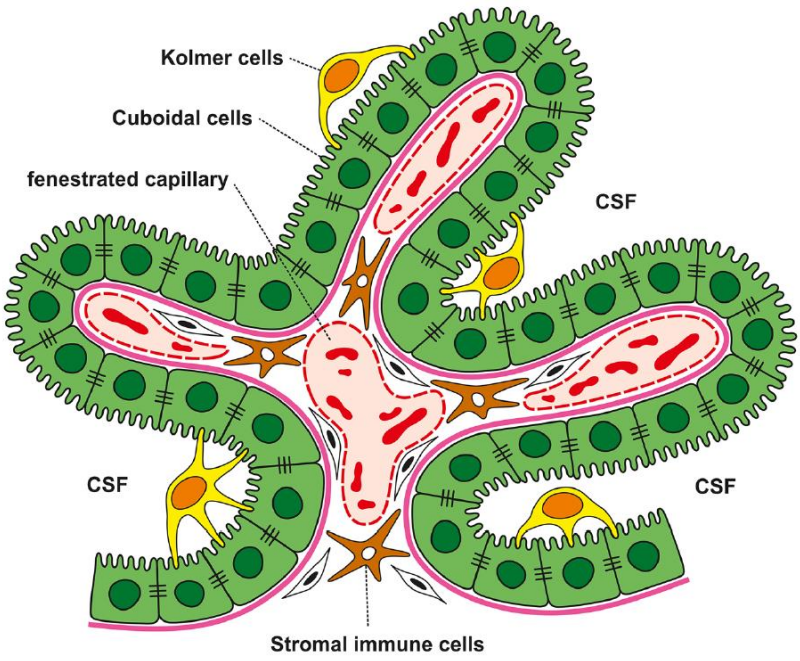

Figure 3. Choroid Plexus (CP) structure. Each CP is composed of a monolayer of cuboidal epithelial cells with numerous microvilli in the apical surface. The epithelial cells lay in a basement membrane which below resides a highly vascularized connective tissue with fenestrated capillaries, rich in fibroblasts and immune system cells. The phagocytic Kolmer cells are anchored to the apical surface of epithelial cells and may act as scavengers for the brain ventricular system. Adapted from (Solár et al., 2020).

2.2. Cerebrospinal fluid secretion

CPs are the main sites of CSF production. CSF is renewed 4 to 5 times a day, and 60 to 70% of the daily production is driven by the CPs of lateral ventricles (Hladky and Barrand, 2014; Sakka et al., 2011). CSF is actively secreted by the CP, where the active transport of ions into the CSF creates an osmotic gradient that drives the transport of water across the choroidal epithelium (Damkier et al., 2013; Hladky and Barrand, 2016). According to the classical hypothesis, CSF flux is unidirectional (Figure 4). Starting from the lateral ventricles, CSF flows for the third ventricle through the foramen of Monro, and therefrom for the fourth ventricle through the aqueduct of Sylvius. Lastly, CSF leaves the ventricular system to the subarachnoid space through the foramina of Luschka and Magendie, and its reabsorbed via arachnoid granulations into the meningeal sinus, and drained via meningeal and nasal mucosa lymphatics to the cervical lymph nodes (Da Mesquita et al., 2018; Damkier et al., 2013; Segal, 2001).

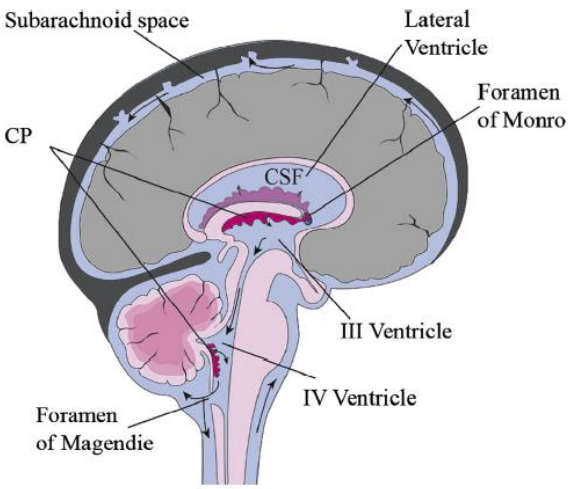

Figure 4. Brain ventricular system and cerebrospinal fluid (CSF) circulation. CSF is produced by the choroid plexus (CPs) on each ventricle. CSF circulates from the lateral ventricles to the third ventricle through the foramen of Monro and then from the fourth ventricle through the aqueduct of Sylvius. In the fourth ventricle, CSF leaves the ventricular space to the subarachnoid space through the Foramina of Luschka and Magendie. Adapted from (Santos et al., 2017).

CSF is composed of water (99%), ions, vitamins, and proteins that, like vitamins, can be synthesized in CP or transferred from the blood into the CSF. According to its composition, CSF is a source of ions and molecules, in which many of them contribute to a proper and supportive environment for CNS (Skipor and Thiery, 2008; Spector et al., 2015). CSF also participates in the clearance of brain metabolic waste and deleterious compounds. Brain metabolites can diffuse from brain interstitial fluid into the CSF, and then are carried out of the CNS via arachnoid granulations into meningeal sinus, via nasal mucosa and meningeal lymphatics, and through the receptors and transporters in the apical membrane of CPEC (Da Mesquita et al., 2018; Ghersi-Egea et al., 2018; Jessen et al., 2015). Overall, CSF's function is to maintain the CNS homeostasis, which depends both on CSF composition and clearance ability. However, this is only possible because CPs are selective barriers, which control the traffic of ions and molecules across the choroidal epithelium (Strazielle and Ghersi-Egea, 2015, 2016).

2.3. Blood-Cerebrospinal fluid barrier

The principal function of the brain barrier is to regulate the exchange of substances between blood and brain. For that, three specialized structures lay in the CNS. (i) The blood-brain barrier (BBB), composed of brain capillary endothelial cells, pericytes, and astrocytes endfeet. (ii) The blood-cerebrospinal fluid barrier (BCSFB) formed by the

CPs epithelium (Figure 5) and (iii) the blood-arachnoid barrier constituted by the epithelial cells of the arachnoid layer (Abbott et al., 2010; Gomez-Zepeda et al., 2019; Strazielle and Ghersi-Egea, 2013).

For a barrier to function properly its permeability must be selective. Thus, to maintain the normal function of the brain, brain-barriers must allow the passage of nutrients, ions, and brain metabolites, and must be impermeable to xenobiotics and deleterious compounds (Abbott, 2013; Santos et al., 2019). These "traffic selectivity" is ensured by the presence of tight junctions, uptake and efflux transporters, and detoxification enzymes in the barrier epithelial cells (Figure 5). However, besides this protective role, these three barrier hallmarks can also modulate the availability of therapeutic drugs in the brain (Abbott, 2013; Ghersi-Egea and Strazielle, 2001; Ghersi-Egea et al., 2018; Morris et al., 2017).

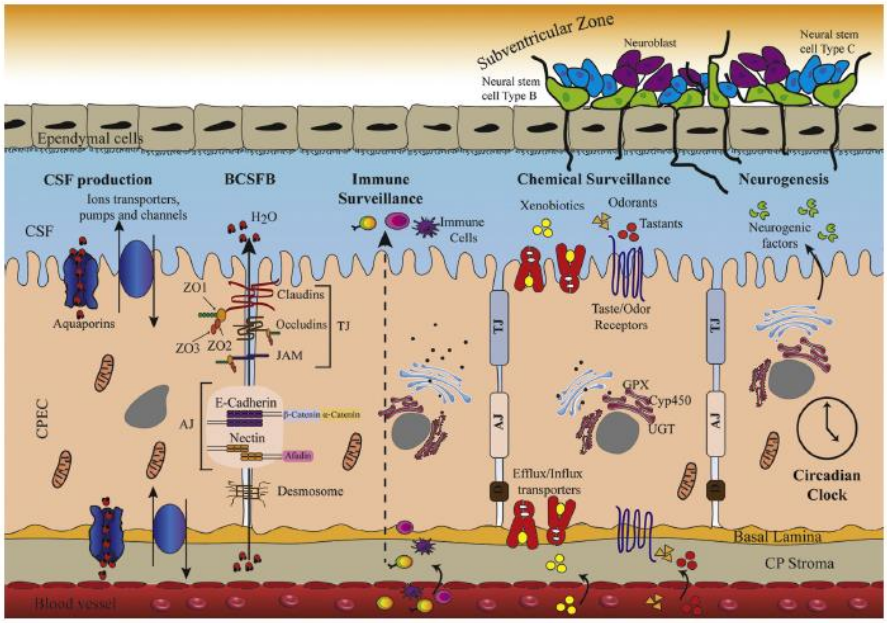

Figure 5. Choroid plexus physiology and functions. Choroid plexuses (CPs) are the main sites of cerebrospinal fluid production and, are composed of a monolayer of cuboidal epithelial cells resting in a basal lamina and escheat a highly vascularized connective stroma. Choroid plexus epithelial cells (CPEC) are connected by apical tight junctions and have numerous microvilli in the apical surface. CPEC also have numerous mitochondria, Golgi apparatus, lysosome-like vesicles, and smooth endoplasmic reticulum, which are important hallmarks of its intense synthetic and secretory activity (Marques et al., 2013). Together with tight junctions, CPEC express membrane transporters and detoxification enzymes, thus constituting a barrier for xenobiotics. Besides this barrier function, CPs contribute to the development of subventricular zone neurogenic niche, and for the migration of immune cells during some central nervous system disease states. Adapted from (Santos et al., 2017).

More specifically, in BCSFB, claudins-1, -2 and -3, are the main claudins involved in tight junctions of CPEC. Claudins-1 and -3 are barrier-tightening claudins, i.e., they form impermeable barriers, while claudin-2, forms paracellular pores permeable to

some ions and water. Contrarily to BCSFB, the major claudin expressed in BBB is claudin-5, which is a barrier-tightening claudin. So, the differential expression of claudins between these two barriers explains why BCSFB is less restrictive than BBB (Ghersi-Egea et al., 2018; Gonçalves et al., 2013).

Membrane transporters present in the CP (Supplementary tables 1 and 2) modulate the transport of substances across the BCSFB, influencing the availability of drugs in the brain, as well as the clearance of harmful compounds from brain tissue (Santos et al., 2019; Strazielle and Ghersi-Egea, 2015). These transport systems include members from the ATP-binding cassette (ABC) and Solute-Carrier superfamilies (SLC). The ABC family transporters are conserved in structure and hydrolyze ATP to efflux substances against their electrochemical gradient (primary active transport) (Figure 6) (Chen et al., 2016; Robey et al., 2018; Szöllősi et al., 2018; ter Beek et al., 2014). The main ABC transporters involved in the efflux of therapeutic drugs are ABCB1 (also known as P-glycoprotein (P-GP or multidrug resistance protein 1 (MDR1), the multidrug resistance-associated proteins (MRPs) of ABCC subfamily, and ABCG2, also known as Breast cancer resistance protein (BCRP). Contrarily to the ABC, SLC transporters are mainly involved in the influx of substances by either facilitated diffusion or secondary active transport (utilization of an ion gradient as a driving force) (Hediger et al., 2013; Yacovino and Aleksunes, 2012) and, the SLCO, SLC22, and SLC47 are the main families involved in the transportation of therapeutic drugs (Nigam, 2015).

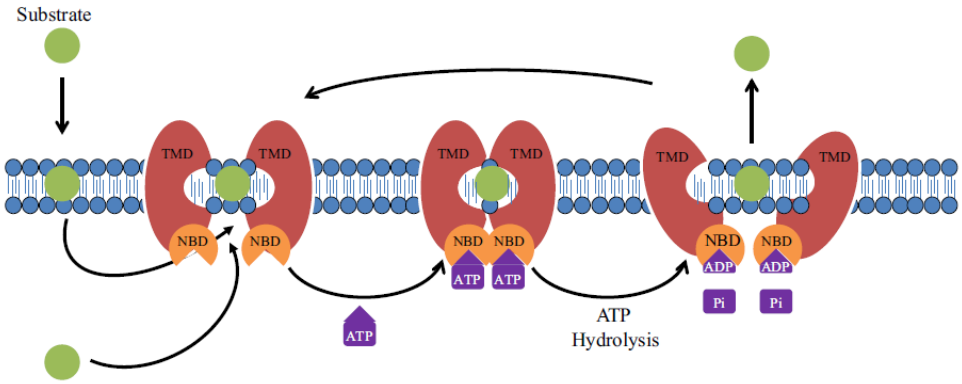

Figure 6. Structure and mechanism of ATP-binding cassette (ABC) exporters. ABC exporters are formed by two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). At the inward-facing conformation, the substrate binds to the TMDs from the cytoplasm or the inner leaflet of the lipidic bilayer. Two ATP molecules bind and induce the NBDs dimerization leading to an occluded state. The ATP is hydrolyzed, and the TMDs adopt an outward-facing conformational allowing the substrate release. Finally, ADP and Pi dissociate from the NBDs and the exporter return to the initial inward-facing conformation. Adapted from (Chen et al., 2016).

Particularly in CPEC, the most preponderant therapeutic drug transporters (supplementary tables 1 and 2) are ABCB1, ABCC1, ABCC4, and ABCG2 from ABC

superfamily, and organic anion transporters 1 and 3 (OAT1 and OAT3) from SLC22 family. ABCC1 and ABCC4 are identified in the basolateral membrane of CPEC, while, ABCB1, ABCG2, OAT1, and OAT3 are located on the apical side of the CPEC membrane (Gomez-Zepeda et al., 2019; Leggas et al., 2004; Nigam, 2015; Rao et al., 1999). Lastly, but not less important CPEC also express a variety of phase I and phase II detoxification enzymes, which are summarized by (Ghersi-Egea and Strazielle, 2001)

2.4. Choroid Plexus as a circadian oscillator

The first study to report a rhythmic event in CPs was conducted in the early '70s, where was demonstrated a slightly lower activity of carbonic anhydrase during the light phase in rat CPs of the fourth and lateral ventricles (Quay, 1972). Almost 50 years later, already with the molecular mechanism of mammalian circadian clock deciphered (Shearman et al., 2000), *Quintela et al.* showed that the CP express the canonical clock genes, and its circadian rhythmicity differed between sex. This study reported that Bmal1, Cry2, Per1, and Per2 are rhythmically expressed in CP of female rats, while, in the CP of male rats only Cry2 and Per2 expression showed daily oscillations (Quintela et al., 2015). However, a recent study showed circadian oscillations in the expression of Per1 and Bmal1 in the CP of male rats (Yamaguchi et al., 2020). These data indicate the existence of a functional molecular clock in CP. In addition to this initial characterization, it was demonstrated that the CP molecular clock is regulated by estrogens. The treatment of cultured CPEC with estradiol (E_2) leads to an upregulation of Bmal1, Per1, and Per2. However, for Per1 and Per2 this effect was attenuated in the presence of the estrogen antagonist fulvestrant (ICI 182,780), indicating that, in the CP, E2 modulates Per1 and Per2 expression through estrogen receptors (ER) activation. (Quintela et al., 2018a). Besides, *Myung et al.* found that the CP molecular clock is more robust than the SCN clock and reported that the communication between CPEC via gap junctions support fast and high amplitude rhythms in clock genes, suggesting that the gap junctions are responsible for the robustness of CP molecular clock (Myung et al., 2018).

Two studies tried to connect the molecular clock with CP functions. One study has characterized the CP as an alternative source of melatonin and showed that the Arylalkylamine N-acetyltransferase expression undergoes circadian variations in rat CP. In the same study, an *in vitro* assay with porcine CP explants showed that melatonin secretion did not follow a circadian rhythmic pattern (Quintela et al., 2018b). Moreover, it is well documented that the CSF is secreted in a circadian way

with peak production during the nighttime (Nilsson et al., 1992), but the expression of aquaporin 1, a water channel important for CSF formation, doesn't show circadian oscillations (Yamaguchi et al., 2020). So, taking this into account, and the fact that CP plays a diversity of functions (Ghersi-Egea et al., 2018; Santos et al., 2017) is necessary to understand the influence of the molecular clock on CP functions, and the outcomes of that influence in CNS therapeutics and disease states.

2.5. Choroid plexus as a sex hormone target

In the CP several sex hormones receptors were identified, namely nuclear (alpha and beta) and membrane-bound estrogen receptors (Hong-Goka and Chang, 2004; Quintela et al., 2016; Santos et al., 2017), androgen receptor (Alves et al., 2009), and the nuclear and membrane-bound progesterone receptors (Quadros et al., 2007) The presence of these receptors make the CP a sex hormone target, abling the sex steroids to modulate the CP functions and physiology. A microarray study performed by *Quintela et al.* with sham-operated and gonadectomized female and male rats revealed that 25% and 15% of rat's transcriptome is modulated by ovarian hormones and androgens, respectively (Quintela et al., 2013).

Among the different pathways involved in the CP functions, some components of the BCSFB and the molecular clock were shown to be affected by the SH background (Quintela et al., 2013; Quintela et al., 2016; Santos et al., 2017). Between the three major claudins expressed in the BCSFB, only claudin 1 expression showed a down-regulation induced by ovariectomy. In claudins less expressed in the CP, claudin 5 and claudin 7, were differentially expressed in the CP of male and female rats, in which claudin 5 was up-regulated, and claudin 7 was down-regulated in the CP of male rats in comparison with the CP of female rats (Quintela et al., 2016; Santos et al., 2017). In addition, claudin 12 was down-regulated in the CP of orchidectomized (OOX) rats (Quintela et al., 2013; Santos et al., 2017).

In detoxification enzymes, cytochrome P450 (CYP450) 2a2 was differently expressed in the CP of male and female rats with an up-regulation in the CP of male rats (Quintela et al., 2016; Santos et al., 2017). CYP450 2a2 also showed an up-regulation in ovariectomized (OVX) rats when compared with sham-operated female rats. CYP450 11b3 showed an up-regulation in OOX rats when compared to sham-operated male rats, and also was up-regulated upon ovariectomy. The phase II metabolizing enzyme UDP-glucuronosyltransferase 1A6 was up-regulated in OOX rats in relation to sham-

operated male rats (Quintela et al., 2013; Santos et al., 2017). Regarding membrane transporters, Abcc1 and Oat3 are differentially expressed in the CP of male and female rats, where Abcc1 was up-regulated and Oat3 was down-regulated in the CP of male rats in comparison with the CP of female rats. Abcc1 was also down-regulated in OOX rats when compared with sham male rats. An *in vitro* study reported that the treatment of rabbit CP explants with E2 plus progesterone (P4) decreased the uptake of choline by the CP (Lindvall-Axelsson and Owman, 1989, 1990), and this may be due to the regulation of membrane transporters in the CP by the SH.

Besides the regulation of Per1, Per2, and Bmal1 by E2 in the CP molecular clock (Quintela et al., 2018a), there is additional evidence that SH background affects the clock genes in CP. Bmal1, Per2, and Per3 shown to be differentially expressed in the CP of male and female rats. Bmal1 showed to be down-regulated, while Per2 and Per3 were up-regulated in the CP of male rats in comparison with the CP of female rats (Quintela et al., 2016; Quintela et al., 2015; Santos et al., 2017). Furthermore, in OVX rats, it was observed a Per2 and Per3 up-regulation and a Bmal1 down-regulation when compared with sham-operated female rats. Notwithstanding, in OOX rats comparing with sham-operated male rats, Per2 and Per3 showed to be down-regulated and Bmal1 up-regulated (Quintela et al., 2013; Santos et al., 2017). In addition to the core clock genes, Dbp was up-regulated in the CP of male rats when compared with the CP of female rats and was up-regulated upon ovariectomy, and down-regulated upon orchidectomy (Quintela et al., 2013; Quintela et al., 2016; Santos et al., 2017).

Chapter II – Aim

Sex hormones regulate several biological functions of the CP (Santos et al., 2017). Recently, a functional molecular clock modulated by E2 was identified in CP (Quintela et al., 2018a; Quintela et al., 2015) and, given the importance of that discovery, it became imperative to understand the impact of circadian rhythms in CP functions. BCSFB is formed by the CPEC, that through membrane transporters, receptors, and detoxification enzymes regulate the trafficking of substances, including therapeutic drugs, in and out of the CNS (Ghersi-Egea et al., 2018). Thus, understanding the impact of SH and circadian rhythms in that pathways would be important for the development of novel therapeutic strategies for drug delivery to the brain. For that purpose, the following objectives were designed:

- Evaluate the effect of sex differences in the circadian expression of Abca1, Abcc1, Abcc4, Abcg4, and Oat3 in the CP.
- Analyze the influence of SH in Abca1, Abcc1, Abcc4, Abcg4, and Oat3 circadian expression in the CP.
- Investigate whether methotrexate (MTX) is transported in a circadian way across the BCSFB, and evaluate the involvement of the efflux transporter ABCC4 in the circadian MTX transport across the BCSFB.

Chapter III – Materials and methods

3.1. Animals and cell lines

In the present study proestrus female and male Wistar rats at the age of 8-10 weeks old were used. Wistar rats were divided in four experimental groups: intact females (n=24), intact males (n=24), OVX (n=24), and sham-operated female rats (n=24). All animals were housed with standard laboratory chow and water *ad libitum* and were maintained under constant temperature and 12 hours light (07:00 h-19:00 h)/dark (19:00 h-07:00 h) cycles. Zeitgeber time (ZT) – 0 was defined as lights on and ZT12 as lights off. Intact female and male rats were euthanized under a ketamine/xylazine anesthetic mixture. OVX and sham-operated female rats were operated under the administration of a ketamine/medetomidine solution, and two weeks after surgery rats were euthanized. The CPs from lateral ventricles were collected at four different time points (ZT1, ZT7, ZT13, and ZT19) and immediately frozen in liquid nitrogen.

All animal procedures followed the NIH guidelines and the European rules for care and handling laboratory animals (Directive, 2010/63/EU).

The human epithelial CP 228 papilloma (HIBCPP) cell line derived from a human malignant CP papilloma and was kindly offered by C Schwerk. This cell line was originally obtained by the culture of cells from a surgically removed CP papilloma of a 29 years old woman (Ishiwata et al., 2005). These cells preserve the polygonal morphology and the phenotype of the CPEC. The HIBCPP cells grow in a continuous monolayer with some papillary structures and form a functional epithelial barrier with high transepithelial electric resistance (TEER) values (Bernd et al., 2015; Ishiwata et al., 2005; Schwerk et al., 2012).

3.2. HIBCPP cell culture

HIBCPP cells were seeded in 24-well plates with Dulbecco's modified eagle medium (DMEM) /F12 (Gibco) supplemented with 10 % Fetal Bovine Serum (FBS) (Life Technologies), 1% penicillin/streptomycin (MP Biomedicals), and with 5 μg/mL insulin (Sigma-Aldrich, Portugal). The cells were kept in a humidified incubator at 37 °C and 5% CO_2. One day after seeding, the medium was changed, and from here, the medium was replaced every two days. After getting 70% confluent, the cells were synchronized with 1% of dexamethasone for 2 h at 37 °C and 5% CO_2. After the synchronization, the medium was changed, and the cells were harvested for total RNA extraction described in section 3.3) at the following time points: 04:00 h, 08:00 h, 12:00 h, 16:00 h, 20:00 h, and 24:00 h after synchronization. To validate the synchronization of the HIBCPP cells the circadian expression of Bmal1 was confirmed by real-time quantitative PCR (described in section 3.5).

3.3. Total RNA extraction

Total RNA was isolated from the CPs and HIBCPP cells using TRIzol (Grisp, Portugal). For the CP samples, TRIzol was added to the microtubes and the samples were homogenized with a mini pestle. In the HIBCPP cells, TRIzol was added to each cultured well followed by an up and down homogenization and then, the samples were transferred into sterile microtubes. The samples were incubated for 5 min at room temperature. Chloroform was added in a proportion of 1:5 (chloroform:TRIzol), and the samples were mixed by inversion and incubated for 15 min at room temperature. After the incubation, the samples were centrifuged for 15 min at 4 °C and 12 000 g, and the upper aqueous phase was collected to a new microtube. In the next step, isopropanol was added in a proportion of 1:2 (isopropanol:TRIzol), and the mixtures were homogenized by inversion. A 10 min incubation at room temperature was performed followed by a 10 min centrifugation at 4 °C and 12 000 g. The supernatant was rejected, and the pellet was washed with 75% ethanol in DPEC water and a 5 min centrifugation at 4 °C and 7500 g was performed. Then, the supernatant was rejected and the excess of ethanol was removed. Total RNA was rehydrated in DEPC water and stored at -80 °C. Total RNA quantification and purity were accessed by the values of the absorvance at 260 nm and the ratio of the absorvances at 260 and 280 nm (260/280), respectively, which were determined using a NanoPhotometer™ (Implen, Germany). The integrity of total RNA was validated by an agarose gel electrophoresis.

3.4. cDNA synthesis

For cDNA synthesis, a reverse transcription was performed using an M-MLV reverse transcriptase (NZYTech, Portugal). Each reaction was made in a total of 20 µL. First, a reaction mixture with 1 µL of dNTPs, 2 µL of Random hexamers, and 14µL of RNA and sterile water together, in specific volumes to have 500 ng of RNA per reaction, undergo a denaturation step for 5 min at 65 °C. Then, 2 µL of enzyme buffer and 1 µL of M-MLV reverse transcriptase were added to the reaction mixture and, the reverse transcription was performed by 10 min at 25 °C followed by 50 min at 37 °C and 15 min at 70 °C, and finally, the newly synthesized cDNA was stored at -20 °C.

3.5. Real-time quantitative RT-PCR

Real-time quantitative reverse transcription PCR (RT-qPCR) was used to quantify the mRNA expression of rAbca1, rAbcc1, rAbcc4, rAbcg4, rOat3, hAbcc4, the two reference genes: rat *Cyclophilin A* (CycA) and human *glyceraldehyde 3-phosphate dehydrogenase* (GADPH) and hBMAL1. RT-qPCR were performed on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using an Xpert Fast SYBR 2X mastermix (Grisp, Portugal). Each reaction was made in a total of 10 µL with 1 µL of cDNA, 5 µL of SYBR Green, 0.4 µL of each primer (forward and reverse) diluted in 1:10, and 3.2 µL of sterile water. qPCRs were carried out with an initial denaturation step at 95 °C for 3 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 10 s. The amplification of all transcripts was validated by the profiles of melting curves where, as expected, each curve showed only a single peak while, the curves of the non-template controls did not show any peak. The relative expression of selected genes was calculated according to ΔΔct method (Pfaffl, 2001). The efficiency of all primers was previously tested with the following cDNA dilutions (1; 1:2; 1:4; 1:8), and its sequences and optimized conditions were listed in Table 1.

Table 1. Real-time quantitative PCR conditions

Gene	Primer sequence (5'-3')	Amplicon size (bp)	Annealing temperature (ºC)	Cycles
hABCC4	FW: TGTGGCTTTGAACACAGCGTA RV: CCAGCACACTGAACGTGATAA	105	60	40
hBMAL1	FW: GACAAAGATGACCCTCATG RV: CATGTTGGTACCAAAGAAG	149	60	40
hGADPH	FW: ATGGGGAAGGTGAAGGTCG RV: GGGGTCATTGATGGCAACAATA	108	60	40
rAbca1	FW: CGGCGGAGTAGAAAGGGTTT RV: CACGATCAGGCTGAAGACCAG	84	60	40
rAbcc1	FW: TTCATATCTGCTTCGTCACCG RV: CGTAAACAGCACCCACCACAGC	60	60	40
rAbcc4	FW: TTCCCCTTCGACCTTATCCT RV: TAGGCAGCTGTTGTCAGTGG	124	60	40
rAbcg4	FW: ATGGCTGATGTACCCTTCCAGGTT RV: ATCAAGAGTCCCAAAGACTGGGCA	155	60	40
rOat3	FW: GAGGACCTGTGATTGGAGAACTG RV: CTG GCT GCC AGC ATG AGA TA	82	60	40
rCycA	FW: CAAGACTGAGTGGCTGGATGG RV: GCCCGCAAGTCAAAGAAATTAGAG	163	60	40

3.6. MTX uptake assay

To accesses the putative ABCC4 circadian function in the BCSFB, MTX was used as a specific subsract of this efflux transporter, in *transwell* assays using HIBCPP cells. The HIBCPP cells were seeded in the apical compartment of culture inserts (pore diameter 0.4 μm and insert area 0.33 cm^2; VWR, Portugal) in a density of 1.5 x 10^5 cells/insert in DMEM/F12 supplemented with 10% FBS, 5 μM/mL insulin, and 1% penicillin/streptomicin. Culture medium was added to the basal compartment only two days after seeding and, from that day, the medium was changed every two days. The paracellular permeability was accessed every day by the measurement of TEER values using an Epithelial Volt/Ohm Meter (WPI, Florida, U.S.A.). On the fourth day of culture, TEER values reached the 300 Ω.cm^2 and then, the medium was changed and maintained from that day on with 1% FBS. On the seventh day of culture, the cells were synchronized with 1% dexamethasone, for 2 h at 37 °C and 5% CO_2. To verify the rhythmicity of the MTX transport across BCSFB, after the synchronization and before

the incubation with the substrate, the cells were washed three times and were preincubated with Krebs buffer (KRB). Next, the cells were incubated with fluorescein-MTX (FL-MTX, Biotium, USA), at six different time points, for 3 h at 37 °C and 5% CO_2. After the incubation with the substrate, the apical and basal mediums were collected and pipetted into a black plate. The remaining cells were washed three times with KRB and were lysed by the incubation with a Triton X-100 1% solution for 30 min at 37 °C. After the incubation, the lysis solution was homogenized and pipetted into the black plate. Lastly, the FL-MTX concentration was determined by fluorescence reading using a SpectraMax Gemini spectrofluorometer (Molecular Devices) at the excitation wavelength of 490 nm and the emission wavelength of 520 nm. Finally, to confirm the involvement of ABCC4 and ABCG2 in the circadian rhythmicity of MTX transport across the BCSFB, two additional assays were performed but at this time, before the FL-MTX incubation the cells were preincubated with the inhibitors of ABCC4 (applied in the basal compartment) and ABCG2 (applied in the basal and apical compartments), ceefourin 1 (5 μM; Tocris, UK) or Ko143 (100 nm; Tobu-bio, UK), respectively.

3.7. Statistical analysis

The rhythmicity in the mRNA expression of CP transporters and, in the FL-MTX concentration in the three compartments (apical, basal, and intracellular) were analyzed by a harmonic regression method, with an assumed period of 24 h and with alpha set at 0.05, using the CircWave v1.4 software (Dr. Roelof A. Hut). Statistically significant rhythms were considered when $p<0.05$. Points and full bars represent the mean, and error bars represent the standard error of the mean (± SEM).

Chapter IV – Results

4.1. Sex-dependent mRNA circadian oscillations of membrane transporters in rat choroid plexus

To analyze whether the circadian expression of membrane transporters in the CP is sex-dependent, the temporal expression profile of several transporters of male and female rats was accessed by RT-qPCR. The circadian mRNA expression profiles were obtained for Abca1, Abcc1, Abcc4, Abcg4, and Oat3. Abca1 and Abcc1 expression was not rhythmic either in the CP of male and female rats (Figure 7a). Abcc4 expression in female rats showed a statistically significant rhythm (CircWave, $p<0.05$), with a maximum of expression, during the dark phase, between ZT14 and ZT15 (Figure 7b). Abcg4 mRNA 24 h variation in male rats was statistically rhythmic (CircWave, $p <0.05$), showing a peak around ZT2, during the light phase (Figure 7c). Oat3 mRNA levels in female rats were also statistically rhythmic (CircWave, $p<0.05$), where it's observed a peak of expression during the first half of the dark phase around ZT17 (Figure 7d).

4.2. Effects of ovariectomy in the mRNA circadian oscillations of membrane transporters in rat choroid plexus

As there are differences between sexes in the circadian expression of membrane transporters in rat CP it was hypothesized if SH are the origin of those differences. So, the effect of ovarian hormones in the rhythmicity of membrane transporters was analyzed in the CP, by comparing the temporal expression profiles of genes encoding membrane transporters between sham-operated and OVX female rats. Oat3 mRNA levels showed significant rhythmicity in sham-operated animals (CircWave, $p<0.05$), with an expression peak around ZT15 (Figure 7d). Oat3 diary expression in OVX rats and the other genes studied in both groups did not show significant circadian rhythms (Figure 7).

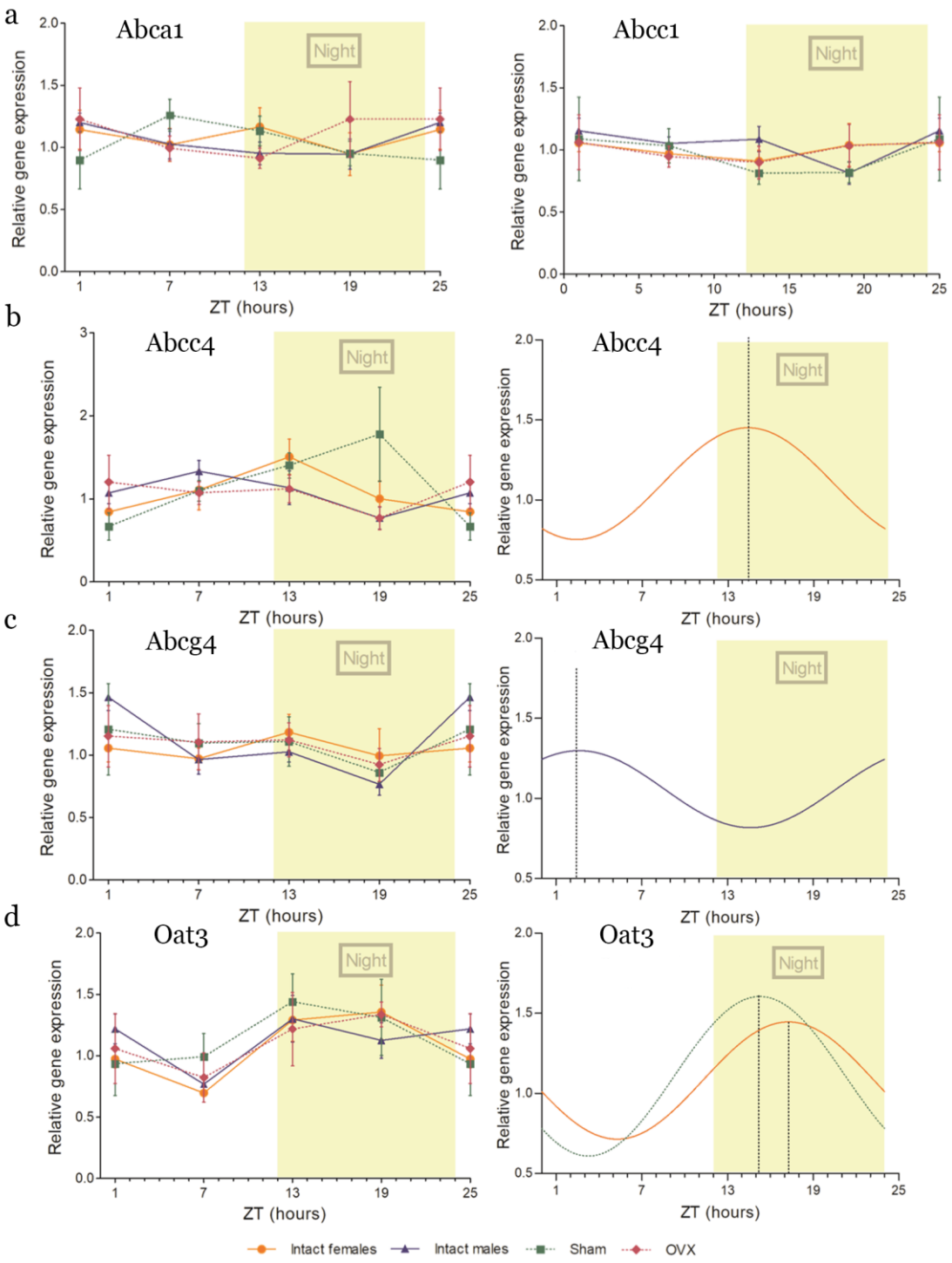

Figure 7. Circadian transcription profiles of membrane transporters in the CP of intact males, intact females, OVX, and sham-operated female rats. Abca1 (a), Abcc1 (a), Abcc4 (b) Abcg4 (c), and Oat3 (d) mRNA circadian expression were analyzed. White and yellow backgrounds represent the day and night periods, respectively. The left side and the first graph from the right side of the panel shows the mean ± SEM transcript levels (n=3-5), and data from ZT1 and ZT25 are double plotted. The CircWave analysis is represented by the three last graphs from the right side of the panel. Dotted lines indicate the Center of Gravity (COG). Absence of the CircWave curve indicates absence of significant rhythmicity as analyzed by CircWave.

4.3. Circadian oscillations in MTX transport across the BSCFB

An *in vitro* transport assay using the HIBCPP cell line was performed to evaluate if the MTX is transported in a circadian way across the BCSFB. According to the results shown in figure 8, FL-MTX concentration in the basal compartment showed a significant statistically rhythm (CircWave, $p<0.05$) with a peak at 21 hours after synchronization (Figure 8a). In the apical compartment is also observed a statistically significant rhythm (CircWave, $p<0.05$), with a well-sustained peak around 3 hours after synchronization, and a less pronounced peak at 16 hours after synchronization (Figure 8b). In the intracellular compartment, FL-MTX concentration is also statistically rhythmic (CircWave, $p<0.05$) showing 2 peaks, a more pronounced one at 7 hours, and a second one at 19 hours after the synchronization (Figure 8c). With these results, we can infer that FL-MTX is transported across the apical and the basal membranes in a circadian way.

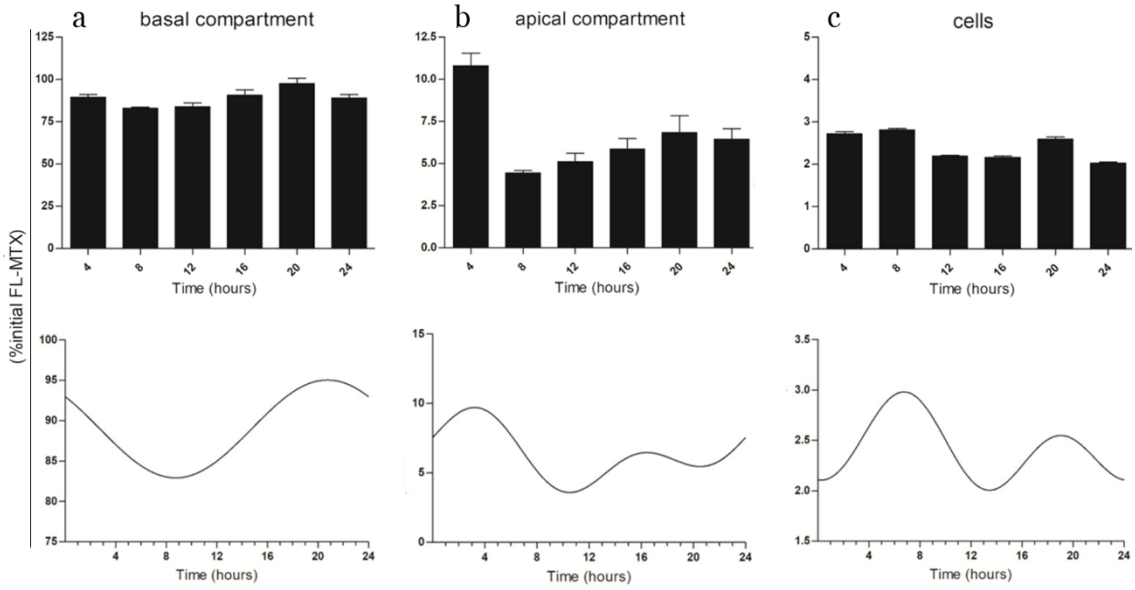

Figure 8. MTX transport across the BCSFB. (top) FL-MTX levels from basal (a), apical (b), and intracellular (c) compartments at the following time points: 4h, 8h, 12h, 16h, 20h, 24h, after synchronization. Error bars indicate mean ± SEM (n=4). (Bottom) CircWave analysis of the levels of FL-MTX in the three compartments. The represented curves indicate a statistically significant rhythm (CircWave, $p<0.05$).

As Abcc4 showed circadian oscillations in intact female rats, and the fact that MTX is a known substrate for Abcc4, we examine whether this membrane transporter is responsible for the MTX circadian transport across the BSCFB. For that, first, we accessed the temporal expression of Abcc4 mRNA in the HIBCCP cells and, the results showed a significant rhythm (CircWave, $p<0.05$) with a peak at 8 hours after synchronization (Figure 9).

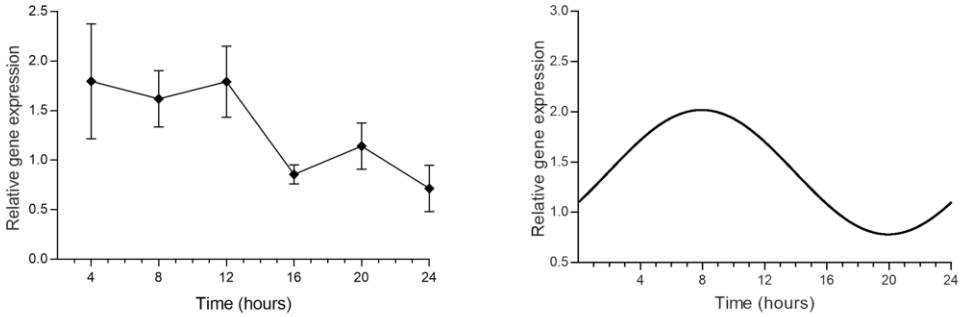

Figure 9. Circadian profile of Abcc4 in the HIBCPP cells**.** (left) Abcc4 24 hours expression profile. Data points indicate the mean ± SEM (n=5-6) (right) CircWave analysis of ABCC4 expression profile. The represented curve indicates a statistically significant rhythm (CircWave, $p<0.05$).

To evaluate the involvement of ABCC4 in the circadian transport of MTX across the BCSFB, the *in vitro* assay was repeated with a selective ABCC4 inhibitor. In the basal compartment no statistically significant rhythm was detected (Figure 10a). In the apical compartment, FL-MTX concentration showed a statistically significant rhythm (CircWave, $p<0.05$), with a peak around 21 hours after synchronization (Figure 10b). At the intracellular compartment, a statistically significant rhythm in the FL-MTX concentration was also observed (CircWave, $p<0.05$), with a major peak at 22 hours, and a less pronounced peak between 9 and 10 hours after synchronization (Figure 10c). These results indicate that ABCC4 participates in the FL-MTX circadian transport across the BCSFB.

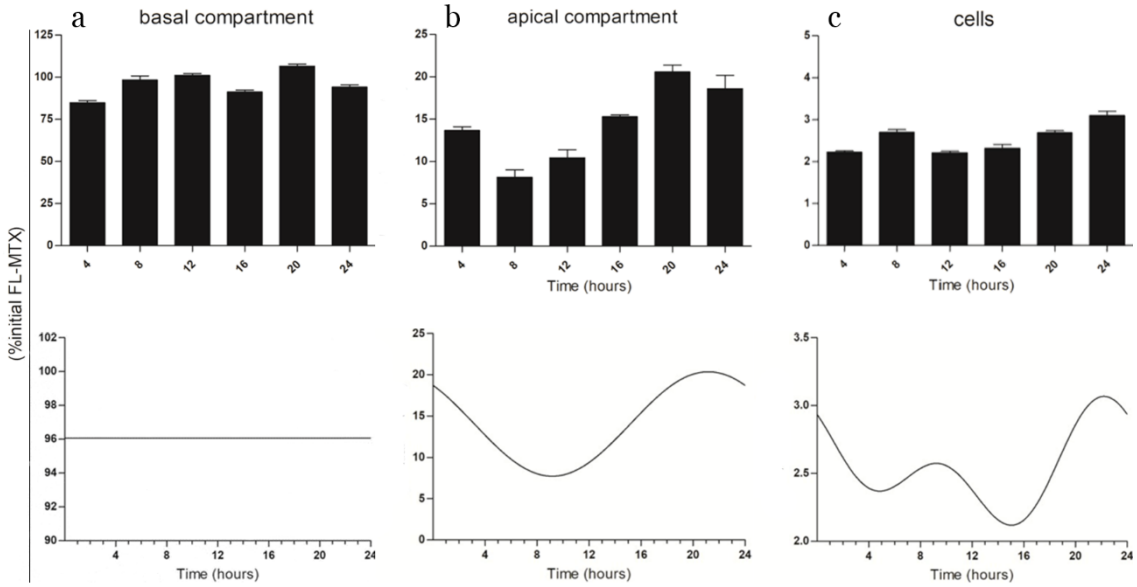

Figure 10. Effects of ABCC4 inhibition in the MTX circadian transport across the BCSFB. (top) FL-MTX levels from basal (a), apical (b), and intracellular (c) compartments after the inhibition of ABCC4 at the following time points: 4h, 8h, 12h, 16h, 20h, 24h, after synchronization. Error bars indicate mean ± SEM (n=3-4). (Bottom) CircWave analysis of the levels of FL-MTX in the three compartments. The represented curves indicate a statistically significant rhythm (CircWave, $p<0.05$).

To verify if the ABCC4 is the only transporter responsible for the circadian rhythm in MTX transport across the basal membrane, a third assay with an inhibitor of ABCG2 was performed. In the basal compartment, like in the previous assay, no statistically significant rhythm in the FL-MTX concentration was reported (Figure 11a). In the apical compartment, the FL-MTX concentration showed a significant rhythm (CircWave, $p<0.05$), where a well-sustained peak at 20 hours after synchronization can be observed (Figure 11b). Finally, in the intracellular compartment, the FL-MTX concentration also showed a statistically significant rhythm (CircWave, $p<0.05$), where a major peak can be observed between 21 and 22 hours after synchronization. (Figure 11c).

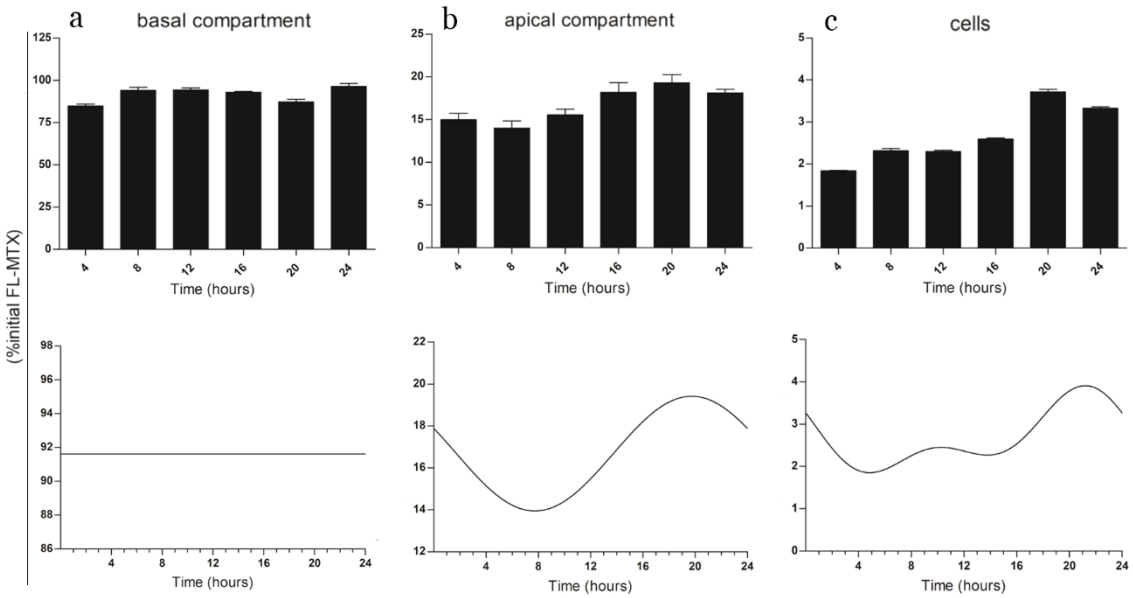

Figure 11. Effects of ABCG2 inhibition in the MTX circadian transport across the BCSFB. (top) FL-MTX levels from basal (a), apical (b), and intracellular (c) compartments after the inhibition of ABCG2 at the following time points: 4h, 8h, 12h, 16h, 20h, 24h after synchronization. Error bars indicate mean ± SEM (n=3-4). (Bottom) CircWave analysis of the levels of FL-MTX in the three compartments. The represented curves indicate a statistically significant rhythm (CircWave, $p<0.05$).

Chapter V – Discussion

Biological rhythms affect almost all cellular functions; therefore, the circadian timing clock can affect the pharmacokinetics and the efficacy of many drugs. The processes of absorption, distribution, metabolism, and excretion show circadian variations and, can be partially explained by circadian rhythms in efflux and influx transporters (Ballesta et al., 2017; Gaspar et al., 2019; Sulli et al., 2018). To the further development of chronotherapeutic strategies (administration of a therapeutic drug according to biological rhythms, to improve its efficacy and/or ameliorate its adverse effects), first, we need to fully understand the molecular mechanisms behind the rhythmicity in pharmacokinetics and pharmacodynamics, in order to achieve an optimal timing for the administration of a therapeutic drug (Ballesta et al., 2017). Cyclic daily variations in one gene arise from the molecular clock and/or from multiple stimuli that undergo circadian variations. Hormones are one of that stimuli and, as documented by *Vagnerová et al.* can affect the rhythmicity of a gene in three ways: (i) affecting the expression of genes involved in the TTFLs, (ii) influencing clock controlled nuclear receptors and transcription factors, and (iii) by direct modulation of a gene expression (Vagnerová et al., 2019).

The present study showed that the Abca1 and Abcc1 expression is not rhythmic in the CP. Contrarily, in the liver of male rats, Abca1 is rhythmic with a peak in the dark/light transition and a nadir in the light/dark transition (Chen et al., 2018). In the Caco-2 cell line a circadian rhythm was reported in the expression of Abcc1, with a peak between the 6 and the 12 hours after synchronization (Ballesta et al., 2011). However, another study reported that Abcc1 expression in the jejunal mucosa of male rats was not rhythmic (Stearns et al., 2008), according to our results. On the other hand, we demonstrated circadian rhythmicity in the expression of Abcc4 and Oat3 in the CP of intact female rats and Abcg4 in the CP of intact male rats. The evidence linking circadian rhythms and membrane transporters is still scarce. A microarray study showed reduced Abcc4 expression in the kidney of proline and acidic amino acid-rich basic leucine zipper (PARbZip) transcription factors knockout mice, suggesting that Abcc4 is a clock controlled gene (Gachon et al., 2006). Possibly, like Abcb1 and Abcb4 (Kotaka et al., 2008; Murakami et al., 2008), if the Abcc4 gene possess a D-box response element, its expression may be directly regulated by the PARbZip transcription factors. Abcc4 is also regulated by the xenobiotic receptors (Constitutive

androstane receptor (CAR); Aryl hydrocarbon receptor (AhR); Pregnane X receptor (PXR)) (Assem et al., 2004; Renga et al., 2011; Whyte-Allman et al., 2017; Xu et al., 2010), known to have a circadian expression in the liver (Zhang et al., 2009). Besides, some studies report that CAR and AhR genes are clock controlled (Gachon et al., 2006; Richardson et al., 1998). Therefore, there's a possibility that molecular clock directly or indirectly, through xenobiotic receptors, regulate the circadian expression of Abcc4, however, there's still no evidence of xenobiotic receptors expression in CP. CP functions are regulated by SH, including the molecular clock pathway (Santos et al., 2017). Clock genes in CP are differentially expressed between sexes, and E2 modulates the expression of Per1, Per2, and Bmal1 (Quintela et al., 2018a; Quintela et al., 2015; Santos et al., 2017). The regulation of Abcc4 by estrogens is not well established. A study with porcine endometrium explants showed that neither E2 or P4 can modulate Abcc4 expression (Seo et al., 2014). *Mayer et al.* corroborate these data where no differential results were shown between control, OVX, and OVX replaced with E2 mice (Maher et al., 2006). However, there is a study reporting the involvement of ERs in the expression of Abcc4, showing that the activation of ERs increases the Abcc4 expression (Koraïchi et al., 2013). Contrarily to E2, dihydrotestosterone (DHT) is shown to upregulate Abcc4 in the LNCaP cell line (Cai et al., 2007; Ho et al., 2008). Therefore, these differential effects of SH in the Abcc4 expression may support the rhythmicity verified only in female rats. However, in the present study Abcc4 expression does not show rhythmicity in sham and OVX female rats. These may be due to the use of ketamine since sham and OVX female rats were operated under the administration of a ketamine/xylazine solution and only two weeks later were euthanized, so, these rats, contrarily to the intact females, where ketamine was used only for euthanasia, may suffered secondary effects from the anesthetics and, it is already shown that ketamine can influence over the circadian rhythms. In locomotor activity rhythms, when administered at the resting phase, ketamine causes a phase advance, while, when administered during the active phase, causes a phase delay (Mihara et al., 2012; Orts-Sebastian et al., 2019). At the molecular level ketamine effects were already described. Ketamine induce a phase shift in Bmal1 and Dbp expression (Bellet et al., 2011; Orts-Sebastian et al., 2019), which can be a possible pathway for the observed effects in Abcc4 circadian mRNA fluctuations in sham animals since its expression looks to be controlled by the PARbZip transcription factors (Gachon et al., 2006). Thus, we cannot conclude that Abcc4 rhythmicity in females is dependent on ovarian hormones. A study with Bmal1 KO rats reported a decreased expression of Oat3 in the kidney when compared with control rats, suggesting that Oat3 is a clock-controlled gene (Nikolaeva et al., 2016). Oat3 showed rhythmicity in the CP of female and sham-operated rats, but

not on OVX animals thus, the rhythmicity in Oat3 expression is dependent on ovarian hormones. These results also confirmed that not only the molecular clock is responsible for the rhythmicity of Oat3 in rats. E2 may be responsible or, at least, partially responsible for the rhythmicity in female rats since it can entrain the molecular clock in CP (Quintela et al., 2018a), and there's some evidence reporting that E2 can modulate Oat3 expression in rats (Kudo et al., 2002; Ljubojevic et al., 2004). Abcg4 is a poorly characterized ABC exporter. In the four studied experimental groups, Abcg4 showed rhythmicity only in the CP of male rats, which lead to the hypothesis that androgens are responsible for that rhythmicity.

In terms of transport, MTX is a very unspecific substrate. CP express several membrane proteins capable of transport MTX. ABCB1, ABCC1, ABCC2, ABCC3, ABCC4, ABCC10, ABCG2, OAT1, OAT3, organic anion transporter polypeptide (OATP) 1C1, proton-coupled folate transporter (PCFT), peptide transporter (PEPT) 1, and reduced folate carrier (RFC) are all capable of transporting MTX (Assaraf, 2006; Chen et al., 2003; Inoue and Yuasa, 2014; Lima et al., 2014; Norris et al., 1996; Pizzagalli et al., 2002; Tamai et al., 1999) and are expressed in the CP (Hinken et al., 2011; Morris et al., 2017; Stieger and Gao, 2015; Wollack et al., 2008; Zhao et al., 2009). Its known that ABCC1, ABCC4, and PCFT are expressed in the basolateral membrane of CPEC (Gazzin et al., 2008; Leggas et al., 2004; Zhao et al., 2009), SLCO1C1 in the basolateral and in the apical membrane (Roberts et al., 2008b), and ABCB1, ABCG2, and RFC are located in the apical membrane (Gazzin et al., 2008; Hinken et al., 2011; Tachikawa et al., 2005). Folate receptor α, which mediates the transcytosis of MTX, is also located in the apical membrane of CPEC (Grapp et al., 2013). Using a human BCSFB *in vitro* model with HIBCPP cells, we performed an uptake assay and observed daily oscillations in the FL-MTX concentrations in the three compartments (basolateral, apical, and intracellular), demonstrating that MTX is transported across the basal and the apical membranes of CPEC in a circadian way. To analyze the involvement of ABCC4 in the rhythmic transport of MTX, first, we reported a circadian rhythmicity with a peak at the 8 hours after synchronization in its mRNA levels in the HIBCPP cells. Together with the previous results of the Abcc4 circadian expression in intact females, it was the first time that circadian rhythmicity in the Abcc4 expression was reported. Circadian rhythmicity in mRNA expression does not mean rhythmicity in protein expression either in protein function (Zhang et al., 2018). So, we performed an uptake assay with the inhibition of ABCC4 where the rhythmicity of FL-MTX transport across the basal membrane is loose, while across the apical membrane is conserved. These results indicate that probably ABCC4 is the responsible for the MTX circadian transportation across the

basal membrane, and also indicate a probably relationship between its expression and function with a delay of 13 hours, since the expression peak of Abcc4 was at the 8 hours after synchronization, and the peak of FL-MTX concentration in the basal compartment in the assay without the inhibitor was around 21 hours after synchronization. So, these 13 hours possibly correspond to the time ranging from RNA processing to its translation, protein maturation and migration to the plasma membrane. Moreover, to verify if the ABCC4 is the only transporter responsible for the circadian rhythm in MTX transport across the basal membrane, we performed another uptake assay, but, at this time, with the inhibition of ABCG2. When ABCG2 was inhibited we also observed a loss of rhythmicity in the FL-MTX concentration in the basal compartment. However, the FL-MTX concentration in the apical and in the intracellular compartments remained rhythmic upon the inhibition of ABCG2. So, our results indicate that the rhythmicity of the FL-MTX transport across the basal membrane is not exclusively driven by ABCC4 since the inhibition of ABCG2, an apically located transporter, also impaired the rhythmicity of FL-MTX transport across the basal membrane.

Conclusions & future perspectives

In sum, we demonstrate that Abcc4, Oat3, and Abcg4 have a circadian expression in CP, which is strongly influenced by the SH. Therefore, these data highlight the idea that SH modulate the transport of substances across the BCSFB. We also demonstrate that MTX is transported in a circadian way across the BCSFB and, ABCC4 is partially responsible for the transport rhythmicity across the basal membrane.

Our results can contribute to elucidate the molecular mechanism of the circadian rhythmicity in the pharmacokinetics and pharmacodynamics of several drugs. For a better characterization of the impact of circadian rhythms in CNS drug availability, these studies must also be performed in the BBB, since it is the major pathway for drug delivery into the brain (Strazielle and Ghersi-Egea, 2016). Biological rhythms are affected by many intraindividual and interindividual factors like disease states, sex, age, stress, and others (Chang and Kim, 2019; Hood and Amir, 2017; Koch et al., 2017; Nicolaides and Chrousos, 2020). Thus, after understanding the molecular mechanisms, the influence of those factors in the rhythmicity of drug efficacy must be accessed. In the next phase, the chronotherapeutic strategy must be designed and tested in animal models and, in the final stage must be optimized to be used in the human population. As previously said, biological rhythms are influenced by many factors thus, the chronotherapeutic strategies must be personalized for each individual. Overall, the influence of circadian rhythms in pharmacological processes is poorly understood, and for the final outcome of a personalized and effective chronotherapeutics, a lot of research in this field needs to be made (Ballesta et al., 2017; Sulli et al., 2018).

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Appendix

Supplementary table 1. ABC transporters in choroid plexus

ABC transporters in choroid plexus						
Family	**Transporters**	**mRNA expression**	**Protein expression**	**Localization**	**Function**	**References**
ABCA	ABCA1	m; r	h; r	-	Cholesterol efflux to poor lipidated ApoE in the brain, and ApoA1.	(Fujiyoshi et al., 2007; Matsumoto et al., 2015; Pereira et al., 2018; Tachikawa et al., 2005)
	ABCA4	r	r	-	Flipase for N-retynilidene-phosphatidylethanolamine	(Bhongsatiern et al., 2005; Mohammad et al., 2018; Tachikawa et al., 2005)
	ABCA8	m	h	-	Efflux of hydrophobic drugs	(Matsumoto et al., 2003; Mohammad et al., 2018; Uchida et al., 2015)
ABCB	ABCB1	h; p	d; h; p	A/SA (p; h)	Major player in drugs and xenobiotics efflux. Also involved in lipidic compunds efflux, and Ab-clearence in the brain.	(Baehr et al., 2006; Bernd et al., 2015; Niehof and Borlak, 2009; Rao et al., 1999; Zhang et al., 2017)
	Abcb1a	m; r	r	-	Major player in drug efflux. Also involved in lipidic compunds efflux, and Ab-clearence in the brain.	(Choudhuri et al., 2003; Rao et al., 1999; Reichel et al., 2011; Wang et al., 2016)
	Abcb1b	r	r	-	Major player in drug efflux. Also involved in lipidic compunds efflux, and Ab-clearence in the brain.	(Choudhuri et al., 2003; Kratzer et al., 2013; Rao et al., 1999; Wang et al., 2016)
	ABCB2	r	-	-	Peptide influx to endoplasmatic reticulum (intracelular transporter)	(Choudhuri et al., 2003; Mohammad et al., 2018)
	ABCB4	h	-	-	Efflux of drugs and phosphatadilcoline	(Mohammad et al., 2018; Niehof and Borlak, 2009)
ABCC	ABCC1	h; r	m; r; p; h	B (h; r; m; p)	Major player in drugs and xenobiotics efflux	(Baehr et al., 2006; Bernd et al., 2015; Gazzin et al., 2011; Koehn et al., 2019; Mohammad et al., 2018; Niehof and Borlak, 2009; Soontornmalai et al., 2006)
	ABCC2	h; m; r	h	-	Major player in drugs and xenobiotics efflux	(Bernd et al., 2015; Flores et al., 2017; Koehn et al., 2019; Mohammad et al., 2018; Niehof and Borlak, 2009)

(Continued)

Supplementary table 1. Continued

ABC transporters in choroid plexus						
Family	**Transporters**	**mRNA expression**	**Protein expression**	**Localization**	**Function**	**References**
	ABCC3	h; r	m; h; p	-	Major player in drugs and xenobiotics efflux	(Bernd et al., 2015; Koehn et al., 2019; Mohammad et al., 2018; Niehof and Borlak, 2009)
	ABCC4	h; m; r	d; h; p; r	B (h; m; r)	Major player in drugs and xenobiotics efflux	(Choudhuri et al., 2003; Flores et al., 2017; Leggas et al., 2004; Mohammad et al., 2018; Niehof and Borlak, 2009; Wang et al., 2016)
	ABCC5	r	r	-	Major player in drugs and xenobiotics efflux	(Choudhuri et al., 2003; Koehn et al., 2019; Mohammad et al., 2018; Reichel et al., 2011)
	ABCC6	r	-	-	Major player in drugs and xenobiotics efflux	(Mohammad et al., 2018; Niehof and Borlak, 2009)
	ABCC10	r	-	-	Major player in drugs and xenobiotics efflux	(Kratzer et al., 2013; Mohammad et al., 2018)
ABCG	ABCG1	r	r	-	Cholesterol efflux to HDL particles and sphingomielin and phospholipids transportation	(Fujiyoshi et al., 2007; Mohammad et al., 2018)
	ABCG2	m; r	m; h; p; r	A (r; m)	Major player in drugs and xenobiotics efflux, and flipase activity for phospholipids	(Halwachs et al., 2011; Koehn et al., 2019; Mohammad et al., 2018; Tachikawa et al., 2005; Verscheijden et al., 2020; Yasuda et al., 2013; Zhang et al., 2017)
	ABCG4	r	h	-	Cholesterol efflux to HDL particles; AB clearance in the brain	(Fujiyoshi et al., 2007; Matsumoto et al., 2015; Pereira et al., 2018; Wang et al., 2004a)
	ABCG5	r	-	-	Sitosterol and cholesterol transportation	(Choudhuri et al., 2003; Fujiyoshi et al., 2007; Mohammad et al., 2018)
	ABCG8	r	-	-	Sterolin transport	(Choudhuri et al., 2003; Mohammad et al., 2018)

d - dog; h - human; m - mouse; p - porcine; r - rat; A - Apical; B- Basolateral; SA - Subapical

A - Apical; B- Basolateral; SA - Subapical

Supplementary table 2. SLC transporters in choroid plexus

SLC transporters in choroid plexus						
Family	**SLC transporters**	**mRNA expression**	**Protein expression**	**Localization**	**Functions**	**References**
SLC1	SLC1A1/EAAT3	-	r	A (r)	Glutamate and aspartate active transport dependent on sodium, potassium, and proton gradients.	(Akanuma et al., 2015; Bjørn-Yoshimoto and Underhill, 2016)
SLC2	SLC2A1/GLUT1	b; r	b; h; p; m; mk; r; ra	B (h; m; mk; r; ra)	Glucose facilitated diffusion	(Castañeyra-Ruiz et al., 2016; Cornford et al., 1998; Dobrogowska and Vorbrodt, 1999; Dwyer and Pardridge, 1993; Kumagai et al., 1994; Pragallapati and Manyam, 2019; Saunders et al., 2015; Uchida et al., 2019)
	SLC2A3/GLUT3	-	h	-	Glucose facilitated diffusion	(Saunders et al., 2015; Simpson et al., 2008)
	SLC2A4/GLUT4	m	-	-	Glucose facilitated diffusion	(Richter and Hargreaves, 2013; Vannucci et al., 2000)
	SLC2A5/GLUT5	-	m	A (m)	Fructose facilitated diffusion	(Douard and Ferraris, 2008; Ueno et al., 2014)
	SLC2A8/GLUT8	-	h; m	C (h; m)	Intracellular hexose transporter	(Mueckler and Thorens, 2013; Murakami et al., 2016)
	SLC2A9/GLUT9	-	h	A (h)	Urate transportation	(Uemura et al., 2017; Wang et al., 2019)
SLC4	SLC4A1/AE1	r			Chloride, bicarbonate exchanger	(Inohana et al., 2018; Reithmeier et al., 2016)
	SLC4A2/AE2	r	h; r	B (h; m; r)	Chloride, bicarbonate exchanger	(Lindsey et al., 1990; Praetorius and Nielsen, 2006; Reithmeier et al., 2016)
	SLC4A4/NBCe1	h	-	-	Sodium, bicarbonate cotransporter	(Damkier et al., 2007; Kurtz and Zhu, 2013)
	SLC4A5/NBCe2	m; h	r	A (m; r)	Sodium, bicarbonate cotransporter	(Bouzinova et al., 2005; Hladky and Barrand, 2016; Praetorius et al., 2004)
	SLC4A7/NBCn1	h; r	h; m; r	B (h; m; r); A (h; m)	Sodium, bicarbonate cotransporter	(Damkier et al., 2006, 2007; Praetorius et al., 2004)
	SLC4A8/NDCBE	h	r	B (r)	Sodium, chloride-bicarbonate exchanger	(Christensen et al., 2013; Damkier et al., 2007; Hladky and Barrand, 2016)

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Supplementary table 2. Continued

SLC transporters in choroid plexus						
Family	**SLC transporters**	**mRNA expression**	**Protein expression**	**Localization**	**Functions**	**References**
	SLC4A10/NBCn2	h; m	h; m	B (h; m; r)	Sodium, bicarbonate cotransporter	(Bouzinova et al., 2005; Chen et al., 2008; Damkier et al., 2007; Damkier and Praetorius, 2012; Jacobs et al., 2008; Praetorius et al., 2004)
	SLC4A11/BTR1	h	-	-	Sodium, bicarbonate symporter	(Damkier et al., 2007; Hübner and Holthoff, 2013)
SLC5	SLC5A6/SMVT	-	p	-	Co-transport of sodium ions with some vitamins (biotin and pantothenic acid)	(Vadlapudi et al., 2012; Zhang et al., 2017)
	SLC5A11/SMIT1	-	r	A (r)	Sodium, myo-inositol antiporter	(Abbott et al., 2014; Neverisky and Abbott, 2015)
SLC6	SLC6A4/SERT	b	b	A (b)	Serotonin transport dependent on chloride, sodium, and potassium gradients	(De Felice, 2016; Pavone et al., 2007)
	SLC6A13/GAT2	-	r	-	y-aminobutyric acid transport dependent on chloride and sodium gradients	(Conti et al., 1999; Zhou and Danbolt, 2013)
	SLC6A15/B^{0}AT2	-	m	A (m)	Active transport of branched-chain amino acids and methionine using sodium gradient	(Hägglund et al., 2013; Takanaga et al., 2005a)
	SLC6A20/SIT1	m; r	-	-	Imino-acids transport accoupled with sodium gradient	(Dolgodilina et al., 2020; Takanaga et al., 2005b)
SLC7	SLC7A5/LAT1	m; r	r	B (r)	Transport of histidine, isoleucine, methionine, tryptophan, phenylalanine, leucine, cysteine, tyrosine, and glutamine. Also transports therapeutic drugs, including L-Dopa, and thyroid hormones	(Duelli et al., 2000; Müller and Heuer, 2014; Puris et al., 2020; Roberts et al., 2008a)
	SLC7A8/LAT2	m; r	m	A (m)	Amino acid exchanger	(del Amo et al., 2008; Dolgodilina et al., 2020; Müller and Heuer, 2014; Wittmann et al., 2015)
	SLC7A11/xCT	m	p; m	-	Cysteine and glutamate antiport	(Burdo et al., 2006; Saunders et al., 2015; Uchida et al., 2019)
	SLC7A10/ASC-1	r	-	-	Antiport of alanine, serine, cysteine, and glutamine	(Jiang et al., 2020; Kasai et al., 2011)

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Supplementary table 2. Continued

SLC transporters in choroid plexus						
Family	**SLC transporters**	**mRNA expression**	**Protein expression**	**Localization**	**Functions**	**References**
SLC9	SLC9A1/NHE1	h	h; r; m	A (h; m); B (m)	Sodium, proton exchanger	(Damkier et al., 2009; Kalaria et al., 1998; Sakae et al., 2008; Slepkov and Fliegel, 2002)
	SLC9A6/NHE6	m	m	A (m)	Sodium, proton exchanger	(Damkier et al., 2018)
SLC10	SLC10A1/NTCP	r	-	-	Co-transport of sodium and taurocholate. Also transport statins	(Choudhuri et al., 2003; Stieger, 2011)
SLC11	SLC11A2/DMT1	r	r; h	C (h; r)	Divalent metal ions transport accoupled with proton gradient	(Siddappa et al., 2003; Wang et al., 2006; Wang et al., 2008; Zheng et al., 2014)
SLC12	SLC12A2/NKCC1	m; r	h; m; r	A (m; h)	Sodium, potassium, and chloride co-transport	(Damkier et al., 2006; Kim and Jung, 2012; Piechotta et al., 2002; Steffensen et al., 2018)
	SLC12A4/KCC1	r	-	-	Potassium and chloride co-transport	(Hladky and Barrand, 2016; Kanaka et al., 2001)
	SLC12A6/KCC3	r	m	B (m)	Potassium and chloride co-transport	(Le Rouzic et al., 2006; Pearson et al., 2001)
	SLC12A7/KCC4	r	m	A (m)	Potassium and chloride co-transport	(Karadsheh et al., 2004; Le Rouzic et al., 2006; Li et al., 2002)
SLC13	SLC13A4/NaS2	p	-	-	Sodium and sulphate co-transport	(Barnes et al., 2017; Bergeron et al., 2013)
SLC14	SLC14A1/UT-B	r	r	-	Urea facilitated diffusion	(Guo et al., 2015; Hou et al., 2017)
	SLC14A2/UT-A	r	-	-	Urea facilitated diffusion	(Bankir and Trinh-Trang-Tan, 2000; Guo et al., 2015)
SLC15	SLC15A1/PEPT1	r	-	-	Di, and tri-peptides transport accoupled with proton gradient. Also transports therapeutic drugs (B-lactam antibiotics, antiviral drugs, Angiotensin converting enzyme inhibitors, L-Dopa pro drugs, and polymixins)	(Foley et al., 2010; Morris et al., 2017; Smith et al., 2013)

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Supplementary table 2. Continued

SLC transporters in choroid plexus						
Family	**SLC transporters**	**mRNA expression**	**Protein expression**	**Localization**	**Functions**	**References**
	SLC15A2/PEPT2	r	m; r	A (m; r)	Di, and tri-peptides uptake accoupled with proton gradient. Also transports therapeutic drugs (B-lactam antibiotics, antiviral drugs, Angiotensin converting enzyme inhibitors)	(Daniel and Kottra, 2004; Kennedy et al., 2002; Shen et al., 2004; Shu et al., 2002)
	SLC15A4/PHT1	r	-	-	Transport of di, tri-peptides, and histidine accoupled to proton gradient. Normally identified as a vesicular transport	(Song et al., 2018; Yamashita et al., 1997)
SLC16	SLC16A1/MCT1	h; m; r	p	-	Proton-coupled monocarboxylates transport. Also involved in the uptake of some therapeutic drugs	(Koehler-Stec et al., 1998; Uchida et al., 2015; Vijay and Morris, 2014; Zhang et al., 2017)
	SLC16A2/MCT8	-	h; m; r	A (h; m; r); B (h)	Thyroid hormones proton-coupled transport	(Grijota-Martínez et al., 2011; Heuer et al., 2005; Iwayama et al., 2016; Vijay and Morris, 2014; Wilpert et al., 2020)
	SLC16A8/MCT3	-	r	B (r)	Proton-coupled lactate transport	(Bergersen et al., 1999; Vijay and Morris, 2014)
SLC19	SLC19A1/RFC	-	r; m	A (r, m)	Folates transport accoupled with inorganic phosphate gradient. Also responsible for transport antifolate chemotherapeutic agents (e.x. methotrexate and pemetrexed)	(Hinken et al., 2011; Vergote et al., 2015; Wang et al., 2001)
SLC20	SLC20A2/PiT2	-	r	A (r)	Sodium and inorganic phosphate co-transport	(Guerreiro et al., 2014)
SLC22	SLC22A1/OCT1	r	-	-	Facilitated diffusion of cationic and zwitterionic organic ions. Also involved in the transportation of therapeutic drugs	(Choudhuri et al., 2003; Koepsell, 2020)
	SLC22A2/OCT2	-	r	A (r)	Transport of cationic and zwitterionic organic ions, and some neutral substances. Also Involved in the transportation of therapeutic drugs	(Koepsell, 2020; Sweet et al., 2001)

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Supplementary table 2. Continued

SLC transporters in choroid plexus						
Family	**SLC transporters**	**mRNA expression**	**Protein expression**	**Localization**	**Functions**	**References**
	SLC22A3/OCT3	r	-	-	Transport of cationic and zwitterionic organic ions, and some neutral substances (mechanism dependent on the substrate). Also Involved in the transportation of therapeutic drugs	(Koepsell, 2020; Sweet et al., 2001)
	SLC22A4/OCTN1	r	m	-	Transport of cationic and zwitterionic organic ions, and some neutral substances (mechanism dependent on the substrate). Also Involved in the transportation of therapeutic drugs	(Choudhuri et al., 2003; Koepsell, 2020; Lamhonwah et al., 2008)
	SLC22A5/OCTN2		m; p	-	Transport of cationic and zwitterionic organic ions, and some neutral substances (mechanism dependent on the substrate). Also Involved in the transportation of therapeutic drugs	(Koepsell, 2020; Uchida et al., 2019)
	SLC22A6/OAT1	m	h; r	A (h; r)	Transport of organic anions by exchange with di-carboxylates, mainly a-ketoglutarate. Also involved in the transportation of therapeutic drugs	(Alebouyeh et al., 2003; Ivanyuk et al., 2017; Nagata et al., 2002; Nagle et al., 2013; Yasuda et al., 2013)
	SLC22A8/OAT3	m	h; p; r	A (r)	Transport of organic anions by exchange with di-carboxylates, mainly a-ketoglutarate. Also involved in the transportation of therapeutic drugs	(Alebouyeh et al., 2004; Ivanyuk et al., 2017; Nagle et al., 2013; Uchida et al., 2019; Yasuda et al., 2013)
	SLC22A12/URAT1	-	h	B (h)	Urate, anion exchanger	(Uemura et al., 2017; Wang et al., 2019)
SLC23	SLC23A2/SVCT2	-	b; h; r	B (r)	Vitamin C transport dependent on sodium gradient	(Astuya et al., 2005; Ulloa et al., 2013; Ulloa et al., 2019)
SLC28	SLC28A1/CNT1	r	-	-	Transport of pyrimidine nucleosides dependent of sodium gradient. Also involved in the transportation of antiviral drugs	(Choudhuri et al., 2003; Pastor-Anglada et al., 2018)

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Suplementary table 2. Continued

SLC transporters in choroid plexus						
Family	**SLC transporters**	**mRNA expression**	**Protein expression**	**Localization**	**Functions**	**References**
	SLC28A2/CNT2	r	r	-	Transport of purine nucleosides and uridine dependent of sodium gradient. Also involved in the transportation of antiviral drugs	(Pastor-Anglada et al., 2018; Redzic et al., 2005)
	SLC28A3/CNT3	r	h	-	Transport of purine and pyrimidine nucleosides dependent on sodium gradient. Also involved in the transportation of antiviral drugs	(Pastor-Anglada et al., 2018; Redzic et al., 2005; Redzic et al., 2010)
SLC29	SLC29A1/ENT1	m; r	h; r	-	Facilitated diffusion of nucleosides and nucleotides. Also involved in the transportation of antiviral drugs	(Choudhuri et al., 2003; Nagata et al., 2002; Pastor-Anglada et al., 2018; Redzic et al., 2010; Yasuda et al., 2013)
	SLC29A2/ENT2		h	-	Facilitated diffusion of nucleosides and nucleotides. Also involved in the transportation of antiviral drugs	(Pastor-Anglada et al., 2018; Redzic et al., 2010)
	SLC29A3/ENT3	h	h	-	Adenosine and Uridine transport. Normally located in intracellular compartments	(Pastor-Anglada et al., 2018; Redzic et al., 2010)
	SL29A4/PMAT	m	m	A (m)	Transport of organic cations (mainly biogenic amines). Also involved in the transportation of therapeutic drugs.	(Dahlin et al., 2007; Duan and Wang, 2013; Wang, 2016)
SLC30	SLC30A1/ZnT1	r	m		Zinc transport	(Shi et al., 2008; Wang et al., 2004b)
	SLC30A2/ZnT2	r	r	A (r); B (r)	Zinc transport	(Fu et al., 2014; Wang et al., 2004b)
	SLC30A3/ZnT3	-	m	Amv (m)	Zinc transport	(Wang et al., 2004b)
	SLC30A4/ZnT4	-	m	-	Zinc transport	(Wang et al., 2004b)
	SLC30A6/ZnT6	-	m	-	Zinc transport	(Wang et al., 2004b)

(Continued)

Suplementary table 2. Continued

SLC transporters in choroid plexus						
Family	**SLC transporters**	**mRNA expression**	**Protein expression**	**Localization**	**Functions**	**References**
SLC31	SLC31A1/CRT1	m; r	m; r	A (m)	Copper transport	(Chen et al., 2012; Choi and Zheng, 2009; Fu et al., 2015; Kuo et al., 2006; Kuo et al., 2001; Monnot et al., 2012)
SLC36	SLC36A1/PAT1	r	r	-	Proton-coupled glycine, proline, and alanine transport. Also transport therapeutic drugs	(Agulhon et al., 2003; Metzner et al., 2006)
	SLC36A4/PAT4		m	A (m)	Facilitated diffusion of proline, tryptophan, and alanine	(Pillai and Meredith, 2011; Roshanbin et al., 2014)
SLC38	SLC38A1/SNAT1	m	m; r	B (m)	Transport of amino acids accoupled with sodium gradient	(Dolgodilina et al., 2020; Mackenzie and Erickson, 2004; Melone et al., 2004)
	SLC38A2/SNAT2	m	r	-	Transport of amino acids accoupled with sodium gradient	(Dolgodilina et al., 2020; Mackenzie and Erickson, 2004; Melone et al., 2006)
	SLC38A3/SNAT3	m	m	A (m)	Transport of amino acids accoupled with sodium and proton gradients	(Dolgodilina et al., 2020; Mackenzie and Erickson, 2004)
	SLC38A4/SNAT4	m	-	-	Transport of amino acids accoupled with sodium gradient	(Dolgodilina et al., 2020; Mackenzie and Erickson, 2004)
	SLC38A6/SNAT6	m	-	-	Orphan	(Dolgodilina et al., 2020; Mackenzie and Erickson, 2004)
SLC39	SLC39A1/ZIP1	r	-	-	Zinc transport	(Belloni-Olivi et al., 2009)
	SLC39A4/ZIP4	r	-	-	Zinc transport	(Belloni-Olivi et al., 2009)
SLC40	SLC40A1/IREG1	-	m; h	-	iron transport	(Boserup et al., 2011; Clardy et al., 2006; Wu et al., 2004)

(Continued)

Supplementary table 2. Continued

SLC transporters in choroid plexus						
Family	**SLC transporters**	**mRNA expression**	**Protein expression**	**Localization**	**Functions**	**References**
SLC46	SLC46A1/PCFT	r	m	B (m)	Proton-coupled folates transport. Also involved in transport of antifolate chemotherapeutic agents	(Vergote et al., 2015; Wollack et al., 2008; Zhao et al., 2009)
SLC47	SLC47A1/MATE1	-	h; p; r	-	Proton antiport of organic cations, zwitterions, and noncharged substances. Very relevant in therapeutic drugs efflux	(Koepsell, 2020; Uchida et al., 2019; Uchida et al., 2015)
SLCO	Slco1a1/oatp1a1	r	r	A (r)	Transport of a diversity of amphipathic compounds. Also mediate the transport of a wide range of therapeutic drugs.	(Angeletti et al., 1997; Hagenbuch and Meier, 2003)
	Slco1a3/Oatp1a3	r	r	A (r)	Transport of a diversity of amphipathic compounds. Also mediate the transport of a wide range of therapeutic drugs.	(Hagenbuch and Meier, 2003; Ohtsuki et al., 2003; Sathyanesan et al., 2012)
	Slco1a4/Oatp1a4	m; r	m; r	B (r)	Transport of a diversity of amphipathic compounds. Also mediate the transport of a wide range of therapeutic drugs.	(Gao et al., 1999; Hagenbuch and Meier, 2003; Ohtsuki et al., 2004; Ose et al., 2010)
	Slco1a5/Oatp1a5	m; r	m; r	A (m; r)	Transport of a diversity of amphipathic compounds. Also mediate the transport of a wide range of therapeutic drugs.	(Hagenbuch and Meier, 2003; Kusuhara et al., 2003; Ohtsuki et al., 2004)
	Slco1a6/Oatp1a6	m	-	-	Transport of a diversity of amphipathic compounds. Also mediate the transport of a wide range of therapeutic drugs.	(Hagenbuch and Meier, 2003; Ohtsuki et al., 2004)
	SLCO1C1/OATP1C1	h	h	A (h); B (h)	Transport of a diversity of amphipathic compounds. Also mediate the transport of a wide range of therapeutic drugs.	(Hagenbuch and Meier, 2003; Müller and Heuer, 2014; Roberts et al., 2008a)
	Slco1c1/Oatp1c1	m; r	m; r	A (m; r); B (m; r)	Transport of a diversity of amphipathic compounds. Also mediate the transport of a wide range of therapeutic drugs.	(Hagenbuch and Meier, 2003; Ohtsuki et al., 2004; Roberts et al., 2008a; Sathyanesan et al., 2012; Sugiyama et al., 2003)
	Slco2a1/Oatp2a1	r	r	A (r)	Transport of a diversity of amphipathic compounds. Also mediate the transport of a wide range of therapeutic drugs.	(Hagenbuch and Meier, 2003; Kis et al., 2006)

(Continued)

Supplementary table 2. Continued

SLC transporters in choroid plexus						
Family	**SLC transporters**	**mRNA expression**	**Protein expression**	**Localization**	**Functions**	**References**
	Slco2b1/Oatp2b1	r	r	A (r)	Transport of a diversity of amphipathic compounds. Also mediate the transport of a wide range of therapeutic drugs.	(Choudhuri et al., 2003; Hagenbuch and Meier, 2003; Roberts et al., 2008a)
	Slco3a1/Oatp3a1	r	-	-	Transport of a diversity of amphipathic compounds. Also mediate the transport of a wide range of therapeutic drugs.	(Hagenbuch and Meier, 2003; Kratzer et al., 2013)
	SLCO3A4/OATP3A4	-	h	B (h)	Transport of a diversity of amphipathic compounds. Also mediate the transport of a wide range of therapeutic drugs.	(Hagenbuch and Meier, 2003; Huber et al., 2007)
	Slco4a1/Oatp4a1	r	-	-	Transport of a diversity of amphipathic compounds. Also mediate the transport of a wide range of therapeutic drugs	(Choudhuri et al., 2003)

b – bovine; h – human; mk – monkey; m-mouse r – rat; ra - rabbit

A – apical; Amv – Apical microvili vesicules B – basolateral; C - Citoplasmatic

AE - anion exchanger; ASC-1 – Asc-type amino acid transporter 1 B^{0}AT2 - Sodium-dependent neutral amino acid transporter**;** BTR1 - bicarbonate transporter-related protein**;**

CNT - concentrative nucleoside transporter; CRT1 - Copper transporter 1**;** DMT1 - Divalent Metal Transporter 1; EAAT - excitatory amino acid transporter**;**

ENT - Equilibrative nucleoside transporter**;** GAT2 – GABBA transporter 2; GLUT – **G**lucose transporter**;** IREG - Iron-regulated transporter**;**

KCC - Electroneutral potassium-chloride cotransporter; LAT - L-type amino acid transporter; Mate - Multidrug and toxin extrusion; MCT – Monocarboxylate transporter**;**

NaS2 - sodium sulfate cotransporter-2; NBCe - Electrogenic sodium bicarbonate cotransporter; NBCn - Electroneutral sodium bicarbonate cotransporter**;**

NDCBE - Sodium-Driven Chloride/Bicarbonate Exchanger**;** NHE - Sodium/hydrogen exchanger NKCC1 - sodium/potassium/chloride transporter 1;

NTCP – Sodium/bile acid cotransporter; OAT – Organic anion transporter; OATP – Organic anion transporting polypeptide; OCT – Organic cation transporter;

OCTN - Organic zwitterions/cation transporter; PAT - Proton-coupled Amino acid Transporter; PCFT - Proton-coupled folate transporter;

PIT2 - Sodium-dependent phosphate transporter 2; PEPT – Peptide transporter; PMAT - Plasma membrane monoamine transporter; RFC - reduced folate carrier;

SIT1 - sodium/imino-acid transporter 1; SERT – Serotonin transporter; SMIT1 – Osmoregulatory inositol transporter; SMVT - Sodium Dependent Multivitamin Transporter;

SNAT - Sodium-coupled neutral amino acid transporter; SVCT – Sodium ascorbic acid cotransporter UT-A - urea transporter 2; UT-B - urea transporter 1;

URAT-1 Urate anion exchanger 1; xCT – Cystine/glutamate transporter ZIP – Zinc/iron-regulated transporter-like; ZnT – Zinc transporter