Chronotherapy of Brain Diseases:
Assessment of the Circadian Rhythms of Efflux Transporters at the Blood-cerebrospinal Fluid Barrier

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Dedicatory

To my family and girlfriend who always fought to give me the best they could and made me realize that quitting wasn't an option.

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Resumo

A barreira sangue-líquido cefalorraquidiano (BSLCR) é formada por uma estrutura denominada de Plexo Coróide (PC). O PC é constituído por uma monocamada de células epiteliais unidas por junções apertadas. Na membrana apical, estas células apresentam microvilosidades e estão em contacto com o líquido cefalorraquidiano (LCR). Na membrana basal, as células estão rodeadas por capilares sanguíneos. O PC é responsável por diversas funções indispensáveis ao funcionamento do sistema nervoso central (SNC) onde se inclui a produção de LCR, a síntese de diversas proteínas, proteção do SNC contra elementos estranhos, destoxificação do LCR de compostos nocivos resultantes do metabolismo normal do SNC e transporte de diversas moléculas através da BSLCR.

O PC apresenta um papel essencial no transporte de moléculas terapêuticas destinadas ao SNC através da BSLCR. Para isso, expressa proteínas transportadoras de membrana que estão descritas como essenciais no transporte de fármacos através das barreiras do cérebro.

Recentemente, foi descrito no PC um relógio molecular funcional. Como tal, as funções biológicas desta estrutura poderão estar associadas ao ritmo circadiano. Existe a possibilidade deste relógio circadiano influenciar a expressão e atividade de diversos transportadores de membrana no PC, resultando numa modificação da biodisponibilidade de moléculas terapêuticas no SNC ao longo do dia, dependendo da hora a que são administradas.

Como tal, o trabalho desenvolvido nesta tese de doutoramento teve como principal objetivo a análise da influência do ritmo circadiano na expressão de diversos transportadores de membrana localizados no PC. Para além disso, procedeu-se à utilização de compostos terapêuticos, nomeadamente metotrexato (MTX) e donepezil (DNPZ), para avaliar a relação entre a expressão circadiana dos transportadores de membrana e a sua função no transporte de fármacos através da BSLCR.

Em particular, um dos objetivos deste projeto foi avaliar a expressão circadiana de múltiplos transportadores de membrana presentes no PC. Sendo assim, utilizando culturas primárias de PC de ratos neonatais, constatou-se que a expressão dos transportadores de membrana rSlc9a1 e rSlco1a5 apresentava uma ritmicidade circadiana ao contrário da expressão do transportador de membrana rSlc47a1 que se manteve constante ao longo de 24 horas.

Com o objetivo de explorar o impacto das diferenças entre sexos nas oscilações diárias dos transportadores de membrana da família ABC e SLC presentes no PC de rato, foram utilizados PC recolhidos de ratos macho, fêmeas, fêmeas ovariectomizadas e fêmeas controlo. Os resultados obtidos demonstram que o transportador de membrana rAbcc1 é expresso de forma rítmica no PC de ratos macho, e o rAbcg2 em ratos fêmea. Os transportadores rAbcc4 e rOat3 são ambos expressos ritmicamente em machos e fêmeas. Posteriormente, para avaliar a relevância da expressão circadiana do Abcc4 no transporte de MTX através da BSLCR foi utilizado um modelo *in vitro* da barreira. Verificou-se que o transporte de MTX através da BSLCR apresenta ritmicidade. A expressão rítmica do rAbcc4 poderá ser uma das razões para o transporte do MTX, através da BSLCR, ser dependente do ritmo circadiano.

Outro dos objetivos deste projeto era caraterizar a regulação circadiana da expressão do Abcg2 e posteriormente estudar o perfil circadiano de transporte do DNPZ através da BSLCR. Utilizando culturas primárias de PC de ratos neonatais, observou-se uma expressão rítmica do transportador de membrana rAbcg2. Com recurso a um modelo *in vitro* da barreira que tinha por base as culturas primárias de PC de ratos neonatais, observou-se que o transporte de DNPZ através da BSLCR apresentava uma ritmicidade circadiana. É ainda proposto que para além do envolvimento do ABCG2, também o SLC22A4 poderá ter influência no transporte rítmico de DNPZ através da BSLCR.

Os resultados obtidos neste projeto permitem afirmar que os transportadores de membrana apresentam uma expressão rítmica na BSLCR. Foi possível observar a influencia da expressão rítmica destes transportadores de membrana no transporte de moléculas terapêuticas como o MTX e DNPZ. No futuro será importante continuar a explorar o papel do ritmo circadiano na expressão de transportadores de membrana no PC e a sua influência no transporte de fármacos através da BSLCR. Esta informação poderá revelar-se crucial no tratamento de doenças do SNC. Ao adequar o período de administração do fármaco ao ritmo biológico do paciente, estaremos a garantir o máximo de concentração de fármaco no local desejado, e uma redução dos efeitos secundários.

Palavras-chave

Plexo coróide; transportadores de membrana; barreira sangue-líquido cefalorraquidiano; cronoterapia; metotrexato; donepezil.

Resumo Alargado

O comportamento animal, a sua atividade, processos fisiológicos e bioquímicos ocorrem ao longo do dia com diversos ritmos associados. O elemento ambiental que mais contribui para a definição e manutenção destes ritmos biológicos é a luz, e em condições constantes, os animais tendem a manter ritmos biológicos com aproximadamente 24 horas, denominados ritmos circadianos. Relógios internos são responsáveis pelo registo e manutenção destes ritmos, uma vez que são mantidos mesmo na ausência de estímulos externos. O relógio principal localiza-se no núcleo supraquiasmático (NSQ) do hipotálamo, no entanto existem outros relógios secundários no organismo. Um destes relógios secundários está localizado no plexo coróide (PC). O relógio circadiano do PC é regulado pelas hormonas sexuais e encontra-se em estreita comunicação com o NSQ.

O PC é parte integrante da constituição da barreira sangue-líquido cefalorraquidiano (BSLCR), uma de duas barreiras responsáveis pela proteção do sistema nervoso central (SNC). O PC é formado por uma monocamada de células epiteliais unidas por junções apertadas. Esta estrutura localiza-se nos ventrículos cerebrais e é altamente vascularizada ao nível da membrana basal. Na membrana apical estas células apresentam microvilosidades que se encontram em contacto com o líquido cefalorraquidiano (LCR). O PC apresenta várias funções associadas e que são vitais para o bom funcionamento do SNC, tais como a produção do LCR, a síntese e excreção de inúmeras proteínas essenciais ao metabolismo do SNC e a limpeza do LCR de moléculas tóxicas (que podem ser provenientes do normal metabolismo do SNC ou de origem exógena). Para além destas atividades, o PC é também parte integrante da BSLCR, uma vez que expressa transportadores de membrana que controlam o fluxo de fármacos através da BSLCR. As duas principais famílias de transportadores de membrana presentes no PC são a família *ATP-binding cassette* (ABC) *e* a família *solute carrier* (SLC). Os transportadores da família ABC são geralmente responsáveis pelo transporte de efluxo contra um gradiente de concentração enquanto que os transportadores da família SLC estão associados ao influxo de moléculas. Diversos fármacos utilizados no tratamento de patologias do SNC são substratos de transportadores de membrana que integram estas duas famílias. Como tal, a existência de transportadores de membrana de efluxo da família ABC na BSLCR é um dos principais fatores que limita a terapia farmacológica de doenças do SNC, resultando numa deficiente biodisponibilidade no cérebro de compostos terapêuticos. Os transportadores de membrana da família SLC também são parte integrante do sistema membranar de transporte, tornando-os parte ativa no controlo da biodisponibilidade de fármacos no cérebro.

É do conhecimento geral que diversas funções biológicas são reguladas e influenciadas pelo ritmo circadiano. O PC não é exceção. Para além de ser um dos relógios secundários presentes no organismo, algumas das suas funções já foram descritas como sendo dependentes do ritmo circadiano. Sendo assim, podemos sugerir que também a expressão e atividade dos transportadores de membrana expressos no PC possam estar sob influencia de ritmos circadianos. Vários dos transportadores de membrana presentes no PC foram já identificados como apresentando uma expressão e função com caraterísticas circadianas em diversos tecidos.

A cronoterapia, é uma nova corrente de pensamento médico, que pretende alinhar a administração de tratamentos, sejam eles farmacológicos, cirúrgicos, ambientais ou outros, com os ritmos biológicos dos pacientes. O objetivo é aumentar a eficácia do tratamento, reduzir os efeitos secundários e custos económicos, e melhorar de forma significativa a qualidade de vida dos pacientes e cuidadores. Sendo assim, para a aplicação desta estratégia terapêutica no tratamento de patologias do SNC é essencial um maior conhecimento de como os ritmos circadianos influenciam a passagem de fármacos através da BSLCR.

O trabalho desenvolvido nesta tese de doutoramento teve como principal objetivo a caraterização da expressão circadiana de diversos transportadores de membrana no PC, utilizando amostras de PC recolhidas de ratos macho, fêmeas, fêmeas ovariectomizadas e fêmeas controlo (sujeitas a intervenção cirúrgica, mas sem que tenham sido retirados os ovários) e dois modelos *in vitro* do PC, um fazendo uso duma linha celular humana e outro empregando uma cultura primária de células de ratos neonatais. Foi também alvo de estudo a influência dos ritmos no transporte de metotrexato (MTX, fármaco utilizado no tratamento do glioblastoma) e donepezil (DNPZ, fármaco utilizado no tratamento da doença de Alzheimer) pelos transportadores de membrana do PC através da BSLCR.

Em particular, um dos objetivos deste trabalho foi avaliar a expressão circadiana de múltiplos transportadores de membrana presentes no PC em culturas primárias de PC de ratos neonatais. Os transportadores selecionados para este efeito já tinham sido previamente descritos no PC. Os resultados obtidos neste trabalho permitiram assim constatar que a expressão dos transportadores de membrana rSlc9a1 e rSlco1a5 apresentam uma expressão rítmica no PC enquanto que a expressão do transportador de membrana rSlc47A1 se mantem constante ao longo de 24 horas.

Este projeto focou-se também no impacto das diferenças entre sexos nas oscilações diárias dos transportadores de membrana ABC e SLC presentes no PC de rato, utilizando PC de ratos macho, fêmeas, fêmeas ovariectomizadas e fêmeas controlo. O transportador

de membrana rAbcc1 apresentou uma expressão circadiana no PC de ratos macho, já o transportador de membrana rAbcg2 apresentou uma expressão circadiana no PC de ratos fêmea. Os transportadores de membrana rAbcc4 e rOat3 revelaram expressão circadiana no PC de ratos de ambos os sexos. Uma vez que a expressão de rAbcc4 apresentou ritmicidade no PC de ambos os sexos, o objetivo seguinte focou-se na relevância desta expressão no transporte do seu substrato, MTX, através da BSLCR. Para este estudo uma linha celular de PC humana foi cultivada de forma a obter um modelo *in vitro* da barreira. A expressão circadiana de hAbcc4 neste modelo foi confirmada e para além deste transportador de membrana, também o hAbcg2 apresentou expressão rítmica. Utilizando o mesmo modelo *in vitro*, verificou-se que o transporte de MTX através da BSLCR apresenta uma ritmicidade circadiana associada. O hABCC4 (transportador de membrana localizado na membrana basal do PC) quando inibido, levou a uma perda de ritmicidade no transporte de MTX no lado basal do modelo *in vitro*. A inibição do hABCG2 (transportador de membrana localizado na membrana apical do PC) resultou igualmente na perda de ritmicidade no transporte de MTX no compartimento basal.

Finalmente, este projeto teve também como objetivo elucidar de que forma os ritmos circadianos afetam a expressão do rAbcg2 e principalmente o transporte do seu substrato, DNPZ, através da BSLCR. Inicialmente foi necessário confirmar a expressão circadiana do rAbcg2 em culturas primárias de PC de ratos neonatais. Posteriormente, a análise do perfil circadiano de transporte do DNPZ através da BSLCR foi estudada utilizando o mesmo modelo *in vitro*. O transporte do DNPZ através da BSLCR foi descrito como sendo rítmico e esta ritmicidade foi anulada após estímulo com um inibidor do rABCG2. Foi também descrita o possível envolvimento do transportador de membrana rSlc22a4 no transporte de DNPZ através da BSLCR.

No seu conjunto, os resultados obtidos no decorrer dos trabalhos enunciados nesta tese, suportam a hipótese de que o PC expressa de forma rítmica transportadores de membrana envolvidos no transporte de inúmeros fármacos através da BSLCR. Os resultados desta tese também confirmam que para além da expressão rítmica destes transportadores no PC, também o transporte dos seus substratos é controlado pelo ritmo circadiano. No futuro, espera-se que os estudos aqui apresentados sirvam de ponto de partida para o desenvolvimento de novas abordagens terapêuticas, nomeadamente em doenças do SNC. Adicionalmente, espera-se que o PC receba o destaque merecido enquanto porta de entrada e saída para o SNC, que a sua crescente relevância na homeostase do CNS continue a ser explorada e aprofundada e que o seu potencial contributo aquando do desenvolvimento de novas abordagens terapêuticas que tenham

como alvo o SNC, promova uma melhoria das terapias disponíveis para o tratamento de doenças do SNC e consequentemente para a qualidade de vida dos pacientes.

Palavras-chave

Plexo coróide; transportadores de membrana; barreira sangue-líquido cefalorraquidiano; cronoterapia; metotrexato; donepezil.

Abstract

The choroid plexus (CP) is an integral part of the blood cerebrospinal-fluid barrier (BCSFB). The CP is formed by a monolayer of cuboidal epithelial cells united by tight junctions. On the apical side, these cells present microvilli and are in contact with the cerebrospinal fluid (CSF). On the basal membrane, these cells are surrounded by a vast network of capillary blood vessels. The CP is responsible for several functions that are vital to the homeostasis of the central nervous system (CNS) where we include the production of the CSF, synthesis of several proteins, CNS protection against foreign elements, CSF detoxification from noxious compounds that result from normal cell metabolism and the transport of multiple molecules across the BCSFB.

The CP has an essential role on the transport across the BCSFB of therapeutic molecules targeting the CNS. For that, it expresses multiple membrane transporters that have been described in the literature as essential for the transport of therapeutic compounds across CNS biological barriers.

Recently, a functional molecular clock was described in the CP. This means that the biological functions of this structure might have a circadian rhythmicity associated. There's the possibility that this circadian clock influences membrane transporters' expression and activity at the CP which would result in circadian changes of the bioavailability of therapeutic compounds in the CNS depending on the time of administration.

As such, the main goal of this doctoral thesis was to analyse the influence of circadian rhythms on the expression of multiple membrane transporters on the CP. Additionally, we used therapeutic compounds, namely methotrexate (MTX) and donepezil (DNPZ) to assess the relation between the CP's membrane transporters circadian expression and their drug transport function across the BCSFB.

One of the objectives of this project, as mentioned earlier, was to assess the circadian expression of multiple CP’s membrane transporters. For that, CP primary cell cultures of neonate rats were used. We concluded that rSlc9a1 and rSlc1a5 expression was rhythmic during a 24-hour period while rSlc47a1 did not reveal a circadian pattern.

This work also aimed at disclosing the influence of sex on the daily expression oscillations of several ABC and SLC membrane transporters expressed by the CP. For this we used CPs from male, female, ovariectomized and sham-operated female rats. The

results showed that the membrane transporter rAbcc1 is expressed in a circadian manner in the CP of male rats, while rAbcg2 presented circadian rhythmic expression in the CP of female rats. Both rAbcc4 and rOat3 were rhythmically expressed in the CP of male and female rats. Next, we used an *in vitro* model of the CP in order to evaluate the relevance of Abcc4's circadian expression in the transport of MTX across the BCSFB. We demonstrated that MTX transport across the BCSFB was rhythmic. Besides, we also concluded that Abcc4 circadian expression might influence the MTX circadian transport across the BCSFB.

Finally, this project also aimed to describe the impact of circadian rhythms on CP Abcg2 expression and also on the circadian transport profile of DNPZ across the BCSFB. Using CP primary cell cultures of neonate rats, we demonstrated the presence of rAbcg2 circadian expression. Next, using primary cell cultures, an *in vitro* model of the BCSFB was established and we discovered that DNPZ transport across the BCSFB presents circadian rhythmicity. Furthermore, it was also proposed that besides rABCG2, SLC22A4 could also be involved in the DNPZ circadian transport across the BCSFB.

The results obtained in this project demonstrate that membrane transporters present circadian expression in the BCSFB. Moreover, the transport of therapeutic compounds, such as MTX and DNPZ, across the BCSFB is also influenced by the circadian rhythm of CP membrane transporters. In the future, it is essential to further exploit the role of circadian rhythms on the expression of membrane transporters at the CP and its influence on the transport of therapeutic compounds across the BCSFB. This information might prove vital in the treatment of CNS diseases. By timing drug administration with the period when they are more prone to reach the target tissue at the CNS, we are ensuring their maximum target tissue concentration, and a reduction in side effects.

Keywords

Choroid plexus; membrane transporters; blood cerebrospinal-fluid barrier; chronotherapy; methotrexate; donepezil.

Thesis Overview

This Doctoral thesis is organized in 6 chapters.

The first chapter enclose the introductory section and intend to contextualize the putative relevance of circadian rhythms on membrane transporters activity and their relevance at the blood- cerebrospinal fluid barrier. Additionally, the importance of these membrane transporters expression and activity for the pharmacological treatment of central nervous system diseases is also addressed.

The third chapter presents the general and specific aims established for the work plan of this doctoral thesis.

The third, fourth and fifth chapters present the results of the research work developed:

- Research Work 1: Circadian Expression of Membrane Transporters at the BCSFB (Chapter 3);

- Research Work 2: The Daily Expression of ABCC4 at the BCSFB Affects the Transport of its Substrate Methotrexate (Chapter5);

- Research Work 3: Circadian ABCG2 Expression Influences the Brain Uptake of Donepezil across the Brain-Cerebrospinal Fluid Barrier (Chapter 6).

Finally, the sixth chapter contains the concluding remarks highlighting the advances obtained during this research work and discuss the future directions in the central nervous system diseases' chronotherapy.

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Abbreviations

ABC	ATP-binding cassette
Aβ	Amyloid-beta
AD	Alzheimer's disease
AhR	Aryl hydrocarbon receptor
BBB	Blood-brain barrier
BCSFB	Blood-cerebrospinal fluid barrier
BMAL1	Basic helix-loop-helix ARNT-like 1
BT	Brain tumour
CAR	Constitutive and rostane receptor
CCG	Clock controlled genes
CLOCK	Circadian locomotor clock kaput
CNS	Central nervous system
COG	Center of gravity
CP	Choroid plexus
CPEC	Choroid plexus epithelial cells
CRY	Cryptochrome
CSF	Cerebrospinal fluid
CT	Computed tomography
CV	Coefficient of variation
Dbp	Albumin D-box binding protein
DIV1	Day *in vitro* 1
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified eagle medium
DNPZ	Donepezil
ESD	Extreme standardized deviate
ERs	Estrogen receptors
FBS	Fetal bovine serum
FDA	US Food and Drug Administration

FL-MTX	MTX conjugated with fluorescein
FOLR1	Folate receptor alpha
FOLR2	Folate receptor beta
GBM	Glioblastoma
GLUT1	Glucose transporter SLC2A1
hCMED/D3	Human cerebral microvascular endothelial cell line
HIBCPP	Human epithelial CP papilloma
Hlf	Hepatic leukaemia factor
HPLC-FLD	High-performance liquid chromatography- fluorescence detector
KRB	Krebs-Ringer buffer
LLOQ	Linearity limit of quantification
LOD	Limit of detection
MTX	Methotrexate
NPAS2	Neuronal PAS domain-containing protein 2
Nr1d1	Nuclear receptor subfamily 1 group D member 1
OATP	Organic anion transporter polypeptide
OVX	Ovariectomized
PCFT	Proton-coupled folate transporter
PD	Parkinson's disease
PEPT	Peptide transporter
PER	Period
PET	Positron emission tomography
PPARα	Peroxisome proliferator-activated receptor α
PXR	Pregnane X receptor
qPCR	Quantitative real-time PCR
rCyc	Rat cyclophilin A
RE	Relative error
RER	Rev-Erb/ROR-response domain
RFC	Reduced folate carrier
ROR	Retinoic acid receptor-related orphan receptor

SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
Sham	Sham-operated
SLC	Solute carrier
SLCO	Solute carrier organic anion
TEER	Transepithelial electric resistance
Tef	Thyrotroph embryonic factor
TRPM7	Transient receptor potential cation channel (TRPM7)
ZT	Zeitgeber Time

Chapter 1

Introduction
The Role of Circadian Rhythm at the BCSFB: Towards a Personalized Chronotherapy

1. Introduction

Animal behavior, activity, physiological and biochemical processes occur along the day at different rhythms. Light is the strongest environmental cue (Zeitgeber (ZT)) to set and maintain biological rhythms, and is responsible for their alignment with the day and night cycle (entrainment) (Honma, 2018). This results in diverse timed behavioral patterns, such as diurnal, nocturnal, or crepuscular activities. In constant conditions, most organisms continue to maintain an approximate 24-hour period rhythm called the circadian rhythm. In an organism, this conserved periodicity is maintained in various physiological functions independently of external conditions, supporting the hypothesis that circadian rhythms are set by internal clocks (Yan & Silver, 2016).

A central clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus is known to synchronize peripheral clocks and extra-SCN oscillators in the brain. Extensive literature highlighted the relevance of extra-SCN oscillators and described how they communicate with the central clock (reviewed by (Begemann et al., 2020)). Recently, we identified an extra-SCN oscillator in the choroid plexus (CP) which was considered an important circadian clock component (Myung et al., 2018). The CP circadian clock is regulated by sex steroid hormones (Quintela et al., 2018; Quintela et al., 2015) and is in close communication with the SCN (Myung et al., 2018). Despite the identification of the clock machinery in the CP, it is unknown if it regulates the physiological functions of the CP.

Central nervous system (CNS) disorders are a comprehensive category of conditions affecting brain's function and limiting health. Some of the most common conditions are neurodegenerative diseases like Alzheimer's disease (AD) and Parkinson's disease (PD), brain tumours (BT), epilepsy, migraine, strokes, neurological infections and other traumatic conditions such as head injuries and disorders caused by malnutrition. While a cure is still to be found for most of this conditions, medical and surgical therapies can often help managing symptoms. One of the major hurdles when dealing with CNS diseases is the fact that, for pharmacotherapeutic compounds to reach their target tissue, they must go through the brain barriers (blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB)). These are essential for protecting the CNS from pathogens, toxins, and inflammatory molecules circulating in the bloodstream (Kaur et al., 2016), and also in controlling molecular trafficking between the blood and the brain or the cerebrospinal fluid (CSF) depending if we are focusing on the BBB or the BCSFB, respectively. They also constitute a route for elimination of metabolites out of the brain and into the bloodstream. One of the features contributing to the gatekeeper function of both these barriers is the presence of multiple membrane transporters (Sun & Wang, 2021). Emerging therapeutic approaches focus on taking advantage of the existing membrane transport systems for drugs, to bypass the brain interfaces, and thus reaching the target tissue more effectively (Pardridge, 2012). Recently some of these membrane transport mechanism’s function has been associated to circadian rhythms.

Chronotherapy, in recent years, has been proposed to overcome the challenges posed by the brain barriers when treating CNS diseases. This developing therapeutic approach consists on timing drug administration to achieve the optimal efficacy with minimal side effects (Amiama-Roig et al., 2022). Looking at the BCSFB, the objective must be to improve the influx of drugs from the blood stream into the CSF while also reducing their efflux from the CSF to the bloodstream. A chronotherapeutic approach, takes advantage of the circadian pattern of CP membrane transport systems, timing drug administration to better suit these objectives.

2. The BCSFB: structure and function

The BCSFB is comprised by the CP, which is formed by a single layer of cuboidal epithelial cells, and ependymal cells located on the floor of the ventricles (Del Bigio, 1995; Smith et al., 2004). The CP are vascularized structures located in the cerebroventricular system: third, fourth and lateral ventricles (Redzic & Segal, 2004). Tight junctions located between CP epithelial cells, prevent paracellular movement of organic molecules. This monolayer of epithelial cells lays on loose connective tissue containing immune system cells and fibroblasts, and a dense network of fenestrated capillaries, supporting quick delivery of water (Redzic & Segal, 2004; Wolburg & Paulus, 2010). This facilitates the exit of molecules and fluids from the bloodstream to the interstitial fluid (Ballermann & Stan, 2007), thereby rendering the CP as the limiting barrier to the passage of polar compounds from the blood to the CSF (Redzic & Segal, 2004). The most expressed claudins in the CP are claudin-1, -2 and -3. Claudin-1 and -3 are barrier forming proteins involved in the tight junctions connecting the CP epithelial cells (CPEC) while claudin-2 has been described as a pore-forming protein selective for cations (Günzel & Yu, 2013). In addition to the permeability to cations like K^+ and Na^+, claudin-2 expression was also reported to enhance the paracellular permeability of water (Rosenthal et al., 2010). This is essential for CSF secretion which also involves the active transport of Na^+, Cl^- and HCO_3^- to the CSF side. This creates an osmotic gradient favorable to water movement towards the brain ventricles (Damkier et al., 2013; Ghersi-Egea et al., 2018). Together with the BBB, the BCSFB is essential for protecting the CNS from pathogens, toxins, and inflammatory molecules circulating in the bloodstream (Kaur et al., 2016), and also in controlling molecular trafficking between the blood and the CSF, constituting a route for elimination of metabolites out of the brain.

The CP uptake and efflux of drug substrates depends on ATP-binding cassette (ABC) and solute carrier (SLC) transporters (Pardridge, 2012). Efflux transporters facilitate the movement of substrates out of the cells while influx transporters facilitate the movement inwards (Morris et al., 2017). The ABC family usually is responsible for the efflux transport of molecules against a concentration gradient while the SLC family carries out the uptake (Stieger & Gao, 2015). Nevertheless, some SLC family transporters can also present themselves as bidirectional or efflux transporters (Figure 1) (Strazielle & Ghersi-Egea, 2015).

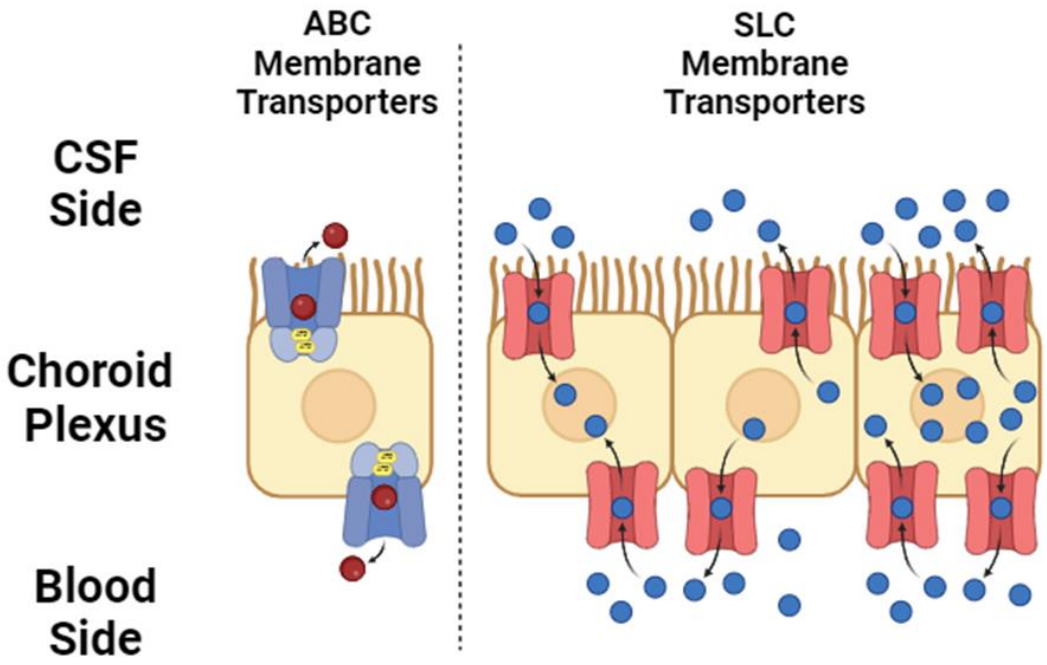

Figure 1. Uptake and efflux transport by the CP epithelial cells membrane transporters. Depending on the membrane transporter's family, transport of the specific substrates may be primarily efflux (ABC family of membrane transporters), or a combination between efflux and influx transport (SLC family of membrane transporters).

The ABC super family has 48 members classified into seven different subfamilies (from A to G) depending on the amino acid sequence and phylogeny (Chaves et al., 2014). These transporters require an energy source (ATP) to execute the active transport of their substrates across a membrane (Morris et al., 2017). In the BCSFB there have been identified several members of this family including ABCB1, ABCG2 and multiple members of the ABCC subfamily (Morris et al., 2017).

The SLC super family is subdivided into 52 subfamilies with a total of 395 individual transporter genes (Stieger & Gao, 2015). A 25% amino acid homology between family members is essential for the classification into these families (Girardin, 2006). These membrane transporters are responsible for facilitated transport, ion-coupled or ion-exchange transport (Redzic, 2011). The electrochemical potential difference is essential to promote transport of substrates, on the other hand, ion-coupled transport requires a sodium or proton gradient in order to transport substrates against a concentration gradient (Morris et al., 2017). Substrates for the SLC super family include organic anions and cations, monocarboxylates, peptides, drugs and drug conjugates and steroids (Girardin, 2006; Strazielle & Ghersi-Egea, 2015). Multiple SLC subfamilies including SLCO, SLC7A, SLC16A, SLC22A, SLC28A and SLC29A have been identified in the BCSFB (Stieger & Gao, 2015).

3. Circadian rhythms

Most living organisms can anticipate changes in their environment, and this can include light, food availability and temperature. Daily circadian rhythms in both behavior and/or physiological

processes have been observed in mammals, bacteria, fungi and plants (Patke et al., 2020). Circadian rhythms result from an intrinsic and autonomous system that has been identified as the circadian clock. This clock is capable of running with a 24-hour period even under constant environmental conditions. The process responsible for aligning the circadian clock with the external time cues (ZTs) is called entrainment. ZTs are responsible for advancing or delaying the internal circadian clock. Thus, unnatural exposure to ZTs such as light (which is very common in today's society) can disrupt circadian homeostasis and have marked consequences in health (Roenneberg & Merrow, 2016). The circadian clock is genetically controlled by a molecular system that is composed by a series of autoregulatory succession of expression, inhibition, accumulation and degradation of clock gene products (Patke et al., 2020). In animals, several physiological processes around the body are dependent of the molecular circadian clock control. As such, normal circadian physiology is dependent of a hierarchical network of central and peripheral clocks (Zhang et al., 2014)

3.1. The molecular circadian clock

The primary circadian pacemaker in mammals is the SCN located in the hypothalamus (Green & Gillette, 1982; Ueyama et al., 1999), which exerts direct control over multiple physiological rhythms. In animals rendered arrhythmic due to SCN damage, implanted grafts of SCN tissue restore circadian rhythmicity (Lehman et al., 1987; Sujino et al., 2003).

The SCN receives external and internal inputs, that are essential for coordinating the internal circadian rhythms with the external environment. The SCN maintains the circadian clock at the cellular level, regulating rhythmic expression of clock genes, in synchronization with the external light/dark cycle (Welsh et al., 2010). The photosensitive retinal ganglion cells in the retina capture time-of-day information, and the retinohypothalamic tract conveys it to the SCN through combinations of neurotransmitters and peptides to adjust the phase of the SCN clock (Atkins et al., 2018). The sensitivity of the light input can be modulated by extra visual stimuli, like the olfactory stimuli, proposing a role of the olfactory stimulation in the light resetting efficacy (Amir et al., 1999). The phase adjustment of the SCN clock, i.e. the adjustment of clock timing to incoming signals, also depends on its own susceptibility to inputs, a phenomenon known as gating (Rusak, 1989). This phenomenon is responsible for the controlled or diverse outputs that might occur from the same input. The gated signal is then synchronized in the SCN through a small-world-like network (Abel et al., 2016) and produces outputs leading to rhythmic locomotor activities (LeSauter & Silver, 1999). The SCN not only sends out circadian signals, but also receives them. These inputs come from extra-SCN neuronal pathways, through neuropeptide Y (Gribkoff et al., 1998), and peripheral clocks can modulate SCN timekeeping via neural connections and humoral signals. Peripheral oscillators are present in the retina, lung, liver, ovary, and even fibroblasts (Sellix, 2015; Stokkan et al., 2001; Tosini & Menaker, 1996; Yamazaki et al., 2000). Their phases are organized primarily by the SCN but also by different non-SCN cues such as food intake, one of the strongest entrainment cues especially in the liver (Damiola et al., 2000; Stokkan et al., 2001). Temperature (Brown et al., 2002; Saini et al., 2012), oxygen (Adamovich

et al., 2017), and glucocorticoids (Balsalobre et al., 2000) also influence entrainment of the peripheral oscillators.

The molecular circadian clock is at the core of these oscillators. It is driven by autoregulatory feedback expression of clock genes that include *Basic Helix-loop-helix ARNT-Like 1* (*Bmal1*), *Circadian Locomotor Output Cycles Kaput* (*Clock*), *Period* (*Per1, Per2,* and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*). These molecules interact and form interlocked transcriptional-translational feedback loops that are composed of primary negative and auxiliary positive loops (Figure 2) (Hastings et al., 2018; Ko & Takahashi, 2006; Logan & McClung, 2019; Welsh et al., 2010). Heterodimerization of the CLOCK protein with BMAL1 originates a positive feedback loop (also called *RORE loop* or *Bmal-Rev loop*), which drives the expression of most of the genes that make up the negative feedback loop (*Per loop*) (Schmal et al., 2019). Neuronal PAS domain-containing protein 2 (NPAS2) can take place as a BMAL1 heterodimerization partner in tissues or cells where CLOCK is not expressed (D. Landgraf et al., 2016). The positive loop component CLOCK/BMAL1 promotes transcription of clock controlled genes as well as negative loop components, including PER and CRY (Hastings et al., 2018). PER and CRY form an heterodimer that shuttles to the nucleus and sequesters CLOCK/BMAL1, thereby inhibiting their own gene transcription and completing a negative feedback loop (Hastings et al., 2018). CLOCK/BMAL1 binds to the E-box promoter sequence and activates the transcription of nuclear retinoic acid receptor-related, orphan receptor (ROR) and nuclear receptor Rev-Erb (Kondratov et al., 2007). These proteins compete for binding the Rev-Erb/ROR-response element (RRE) in the *Clock* and *Bmal1* genes controlling their expression in a positive feedback loop: Rev-Erb represses (Preitner et al., 2002) while ROR promotes *Bmal1* expression (Figure 2) (Jetten, 2009).

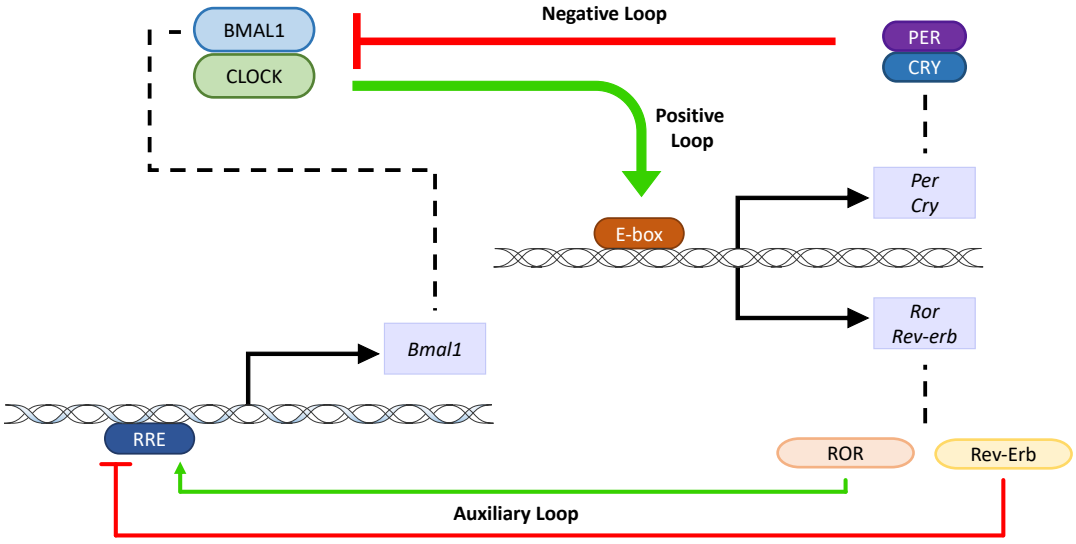

Figure 2. The Molecular Circadian Clock. The CLOCK/BMAL1 heterodimer is responsible for activating the transcription of the negative loop clock genes *Per* and *Cry* and the positive loop clock genes *Ror* and *Rev-erb*, forming interlocked feedback loops. The PER/CRY complex inhibits CLOCK/BMAL1 activity in the primary negative feedback loop, while ROR and Rev-Erb are responsible for forming the auxiliary positive feedback loop of *Bmal1*.

3.2 Clock hierarchy: extra-SCN oscillators in the brain

The SCN is part of the mammalian circadian system and has the responsibility to regulate additional circadian rhythms found in non-SCN tissues throughout the brain and peripheral organs (Mohawk et al., 2012; Yamazaki et al., 2000). The SCN is the only clock that is entrained by light and is responsible to synchronize the peripheral clocks to the same 24-hour cycle, through mechanisms that have still to be fully disclosed. Asynchrony between the SCN and the extra-SCN brain clocks may be a disease inducing factor (Mendoza & Challet, 2009). This can occur when other synchronizing cues, like temperature, daily feeding or social interactions, also exert their action on the CNS circadian clocks and might overrule the SCN synchronizing action, leading to internal conflicts between different clocks and their associated functions (Mendoza & Challet, 2009). The first clock identified in neuronal tissue outside the SCN was in the retina and showed an endogenous and autonomous circadian rhythm that regulates the synthesis of melatonin in this tissue (Tosini & Menaker, 1996). Cellular brain oscillators located outside the SCN also exist in the cerebral cortex (Rath et al., 2013), pineal and pituitary glands (Abe et al., 2002), hippocampus (Harbour et al., 2014; Jilg et al., 2010), hypothalamic nuclei (Guilding et al., 2009; Mieda et al., 2006), the lateral habenula (Baño-Otálora & Piggins, 2017), the *nucleus accumbens* (Dominic Landgraf et al., 2016), the caudate and putamen (Natsubori et al., 2014) of the striatum, the nucleus tractus solitarii, the area postrema of the brainstem (Chrobok et al., 2020), the amygdala (Savalli et al., 2014), the arcuate nucleus (Kriegsfeld et al., 2003) and cerebellum (Mendoza et al., 2010). However, only the olfactory bulb (D. Granados-Fuentes et al., 2004; Daniel Granados-Fuentes et al., 2004), presents all the characteristics of a circadian pacemaker.

More recently, the CP was identified as a circadian oscillator (Myung et al., 2018; Quintela et al., 2018; Quintela et al., 2015). More details concerning this new characterized clock are given in the next section.

4. The choroid plexus as a strong brain clock

One of the primary functions of the CP is the production of CSF. The apical side of the CP in contact with the CSF presents numerous microvilli and cilia which favor the flow of the CSF while blending the different compounds in this fluid (Redzic & Segal, 2004). The CP is also responsible for detoxifying the CSF from harmful products resulting from brain metabolism (Strazielle et al., 2004) and produce numerous proteins with nutritional and neuroprotective properties to the brain (Krzyzanowska & Carro, 2012). The CP also benefits from an olfactory and taste-like chemo sensing system to detect alterations on the chemical composition of blood and CSF in order to respond to alterations that might compromise brain homeostasis (Gonçalves et al., 2016; Tomas et al., 2016). Therefore, the CP plays a crucial role in the brain's metabolism, neuro-signaling, immunological/inflammatory processes, neuroprotection, and if not working properly may as well favor or enhance neurodegeneration (Janssen et al., 2013). Also, it is now recognized that the CP holds a circadian clock. The presence of the core clock genes (*Bmal1*, *Clock*, *Cry1*, *Cry2*, *Per1*, *Per2*, and *Per3*) mRNA expression and corresponding proteins was first reported in the CP

in 2015 (Quintela et al., 2015). Using female and male rats, the authors demonstrated that clock genes are under circadian regulation which differs between male and female rats. *Bmal1*, *Cry2*, and *Per2* are rhythmically expressed in the CP of female rats while only *Cry2* and *Per2* show rhythmic expression along the day in male rats (Quintela et al., 2015). Besides, *Quintela et al.* also demonstrated a sexual dimorphism in the CP circadian clock and showed that estrogens modulate the expression of *Bmal1*, *Per1*, and *Per2* in female rats, most likely via an estrogen receptor-dependent pathway (Quintela et al., 2018). The CP contains estrogen receptors (Hong-Goka & Chang, 2004) but these are scarce in the SCN (Karatsoreos & Silver, 2007). Thus, the CP may act as an extra-SCN site of action for estrogen, communicating with the SCN and adjusting its circadian rhythmicity (Myung et al., 2018; Santos et al., 2017). The variations that occur in the expression of the CP clock genes, resulting from sex hormone actions, can be a part of that regulatory mechanism. The relevance of the circadian clock hold by CP cells was further confirmed by *Myung et al.* that compared the circadian oscillations of *Bmal1* and *Per2* expression from various loci of the brain and observed a higher amplitude and strong, persistent circadian oscillations in the CP than in the SCN. The robustness of the CP circadian oscillation was justified by the synchronization through gap junction coupling (Myung et al., 2018). Furthermore, the results obtained by the same group suggested that communication between CP and SCN clocks is mediated by the CSF flow, supporting the modulation of the central clock by the CP (Myung et al., 2018).

From the literature it is possible to conclude that the CP cannot be seen as just one more circadian clock distributed throughout the brain. In fact, we believe that this oscillator will turn out to occupy a decisive position in the global network structure of the mammalian circadian system. The modulation of the CP circadian clock by sex steroid hormones and the impact of the CP on the SCN, probably via diffusible factors in the CSF, may contribute to the coordination of circadian rhythms in the organism's physiology.

4.1 Involvement of circadian rhythms in choroid plexus functions

It is already known that most physiological functions are strongly influenced by circadian clocks. In a recent review has been highlighted the CP physiological functions that have been proven to be governed and dependent on circadian rhythms and the molecular clock (Quintela et al., 2021). One of the mechanisms possibly under this circadian regulation is the transport of endogenous and exogenous compounds by membrane transporters across the BCSFB. This circadian time dependent transport is believed to be dependent on the circadian regulation of CP membrane transporters expression and function. In order to highlight the mechanisms of rhythmic CP integration and in line with this work's objective, we focus on the influence of the circadian clock on the daily regulation of CP membrane transporters expression and function.

The membrane efflux ABC transporters are recognized as important gatekeepers of the CNS by enabling or limiting drug delivery through the BCSFB and BBB. Consequently, this safety system is responsible for the deficient brain bioavailability of several pharmacological agents to

treat CNS diseases. Several of these transporters have already been demonstrated to be rhythmically expressed by various tissues from multiple species.

ABCB1 expression is much higher in the BBB than the BCSFB (Gazzin et al., 2008) and it is described as being rhythmically expressed in the BBB of both mice and humans (Zhang et al., 2021; Zhang et al., 2018). Its expression has also been proved to be rhythmic in human renal cells (Omata et al., 2021), in the monkey's intestine (Iwasaki et al., 2015), and mouse liver (Zhang et al., 2009). In opposition to Abcb1, Abcc1 is highly expressed in the basolateral membrane of CP epithelial cells (Gazzin et al., 2008). This difference in ABCC1 expression between the BBB and the BCSFB suggests that ABCC1 preferential location and therefore activity is at the BCSFB. The involvement of the circadian clock on Abcc1 mRNA levels was already demonstrated to be rhythmic in various tissues including the synchronized human colorectal adenocarcinoma (Caco-2) cell line (Ballesta et al., 2011; Ozturk et al., 2017) and ovary cell lines (Gaspar et al., 2019). At the BCSFB, Abcc1 is the predominant efflux transporter at the basolateral membrane, with Abcc4 and Abcg2 also involved in restraining therapeutic agents across the barrier (Koehn et al., 2019). As far as we know, no previous research has investigated the involvement of circadian rhythm in Abcc4 expression. On the contrary, the impact of the circadian rhythm on the expression and function of Abcg2 has been highlighted in recent years. Abcg2 mRNA rhythmicity was shown in mouse liver and in rat jejunal mucosa (Ozturk et al., 2017). Data also showed that ATF4, a molecular component of the circadian clock, functions as a circadian regulator of *Abcg2* mRNA expression in mice intestine (Hamdan et al., 2012). There are also other ABC transporters that have been identified in the BCSFB such as ABCC2 and ABCC3 (Bernd et al., 2015; Roberts et al., 2008). ABCC2 has already been described as being rhythmically expressed in the mouse small intestine and liver, but not in the kidneys, and monkey's small intestine, with peak protein expression during the animal active period (Kotaka et al., 2008; Ma et al., 2009; Oh et al., 2017; Stearns et al., 2008; Vagnerová et al., 2019; Yu et al., 2019; Zhang et al., 2011). ABCC3 has been reported as not having a rhythmic expression in mice liver (Wang et al., 2019). The global analysis of the existing research reveals that the circadian rhythm, at least partially, impacts the ABC transporter family expression, which lead us to hypothesize that the circadian regulation of some efflux transporters might interfere with the distribution of drugs.

Besides the ABC efflux transporters, the SLC superfamily are also responsible for neuroprotection, and transporting endogenous and exogenous toxic substances from the CSF to the bloodstream (Strazielle & Ghersi-Egea, 2015). SLCO family transcripts were more abundant during the light phase than the dark phase in mice, suggesting a possible circadian clock dependent regulation (Shen et al., 2014). Glucose transporter SLC2A1 (GLUT1) is also present in the CP basolateral membrane (Ulloa et al., 2019). GLUT1 has a circadian expression with its peak at the beginning of the dark phase in the rat cerebellum (Soltésová et al., 2013) and its rhythmicity was also reported in the human retinal pigment epithelial cell line ARPE-19 (Milićević et al., 2020). Furthermore, it was recently demonstrated that NPAS2 upregulates SLC2A1 expression both at the mRNA and protein levels (Yuan et al., 2020). NPAS2 can regulate the

circadian clock through the formation of the NPAS2/BMAL1 heterodimer which in turn controls the expression of *Per* and *Cry* genes (D. Landgraf et al., 2016). Slc7a1 mRNA, another amino acid membrane transporter expressed on the CP, is circadian rhythmic in the piglets jejunal mucosa (Saunders et al., 2015; Zhang et al., 2017). SLC16A1 was found to be rhythmically expressed in the human retinal pigment epithelial cell line ARPE-19 (Milićević et al., 2020). *Slc27a1* was described as having circadian variations in mouse epididymal adipose tissue (Bray & Young, 2007). SLC22A2 was found to be rhythmically expressed in mouse kidneys (Oda et al., 2014). Peroxisome proliferator-activated receptor α (PPARα) expression is under circadian control, being activated by the CLOCK/BMAL1 complex (Oda et al., 2014; Oishi et al., 2005). Thus, the authors came to the conclusion that blunted rhythms of PPARα observed in CLOCK knockout mice lead to loss of SLC22A2 rhythmic expression in the kidneys (Oda et al., 2014). SLC22A5 and SLC22A8 were also found to present circadian rhythmicity in the mouse kidney (Nikolaeva et al., 2016; Oda et al., 2014) while SLC22A3 circadian expression has been described in murine mast cells (Nakamura et al., 2017) and SLC22A4 is reported as being rhythmically expressed in mice small intestine (Akamine et al., 2015; Wada et al., 2015). SLC27A1 it's another membrane transporter that has also been reported as being highly expressed in the CP and is responsible for the transport of fatty acids (Dahlin et al., 2009; Ho et al., 2012; Huang et al., 2021). It was described as having circadian variations in mouse epididymal adipose tissue (Bray & Young, 2007). Also, acid sensing ion channel Type 1 (ASIC1) was demonstrated to be rhythmically expressed in male fish skin (Lu et al., 2018).

The influence of the circadian clock on CP transporters is supported by the fact that the expression of many ABC and SLC carrier genes is modulated by nuclear receptors such as constitutive and rostane receptor (CAR), Pregnane X receptor (PXR), PPARα, CCAAT, hepatocyte nuclear factor, and peroxisome proliferator-activated receptor gamma coactivator (Dietrich et al., 2007; Fernández-Veledo et al., 2007; Hirai et al., 2007; More et al., 2017; Wagner et al., 2005), and the expression of many of these nuclear receptors is, regulated by the molecular clock (Gachon et al., 2006; Nakamura et al., 2008; Oishi et al., 2005; Yang et al., 2006).

The BCSFB cannot be neglected as a potential brain gateway for therapeutic drugs due to its architecture, strategic position and highly dynamic transport activity, but the majority of studies regarding CNS pharmacotherapy focused on the BBB.

5. Association between circadian rhythms and central nervous system diseases

CNS disease is a broad category that can include multiple conditions caused by numerous factors. The condition can be inherited, as in the case of metabolic disorders, result of damage or infection, a degenerative condition, result from a tumour or other problems, or even of unknown cause. This includes AD, PD, epilepsy, BT, strokes, traumatic brain injury, infections and migraine (McFaline-Figueroa & Lee, 2018). Several of these diseases have been associated with circadian rhythm disturbances which are both a cause and consequence of the underlying CNS disease

(Rijo-Ferreira & Takahashi, 2019). Chronotherapy, specifically chrono-pharmacology is constantly changing our view of pharmacotherapy. Targeting genes that are cycling at specific times of day may be advantageous for disease treatment (Anafi et al., 2017; Selfridge et al., 2016; Zhang et al., 2014). The US Food and Drug Administration (FDA) has described many drug target proteins (including membrane transporter proteins) whose genes were also reported as showing circadian oscillations in primates (Mure et al., 2018) and humans (Ruben et al., 2018).

Behavioural, hormonal and physiologic circadian rhythms, such as the 24-hour sleep-wake cycle are often disrupted in patients with neurological disorders (Turek et al., 2001). For many years, it was assumed that clinical manifestations of circadian disturbances, such as changes in sleep and wakefulness patterns, were secondary to the underlying neurologic disorder thus resulting in therapies being targeted at symptom treatment (Abbott & Zee, 2019). Recently, this theory has been debunked as the role of circadian clock genes in the regulation of neuronal function gets a growing attention as well as the notion that the disruption of clock function can alter the expression and development of CNS pathologies. As such, circadian disfunction not only plays a major role in disease's pathophysiology but also could be manipulated to improve treatment outcomes.

Healthcare resources are under increasing pressure as CNS pathologies become more prevalent. Among neurodegenerative diseases AD alone represents 60-80% of all dementia cases worldwide (Erkkinen et al., 2018). AD is considered to be a disease of the elderly. With the increase of life expectancy, AD incidence is expected to rise worldwide which represents increased healthcare costs, poorer health in an already vulnerable elderly population and additional pressure over caretakers. As for brain tumours, glioblastoma (GBM) accounts for 14,5% of all CNS tumours and for 48.6% of malignant CNS tumours, being also one of the most aggressive malignancies with an overall median survival of 15 months (Grochans et al., 2022). With 250 000 new GBM diagnostics reported every year, and with this number on the rise, it is imperative that new therapeutic approaches are developed to improve survival rate expectancy (Grech et al., 2020). Both AD and GBM are therefore two major players regarding CNS pathologies and a better understanding of the disease mechanisms and treatment opportunities might be crucial to decrease the burden that both these two pathologies represent for society.

5.1 Circadian rhythms in neurodegeneration: Alzheimer's disease

AD and related dementia affects over 5.8 million people in the USA and over 50 million people worldwide being the most common cause of dementia in old age (Association, 2019). This represents a global cost of over 1.3 trillion US dollars and this is expected to rise to 2.8 trillion US dollars by 2030 (Shin, 2022). Cognitive decline is the hallmark of AD. It is characterized by memory loss, language difficulties and visuospatial deficits (Lane et al., 2018). Neurodegeneration is the ultimate pathological mechanism leading to functional decline and is highly correlated with cognitive decline in patients with AD (Timmers et al., 2019). AD is

characterized by the deposition of amyloid-beta (Aβ) plaques and neurofibrillary tangles constituted by hyperphosphorylated tau protein in the brain (Temmerman et al., 2023). Neuropathology may develop as early as 20 years before any manifestation of cognitive symptoms (Jack et al., 2013). AD patients have been described as experiencing increased daytime sleep and night-time wakefulness (Holth et al., 2019). Aβ plaques are linked to circadian behaviour disruption (Musiek et al., 2018). Aβ levels are associated with robust daily oscillations in the mouse hippocampal interstitial fluid (Kang et al., 2009; Roh et al., 2012). SCN Bmal1 expression has been described as essential for the maintenance of Aβ levels circadian rhythm (Kress et al., 2018). Brain *Bmal1* deletion leads to the loss of Aβ oscillations contributing to a steep increase in Aβ plaque formation (Kress et al., 2018). Recently, AD was also demonstrated to disrupt the CP molecular clock circadian rhythm in mice (Furtado et al., 2020). Tau levels also show circadian rhythmicity (Holth et al., 2019). In mice, brain interstitial fluid tau levels appear to be higher during the active period, while also increasing in sleep deprivation conditions (Holth et al., 2019). In humans, sleep deprivation also leads to increased CSF tau levels (Holth et al., 2019).

There are several FDA approved drugs to help manage AD symptoms and progression (*How Is Alzheimer's Disease Treated?*, 2023). Most of these drugs work better in early and middle stages of the disease. Cholinesterase inhibitors are prescribed to reduce or control some cognitive and behavioural symptoms in mild to moderate AD. FDA has approved three cholinesterase inhibitors for the treatment of AD, galantamine, rivastigmine and donepezil (DNPZ). The transport of this drugs across membranes has been described as dependent of membrane transporters (Namanja et al., 2009; Takeuchi et al., 2016). The principal membrane transporter responsible for the transport of galantamine is ABCB1 (Namanja et al., 2009), ABCG2 is the main DNPZ transporter (Takeuchi et al., 2016), Rivastigmine is an hydrophilic compound and it is yet to be described which membrane transport system is responsible for its diffusion to the brain. ABCB1 is an efflux transporter located in the apical side of the CP (Rao et al., 1999; Saunders et al., 2015). Its expression is much higher in the BBB than the BCSFB (Gazzin et al., 2008) contrarily to ABCG2, a membrane transporter located in the CSF side of the BCSFB (Tomioka et al., 2016), whose expression is much higher in the CP. In moderate to severe AD, FDA has approved memantine. Memantine's main membrane transporters are SLC22A2 (Busch et al., 1998; Müller et al., 2017), SLC9A1 (Mehta et al., 2013a, 2013b), SLC22A4 (Mehta et al., 2013a) and SLC47A1 (Müller et al., 2017). SLC22A2 has been described on the apical side of the CP (Saunders et al., 2015; Sweet et al., 2001), SLC9A1 was proposed to be located on the basolateral side of the CPEC (Davson & Segal, 1970; Kalaria et al., 1998; Segal & Burgess, 1974), SLC22A4 is also expressed in the CP (Sweet et al., 2001) as well as SLC47A1 (Uchida et al., 2015). Two immunotherapeutic drugs (lecanemab and aducanumab) have also received a FDA Accelerated Approval to treat early AD. Lecanemab and aducanumab tend to cross the brain barriers through receptor-mediated transcytosis (Kouhi et al., 2021; Pardridge, 2021).

5.2 Circadian rhythms in brain tumours: Glioblastoma

There are multiple forms of BT with the most common being intracranial metastases, meningiomas and GBM (McFaline-Figueroa & Lee, 2018). Gliomas are the most common form of malignancies of the CNS (Louis et al., 2016). These include astrocytomas, oligodendrogliomas, ependymomas, and several other rare histological presentations (Louis et al., 2016). Grade IV astrocytomas are called GBM and are the most common and aggressive form of glioma (McFaline-Figueroa & Lee, 2018). They make up 15% of all primary BT and 45% of all malignant primary BT (Ostrom et al., 2015). GBM's prognosis is notably poor with a 5-year survival rate of only 4 to 5% and a 2-year survival rate of 26 to 33% (Carlsson et al., 2014; Razavi et al., 2016; Van Meir et al., 2010; Weathers & Gilbert, 2015). GBM's clinical signs include headaches, seizures and focal neurological symptoms and these might develop rapidly due to GBM's aggressive nature (McFaline-Figueroa & Lee, 2018). Diagnosis is usually based on brain magnetic resonance imaging with or without contrast and a biopsy or surgical resection (McFaline-Figueroa & Lee, 2018). For an "integrated diagnosis", a combination of histological appearance and molecular information is used (Louis et al., 2016; McFaline-Figueroa & Lee, 2018). There have been several preclinical studies that aimed at assessing if timing chemotherapy or radiotherapy treatment would increase its efficacy and/or reduce its side effects but there is currently no evidence that supports this theory (Nelson & Relógio, 2024; Shuboni-Mulligan et al., 2019).

Circadian disruption has been linked to increased cancer susceptibility (Fu & Kettner, 2013; Fu & Lee, 2003; Yu & Weaver, 2011). *Per1, Per2 and Per3* polymorphisms are often found in human cancers (Fu & Kettner, 2013) and oncogenic MYC was reported to disrupt the molecular clock (Altman et al., 2015). There is also a cause/effect relation between the molecular clock disruption and cancer development (Rijo-Ferreira & Takahashi, 2019). *Per2* and *Bmal1* mice knockout contributed to lung tumorigenesis, and this was followed by an increase in c-Myc expression, proliferation, and metabolic disruption (Papagiannakopoulos et al., 2016). Chronic jet lag was also found to induce hepatocellular carcinoma in mice and disease evolution was very similar to that observed in obese humans (Kettner et al., 2016). *Clock* and *Bmal1* suppression via microRNA miR-211 also promoted tumour progression (Bu et al., 2018) while the use of REV-ERB agonists demonstrated to be beneficial in cancer treatment (Sulli et al., 2018).

Currently, besides surgery and radiotherapy, the chemotherapeutic approach for the treatment of GBM is limited to temozolomide, carmustine and lomustine (Zhang et al., 2019). Bevacizumab, which is a vascular endothelia growth factor ligand approved by the FDA is used as adjuvant therapy for angiogenesis inhibition (Zhang et al., 2019). Temozolomide's membrane transporters are ABCB1 and ABCG2 (de Gooijer et al., 2018; Lin et al., 2014; Munoz et al., 2015) with no membrane transporters reported for the other therapeutic agents. In recent years, there has been an attempt to, besides developing new therapeutic agents, use the existing chemotherapeutic agents in the treatment of GBM. One of the compounds at the forefront of GBM

treatment is methotrexate (MTX) (Battaglia et al., 2017; Madani et al., 2020; Pereira et al., 2018; Z. Ye et al., 2018). MTX is a competitive inhibitor of folic acid synthesis and prevents the synthesis of purines and pyrimidines, decreasing tumour cell growth (Kaye, 1998). MTX transporters are ABCC3, ABCC4, ABCC1, SLC22A6, ABCC10, SLC22A8, ABCC2, ABCB1, SLCO1A2, SLC16A1, ABCC11, SLCO1B3, SLC22A11, SLCO1C1, SLCO3A1, ABCG2, SLC22A7, SLC46A1, SLCO1B1, SLCO4C1, SLC19A1, SLC15A1, SLC36A1, folate receptor alpha and beta (FOLR1 and FOLR2, respectively) (Abe et al., 2001; Adachi et al., 2003; Akita et al., 2002; Bai et al., 2004; Bakos et al., 2000; Breedveld et al., 2004; Cattori et al., 2001; Cha et al., 2001; C. Chen et al., 2003; Chen et al., 2005; Z. S. Chen, E. Hopper-Borge, et al., 2003; Chen et al., 2001; Z. S. Chen, R. W. Robey, et al., 2003; Dai et al., 2009; Han et al., 2001; Heijn et al., 1997; Hirohashi et al., 1999; Hooijberg et al., 1999; Hou et al., 2009; Inoue & Yuasa, 2014; Kusuhara et al., 1999; Kuze et al., 1999; Li et al., 2003; Lu et al., 1999; Masuda et al., 1997; Mikkaichi et al., 2004; Mitomo et al., 2003; Nakai et al., 2007; Norris et al., 1996; Ohtsuki et al., 2004; Oleschuk et al., 2003; Paumi et al., 2003; Pizzagalli et al., 2002; Qiu et al., 2006; Rius et al., 2003; Sekine et al., 1998; Sekine et al., 1997; Sharma et al., 2008; Sun et al., 2001; Suzuki et al., 2009; Suzuki et al., 2003; Takeda et al., 2002; Tamai et al., 1999; Tiwari et al., 2009; Uwai & Iwamoto, 2010; Uwai et al., 1998; van Aubel et al., 2002; van de Steeg et al., 2009; VanWert & Sweet, 2008; Volk & Schneider, 2003; Zehnpfennig et al., 2009; Zeng et al., 2001; Zeng et al., 2000). From these, the ones expressed on the CP are ABCB1, ABCC1, ABCC2, ABCC3, ABCC4, ABCC10, ABCG2, SLC15A1, SLC16A1, SLC19A1, SLC22A6, SLC22A8, SLC36A1, SLC46A1, SLCO3A1, SLCO1C1, FOLR1 and FOLR2 (Agulhon et al., 2003; Alebouyeh et al., 2003; Bernd et al., 2015; Gazzin et al., 2008; Hinken et al., 2011; Ho et al., 2012; Kratzer et al., 2013; Morris et al., 2017; Nagata et al., 2002; Ohtsuki et al., 2003; Olney et al., 2022; Ramaekers et al., 2018; Rao et al., 1999; Roberts et al., 2008; Saunders et al., 2015; Sugiyama et al., 2003; Sykes et al., 2004; Tomioka et al., 2016; Ulloa et al., 2019; Wollack et al., 2008). All this information regarding both AD, BT and all the other CNS diseases is resumed in Attachment 1.

6. Chronotherapy: the key to bypass the BCSFB

Biological rhythms are present in both behaviour and metabolism over a 24-hour period (De Giorgi et al., 2013). One of the processes influenced by the molecular clock is the permeability of the BBB and BCSFB (Christensen et al., 2022; Zhang et al., 2018). Chronotherapy goal is to optimize medical treatments by taking into consideration the individual's circadian rhythms. Chronotherapy has developed into two distinct approaches, one that aims at resynchronizing the normal circadian patterns and another that focus on taking advantage of the organisms' circadian rhythms to improve treatment efficacy and outcome. Both of these chronotherapeutic approaches take advantage of windows of optimal treatment time that are dependent of the bodies circadian rhythms controlling cellular mechanisms essential for treatment sensitivity (Y. Ye et al., 2018). The differences in the phase and amplitude of clock genes rhythms as well as cellular mechanisms under circadian control can change between different tissue types (cancer cells might respond differently than healthy ones to treatment) (Shuboni-Mulligan et al., 2019). Several

reports support the first approach aimed at resynchronizing the normal circadian patterns (Holth et al., 2019). Using a mice model of amyloid pathology, it was possible to demonstrate that pharmacological resynchronization of the molecular clock restored normal circadian patterns of sleep-wake cycles and reduced Aβ burden in both the prefrontal cortex and hippocampus (Sundaram et al., 2019). Using environmental stimuli (light and exercise) or even melatonin to resynchronize circadian patterns in AD patients, proved to be beneficial by reducing the circadian symptoms and ameliorate cognitive parameters (Coogan et al., 2013). When treating GBM and glioma, timing temozolomide administration with *Bmal1* peak expression (morning period), improved treatment efficiency (Damato et al., 2022; Damato et al., 2021; Slat et al., 2017). *Bmal1* expression in GBM and glioma cells is associated with increased temozolomide-induced DNA damage, apoptosis and growth inhibition (Slat et al., 2017). The second chronotherapeutic approach aims at timing drug administration with the patient biological rhythm to improve its bioavailability at the site of action and thus reducing side effects and improving outcome. Chronotherapy proposes that we take advantage of the possible circadian variability of the BCSFB permeability, by timing drug administration, to improve CNS disease treatment. The CP has multiple membrane transporters that are directly involved in the transport across the BCSFB of numerous drugs essential for the treatment of CNS diseases such as AD and BT. Some of these transporters have already been demonstrated to be rhythmically expressed in multiple tissues and have also been implicated in the transport of drugs used in the treatment of both AD and BT. So, by timing drug administration with these circadian rhythms we might improve chronotherapy of CNS's diseases (Figure 3). We aim at further explore this theory along this doctoral thesis.

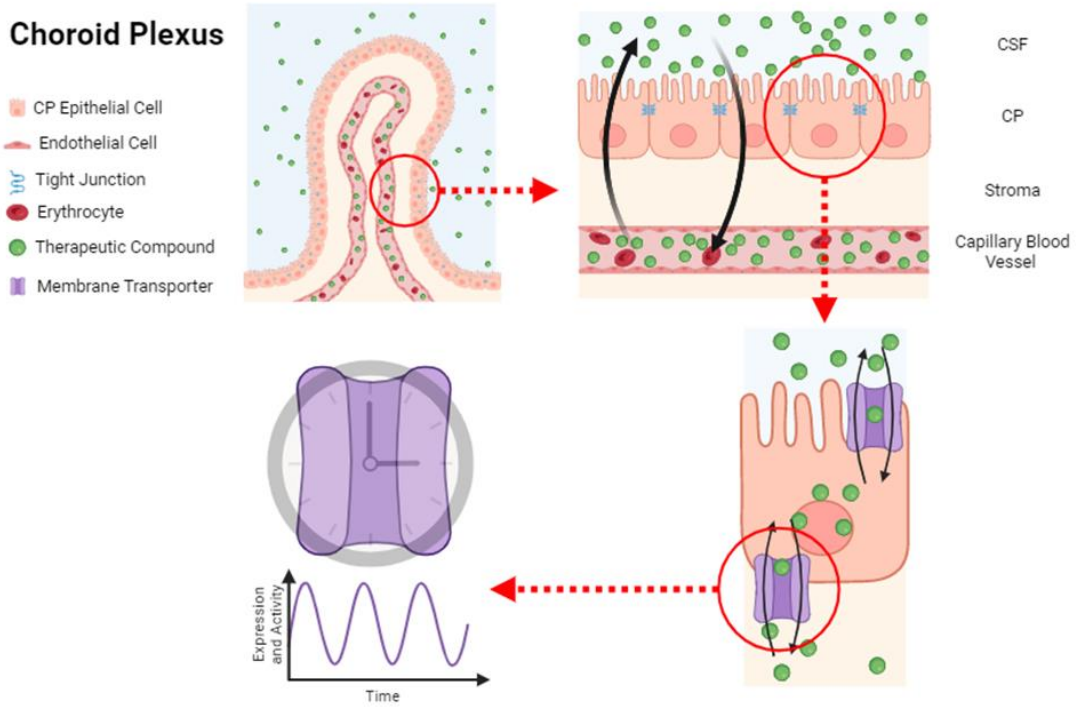

Figure 3. Circadian regulation of CP's membrane transporters expression and activity at the BCSFB. We hypothesize that circadian rhythms influence both the expression and the transport activity of membrane transporters in the CP. This influences the circadian flux of therapeutic compounds across the BCSFB.

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Chapter 2

Global Aims

1. Global aims

Animal behaviour and physiological processes display circadian rhythmicity. This rhythmicity has been proved to be dependent of a molecular clock. Recently, it has been shown that a functional molecular clock is present in the CP. The CP at the BCSFB, together with the blood-brain barrier, are two brain barriers separating the bloodstream from the CNS. These structures act as gatekeepers that protect the CNS from noxious compounds and pathogens but also compromise pharmacological treatment of many CNS diseases. These barriers also possess mechanisms to transport compounds into and out of the CNS. These include membrane transporters which are vital for CNS protection against toxins and exogenous compounds, but also for CNS nourishment and for detoxification of the CNS from metabolism products. The CP has been proved to express a large variety and quantity of membrane transporters, responsible for transporting a variety of substrates into and out of the CNS and some have been also associated with the transport of drugs and therapeutic molecules.

There are multiple brain diseases that compromise health, including neurodegenerative diseases such as AD and BT where GBM is included. Pharmacological treatment of these diseases is hindered by the brain barriers that prevent the therapeutic compounds from reaching their site of action in the CNS. Some of the drugs used in the treatment of these diseases are substrates of several membrane transporters. The CP has also been proved to possess a functional molecular clock that might influence the circadian expression of these membrane transporters. In multiple tissues, this association between membrane transporters and rhythmic transport of therapeutic compounds across membranes, has already been described. Chronotherapy is a novel therapeutic approach intended to take advantage of the organism circadian rhythm to improve disease treatment efficacy and outcome. As such, and considering the most recent chronobiological data, it has been gaining strength as a way to improve current pharmacological therapy of the CNS.

Taking this into account, the main objective of this doctoral thesis is to disclose if circadian rhythms directly influence the expression and function of some relevant membrane transporters expressed in the BCSFB.

The specific aims of this thesis are:

-Confirm which membrane transporters are expressed in the BCSFB;

-Disclose which of the membrane transporters present in the BCSFB are expressed in a circadian way;

-Analyse the circadian transport of some therapeutic compounds, MTX and DNPZ, across the BCSFB;

-Evaluate the influence of the circadian expression of CP membrane transporters in the transport of MTX and DNPZ.

Chapter 3

Research Work 1
Circadian Expression of Membrane Transporters at the BCSFB

1. Introduction

Brain is protected from the peripheral environment by two major barriers, the BBB and the BCSFB. The mechanisms used by these barriers to protect the brain, also hinder the pharmacological treatment of CNS diseases (Kadry et al., 2020; Rubio-Perez et al., 2021). The BBB and the BCSFB are different at both the morphological and functional level (Kung et al., 2022). The BCSFB is constituted primarily by the CP which is comprised by a monolayer of cuboidal epithelial cells that line the brain ventricles. In the apical side it presents microvilli in contact with the CSF and on the basal side it is irrigated by a vast network of fenestrated capillary blood vessels. Besides constituting a selective barrier to the passage of molecules, cells, toxins and other compounds to and from the CNS, it is also responsible for CSF production and secretion, chemical surveillance and detoxification of the CSF, protein production including Aβ scavengers and is also a peripheral circadian clock (Costa-Brito et al., 2021; Duarte, Furtado, et al., 2020; Duarte, Rosado, et al., 2020; Duarte, Santos, et al., 2020; Furtado et al., 2022; Quintela et al., 2021; Quintela et al., 2015).

Circadian timekeeping is an essential mechanism in every living organism (Roenneberg & Merrow, 2016). In accordance with earths 24-hour day period, it gives structure to behavioural and physiological processes. Circadian clocks are autonomous and produce circadian rhythms even in the absence of circadian environmental cues (Roenneberg & Merrow, 2016). At the top of the mammalian circadian clock hierarchical structure is the SCN of the hypothalamus. Positive and negative feedback loops govern the timekeeping process (Takahashi, 2017). BMAL1 forms a dimmer complex with CLOCK and together promote the transcription of multiple protein genes including *Per* (*Per1, Per2* and *Per3*) and *Cry* (*Cry1* and *Cry2*) (Takahashi, 2017). A second dimmer is formed by PER and CRY that inhibits the BMAL1/CLOCK dimer (Takahashi, 2017). This promoter and inhibitory loops also control the transcription of several clock controlled genes (CCG) (Takahashi, 2017).

CNS disorders are a broad group of disorders which encompass conditions such as AD, BT, migraine, neurological infections and multiple others. In most of the cases, the pharmacological treatment of CNS disorders is hindered by the two brain barriers. Focusing on the CP, this barrier function is one of its most recognised roles (Ghersi-Egea et al., 2018). It possesses several membrane transporter proteins, tight junctions and detoxification enzymes, enabling the CP cells to control the traffic of molecules across the BCSFB (Santos et al., 2019). Membrane transporters from SLC and SLCO transporter families have been described in the CP (Quintela et al., 2021). Direction of substrate transport is dependent of the specific membrane transporter at stake (Quintela et al., 2021; Schulz et al., 2023; Sweet, 2021). These transporters are responsible for the pharmacokinetics of multiple therapeutic drugs in a variety of cell types (Mohammad et al., 2018), controlling the drug levels across the body during a therapeutic protocol. At the BCSFB multiple transporters have been identified such as SLC9A1 (NHE1), SLC47A1 (MATE1) and SLCO1A2 (OATP1A2) (homologous to SLCO1A5 transporter in rodents (Song et al., 2020))

(Christensen et al., 2013; Ghersi-Egea et al., 2018; Lamhonwah et al., 2008; Uchida et al., 2015). SLC9A1 is expressed in the luminal membrane of the BCSFB (Damkier et al., 2009). SLC9A1 might also be expressed in the basolateral membrane if there's a need to compensate for high levels of NA^+ at that side of the BCSFB (Damkier et al., 2009). SLC9A1 substrates are transported to the CSF (Barar et al., 2016). SLC47A1 can mediate both influx and efflux transport of its substrates and its localization in the CP membrane is still unidentified (Uchida et al., 2015). Finally, SLCO1A2 (or its homologous SLCO1A5 in rodents) is an uptake transporter and it has been described on the apical membrane of rodent CP (Hagenbuch & Meier, 2003; Kusuhara et al., 2003; Ohtsuki et al., 2004; Urquhart & Kim, 2009). Both SLC9A1 and SLC47A1 have been described as an integral part of memantine's transport across biological barriers (Mehta et al., 2013a, 2013b; Müller et al., 2017). Memantine is a N-methyl-D-aspartate receptor antagonist widely used in the management of AD (Reisberg et al., 2003). On the other hand, SLCO1A2 has been described one of the membrane transporters responsible for the transport of both dabrafenib and MTX across biological barriers (Cattori et al., 2001; Ellens et al., 2017). Both these drugs are used in the treatment of brain tumours, namely intracranial metastases and GBM, respectively (Gorka et al., 2018; Ye et al., 2018). SLC47A1 is also involved in the transport of ciprofloxacin which is an antibiotic used in the treatment of brain abscesses (Bonvin et al., 1998; Ohta et al., 2009; Tanihara et al., 2007) and acyclovir which is a purine analogue used in the treatment of viral meningitis and encephalitis (Davis, 2008; Kennedy, 2004; Nies et al., 2012; Xu et al., 2015) while SLCO1A2 is also responsible for the transport of non-steroidal anti-inflammatory drugs such as ibuprofen and naproxen (Shitara et al., 2002), and sumatriptan which is a serotonin receptor agonist (Lu et al., 1996). All these three drugs are used in the treatment of migraine. Refer to the Attachment 1 for more information regarding CNS diseases, pharmacological treatment and membrane transporters expressed at brain barriers.

These membrane transporters are therefore contributing to the pharmacokinetics of therapeutic drugs for treating CNS diseases. Taking this into account, it becomes essential to study the circadian expression of these membrane transporters at the BCSFB.

2. Material and methods

2.1. Animals

This study was conducted with the approval of the Animal Welfare and Ethics Committee of the Health Science Research Centre of the University of Beira Interior, in compliance with National and European Union rules for the care and handling of laboratory animals. No further licensing was required as this study only required the collection of animal tissues without animal experimentation. The animals used for tissue collection were newborn *Wistar Han* rats which were housed in appropriate cages, at constant room temperature, in a 12-hour light/ 12-hour dark photoperiod and given standard laboratory chow and water *ad libitum*. Efforts were made to minimize the number of used animals and animal suffering.

All CPs included in this work were collected from the lateral ventricles of 2-7 day-old postnatal rats.

2.2. Choroid plexus epithelial culture

Thirty postnatal animals were anesthetized on ice for at least 30 minutes before being decapitated. The CPs were collected from the lateral ventricles and used to establish CPEC primary cultures as previously described by *Gonçalves et al* (Gonçalves et al., 2019). Dissociated cells were seeded into 6-well culture plates and cultured in high-glucose Dulbecco's modified eagle medium (DMEM) supplemented with 5µg/mL insulin (Sigma-Aldrich, Merck, Portugal), 100 U/mL penicillin, 100 µg/mL streptomycin, 10% v/v fetal bovine serum (FBS), 10ng/mL epidermal growth factor (Sigma-Aldrich, Merck, Portugal) and 30 µM cytosine arabinoside (Sigma-Aldrich, Merck, Portugal). Cultures were maintained in a humidified incubator at 37 ºC and 5% CO_2. Culture medium was replaced at day *in vitro* 1 (DIV1) and every 2 days thereafter. All studies were conducted using cultures established for at least 4-5 days.

2.2.1. Membrane transporters circadian pattern

CPEC primary cultures established for at least 4-5 days were trypsinized and seeded in 24-well culture plates (approximately 1,5 x 10^4 cells/well). Culture medium was changed every 2 days and experiments were conducted 8 days after seeding. CPEC were synchronized with 100nM dexamethasone (Sigma-Aldrich, Merck, Portugal) for 2 hours (Woo et al., 2010). The culture medium was changed, and cells were collected 4 hours after synchronization and then every 4 hours during a 24-hour period.

2.2.1.1. Quantitative real-time PCR (qPCR)

Total ribonucleic acid (RNA) was isolated from the CPEC using tripleXtractor reagent (Grisp, Porto, Portugal) according to the manufacturer's instructions. Total RNA purity and integrity were assessed by the measurement of the absorbances at 260 and 280 nm using a NanoPhotometer™ (Implen, Munich, Germany). NZY M-MuLV Reverse Transcriptase (NZYTech Ltd., Portugal), Random hexamer mix (NZYTech Ltd., Portugal), GRS dNTP mix (GRISP Ltd., Portugal) and RNA (500ng) were used for complementary desoxyribonucleic acid (cDNA) synthesis following manufacturer's instructions.

Quantitative real-time PCR (qPCR) was used to assess the daily expression of rSlc9a1, rSlc47a1 and rSlco1a5. Rat cyclophilin A (rCyc) was used as housekeeping gene. Primers sequences are listed in Table 1. qPCR was performed using a CFX-Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using an Xpert Fast SYBR 2x master mix (Grisp, Porto, Portugal). The qPCR protocol consisted of an initial 3-minute denaturation step at 95 ºC, followed by 40 cycles of 5 seconds at 95 ºC, 30 seconds at 62 ºC and 10 seconds at 72 ºC. The transcripts amplification was validated by the profiles of melting curves. All primers were

previously tested with the following cDNA dilutions: stock, 1:2, 1:4, 1:8. The ΔCt was calculated using the housekeeping gene as the reference gene, and the ΔΔCt was calculated between the normalized ΔCt values from each time point and the average Ct of all the time points tested.

Table 1. Primers used in RT-PCR and quantitative real-time PCR

Gene	FW	pb	Ref
rSlc9a1	FW- CACATCAATGAGCTGCTGC RV- GCTGGCAAACTCCTCAAAG	99	(Monazzami et al., 2017)
rSlc47a1	FW- CTCTTCATCAACACCGAGCA RV- ACCCATCACCCCAAGATGTA	249	(Komazawa et al., 2013)
rSlco1a5	FW- CTGAAGAGAAGTCGCTTGGGA RV- CGGGCTCACCACATTTCAGG	138	
rCyc	FW- CAAGACTGAGTGGCTGGATGG RV- GCCCGCAAGTCAAAGAAATTAGAG	163	(Duarte, Furtado, et al., 2020)

2.3. Statistical analysis

A normality test was performed to ensure a normal distribution (Shapiro–Wilk normality test). CircWave v1.4 analysis software (Dr. Roelof A. Hut) was used to analyse the rhythmicity of rSlc9a1's, rSlc47a1's and rSlco1a5's expression by a harmonic regression method, with an assumed period of 24 h and an alpha set at 0.05,

3. Results

3.1. Membrane transporters circadian expression in CPEC

Circadian expression of membrane transporters of Slc9a1, rSlc47a1 and rSlco1a5 was assessed. Both rSlc9a1 and rSlco1a5 showed significant circadian variation (CircWave, $p<0.05$). In opposite to these transporters, rSlc47a1 did not reveal a significant circadian variation (CircWave, $p>0.05$) (Figure 1, Table 2).

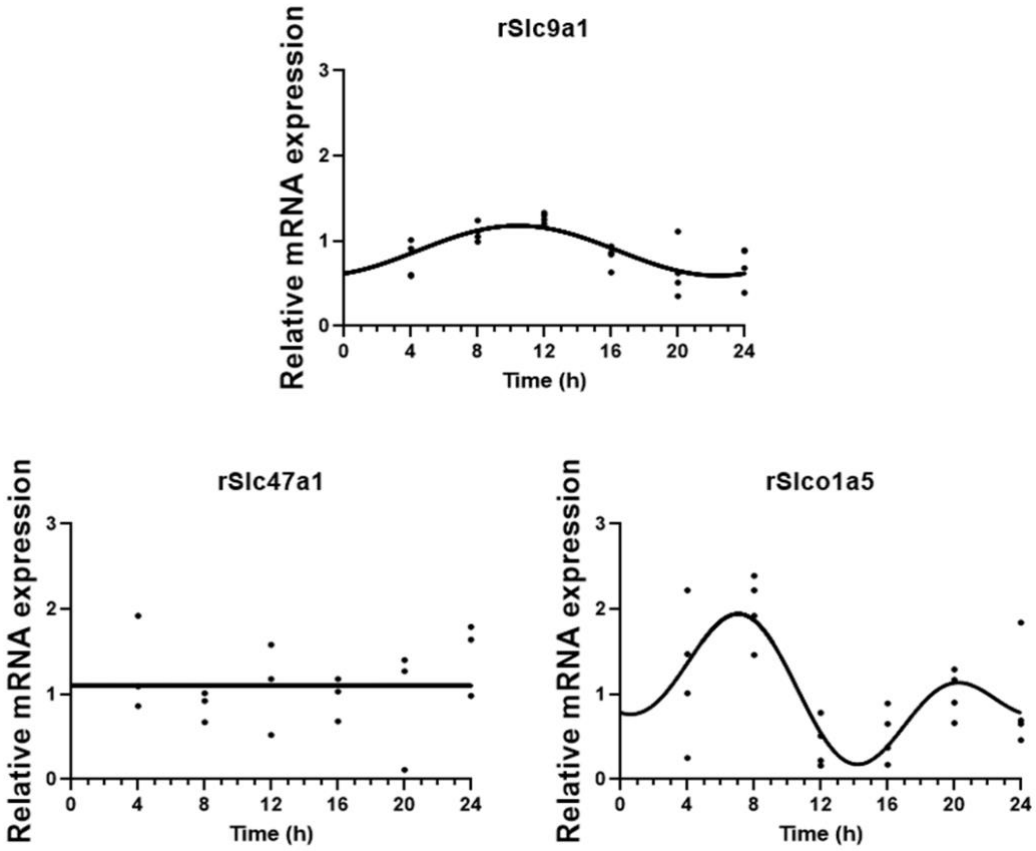

Figure 1. CircWave analysis of rat rSlc9a1, rSlc47a1 and rSlco1a5 membrane transporter genes circadian expression in CPEC. rSlc9a1, rSlc47a1 and rSlco1a5 expression was analysed every 4-hours during a 24-hour period in synchronized CPEC. Sine-cosine fit represents a significant 24-hour period oscillation ($p<0.05$). Statistical analysis is shown in Table 2.

Table 2. Significance (*p*-value) and center of gravity values (COG) for rSlc9a1, rSlc47a1 and rSlco1a5 as determined by CircWave analysis.

Gene	rSlc9a1	*p*-value= 0.0002 COG= 10.41
	rSlc47a1	*p*-value>0.05 COG= 1.47
	rSlco1a5	*p*-value= 0.0028 COG= 5.10

4. Discussion

The transport of therapeutic molecules for CNS diseases therapy across the BCSFB is partially ensured by transporter proteins located in the CPEC membrane. Therefore, if a membrane transporter's activity is controlled by circadian rhythms, pharmacological treatment with their known substrates must be scheduled accordingly. This practice should greatly improve drug delivery and efficacy. Thus, the study of membrane transporters' circadian rhythms and their molecular mechanisms is essential for improving CNS disease treatment and outcome.

In the present study we evaluated the expression of membrane transporters rSlc9a1, rSlc47a1 and rSlco1a5. Both rSlc9a1 and rSlco1a5 display a circadian pattern in CPEC while rSlc47a1 did not show a circadian expression.

rSlc9a1 showed rhythmicity in synchronized CPEC during a 24-hour period, contrary to the results reported by Cheng *et al.* that revealed no circadian rhythmicity of mRNA expression of Slc9a1 in the SCN of rats (Cheng et al., 2019).

The absence of a circadian expression of Slc47a1 has already been reported in mice kidney and (Oda et al., 2014) liver (Henriksson et al., 2017; Zhang et al., 2009). Our results are in agreement, as no significant circadian rhythm were found in rSlc47a1 expression in synchronized CPEC.

No circadian rhythmicity was reported in Slco1a5 expression in the rat jejunum (Vagnerová et al., 2019). Contrary, our results showed a daily circadian expression of rSlco1a5 in synchronized CPEC.

As we know, there are two major barriers protecting the CNS from exogenous compounds present in the bloodstream and this includes not only toxic molecules but also drugs intended for CNS diseases therapy. These two major barriers are the BCSFB and the BBB. Slc9a1, Slc47a1 and Slco1a5 are all expressed in the BBB (Daneman et al., 2010; Liktor-Busa et al., 2020; Suhy et al., 2017).

Slc9a1 has been reported to be expressed in the basolateral membrane of the BBB while in the BCSFB it has been identified in the apical membrane of the epithelial cells (Christensen et al., 2013; Lam et al., 2009). This membrane transporter is associated with the efflux of its substrates (Wen et al., 2023). As such, it seems that at the BCSFB and the BBB, this membrane transporter shares the same function as the direction of transport of its substrates is from the CNS towards the blood stream.

Slc47a1 was described in the abluminal membrane in the BBB (Nilles et al., 2022). While its localization is still to be confirmed in the BCSFB, there is some indications that it might be localized in the basolateral membrane (Damkier & Praetorius, 2020). SLC47A1 is responsible for efflux transport (Damkier & Praetorius, 2020). As such it is possible that this transporter might have antagonistic functions between both barriers. In the BBB it is actively transporting its substrates from the blood stream towards the brain. If its basolateral localization is confirmed in the CP, it should be responsible for the transport of its substrates away from the CNS and into the blood stream.

Slco1a5 has been described as an uptake transporter that is localized in the apical membrane of the BCSFB (Damkier & Praetorius, 2020). Its BBB localization is still to be disclosed while some studies have also reported that its abundance is much greater in the CP epithelium than other tissues such as the kidney, the ileum and the liver (Choudhuri et al., 2003). If the relative

expression of this membrane transporter is also more abundant at the BCSFB than the BBB, this might suggest that this transporter activity has a greater impact at the BCSFB than the BBB.

A recent study has also disclosed the presence of a functional molecular clock at the BBB. Circadian rhythmic expression at the BBB of several molecular clock genes and CCG have been observed (Zhang et al., 2021). BMAL1, PER, nuclear receptor subfamily 1 group D member 1 (Nr1d1), albumin D-box binding protein (Dbp), hepatic leukaemia factor (Hlf) and thyrotroph embryonic factor (Tef) circadian expression was verified in mouse brain endothelial cells (Zhang et al., 2021). Bmal1 circadian rhythmic expression was also documented in the brain microvessels of Wistar rats (Szczepkowska et al., 2021). At the moment there are a limited number of studies focusing on the analysis of circadian rhythms at the BBB. This means that none of the membrane transporters studied in this thesis have been targeted for the assessment of circadian rhythmic expression or activity at the BBB. In the future, this shortcoming must be addressed to give a better understanding on how the BCSFB and BBB membrane transporters circadian expression and activity influence the brain homeostasis and the transport of drugs for the treatment of CNS diseases.

Brain barriers are known to be an obstacle for CNS diseases' pharmacotherapy. In this study we have found that several SLC transporters that are fundamental to drug uptake and excretion from the CNS are rhythmically expressed in CPEC. The circadian rhythmicity of these membrane transporters is possibly tissue and species dependent (Pácha et al., 2021). Several drugs essential for the management of CNS diseases such as AD and GBM have been demonstrated to be transported across biological membranes by these transporters. Estimation of each membrane transporter's specific circadian rhythm is then crucial to better understand the mechanisms governing BCSFB permeability along a 24-hour period. It is also well documented that drugs pharmacokinetic, pharmacodynamic and toxicological profiles vary depending on the time of the day of administration (Pácha et al., 2021). This might not only reflect time dependent blood-flow changes and drug metabolism but also the role of the circadian changes in membrane transporters expression. It is already well described the influence of circadian clocks over several biological processes. Considering this, CP circadian clock seems to have a marked impact on membrane transporters expression which in turn could influence drugs flux through the BCSFB. This could be another clue regarding drugs apparent time-dependent efficacy and toxicity when treating CNS diseases. Thus, a better understanding of drug transporters and their respective tissue-dependent circadian expression and activity are essential for a better and safer drug therapy.

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Chapter 4

Research Work 2
The Daily Expression of ABCC4 at the BCSFB Affects the Transport of its Substrate Methotrexate

This chapter corresponds to the original research article:

Furtado, A., Mineiro, R., Duarte, A. C., Gonçalves, I., Santos, C. R., & Quintela, T. (2022). The Daily Expression of ABCC4 at the BCSFB Affects the Transport of Its Substrate Methotrexate. *Int J Mol Sci*, *23*(5). https://doi.org/10.3390/ijms23052443

1. Introduction

To adapt to the environmental changes, living organisms have developed circadian rhythms, which, ultimately, correspond to daily oscillations in biological processes (Bell-Pedersen et al., 2005; Bhadra et al., 2017; Panda et al., 2002). The mammalian circadian system is conceptualized in a hierarchical way, where the SCN of the hypothalamus operates as the master clock. The SCN is responsible for receiving light information via the optic nerve and for synchronizing the remaining clocks in the body (Hastings et al., 2018). At the cellular level, CLOCK and BMAL1 proteins, form a complex which promotes the transcription of many genes, including the negative regulators, *Per* (*Per1*, *Per2* and *Per3*) *and Cry* (*Cry1* and *Cry2*). Subsequently, PER and CRY proteins interact with CLOCK-BMAL1 complexes repressing its own transcription and the transcription of many clock-controlled genes (Takahashi, 2017).

The circadian system has impact on the disposal and action of drugs, determining the efficacy and toxicity of several therapeutic agents (Pácha et al., 2021; Zhao et al., 2020). This evidence lend support to the idea that daily variations of drug-metabolizing enzymes and transport systems may interfere with drug pharmacokinetics, particularly in the absorption, distribution, metabolism, and elimination mechanisms (Ballesta et al., 2017; Baraldo, 2008; Gaspar et al., 2019). In consequence, some authors have raised the interest in studying and understanding the molecular pathways for circadian control of detoxifying enzymes and specific influx/efflux transporters to enhance therapeutic efficacy and minimize side effects (Ayyar & Sukumaran, 2021; Ballesta et al., 2017; Gaspar et al., 2019; Zaki et al., 2019) by adjusting drug administration scheduling to circadian rhythms.

The CPs are part of the ventricular system of the brain, located in each of the four brain ventricles. Each CP is composed by a monolayer of cuboidal epithelial cells that lay in a basement membrane. Below, in the stroma resides a network of fenestrated capillaries surrounded by connective tissue and immune system cells (Santos et al., 2017). The primary role of CP is to produce the CSF, but the CP also forms an important barrier between blood and CSF, the BCSFB (Ghersi-Egea et al., 2018), which has been overlooked for years. The presence of tight junctions, detoxification enzymes, and membrane transporters in the epithelial cells, enable the CP epithelia to control the traffic of molecules, including therapeutic agents, into the CNS (Santos et al., 2019). Another important function of the CP, is that it holds a circadian clock composed by clock genes which are under circadian regulation with pronounced differences between male and female rats (Quintela et al., 2018; Quintela et al., 2015), and overall susceptibility to sex hormones.

ABC and SLC transporters are two families of membrane proteins responsible for the extrusion of molecules out of the cells, and are widely known by their contribution for pharmacoresistance in many cell types, where they are able to recognize and extrude a vast array of therapeutic drugs (Mohammad et al., 2018). At the BCSFB, previous literature described the presence of several multispecific ABC (ABCB1, ABCG2, ABCC1 and ABCC4) and SLC (OAT3) transporters (Morris et al., 2017). The selective expression of these transporters at the basolateral

membrane (ABCC4; ABCC1) are thought to be involved in the extrusion of molecules back to the blood stream preventing their entry to the CNS (Ishiwata et al., 2005; Markos Leggas et al., 2004). At the apical membrane ABCB1 and ABCG2 transporters transfer molecules from the epithelial cells to the CSF.

The presence of a circadian oscillator at the CP and the involvement of this tissue in the traffic of molecules from blood to the CSF and consequently to the brain due to the presence of membrane transporters, holds promise for the concertation of the circadian pattern of efflux transporters with the best timing for drug administration. For that, a deep knowledge of how the circadian rhythms tune the expression and function of CP membrane transporters is required. Thus, in the present study we explored the daily oscillation of ABC and SLC transporters in rat CP in a sex dependent way and investigated the relevance of Abcc4 circadian expression in the transport of MTX across the BCSFB.

2. Results

2.1. Sex-dependent daily oscillations of membrane transporters in rat choroid plexus

To analyze if the CP membrane transporters exhibit a gender-specific daily oscillation, the temporal expression profile of several transporters was assessed by qPCR in the CP of male and female rats.

The circadian mRNA expression profiles were obtained for rAbca1, rAbcb1, rAbcc1, rAbcc4, rAbcg2, rAbcg4, and rOat3 (Figure 1). rAbca1, rAbcb1 and rAbcg4 mRNA expression did not show significant daily oscillation. On the contrary, Abcc1 mRNA expression in male rats showed a significant daily oscillation (CircWave, $p<0.05$), with a peak during the light phase, between ZT4 and ZT5 (Figure 2, Table 1). The mRNA levels of Abcc4 also showed a significant daily oscillation, but in this case in both intact male and female rats (CircWave, $p <0.05$), with a peak level around ZT14 and ZT7, respectively. A significant daily oscillation of rAbcg2 was also verified in intact female rats (CircWave, $p <0.05$), with a peak level around ZT14. Last, rOat3 mRNA levels in intact male and female rats exhibited a significant daily oscillation (CircWave, $p<0.05$), with a peak of expression during the first half of the dark phase between ZT17 and ZT19 (Figure 2, Table 1).

rAbca1, rAbcb1, rAbcc1, rAbcc4, rAbcg2, rAbcg4 and rOat3in both Sham and OVX groups, did not show significant daily oscillations.

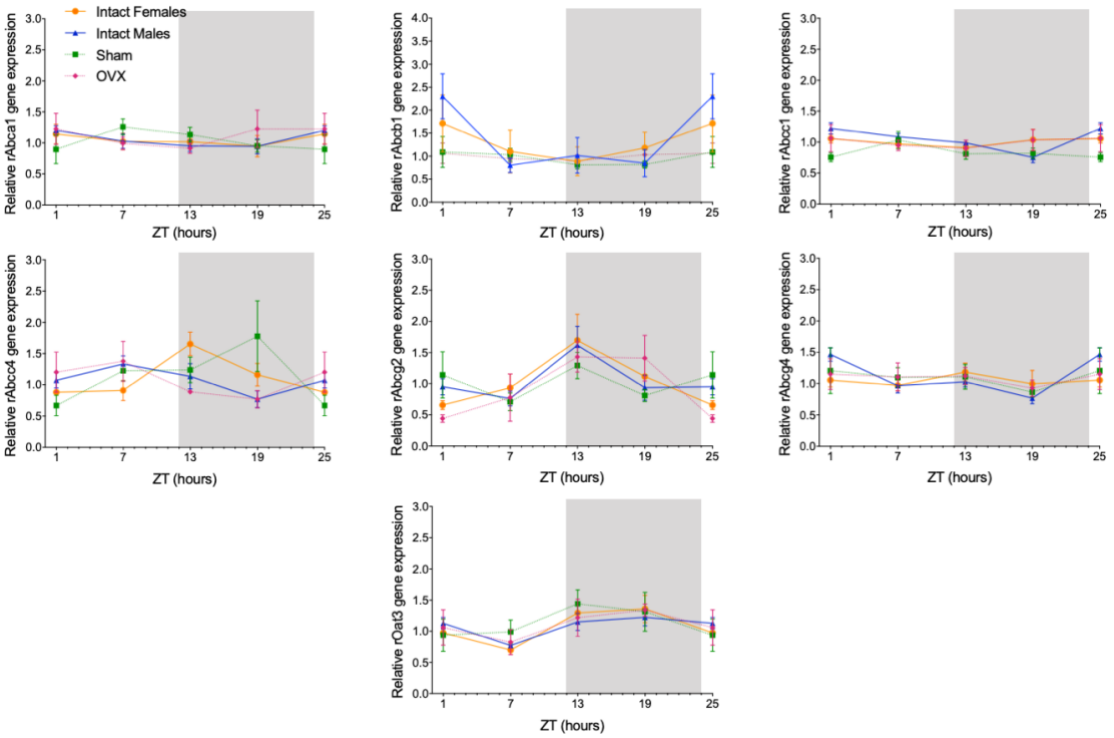

Figure 1. Circadian transcription profiles of membrane transporters in the CP of intact males, intact females, ovariectomized (OVX), and sham-operated female rats (Sham). rAbca1, rAbcb1, rAbcc1, rAbcc4, rAbcg2, rAbcg4, and rOat3mRNA circadian expression were analyzed. White and grey backgrounds represent the day and night periods, respectively. Panel shows the mean ± SEM transcript levels (n=3-6), and data from ZT1 and ZT25 are double plotted.

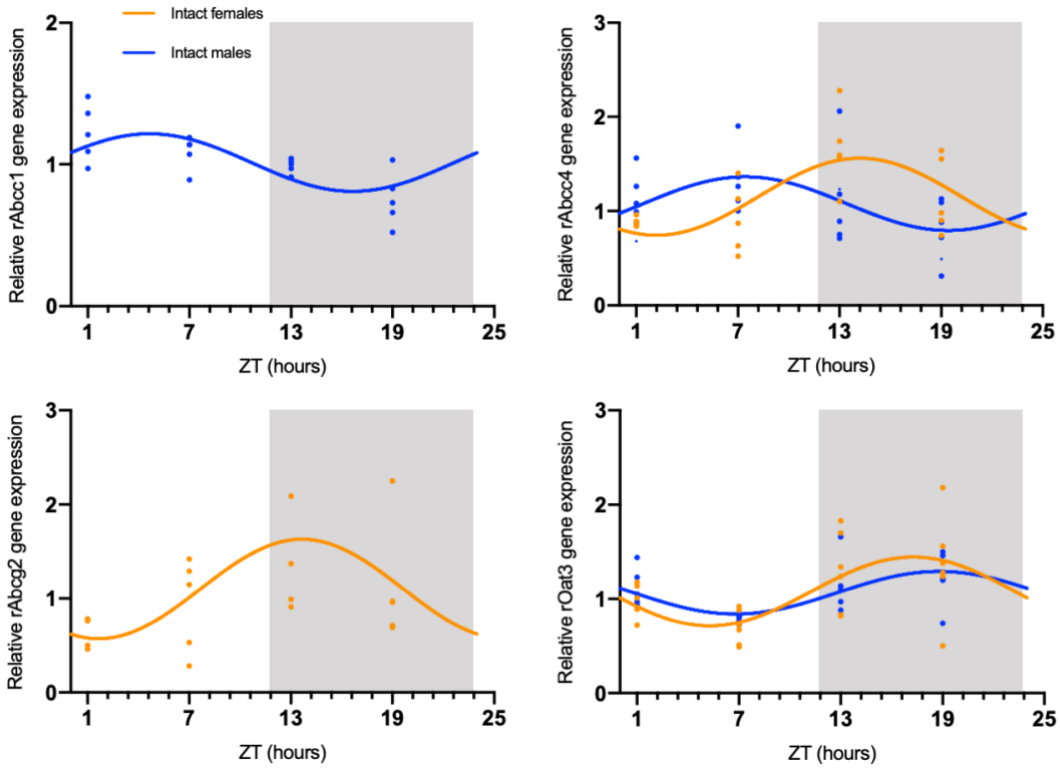

Figure 2. CircWave analysis of membrane transporters rAbcc1, rAbcc4, rAbcg2, and rOat3 mRNA circadian expression in the CP of intact male and female rats. Absence of the CircWave curve indicates absence of significant rhythmicity as analyzed by CircWave. White and grey backgrounds represent the day and night periods, respectively.

Table 1. Significance (*p*-value) and center of gravity (COG) values for rAbcc1, rAbcc4, rAbcg2 and rOat3 as determined by CircWave analysis.

rAbcc1	**Intact males**	*p*-value= 0,0086 COG= 4,65
rAbcc4	**Intact females**	*p*-value= 0,0080 COG= 14,20
	Intact males	*p*-value= 0,0429 COG= 7,43
rAbcg2	**Intact females**	*p*-value= 0,0447 COG= 13,66
rOat3	**Intact females**	*p*-value= 0,0079 COG= 17,27
	Intact males	*p*-value= 0,0312 COG= 18,80

2.2. hAbcc4 and hAbcg2 mRNA circadian expression in the HIBCPP cell line

Since rAbcc4 showed daily oscillations in intact male and female rats and rAbcg2 in intact female rats we also assessed the temporal expression of hAbcc4 and hAbcg2 mRNA in the human HIBCCP cell line. The results showed a circadian variation (CircWave, $p<0.05$) with a peak around 10 hours after synchronization (Figure 3, Table 2).

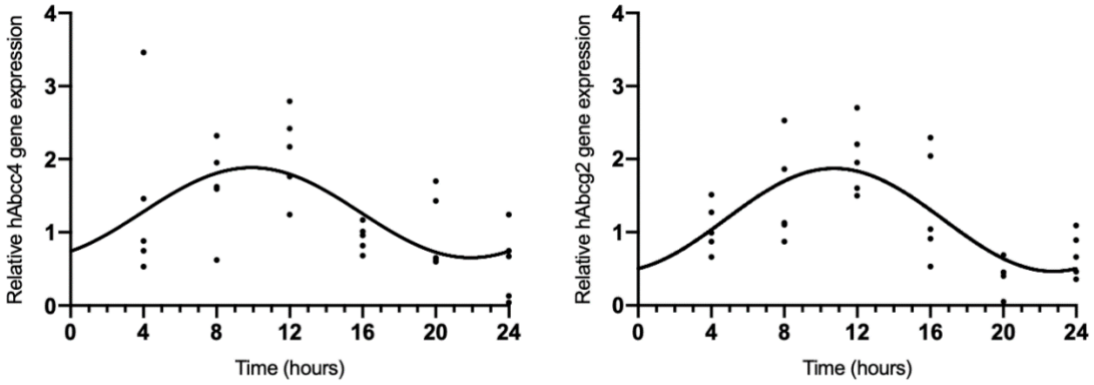

Figure 3. CircWave analysis of hAbcc4 and hAbcg2 expression profile in the HIBCPP cells. The represented curve indicates a statistically significant rhythm (CircWave, $p<0.05$).

Table 2. Significance (*p*-value) and center of gravity (COG) values for hAbcc4 and hAbcg2 determined by CircWave analysis.

hAbcc4	*p*-value= 0,0068 COG= 9,91
hAbcg2	*p*-value= 0,0001 COG= 10,74

2.3. Circadian oscillations in MTX transport across BCSFB

The presence of a circadian pattern of Abcc4 in intact male and female rats CP, led us to examine if the transport of MTX across de BCSFB is circadian dependent. According to the

results shown in Figure 4, MTX conjugated with fluorescein (FL-MTX) concentration in the basal compartment oscillated (CircWave, $p<0.05$) with a peak at 21 hours after synchronization (Figure 4a). In the apical compartment is also observed a significant oscillation in MTX concentration (CircWave, $p<0.05$), with a well-sustained peak around 3 hours after synchronization (Figure 4b). Finally, in the intracellular compartment, FL-MTX concentration also displayed circadian variation (CircWave, $p<0.05$) with a pronounced peak at 7 hours after the synchronization (Figure 4c).

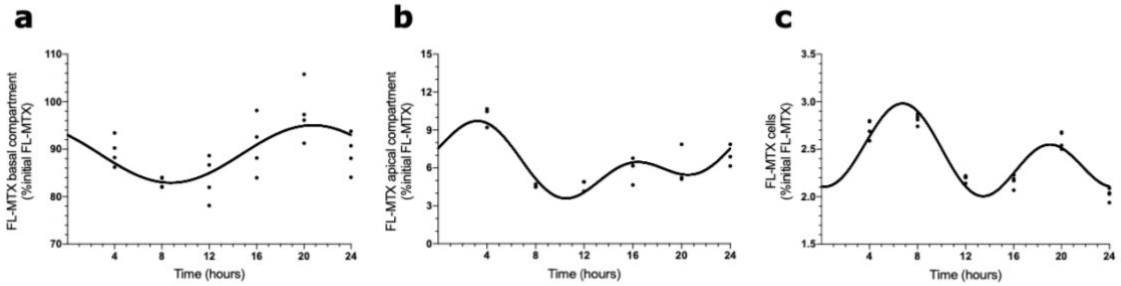

Figure 4. FL-MTX transport across the BCSFB. CircWave analysis of the levels of FL-MTX levels from basal (a), apical (b), and intracellular (c) compartments. The represented curves indicate a statistically significant rhythm (CircWave, $p<0.05$).

2.4. Effects of hABCC4 and hABCG2 inhibition in the MTX circadian transport across BCSFB

To evaluate the involvement of hABCC4 in the circadian transport of FL-MTX across the BCSFB, an analogous *in vitro* transport assay was carried out using a selective hABCC4 inhibitor (ceefourin). We found that in the basal compartment MTX concentration did not reveal a circadian variation (Figure 5a). On the contrary, in the apical compartment, FL-MTX concentration oscillated (CircWave, $p<0.05$), with a peak around 21 hours after synchronization (Figure 5b). At the intracellular compartment, a circadian variation was also observed in FL-MTX concentration (CircWave, $p<0.05$), with a peak at 22 hours (Figure 5c).

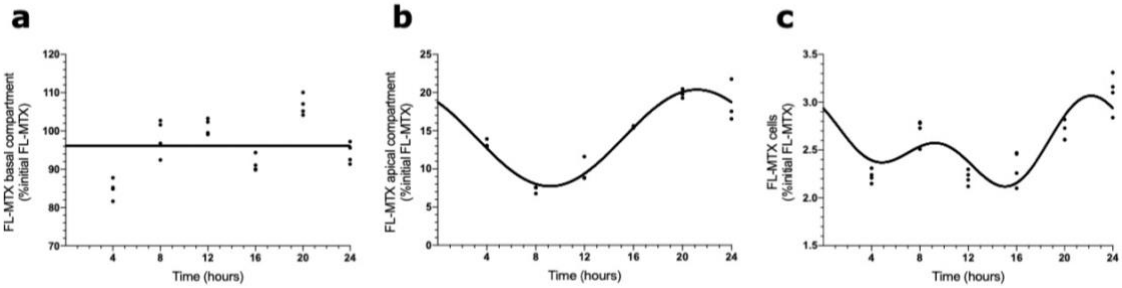

Figure 5. **Effects of hABCC4 inhibition in the FL-MTX circadian transport across the BCSFB**. CircWave analysis of the levels of FL-MTX levels from basal (a), apical (b), and intracellular (c) compartments after the inhibition of hABCC4. The represented curves indicate a statistically significant rhythm (CircWave, $p<0.05$).

ABCC4 is not the only membrane transporter involved in the brain efflux of MTX. For that reason, we assessed the influence of an apical transporter, hABCG2, in MTX transport across BCSFB using its specific inhibitor. ABCG2 is localized in the apical membrane of CPEC and also

revealed a circadian pattern in the CP of intact females. In the basal compartment, like in the previous assay, no circadian variation was reported in the FL-MTX concentration (Figure 6a). In the apical compartment, the FL-MTX concentration oscillated (CircWave, $p<0.05$), with a well-sustained peak at 20 hours after synchronization (Figure 6b). Finally, in the intracellular compartment, the FL-MTX concentration also oscillated (CircWave, $p<0.05$) and a peak can be observed between 21 and 22 hours after the synchronization (Figure 6c).

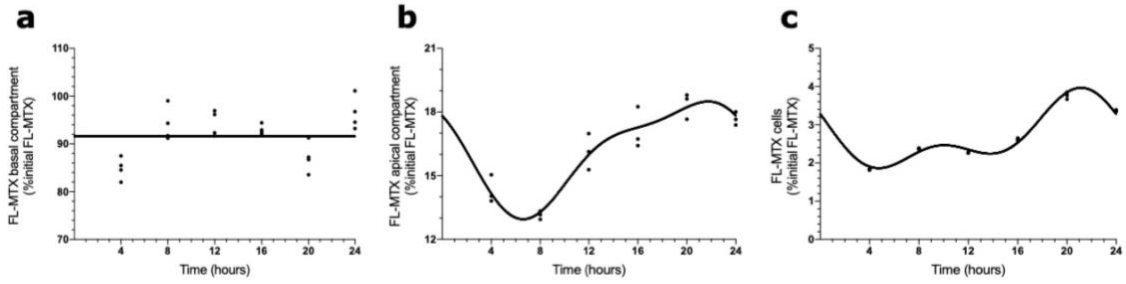

Figure 6. **Effects of hABCG2 inhibition in the FL-MTX circadian transport across the BCSFB.** CircWave analysis of the levels FL-MTX levels from basal (a), apical (b), and intracellular (c) compartments after the inhibition of hABCG2. The represented curves indicate a statistically significant rhythm (CircWave, $p<0.05$).

3. Discussion

The CP limits the passage of molecules from the blood to the CSF and consequently to the brain. This is partially ensured by transporter proteins located in the CPEC membrane. Thus, if the activity of a given transporter is subjected to circadian oscillations, drug administration of its known substrates must be scheduled according to those biological rhythms to improve drug delivery and efficacy of the therapeutic agent. It is thus crucial to study the circadian rhythmicity of drug transporters, and the molecular mechanisms associated.

In the present study we showed that the rAbca1, rAbcb1 and rAbcg4 expression is not rhythmic in the rats CP. In opposition, rAbcc1 showed circadian rhythmicity in intact males, rAbcg2 in intact females, and rAbcc4 and rOat3 in both intact male and female rats. This is the first study addressing the circadian expression of any of these transporters in a brain barrier, but there are some studies showing that ABC transporters are subject to circadian regulation in other tissues. The circadian expression of Abcc1 was studied in the Caco-2 cell line (derived from human colon adenocarcinoma) with a peak between the 6th and the 12th hour after synchronization (Ballesta et al., 2011). Conversely, no rhythmicity was seen in the expression of Abcc1 in the jejunal mucosa of male rats (Stearns et al., 2008), which is not in accordance with our results. In a PARbZip transcription factors knockout mice, Abcc4 expression was reduced in the kidney, suggesting that Abcc4 is a clock-controlled gene (Gachon et al., 2006). The Abcb1 and Abcb4 genes contain a D-box response element (Kotaka et al., 2008; Murakami et al., 2008) and, an analysis of the 2000 bp upstream the transcription start site of the human Abcc4 gene with JASPAR CORE 2018 Vertebrates library revealed three putative DBP binding sites, so, it is possible that its expression may be directly regulated by the PARbZip transcription factors. Abcc4 is also regulated by three xenobiotic receptors: CAR, aryl hydrocarbon receptor (AhR) and PXR

(Assem et al., 2004; Renga et al., 2011; Whyte-Allman et al., 2017; Xu et al., 2010) which show circadian expression in the liver (Zhang et al., 2009), what is supported by additional studies demonstrating that CAR and AhR genes are clock controlled (Gachon et al., 2006; Richardson et al., 1998; Zhang et al., 2009). Thus, there is a possibility that the molecular clock directly or indirectly, through xenobiotic receptors, also regulate the circadian expression of Abcc4.

The molecular clock controlling CP functions is regulated by sex hormones (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., 2018; Quintela et al., 2015). There is also the possibility that Abcc4 might be regulated by estrogens. A study with porcine endometrium explants showed that neither E2 nor P4 modulate Abcc4 expression (Seo et al., 2014). *Maher et al.* corroborate these data where no differential results were shown between control, OVX and OVX mice replaced with E2 (Maher et al., 2006). However, there is a study reporting the involvement of estrogen receptors (ERs) in the expression of Abcc4, showing that the activation of ERs increases Abcc4 expression (Koraïchi et al., 2013). Dihydrotestosterone (DHT), on the other hand, upregulates Abcc4 in the LNCaP cell line (Cai et al., 2007; Ho et al., 2008). However, in the present study, the rAbcc4 circadian pattern observed in intact female rats was lost in Sham and OVX females, as in the other transporters analyzed (rAbcg2 and rOat3). It is likely that the reason for this was the side effects of ketamine in Sham and OVX groups. In fact, there is experimental evidence that ketamine can influence the circadian oscillations in different systems. For instance, ketamine causes a phase advance in the rhythms of rats locomotor activity when administered at the resting phase, while, when administered during the active phase, a phase delay is caused (Mihara et al., 2012). In addition, at the molecular level ketamine induces a phase shift in Bmal1 and Dbp expression. Thus, it is possible, that the observed alterations in rAbcc4 circadian mRNA fluctuations in Sham and OVX female animals, are a consequence of ketamine anesthesia since its expression is possibly controlled by the PARbZip transcription factors (Gachon et al., 2006). So, we cannot conclude that rAbcc4 rhythmicity in females is dependent on SH.

With respect to Abcg2, *Hamdan et al.* demonstrated a circadian rhythm oscillation in the small intestine of mice, with a peak expression occurring during the light phase. This circadian expression is thought to be dependent of the molecular clock since Abcg2 rhythmic expression is completely abolished in *Clock* mutant mice (Hamdan et al., 2012). Later, in another study, it was also described that Abcg2 presented a circadian expression in the mouse liver and again this expression was dependent on the molecular clock, as confirmed in a *Per1 and Per2* double transgenic mouse (Oh et al., 2017). Contrarily to the small intestine, peak expression in the liver was reported during the dark phase (Oh et al., 2017). In our study rAbcg2 was rhythmic only in intact females with its peak expression in the first half of the dark phase. Like rAbcc4, rAbcg2 lost the circadian pattern in Sham and OVX females, and for the same reason we cannot conclude that the rhythmic expression of rAbcg2 in the rats' CP is dependent on female sex hormones.

In relation to rOat3, we showed rhythmicity in the CP of intact male and female rats, but not in OVX or Sham-operated females. Our results are in line with a study that reported a decreased expression of Oat3 in kidney in Bmal1 KO rats compared with controls, suggesting that Oat3 is a clock-controlled gene (Nikolaeva et al., 2016). Overall, our results seem to show that the molecular clock is responsible for the rhythmicity of rAbcc1, rAbcc4, rAbcg2 and rOat3 in rat CP. However, the reasons for the absence of circadian rhythmicity in Sham and OVX-operated female rats are not entirely understood and further work needs to be done to elucidate the causes of these observations.

MTX is a substrate of several membrane transporters expressed in the CP, namely 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) (Assaraf, 2006; Chen et al., 2003; Hinken et al., 2011; Inoue & Yuasa, 2014; Lima et al., 2014; Morris et al., 2017; Norris et al., 1996; Pizzagalli et al., 2002; Stieger & Gao, 2015; Tamai et al., 1999; Wollack et al., 2008; Zhao et al., 2009). Besides, it is well documented that ABCC1, ABCC4, and PCFT are expressed in the basolateral membrane of CPEC (Gazzin et al., 2008; M. Leggas et al., 2004; Zhao et al., 2009), SLCO1C1 in the basolateral and in the apical membrane (Roberts et al., 2008), and ABCB1, ABCG2, and RFC are located in the apical membrane (Gazzin et al., 2008; Hinken et al., 2011; Tachikawa et al., 2005). FOLR1, which mediates the transcytosis of MTX, is also located in the apical membrane of CPEC (Grapp et al., 2013).

To explore the idea that the ABCC4 function vary according to the time of day, we studied the transport of MTX across the BCSFB using an *in vitro* uptake assay. Interestingly, we 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. This result correlates favorably with few authors that demonstrated the circadian rhythm on MTX pharmacokinetics in animal and human studies (Bjarnason, 1995; Ferrazzini et al., 1991; Premaud et al., 2002), supporting the idea of the effect of timing of drug administration to the pharmacokinetic parameters of MTX (Gumustekin et al., 2005). It is interesting to note that the peak observed in MTX transport in the basal compartment occurred around 20 hours after synchronization, and the hAbcc4 mRNA peak expression in HIBCPP cells occurred around 10 hours after synchronization. The10-hour lag is in line with the dynamic phases occurring from mRNA to protein expression. MTX secondary peaks were also detected in the apical and cellular compartments. As described above MTX is a substrate for many of the CP's membrane transporters. Such transporters circadian expression might vary from one to the other as reported by *Zhang et al.* (Zhang et al., 2009). These different timings of expression and consequently different peaks of activity might be the answer to why we see this secondary peak in MTX uptake in both apical and cellular compartments. In the basal compartment such peaks are not present possibly since ABCC4 is MTX's major transporter in basolateral membrane. This

is supported by the fact that hABCC4 inhibition led to the loss of MTX uptake rhythmicity in this compartment.

To further explore the involvement of hABCC4 in the circadian transport of MTX across the BCSFB, we performed an *in vitro* uptake assay using an hABCC4 inhibitor. The rhythmicity of FL-MTX transport across the basal membrane was lost, while across the apical membrane and intracellularly was conserved. We believe that hABCC4 is, in part, responsible for the MTX circadian transportation across the basal membrane.

In a previous study it was demonstrated that the brain exposure to MTX through the BBB, is coordinated by ABCC4 and ABCG2 transporters (Sane et al., 2014). We thus examined whether the MTX transport depends on ABCG2. Curiously, we also observed a loss of rhythmicity in the FL-MTX concentration in the basal compartment using an hABCG2 inhibitor. However, the FL-MTX concentration in the apical and in the intracellular compartments maintained the circadian pattern. Thus, it seems that the rhythmicity of the FL-MTX transport across the basal membrane is not exclusively driven by hABCC4 since the inhibition of hABCG2, also impaired the rhythmicity of FL-MTX transport across the basal membrane. As put forward by Sane *et al.* (Sane et al., 2014), the evidence we found points to the idea of a contributory role of ABCC4 and ABCG2 to the circadian transport of MTX across the BCSFB. To sum up, our work reveals that there is a strong probability that the rhythmicity of MTX across the basolateral membrane is conferred by these membrane transporters present in CPEC that have a circadian dependent expression.

Brain barriers have always presented themselves as an obstacle for CNS pharmacotherapy. Circadian rhythms play a crucial role in BCSFB functions where drug transports and clearance are included (Quintela et al., 2021). We have not only demonstrated circadian rhythmicity of rAbcc4 and rAbcg2 in the rat's CP but also that these are also rhythmically expressed in a human cell line. Not only this but ABCC4 circadian transport of MTX is connected to its circadian expression. This is a first major step into understanding how the transport of therapeutic drugs is directly related with the circadian system. Chronotherapy is a branch of medicine specialized in treating patients at the optimal time of day in order to achieve maximum therapeutic effect with the least side effects and thus improving patient’s outcome (Damato & Herzog, 2021). By understanding how the circadian system controls the circadian expression of ABC and SLC carriers and thus their circadian activity we might get closer to improving SCN chronotherapeutic strategies. This might mean the timing of drug administration for the expected peak plasma levels to be in sync with the peak brain barrier’s permeability. Doing this we reduce the dose and consequent side effects and improve the efficacy.

4. Material and methods

4.1. Animals and cell line

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 males (n=24), intact females (n=24), ovariectomized (OVX; n=24), and sham-operated female rats (Sham; n=24). All animals were housed with standard laboratory chow and water *ad libitum*, and were maintained under constant temperature and in 12 hours light (07:00 h-19:00 h)/dark (19:00 h-07:00 h) cycles. ZT 0 was defined as lights on and ZT12 as lights off. Intact male and female rats were euthanized under a ketamine/xylazine anesthetic mixture. OVX and Sham rats were operated under the administration of a ketamine/medetomidine solution, and two weeks after surgery rats were euthanized. The CP from lateral ventricles were collected at four different time points (ZT1, ZT7, ZT13, and ZT19) and were immediately frozen in liquid nitrogen for qPCR analysis of ABC and SLC transporters.

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

The Human epithelial CP papilloma (HIBCPP) cell line derived from a human malignant CP papilloma was kindly made available by C Schwerk (Ishiwata et al., 2005). These cells preserve the polygonal morphology and the phenotype of the CPEC. The HIBCPP cell line form a functional epithelial barrier with high transepithelial electric resistance (TEER) values (Bernd et al., 2015; Ishiwata et al., 2005; Schwerk et al., 2012). Therefore, it was used for functional studies.

4.2. HIBCPP cell culture

HIBCPP cells were seeded in 24-well plates with DMEM /F12 (Gibco) supplemented with 10 % FBS (Life Technologies), 1% penicillin/streptomycin (MP Biomedicals), and with 5µg/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 reaching 70% confluence, the cells were synchronized with 1% of dexamethasone for 2 h at 37 °C and 5% CO_2.

To analyze the circadian pattern of hAbcc4 and hAbcg2 in the HIBCPP cell line, the cells were harvested for total RNA extraction at the following time points: 4, 8, 12, 16, 20, and 24 h after synchronization.

4.3. Quantitative real-time PCR (qPCR)

Total RNA was isolated from the rat CP and HIBCPP cells using TRIzol reagent (Grisp, Portugal) according to the manufacturer's instructions. Total RNA purity and quantification were assessed by the measurement of the absorbances at 260 and 280 nanometers using a NanoPhotometer™ (Implen, Germany). cDNA was synthetized using a NZY M-MuLV First-Strand synthesis kit (NZYTech, Portugal) according with the manufacturer's protocol.

In animal experiments, quantitative real-time PCR (qPCR) was performed to assess the daily expression of rAbca1, rAbcb1, rAbcc1, rAbcc4, rAbcg2, rAbcg4 and rOat3. In the HIBCPP cells qPCR was implemented to determine the daily expression of hAbcc4 and hAbcg2.qPCRs were performed on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using an Xpert Fast SYBR 2X mastermix (Grisp, Portugal). qPCRs were carried out with an initial denaturation step at 95 °C for 3 min followed by 40 cycles of 95 °C for 5s, 60 °C for 30s, and 72 °C for 10s. The amplification of all transcripts was validated by the profiles of melting curves. 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 amplicon sizes are listed in Table 3.

Table 3. Primers and amplicons' sizes used for real-time quantitative PCR

Gene	Primer sequence (5'-3')	Amplicon size (bp)
hAbcc4	FW: TGTGGCTTTGAACACAGCGTA	105
	RV: CCAGCACACTGAACGTGATAA	
hAbcg2	FW: ACGAACGGATTAACAGGGTCA	93
	RV: CTCCAGACACACCACGGAT	
hGapdh*	FW: ATGGGGAAGGTGAAGGTCG	108
	RV: GGGGTCATTGATGGCAACAATA	
rAbca1	FW: CGGCGGAGTAGAAAGGGTTT	84
	RV: CACGATCAGGCTGAAGACCAG	
rAbcb1	FW: AAGGGGCTACAGGGTCTAGG	100
	RV: AGTGTCAATTGCCAGCCGTA	
rAbcc1	FW: TTCATATCTGCTTCGTCACCG	60
	RV: CGTAAACAGCACCCACCACAGC	
rAbcc4	FW: TTCCCCTTCGACCTTATCCT	124
	RV: TAGGCAGCTGTTGTCAGTGG	
rAbcg2	FW: GGCCTGGACAAAGTAGCAGA	137
	RV: GTTGTGGGCTCATCCAGGAA	
rAbcg4	FW: ATGGCTGATGTACCCTTCCAGGTT	155
	RV: ATCAAGAGTCCCAAAGACTGGGCA	
rOat3	FW: GAGGACCTGTGATTGGAGAACTG	82
	RV: CTG GCT GCC AGC ATG AGA TA	
rCycA*	FW: CAAGACTGAGTGGCTGGATGG	163
	RV: GCCCGCAAGTCAAAGAAATTAGAG	

* Were used as a reference genes

4.4. MTX uptake assay

The assessment of the MTX transport in HIBCPP cells was chosen to investigate if the daily oscillations of Abcc4 expression seen in the CP of intact male and female rats and in the CP cell line would affect the transport of its substrate MTX across the BCSFB. Of interest, ABCC4 is also involved in the transport of several other compounds of pharmacological importance such as

antiviral, antibacterial, antihypertensive and antineoplastic agents, as well as for the efflux of endogenous substrates, like cAMP and prostaglandins (Gil-Martins et al., 2020). HIBCPP cells were seeded in a transwell filter system (Figure 4.1) and used as an *in vitro* model of the BCSFB. For that, the HIBCPP cells were seeded in the apical compartment of cell 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/streptomycin. 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 assessed 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 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-Ringer buffer (KRB). Next, the cells were incubated with FL-MTX for 3 hours with the start of the incubation at six different time points (1, 5, 9, 14, 17, and 21 hours after synchronization) (Figure 7). After the 3h 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. The FL-MTX concentration was then 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. Next, to explore the involvement of ABCC4 in the circadian rhythmicity of FL-MTX transport across the BCSFB, an additional assay was performed with the inhibitor of ABCC4 (applied in the basal compartment) ceefourin1 (5 µM; Tocris, UK). Finally, since ABCG2 was also described as an important apical transporter in limiting the distribution of MTX, a similar assay was performed using an ABCG2 inhibitor (Ko143100 nm; Tebu-bio, UK).

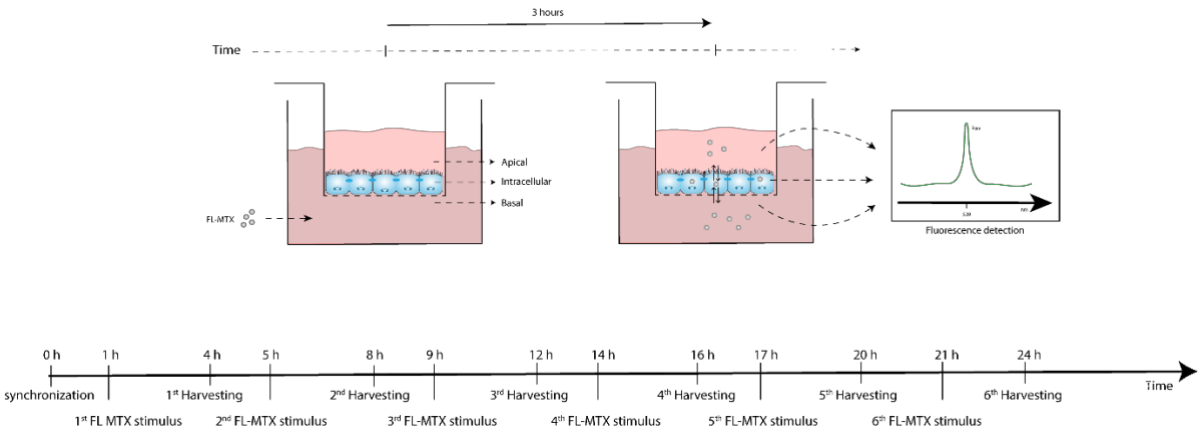

Figure 7. Scheme of the MTX uptake assay. At time point 0 hours the cells were synchronized using dexamethasone. At six different time points (1, 5, 9, 14, 17, and 21 hours after synchronization) the transwell inserts were incubated with

MTX in the basal compartment for 3h. After the incubation, the MTX level in the basal, intracellular, and apical compartments were assessed by fluorescence analysis.

4.5. 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).

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Chapter 5

Research Work 3
Circadian ABCG2 Expression Influences the Brain Uptake of Donepezil across the Blood-Cerebrospinal Fluid Barrier

This chapter corresponds to the original research article:

Furtado, A., Duarte, A. C., Costa, A. R., Gonçalves, I., Santos, C. R. A., Gallardo, E., & Quintela, T. (2024). Circadian ABCG2 Expression Influences the Brain Uptake of Donepezil across the Blood-Cerebrospinal Fluid Barrier. *Int J Mol Sci*, *25*(9). https://doi.org/10.3390/ijms25095014

1. Introduction

AD is the most common cause of dementia in humans. It is a primary degenerative disease and is clinically characterized by memory loss, the impairment of multiple cognitive functions and dementia. Among the few drugs approved by the FDA for the treatment of AD, DNPZ, a second-generation acetylcholinesterase inhibitor, is widely used for the treatment of mild, moderate and severe AD (Birks & Harvey, 2018). Currently, DNPZ is used as a standard symptomatic treatment, with an important contribution to slowing disease progression. However, the side effects resulting from the higher doses prescribed to contradict the insufficient drug delivery to the brain are limiting the efficacy of medication for AD treatment (Shin et al., 2018). In fact, brain barriers are restricting the drug access to the brain contributing to the low bioavailability (Christodoulou et al., 2006).

The CNS maintains the homeostasis of the brain by the presence of two main barriers: the BBB, which separates the systemic circulation from the CNS; and the BCSFB that separates the cerebrospinal compartment from the blood circulation (Zheng et al., 2003). The BCSFB is established by tight junctions in the epithelium of the CP, and the arachnoid membrane. It is in contact with the CSF in the apical side and there is a vast network of fenestrated capillary blood vessels on its basal side (Ghersi-Egea et al., 2018). Since it may regulate the distribution of certain drugs and neurotoxic agents between the blood and the CSF, the CP epithelium of the BCSFB is considered a pharmacologic and toxicologically important barrier (Wang & Zuo, 2018). The delivery of drugs to the CNS is primarily impeded by the BBB. However, we are not alone in our view that the BCSFB could be distinguished as a potential gateway to the brain due to its architecture, strategic position and highly dynamic transport activity (Schwerk et al., 2015). The CP has been associated with numerous functions, including CSF production and secretion, chemical surveillance and detoxification of the CSF and barrier function, as it constitutes a selective physical barrier to the passage of compounds, toxins, cells and molecules to and from the CNS (Costa-Brito et al., 2021; Duarte, Furtado, et al., 2020; Duarte, Rosado, et al., 2020; Duarte, Santos, et al., 2020; Furtado et al., 2022; Quintela et al., 2021; Quintela et al., 2015).

As a selective barrier between the bloodstream and the CSF, CP epithelial cells display several membrane transporter proteins, tight junctions and detoxification enzymes, enabling CP cells to control the traffic of molecules across the BCSFB (Santos et al., 2019). ABC, SLC and SLCO transporter families are expressed in the CP (Quintela et al., 2021). Depending on the transporters, they can be responsible for the efflux or uptake of molecules by CP cells (Quintela et al., 2021; Schulz et al., 2023; Sweet, 2021), affecting the pharmacokinetics of multiple therapeutic drugs (Mohammad et al., 2018). Therefore, drug transporters are in part responsible for the drug delivery into the brain and the effective drug concentrations at the target tissue. At the BCSFB, multiple transporters have been identified, namely ABCG2 (BCRP), which is expressed on the apical/subapical side of the BCSFB, contributing to the transport of its substrates to the CSF (Bernd et al., 2015; Furtado et al., 2022; Ghersi-Egea et al., 2018). ABCG2 was described as a transporter for DNPZ in heart and brain tissues, with a possible clinical application (Takeuchi et al., 2016). Curiously, the location of ABCG2 at the apical BCSFB side,

will imply a function that will be the opposite of what occurs at the blood–brain barrier, involved in limiting the drug distribution to the CNS (Agarwal et al., 2011).

The circadian clock has been demonstrated to interact with drug transporters, influencing the pharmacokinetics and pharmacodynamics of the drugs, reducing side effects and improving therapeutic potential (Nahmias & Androulakis, 2021). Recently, it was demonstrated that CP epithelial cells that compose the BCSFB harbor a functional circadian clock, considered an important component of the circadian clock hierarchy (Myung et al., 2018; Quintela et al., 2015). In addition, the circadian expression of BCSFB transporters, such as ABCC1, ABCG2, ABCC4 and OAT3, was described in the CP of Wistar rats (Furtado et al., 2022). Hence, the widespread concern in understanding how circadian rhythms modulate many processes of drug transport into the brain in order to predict the variability in drug safety and efficacy is the next step to overcome brain barriers and improve effective treatments (Kreuter, 2015). Thus, characterizing the circadian regulation of ABCG2 at the BCSFB and the circadian profile of DNPZ transport across the barrier will strongly impact on the capacity to modulate the BCSFB in order to control the penetration of DNPZ into the brain.

2. Results

2.1. Circadian expression of rBMAL1 and rABCG2 in CPEC

The circadian expression of the clock gene *rBMAL1* was assessed to confirm the synchronization of CPEC primary cultures. The results show a significant circadian variation (CircWave, $p<0.05$), with peak expression at around 7 h after synchronization (Figure 1).

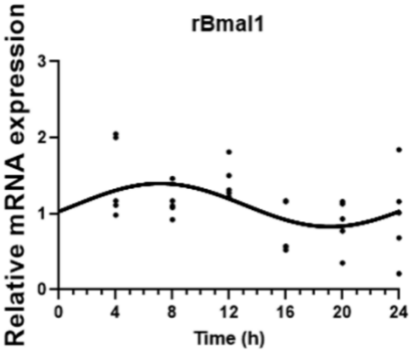

Figure 1. CircWave analysis of rat basic helix–loop–helix ARNT-like 1 (rBMAL1) clock gene circadian expression in CPEC. rBMAL1 expression was analyzed every 4h during a 24h period in synchronized CPECs. The sine–cosine fit represents a significant 24h period oscillation ($p<0.05$). Statistical analysis is shown in Table 1.

The daily profile of rABCG2 membrane transporter was also evaluated and showed a significant circadian variation (CircWave, $p<0.05$), with a peak at expression around 19 h after synchronization (Figure 2).

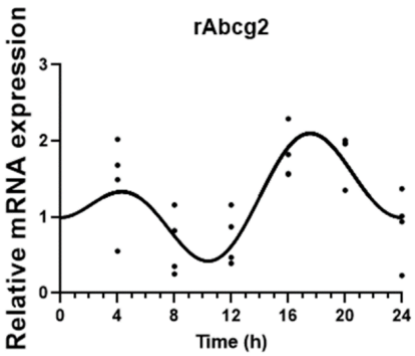

Figure 2. CircWave analysis of rat rABCG2 membrane transporter gene circadian expression in CPEC. rABCG2 expression was analyzed every 4h during a 24h period in synchronized CPECs. The sine–cosine fit represents a significant 24h period oscillation (p<0.05). Statistical analysis is shown in Table 1.

Table 1. Significance (p-value) and center of gravity (COG) values for rBMAL1 and rABCG2 determined by CircWave analysis.

Gene	rBMAL1	p-value= 0.0418 COG= 7.13
	rABCG2	p-value= 0.0012 COG= 19.52

2.2. Circadian profile of DNPZ transport in an *in vitro* model of the BCSFB

The presence of the circadian expression of ABCG2 in synchronized CPECs led us to examine if the transport of DNPZ across an in vitro model of the BCSFB is circadian-dependent. According to the results shown in Figure 5.3, the DNPZ concentration in the apical compartment oscillated (CircWave, p<0.05), with a peak at approximately 22 h after synchronization (Figure 3). In the basal and cell compartment, no significant oscillation in the DNPZ concentration was observed (Figure 3).

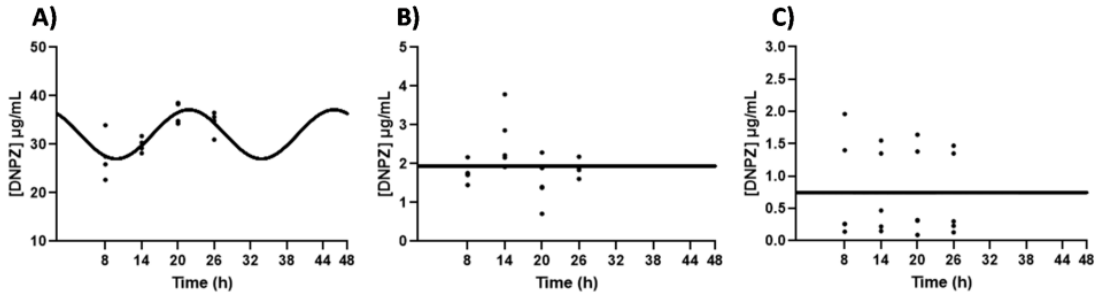

Figure 3. DNPZ transport across an *in vitro* model of the BCSFB. CircWave analysis of DNPZ levels in apical **A**), basal **B**) and intracellular **C**) compartments. The represented curves indicate a statistically significant rhythm (CircWave,p<0.05). Statistical analysis is shown in Table 2.

2.3 The role of ABCG2 in the transport of DNPZ across the BCSFB

To study the role of ABCG2 in the circadian transport of DNPZ across an in vitro model of BCSFB, a transport assay was carried out using an ABCG2 inhibitor (Ko143). We found that in the presence of Ko143, the DNPZ concentration in the apical compartment lost the circadian rhythmicity observed in its absence (Figure 4). On the contrary, in the basal compartment, the DNPZ concentration oscillated (CircWave, $p<0.05$), with a peak at around 12 h after synchronization (Figure 4). Within the intracellular compartment, circadian variation was also observed in the DNPZ concentration (CircWave, $p<0.05$), with a peak between 8 and 9 h after synchronization (Figure 4).

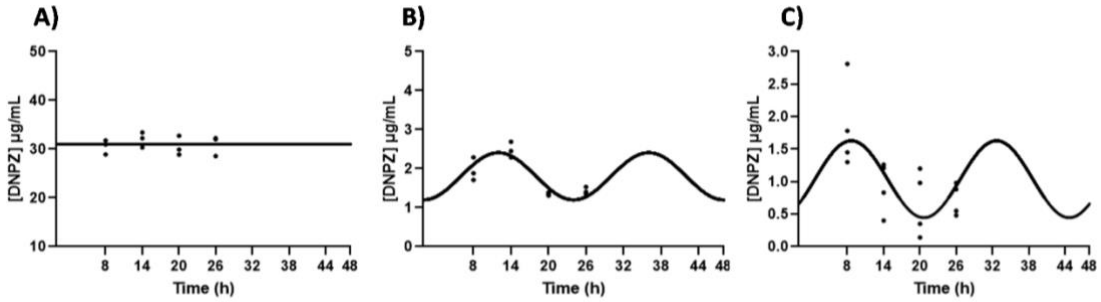

Figure 4. Effects of ABCG2 inhibition in the DNPZ circadian transport across an *in vitro* model of the BCSFB. CircWave analysis of the DNPZ levels from apical **A**), basal **B**) and intracellular **C**) compartments after the inhibition of ABCG2. The represented curves indicate a statistically significant rhythm (CircWave,$p<0.05$). Statistical analysis is shown in Table 2.

Table 2. Significance (p-value) and center of gravity (COG) values for DNPZ concentrations in an *in vitro* model of the BCSFB determined by CircWave analysis.

DNPZ	Apical	p-value = 0.0010 COG= 21.82
	Basal	p-value >0.05 COG= 12.78
	Cells	p-value >0.05 COG= 10.86
DNPZ + Ko143	Apical	p-value >0.05 COG= 13.85
	Basal	p-value = 0.007 COG= 12.01
	Cells	p-value = 0.0224 COG= 8.66

3. Discussion

The BCSFB is found in the CP of the ventricular system of the brain and works as a highly selective barrier to the passage of molecules from the bloodstream to the CSF, providing an obstacle to the delivery of therapeutics for CNS disorders. Consequently, this security system is

responsible for the deficient brain bioavailability of several pharmacological agents (Strazielle & Ghersi-Egea, 2016). Thus, the development of delivery strategies to cross brain barriers requires a better knowledge of the circumstances that change the drug concentration–time profile in the brain. Among the several factors that have been studied, the circadian rhythm, particularly the modulation of membrane transporters according to the time of day, is increasingly shaping our view of future research in the improvement of AD therapy. In approaching this issue, in the present study, we analyze the effects of the circadian clock in ABCG2 expression and its possible involvement in the transport of DNPZ across the BCSFB. Our results confirmed, for the first time, that the circadian expression of ABCG2 controls DNPZ bioavailability in the CSF.

In our study, we observed that ABCG2 displays a circadian pattern in CPEC primary cultures. This result was not surprising, as in our previous work, we also showed that ABCG2 displays a circadian pattern of expression on the female rat CP and in synchronized HIBCPP (Furtado et al., 2022). Additionally, in other tissues such as the mouse small intestine and liver, ABCG2 also displayed circadian rhythmicity (Hamdan et al., 2012; Oh et al., 2017). In the small intestine, ABCG2 rhythmic expression was described as being dependent of the molecular clock as *Clock*-mutant mice lacked ABCG2 rhythmic expression (Hamdan et al., 2012). In the liver, ABCG2 circadian expression was lost in *Per1* and *Per2* double-transgenic mice (Oh et al., 2017).

DNPZ is an approved therapy for the treatment of AD. Its transport across brain barriers is dependent of membrane transporters which play an important role in drug disposition and toxicity (Kim et al., 2010). ABCG2, SLC5A7, SLC22A1, SLC22A2, SLC22A3, SLC22A4 and SLC22A5 have been identified as membrane transporters for DNPZ (Kim et al., 2010; Takeuchi et al., 2016). Furthermore, ABCG2, SLC22A2, SLC22A3, SLC22A4 and SLC22A5 have all been described at the BCSFB (Ghersi-Egea & Strazielle, 2002; Tsuchiya et al., 2014). ABCG2 is located in the apical/ subapical choroidal membrane in human and mouse CP cells and contributes to the transport of DNPZ into the CSF (Chiba et al., 2020; Ghersi-Egea et al., 2018). SLC22A2 and SLC22A3 are located in the apical membrane of the CP and mediate the entry of molecules into the cell cytoplasm (Sweet et al., 2001). SLC22A4 and SLC22A5 localization is still imprecise, with reports showing their expression in the apical, as well as basal membrane of the CP, and they are responsible for the choroidal uptake of their respective substrates (Betterton et al., 2021; Gründemann et al., 2005; Sweet et al., 2001).

To characterize the role of ABCG2 in the circadian transmembrane transport of DNPZ, an in vitro model of the BCSFB using primary cultures of CPECs was implemented. Interestingly, we observed daily oscillations in DNPZ concentrations in the apical compartment. Peak ABCG2 expression at the CPEC occurs around 19 h, 2 h before the peak expression of DNPZ transport across the BCSFB. This finding points to the idea that an increase in the ABCG2 expression at a specific time point of the day will intensify the activity of ABCG2, and DNPZ is transported at an increased rate into the CSF. In the basal and intracellular compartments, the circadian transport profile of DNPZ was not observed. The role of ABCG2 as a multidrug transporter that affects drug

pharmacokinetics was also demonstrated in the intestine. ABCG2 circadian oscillation in mouse intestine was a determinant for the circadian bioavailability of an ABCG2 substrate, sulfasalazine (Hamdan et al., 2012). More recently, the involvement of murine ABCG2 in the transport and secretion of melatonin metabolites to the intestine and kidney also highlights the idea that changes in ABCG2 expression might affect melatonin therapeutic activity (Alvarez-Fernandez et al., 2023). Targeting ABCG2 regulation to successfully deliver drugs to the brain was also widely considered at the blood–brain barrier over the past few years. In fact, the increase in transporter expression and/or activity at the blood–brain barrier was studied to treat neurological disorders, as well as protect the brain (Schulz et al., 2023).

The specificity of ABCG2-mediated circadian transport of DNPZ across the BCSFB was further studied, carrying out the inhibition of the efflux transporter with Ko143. Interestingly, the abolishment of the daily oscillations of DNPZ transport in the apical compartment and the emergence of oscillations in the basal and intracellular compartments was observed. This finding may point to an alternative mechanism of DNPZ transport across the BCSFB. As mentioned before, DNPZ is transported by other BCSFB membrane transporters. Of these, two were described as possibly located in the basal compartment of CP, i.e., SLC22A4 and SLC22A5. Furthermore, both genes were considered the circadian targets for several drugs, namely SCL22A4 for Androgel and Combivent, and SLC22A5 for Lidoderm, Niaspan and Combivent (Zhang et al., 2014). Considering these observations, we hypothesized that SLC22A4 and SLC22A5 might contribute to the circadian oscillation of DNPZ concentrations in both the basal and intracellular compartments. Among these two transporters, SLC22A4 was involved in the suppression of oxidative stress in PC12 cells through the uptake of an antioxidant under saturable conditions (Nakamura et al., 2008). In addition, the circadian expression of mouse SLC22A4 induces dosing–time-dependent differences in the absorption of gabapentin from the intestine (Wada et al., 2015).

Thus, it will be possible that with the inhibition of ABCG2, an increase in DNPZ bioavailability in basal and intracellular compartments might be sufficient to trigger a saturable transport of DNPZ by SLC22A4, with circadian oscillations taking place in the basal and intracellular compartments.

Despite this interpretation, the involvement of ABCG2 in the transport of DNPZ into the CSF is crucial to optimize therapy. This assumption is corroborated by a recent study that measured DNPZ concentrations in the plasma and CSF of AD patients at four different time points and calculated the plasma/CSF ratio. They observed that the plasma and CSF DNPZ levels increase over 24h without statistical significance. Interestingly, the plasma/CSF ratio significantly increased overtime (Nakamura et al., 2008), pointing to the importance of CSF DNPZ concentration levels to optimize DNPZ dosage.

In summary, the present findings reveal the regulation of ABCG2 expression by the circadian rhythm which impacts the circadian-dependent transport of DNPZ across the BCSFB into the

CSF. It will be important to mention that in situations where several drugs are administered, it is possible that competition for the DNPZ receptor may occur, which might affect the rhythmicity of the drug's transport. The present study will have important implications in the modulation of the BCSFB in order to control the penetration of DNPZ into the CSF. Consequently, it will provide powerful data to optimize AD therapy, taking into account the circadian clock, increasing efficacy and reducing side effects commonly associated with some pharmacological interventions. Predicting a priori the most appropriate timing when DNPZ transport equilibrium occurs at the BCSFB to match the therapeutic approach to the patient characteristics will be important for the treatment of AD, improving personalized medical interventions.

4. Material and methods

4.1. Animals

This study was conducted with the approval of the Animal Welfare and Ethics Committee of the Health Science Research Centre of the University of Beira Interior, in compliance with the National and European Union rules for the care and handling of laboratory animals. The CPs were collected from the lateral ventricles of 2–7-day-old postnatal *Wistar Han* rats, which were housed in appropriate cages at constant room temperature in a 12h light/12h dark photoperiod and given standard laboratory chow and water ad libitum. Efforts were made to minimize the number of animals and animal suffering.

4.2. Choroid plexus epithelial primary culture

Thirty postnatal animals were anesthetized on ice for at least 30 min before being euthanized. The CPs were collected from the lateral ventricles and used to establish CPEC primary cultures as previously described by *Gonçalves et al.* (Gonçalves et al., 2019). Briefly, the dissociated cells were seeded into 6-well culture plates and cultured in a high-glucose DMEM supplemented with5 µg/mL insulin (Sigma-Aldrich, Merck, Algés, Portugal), 100 U/mL penicillin, 100 µg/mL streptomycin, 10% *v*/*v* FBS, 10ng/mL epidermal growth factor (Sigma-Aldrich, Merck, Portugal) and 30 µM cytosine arabinoside (Sigma-Aldrich, Merck, Portugal).The cultures were maintained in a humidified incubator at 37 °C and 5% CO_2. The culture medium was replaced at DIV1 and every 2 days thereafter. All studies were conducted using cultures established for 4–5 days.

4.2.1. ABCG2 circadian pattern

CPEC primary cultures established for at least 4–5 days were trypsinized and seeded in 24-well culture plates (approximately 1.5 ×10^4 cells/well). The culture medium was changed every 2 days and experiments were conducted 8 days after seeding. CPECs were synchronized with 100nM dexamethasone (Sigma-Aldrich, Merck, Portugal), an artificial glucocorticoid that resets the circadian clock in the culture, for 2 h. The culture medium was changed, and cells were collected 4 h after synchronization and then every 4 h during a 24h period for total RNA extraction.

4.2.2. Quantitative real-time PCR (qPCR)

Total RNA was isolated from the CPEC using triple Xtractor reagent (Grisp, Porto, Portugal) according to the manufacturer's instructions. Total RNA purity and integrity were assessed by the measurement of the absorbances at 260 and 280 nm using a NanoPhotometer^{TM} (Implen, Munich, Germany). NZY M-MuLV Reverse Transcriptase (NZYTech Ltd.,Lisboa, Portugal), Random hexamer mix (NZYTech Ltd., Portugal), GRS dNTP mix (GRISP Ltd., Portugal) and RNA (500ng) were used for cDNA synthesis following manufacturer's instructions.

qPCR was performed to assess the daily expression of rBmal1 and rABCG2. rCyc was used as a housekeeping gene. Primers sequences are listed in Table 3. qPCR was performed using a CFX-ConnectTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using an Xpert Fast SYBR 2x master mix (Grisp, Porto, Portugal). The qPCR protocol consisted of an initial 3-min denaturation step at 95 °C, followed by 40 cycles of 5 s at 95 °C, 30 s at 62 °C and 10 s at 72 °C. The transcripts amplification was validated by the profiles of melting curves. All primers were previously tested with the following cDNA dilutions: stock, 1:2, 1:4, 1:8. To calculate ΔCt we used the average of the Cts of a sample from which we subtracted the average of the Cts of the housekeeping gene for the same sample. To calculate ΔΔCt we first subtract the average of the ΔCt from the average of the Cts of the housekeeping gene of all the samples. Finally, we subtract the previous result from the ΔCt of the desired sample.

Table 3. Primers used in RT-PCR and Real-time qPCR.

Gene	Primers	Bp	Ref
rBmal1	FW-ACACTGCACCTCGGGAGCGA RV-CGCCGAGCTCCAGAGCACAA	100	(Wharfe et al., 2011)
rABCG2	FW-GGCCTGGACAAAGTAGCAGA RV-CACAGTTGTGGGCTCATCCAGGAA	141	(Jones et al., 2015)
rCyc	FW-CAAGACTGAGTGGCTGGATGG RV-GCCCGCAAGTCAAAGAAATTAGAG	163	(Duarte, Furtado, et al., 2020)

4.3. Donepezil transport assay

The DNPZ transport assay in CPEC was determined to investigate whether the daily oscillations in rABCG2 would affect the transport of its substrate across the BCSFB. CPEC primary cultures established for at least 4–5 days were trypsinized and seeded in a transwell filter system and used as an in vitro model of the BCSFB. Cell culture inserts apical compartment were previously coated with collagen following *Monnot et al.* protocol (Monnot & Zheng, 2013). CPEC were seeded on the apical compartment of cell culture inserts (pore diameter 0.4 µm and insert area 0.33 cm^2; VWR, Alfragide, Portugal) at a density of 2.5×10^4 cells per insert in high-glucose DMEM supplemented with 5 µg/mL insulin, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% *v/v* FBS, 10ng/mL epidermal growth factor and 30 µM cytosine arabinoside. The culture medium was changed every 2 days. The paracellular permeability was assessed every day by the

measurement of TEER values using an Epithelial Volt/Ohm Meter (WPI, Sarasota, FL, USA). On the 8th day of culture, TEER values reached 65–80 Ω·cm^2. Two other criteria of membrane integrity were used: appearance of a confluent monolayer on the insert under the microscope; height of the culture medium on the inner chamber (had to be at least 2 mm higher than that on the outer chamber for at least 24 h) (Monnot & Zheng, 2013). At this point, cells were synchronized with 100nM dexamethasone for 2 h. The culture medium was changed, and the cells were placed in a humidified incubator at 37 °C and 5% CO_2. At 4 different time points after synchronization (4, 10, 16, 22 h) cells were washed 3 times with KRB and placed in the incubator in KRB for another hour. Next, cells were incubated on the apical side with DNPZ 40 µg/mL (Sigma-Aldrich, Merck, Portugal) for 3 h. After incubation, both the apical and basal mediums were collected and the remaining cells were washed 3 times with KRB, trypsinized and collected.

The role of rABCG2 in the transport of DNPZ across the BCSFB was analyzed, using a similar assay with an inhibitor of rABCG2 (Ko143 100nM; Tebu-bio, Lisbon, Portugal).

4.3.1. DNPZ quantification

The quantification of DNPZ was performed on an HPLC 1290 with a binary pump coupled to a fluorescence multi-wavelength detector (FLD-3400 RS detector), both from Agilent Technologies (Soquímica, Lisboa, Portugal). The separation was performed in an Eclipse Plus C_{18} (3.5 µm, 4.6 × 100 mm) analytical column from Agilent Technologies (Soquímica, Lisboa, Portugal) protected by a pre column. The flow rate was 1 mL/min, with a mobile phase consisting of 0.1% methanol as solvent A and acetic acid (70:30, *v*/ *v*) as solvent B. The elution was carried out in gradient mode. The temperature of the sampler was set at 4 °C and the injection volume was 20 µL. The determination of DNPZ was performed at λ ex269 nm and λ em390 nm (Figure 5).

4.3.2. Validation procedure

The described method was validated according to the guiding principles of the Food and Drug Administration (*Guidance for Industry: Bioanalytical Method Validation*, 2018), within the linearity range between 0.04 and 40 µg/mL, and an additional four quality controls (0.04, 1.25, 10, and 40 µg/mL) (n=3) included. The criteria used to assess the fitness of this linear model included a weighted determination coefficient (R^2) higher than 0.99, and the accuracy of the calibrators within ±15% from the nominal value (except at the LLOQ, where ±20% was accepted) was adopted as the acceptance criteria. The method's LLOQ was defined as the lowest concentration that could be precisely and accurately measured, with a coefficient of variation (CV) equal to or lower than 20% and a relative error (RE) within ±20% of the nominal concentration. To evaluate sensitivity, the limit of detection (LOD) as a signal-to-noise ratio >3 was calculated, with 10 replicates performed at a concentration of 0.04 µg/mL.

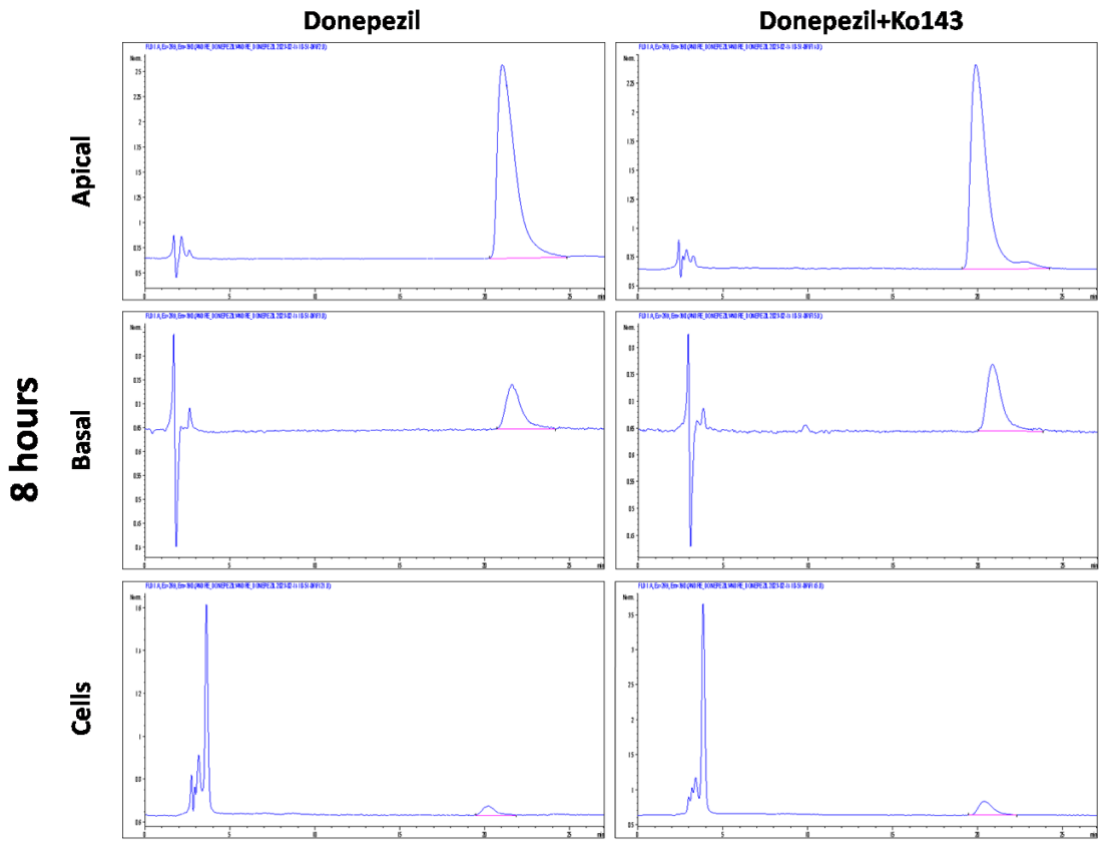

Figure 5. Representative chromatogram obtained for Donepezil (10 µg/mL) and Donepezil + inhibitor (Ko143) in apical, basal and cell compartments. Chromatogram for Donepezil (10 µg/mL) and Donepezil + inhibitor (Ko143) at λ ex269 nm and λ em390 nm.

Precision and accuracy were evaluated during the 5-day protocol, adopting the same concentrations used for the quality controls. Coefficients of variation (CV) equal to or lower than 15% were accepted for precision at all studied concentration levels, while for accuracy, a mean relative error (RE) of ±15% (from the nominal concentration) was accepted for all concentrations, except the LLOQ (±20%).

The CVs obtained in the study of inter-day precision and accuracy (RE) were typically lower than 8%, with an accuracy ranging from 0.3 to 3.4% (Table 4). As for intra-day precision and accuracy, it was evaluated on the same day by the analysis of five replicates at 0.04 (LLOQ), 1.25, 10 and 40 µg/mL. The obtained CVs were once again within the accepted criteria, with the CVs lower than ±14%, and accuracy ranging from 0.5 to 14% (Table 4). The outliers were calculated considering these FDA criteria at the level of each parameter, considering the permitted CV and RE.

Table 4. Inter-day (n = 5), intra-day (n = 5) precision and accuracy.

Concentration	Inter-Day Precision			Intra-Day Precision		
(µg/mL)	Measured*	CV (%)	RE	Measured*	CV (%)	RE
0.04	0.04 ± 0.003	7.24	3.94	0.04 ± 0.005	13.78	−2.00
1.25	1.22 ± 0.050	4.07	−2.40	1.24 ± 0.056	4.53	−0.48

10	9.97 ± 0.652	6.54	−0.32	11.35 ± 0.616	5.43	13.54
40	39.80 ± 2.195	5.52	−0.51	40.96 ± 2.256	5.51	2.40

*Mean values ± standard deviation.

Parameters that were evaluated consisted of selectivity, linearity limit of quantification (LLOQ), limit of detection (LOD), accuracy and precision. The selectivity method was evaluated in the presence of potential interferences (e.g., compounds of the medium of culture) and no signals at the retention time of DNPZ were detected. Linearity was tested in the concentration range from 0.04 (LLOQ) to 40 µg/mL. The acceptance criteria of acceptance included the determination coefficient value (R^2) > 0.99, as well as the calibrators' accuracy within a ±15% (except at the LLOQ, where ±20% was considered acceptable). Table 5 shows the calibration data.

Table 5. Linearity data (n=5), LOD and LLOQ (n=10).

Linear Range	**Linearity**		**R^{2a}**	**LOD**	**LLOQ**
(µg/mL)	**Slope [a]**	**Intercept [a]**		**(µg/mL)**	**(µg/mL)**
0.04–40	4.088 ± 0.034	0.050 ± 0.131	0.9994 ± 0.0004	0.04	0.04

[a] Mean values ± standard deviation.

4.4. Statistical analysis

A normality test was performed to ensure a normal distribution (Shapiro–Wilk normality test). CircWave v1.4 analysis software (Dr. Roelof A. Hut) was used to analyze the rhythmicity of rBMAL1, rABCG2 and DNPZ concentrations in all three compartments (apical, basal and cells) by a harmonic regression method, with an assumed period of 24 h and an alpha set at 0.05 for the expression data, and an assumed period of 48 h and an alpha set at 0.05 for the transport data. Oscillations were considered statistically significant when $p<0.05$. The outliers were calculated using the ESD method (extreme studentized deviate) with an alpha = 0.05.

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Chapter 6

Concluding Remarks and Future Trends

1. Concluding remarks and future trends

1.1. Concluding remarks

Multiple challenges arise when developing therapeutic agents to treat CNS diseases. One of these is the existence of two major brain barriers that limit the traffic of molecules into the brain (Howes & Mehta, 2021). These two barriers are the BBB, which is the largest brain barrier, and the BCSFB. Both these barriers possess membrane transporters and receptors, synthesize detoxification enzymes and have their cells united by tight junctions. Together, these characteristics and mechanisms limit the passage of substances into the brain and make them a major hurdle for CNS diseases pharmacotherapy. The BBB is located in the brain capillaries and presents an endothelial-like structure that protects the brain from physiological fluctuations in the concentration of multiple plasma solutes and from blood-borne compounds that could be harmful for the brain tissue and hinder its functions. Besides, it also presents multiple mechanisms that enable the exchange of metabolic waste products, nutrients, ions and molecular signals between the blood and the brain's interstitial fluid. In contrast, the CP, which forms the BCSFB, is comprised by a monolayer of modified cuboidal epithelial cells, and it separates a network of fenestrated blood capillaries from the CSF. The BBB is much less permeable than the BCSFB and this is due to the fact that the BCSFB is characterized, such as some segments of the kidneys or gut, as a leaky epithelium (Redzic, 2011). This characteristic enables the secretion of large volumes of liquid with little energy expenditure which is essential for the production of CSF. More recently, the BCSFB has been object of increasing attention by the scientific community. For many years, CSF production was the only relevant function attributed to the CP, and although a very important one, limited the scientific research of this structure that has been done in the past, when compared to the BBB (Strazielle & Ghersi-Egea, 2016). As such, the mechanisms governing the transport of drugs across the BCSFB is still poorly studied and understood.

This project focused on the characterisation of the circadian expression of the CP membrane transporters as well the circadian rhythmicity of drug transport across BCSFB (namely MTX and DNPZ) to determine the best time of the day to improve the delivery of medicines into the brain.

Overall, the results presented in this doctoral thesis demonstrate that CP membrane transporters are expressed with a circadian rhythmicity. This rhythmicity is present in both *in vitro* models (using rat and human CP cells) and *in vivo* (rat's CP). This *in* vivo rhythmic expression was also found to be influenced by sex. Besides, membrane transporters activity, namely the transport of drugs across the BCSFB is also associated to circadian rhythms. It was demonstrated that the transport of MTX and DNPZ across the BCSFB is circadian rhythm dependent and that this circadian transport is sustained by CP's membrane transporters.

The surface area of the BBB in the human brain is estimated to be 10-fold higher than the BCSFB's surface area (Pardridge, 2016). The permeability coefficient to a broad spectrum of drugs has also been estimated for both these membranes. It was found that, when drug

permeation across the membrane is low, the clearance rate was limited by surface area which makes drug clearance across the BBB 10-fold greater than drug clearance across the BCSFB (Pardridge, 2016). On the other hand, when drug permeation across the membrane is high, drugs clearance is limited by blood flow making BBB's drug clearance 130-fold higher than BCSFB's (Pardridge, 2016). Drug permeation across the BBB and the BCSFB may be comparable for drugs that use lipid-mediated diffusion to cross the brain barriers, but for drugs that are dependent on membrane transporters activity, the permeability coefficient is dependent on the relative expression of those membrane transporters across the BBB and the BCSFB (Pardridge, 2016). As such, the permeability of both these barriers to different therapeutic agents will be variable as it will depend on the relative abundance of the drug specific membrane transporters and receptors. This is also relevant as CSF drug measurements shouldn't be considered to estimate drug transport across the BBB. Another consideration that needs to be taken into account is that the CSF and brain's interstitial fluid drug concentrations are not in equilibrium. There are three reasons for this: the rapid turnover of CSF-borne drugs into the blood stream, the accelerated CSF turnover (approximately every 5 hours) when compared to the velocity of diffusion of therapeutic compounds into the brain parenchyma, and the drug diffusion from the CSF into the brain parenchyma is a logarithmic function, which means that, the outer layers of the brain in contact with the CSF will present higher concentrations of drug when compared to the inner layers (Pardridge, 2016). Taking together, it is important to acknowledge that the BBB is probably a more viable rout for CNS therapy.

At the moment only a few studies focus on BBB circadian rhythms (Mineiro et al., 2023). Despite this, a functional molecular clock in the BBB was recently identified. Multiple molecular clock genes, such as Bmal1 and Per2, and CCGs have been found to be rhythmically expressed in mouse brain endothelial cells (Zhang et al., 2021). Bmal1 expression is rhythmic in the brain microvessels of Wistar rats (Szczepkowska et al., 2021). Few studies addressed the circadian rhythms of membrane transporters expression and activity in the BBB. Most of the data available is focused on the ABCB1 efflux transporter, whose expression was not rhythmic in either control mice or in endothelial Bmal1 knock out mice (Zhang et al., 2021). However, brain permeability to MC225 (a substrate of ABCB1) increased in Wistar rats at ZT15, matching the active phase of these animals (Savolainen et al., 2016). Bmal1 deletion also impaired the rhythmic Rhodamine 123 and Rhodamine B efflux by mice endothelial cells (Pulido et al., 2020). ABCB1 protein expression also revealed no circadian oscillation in mice brain capillaries (Ogata et al., 2022). This is indicative of other mechanisms that are controlling ABCB1 circadian activity that are independent of this membrane transporters expression. As such, the circadian oscillations of ABCB1 might be dependent of Mg^{2+} rhythms as suggested by *in vitro* experiments using the human cerebral microvascular endothelial cell line (hCMED/D3) (Zhang et al., 2021). Mg^{2+} is a cofactor for ABC transporters. Transient receptor potential cation channel (TRPM7) gene is under direct circadian control by the molecular clock (Zhang et al., 2021). In hCMED/D3, the circadian rhythms of Mg^{2+} are mediated by TRPM7. Mg^{2+} circadian regulation of ABCB1 activity has also been reported in *Drosophila* (Zhang et al., 2018). Regarding other ABC family transporters, a

microarray study of the mouse brain endothelial cells circadian transcriptome revealed that only Abcg2 seems to present circadian rhythmic expression (Mineiro et al., 2023; Zhang et al., 2021). At the protein level, neither ABCC4, ABCC9 or ABCG2 showed significant circadian rhythmic levels in mouse brain capillaries (Ogata et al., 2022). In regard to the SLC family transporters, only *Slc7a5* gene expression showed circadian rhythmicity in mouse brain endothelial cells (Zhang et al., 2021). At the protein level, this rhythmicity is lost as revealed by a proteomics study of the mouse brain capillaries (Ogata et al., 2022). This study also reported no circadian rhythmicity for SLC2A1, SLC3A2, SLC16A1, SLC16A2 or SLC22A8 (Ogata et al., 2022), The only transporter showing circadian rhythmic protein levels was SLC9A3R2 (Ogata et al., 2022). SLC9A3R2 is a regulatory protein that interacts with the SLC9A3 membrane transporter (Jiang et al., 2022). Putting this together, with the already hypothesized circadian regulation of ABCB1 by Mg^{2+}, it is possible that circadian-dependent transport at the BBB by membrane transporter proteins is not dependent of direct circadian regulation of membrane transporters' expression but by indirect circadian control. Nevertheless, this needs to be analysed carefully as both these studies have been performed in murine models and there is also a known species-dependent effect of circadian rhythms in membrane transporters expression.

Currently there are multiple studies evidencing the impact of sex hormone background on membrane transporters expression and activity in multiple tissues including the BBB (Dalla et al., 2022). There is evidence of sex hormone background impacting ABCB1 activity (Kanado et al., 2019). ABCG2 expression seems to be induced by testosterone and inhibited by estradiol in peripheral tissues but this trend appears to be reversed in the brain as demonstrated by a study using female mice which revealed higher expression of this transporter in females' brain than males (Fu et al., 2012; Tanaka et al., 2005). ABCC1, ABCC2 and ABCC4 and SLCO1A2 also seem to be more expressed in the female BBB (Brzica et al., 2018; Flores et al., 2017; Zhang et al., 2013). SLC22A8 showed no circadian rhythmic expression in the BBB in an *in vivo* study using rats (Ohtsuki et al., 2005). These membrane transporters and multiple others involved in therapeutic drugs transport across the brain barriers have also revealed sex dependent expression and activity in other peripheral tissues (Dalla et al., 2022). Further research needs to be done regarding sex hormone background impact on these membrane transporters expression and activity in the BBB. Still, from the data available from this thesis we have also demonstrated the impact of sex over the circadian expression of membrane transporters at the BCSFB. As such, it clearly seems to be a direct relation between sex hormone background, membrane transporters expression levels and membrane transporters circadian rhythmic expression and activity at the BCSFB and possibly also at the BBB.

Nevertheless, the BCSFB is still a very important player in this subject. The fact that certain drugs depend exclusively of membrane transporters activity to cross biological barriers and that the membrane transporters relative expression is variable between the BBB and the BCSFB makes the BCSFB an important route to be considered for certain therapeutic approaches.

We expect that the work here presented can be useful in the future to better understand how circadian rhythms influence brain barriers, namely BCSFB, membrane transporters expression and activity. A deeper knowledge of transporters circadian pattern and activity will certainly give us a better understanding on how the transport of drugs and other compounds through the BCSFB occurs along the day. It should also spark greater research of the mechanisms governing the circadian transport of therapeutic agents across the BBB as the BBB and the BCSFB might complement each other. The involvement of circadian rhythms on the activity of ABC and SLC family of transporters at the BBB still needs to be thoroughly researched (Cuddapah et al., 2019). Nevertheless, there have already been disclosed some clues that might suggest that at least some sort of circadian regulation is exerted over these membrane transporters expression and activity. ABCB1's activity, tight junction proteins' expression, endocytic activity and perivascular clearance have all been demonstrated to be circadian dependent at the BBB (Cuddapah et al., 2019).

This work is also paving the way for the circadian modulation of CNS pharmacological therapy. The results and data from this work might prove to be essential to optimize CNS therapy by considering the BCSFB circadian rhythms. The objective is to control and improve the penetration of drugs into the CNS and thus improving the therapy's outcome while also reducing side effects. This is in line with an increasing advance for the transition to personalized therapies. By predicting the optimal time of day for drug administration according to the patient's own circadian rhythms we can provide a groundbreaking strategy to improve the overall success of CNS disease therapy.

1.2. Future Trends

Beyond the scientific advances achieved with the experimental work carried out in this thesis, several new hypotheses for disclosing CP circadian rhythm influence on CNS therapy were questioned:

Where are CP's membrane transporters located within the BCSFB?

Many membrane transporters have been described in the CP. There have been identified a total of 110 membrane transporters in the CP from both the ABC and SLC superfamilies (Mineiro, 2020). From these, only 34 have been identified in human samples (approximately 30%). In addition, only 51 have a known cellular localization in the BCSFB epithelium, which represents less than 50% of the total number of identified membrane transporters and is mostly described for animal models (Mineiro, 2020). Therefore, there is still a significant amount of membrane transporters to be described in human CP samples and their respective localization within the BCSFB structure (basal membrane, apical membrane, subapical membrane and/or cytoplasm). Using the HIBCPP cell line and resorting to both PCR and immunocytochemistry followed by confocal fluorescence microscopy, will help to study the expression and cellular localization of the membrane transporters that are still to be identified and/or localized.

Which membrane transporters present in the CP have a circadian rhythmic expression?

Having the full view of which membrane transporters are expressed in the CP and their respective cellular localization, it is important to disclose which ones are expressed with an associated circadian rhythmicity. This work is a step into that direction as we were able to demonstrate the circadian expression of multiple membrane transporters. To further explore this subject, we could use synchronized genetically modified bioluminescence reporter-expressing CP cell cultures where the target membrane transporters expressed a specific bioluminescence marker. First, the CP cell cultures would need to undergo genetic edition to attach a bioluminescence reporter gene to the target membrane transporter's gene. With the use of a bioluminescence detection system, we would be able to record in real time the expression of our target membrane transporters. Essential to this is the synchronization of the genetically modified cell cultures to express and sustain the circadian expression of the target membrane transporter at the same time across all the cultured cells. By doing this we would be able to have real time and continuous data of the expression profile of the target membrane transporter.

How does sex hormone background influences membrane transporter's circadian expression?

During this project we have already disclosed that sex is a factor that influences the circadian expression of several membrane transporters. As such, it is important to address the impact of sex hormone background over the membrane transporters circadian expression. To do this, *in vivo* models such as rats are essential. Another crucial aspect of these animals is their genetic modification using CRISPR to associate a radionuclide-fluorescence reporter gene to the target membrane transporter's gene. This would allow us to assess the target membrane transporter's expression using imaging technics such as positron emission tomography (PET) scan. Afterwards animals could be separated into 6 different groups: intact males, intact females, gonadectomized males, gonadectomized females, sham-operated males and sham-operated females. This experimental setup could be replicated for every membrane transporter that was previously found to be rhythmically expressed in the aforementioned assays using *in vivo* models. This should give us a data set of how the sex hormones background impacts the membrane transporter's expression.

Which membrane transporters present in the CP have a circadian rhythmic activity? Is it sex hormone dependent?

It would be also important to evaluate if membrane transporters that have circadian rhythmic expression also present circadian rhythmic activity. Moreover, it is also valuable to understand if the membrane transporters that don't reveal circadian rhythmicity at the CP display circadian rhythmic activity. Another aspect to take into account is the effect of sex hormones over the membrane transporter's activity. To assess this, we could use an *in vivo* model, radiolabelled drugs, specific membrane transporter inhibitors and a computed tomography (CT) scanner. The *in vivo* would allow us to assess how does the target drug behaves when crossing the BCSFB

during the 24-hours. Groups of different sexes (males and females) would enable us to assess the effects of sex hormone background over the membrane transporter's circadian activity. Radiolabelled drugs would allow the CT scanner to track their flow across the BCSFB in real time. Specific membrane transporter inhibitors are also essential. A drug can be a substrate to multiple membrane transporters located in the BCSFB, thus the other membrane transporters involved should be inhibited using specific inhibitors. So, after administering the radiolabelled drug to the *in vivo* model, the CT scanner would allow us to follow circadian transport rhythms associated to our target membrane transporter at the BCSFB. This experimental setup could be used to study every membrane transporter of interest.

How are the previous results relevant for improving chronotherapeutic approaches?

In silico models could be generated to better understand the information that the aforementioned assays would provide. After interpreting these *in silico* models, new chronotherapeutic approaches could be devised and evaluated *in vivo* to determine which approach would be more beneficial for CNS disease treatment.

How does BCSFB's membrane transporters activity complement the BBB's membrane transporters activity?

The study of the impact of circadian rhythms in membrane transporters' expression cannot be limited to the BCSFB as there are two barriers controlling the flux of molecules to and from the CNS. At the moment, it seems that BBB's membrane transporters' circadian activity is not directly regulated by the circadian clock. It appears to be under circadian regulation of other CCG pathways. A human 3-dimensional cell model of the BBB, BBB on-chip models and/or microfluidics BBB models could be used for this study (Shah & Dong, 2022). The impact of circadian rhythms on BBB's membrane transporters activity needs to be addressed and studied to have a better understanding of how circadian rhythms are modulating molecules' traffic at the BBB. This study will help us to understand how both the BCSFB and the BBB interact and complement each other.

How do we envision the future of chronotherapy?

In medicine we are each day moving towards a more personalized therapy. As such, in the future, patients could have their specific circadian rhythms evaluated and the chronotherapeutic approach for the treatment of their pathology will have this into account when administering the required drugs. We have a long way to go, but computer-based models are getting more advanced as each day passes. With the multiple assays described earlier we could gather large amounts of information essential to the development of computer based circadian models of the membrane transporter activity. Having this information, chronotherapy could then be implemented to improve treatments efficacy, reduce its side effects, dosage and associated costs.

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Attachments

Attachment 1

Central nervous system disease's pharmacological treatment and respective membrane transporters.

CNS Disease	Drug	Membrane Transporter	CP	References
Alzheimer's disease	Galantamine			(*How Is Alzheimer's Disease Treated?*, 2023)
		ABCB1	Yes	(Namanja et al., 2009; Rao et al., 1999; Saunders et al., 2015)
	Rivastigmine			(*How Is Alzheimer's Disease Treated?*, 2023)
	Donepezil			(*How Is Alzheimer's Disease Treated?*, 2023)
		ABCG2	Yes	(Takeuchi et al., 2016; Tomioka et al., 2016)
	Memantine			(*How Is Alzheimer's Disease Treated?*, 2023)
		SLC22A2	Yes	(Busch et al., 1998; Müller et al., 2017; Saunders et al., 2015; Sweet et al., 2001)
		SLC9A1	Yes	(Davson & Segal, 1970; Kalaria et al., 1998; Mehta et al., 2013a, 2013b; Segal & Burgess, 1974)
		SLC22A4	Yes	(Mehta et al., 2013a; Sweet et al., 2001)
		SLC47A1	Yes	(Müller et al., 2017; Uchida et al., 2015)
	Lecanemab			(*How Is Alzheimer's Disease Treated?*, 2023)
	Aducanumab			(*How Is Alzheimer's Disease Treated?*, 2023)
Brain Tumours	Dabrafenib			(Davies et al., 2017)
Intracranial metastases		ABCB1	Yes	("FDA Approved Drug Products: TAFINLAR® (dabrafenib) capsules, for oral use," 2013; Mittapalli et al., 2013; Rao et al., 1999; Saunders et al., 2015)
		ABCG2	Yes	(Ellens et al., 2017; "FDA Approved Drug

				Products: TAFINLAR® (dabrafenib) capsules, for oral use," 2013; Mittapalli et al., 2013; Tomioka et al., 2016)
		SLCO1B1		(Ellens et al., 2017; "FDA Approved Drug Products: TAFINLAR® (dabrafenib) capsules, for oral use," 2013)
		SLCO1B3		(Ellens et al., 2017; "FDA Approved Drug Products: TAFINLAR® (dabrafenib) capsules, for oral use," 2013)
		SLCO1A2		(Ellens et al., 2017)
		SLC22A6	Yes	(Alebouyeh et al., 2003; "FDA Approved Drug Products: TAFINLAR® (dabrafenib) capsules, for oral use," 2013)
		SLC22A8	Yes	(Alebouyeh et al., 2003; "FDA Approved Drug Products: TAFINLAR® (dabrafenib) capsules, for oral use," 2013; Nagata et al., 2002; Roberts et al., 2008; Sykes et al., 2004)
		SLC22A2	Yes	(Ellens et al., 2017; Saunders et al., 2015; Sweet et al., 2001)
	Trametinib			(Davies et al., 2017)
	Pembrolizumab			(Goldberg et al., 2016)
Meningiomas	Chemotherapy is mostly only used in refractory disease			(McFaline-Figueroa & Lee, 2018)
Glioblastomas	Temozolomide			(Zhang et al., 2019)
		ABCB1	Yes	(de Gooijer et al., 2018; Lin et al., 2014; Munoz et al., 2015; Rao et al., 1999; Saunders et al., 2015)
		ABCG2	Yes	(de Gooijer et al., 2018; Lin et al., 2014; Tomioka et al., 2016)
	Carmustine			(Zhang et al., 2019)

	Lomustine			(Zhang et al., 2019)
	Bevacizumab			(Zhang et al., 2019)
	Methotrexate			(Battaglia et al., 2017; Madani et al., 2020; Pereira et al., 2018; Ye et al., 2018)
		ABCC3	Yes	(Akita et al., 2002; Bernd et al., 2015; Hirohashi et al., 1999; Li et al., 2003; Oleschuk et al., 2003; Paumi et al., 2003; Zehnpfennig et al., 2009; Zeng et al., 2001; Zeng et al., 2000)
		ABCC4	Yes	(Bai et al., 2004; Bernd et al., 2015; Chen et al., 2001; Rius et al., 2003; Roberts et al., 2008; van Aubel et al., 2002)
		ABCC1	Yes	(Bernd et al., 2015; Gazzin et al., 2008; Heijn et al., 1997; Ohtsuki et al., 2003; Paumi et al., 2003; Roberts et al., 2008; Zeng et al., 2001)
		SLC22A6	Yes	(Alebouyeh et al., 2003; Kuze et al., 1999; Lu et al., 1999; Sekine et al., 1997; Takeda, Khamdang, Narikawa, Kimura, Hosoyamada, et al., 2002; Uwai & Iwamoto, 2010; Uwai et al., 1998)
		ABCC10	Yes	(Z. S. Chen, E. Hopper-Borge, et al., 2003; Kratzer et al., 2013)
		SLC22A8	Yes	(Alebouyeh et al., 2003; Cha et al., 2001; Kusuhara et al., 1999; Nagata et al., 2002; Ohtsuki et al., 2004; Roberts et al., 2008; Sykes et al., 2004; Takeda, Khamdang, Narikawa, Kimura, Hosoyamada, et al., 2002; Uwai & Iwamoto, 2010; VanWert & Sweet, 2008)
		ABCC2	Yes	(Bakos et al., 2000; Bernd et al., 2015; C. Chen et al., 2003; Han et al., 2001;

		Hooijberg et al., 1999; Masuda et al., 1997)
ABCB1	Yes	(Norris et al., 1996; Rao et al., 1999; Saunders et al., 2015)
SLCO1A2		(Cattori et al., 2001)
SLC16A1	Yes	(Tamai et al., 1999; Ulloa et al., 2019)
ABCC11		(Chen et al., 2005)
SLCO1B3		(Abe et al., 2001)
SLC22A11		(Takeda, Khamdang, Narikawa, Kimura, Hosoyamada, et al., 2002)
SLCO1C1	Yes	(Ho et al., 2012; Pizzagalli et al., 2002; Sugiyama et al., 2003)
SLCO3A1	Yes	(Adachi et al., 2003; Kratzer et al., 2013)
ABCG2	Yes	(Breedveld et al., 2004; Z. S. Chen, R. W. Robey, et al., 2003; Dai et al., 2009; Hou et al., 2009; Mitomo et al., 2003; Suzuki et al., 2009; Suzuki et al., 2003; Tiwari et al., 2009; Tomioka et al., 2016; Volk & Schneider, 2003)
SLC22A7		(Sekine et al., 1998; Sun et al., 2001)
SLC46A1	Yes	(Nakai et al., 2007; Wollack et al., 2008)
SLCO1B1		(Abe et al., 2001; van de Steeg et al., 2009)
SLCO4C1		(Mikkaichi et al., 2004)
SLC19A1	Yes	(Hinken et al., 2011; Qiu et al., 2006)
FOLR1	Yes	(Olney et al., 2022; Sharma et al., 2008)
FOLR2	Yes	(Inoue & Yuasa, 2014; Ramaekers et al., 2018)

		SLC15A1	Yes	(Inoue & Yuasa, 2014; Morris et al., 2017)
		SLC36A1	Yes	(Agulhon et al., 2003; Inoue & Yuasa, 2014)
Parkinson's disease	Levodopa			(Dezsi & Vecsei, 2017; Gray et al., 2014)
		SLC15A1		(Han et al., 1999; Tamai et al., 1998)
		SLC16A10	Yes	(Kim et al., 2001; Richardson et al., 2018; Saunders et al., 2015)
		SLC7A5		(Kageyama et al., 2000; Pinho et al., 2004)
		SLC7A8	Yes	(Dolgodilina et al., 2020; Pinho et al., 2004)
	Selegiline			(Dezsi & Vecsei, 2017; Gray et al., 2014)
		ABCB1	Yes	(Mahar Doan et al., 2002; Rao et al., 1999; Saunders et al., 2015)
	Rasagiline			(Dezsi & Vecsei, 2017; Gray et al., 2014)
	Safinamide			(Dezsi & Vecsei, 2017; Gray et al., 2014)
		ABCG2	Yes	(Pevarello & Varasi, 2018; Tomioka et al., 2016)
		SLCO3A1	Yes	(Kratzer et al., 2013; Leuratti et al., 2013)
	Bromocriptine			(Brooks, 2000)
		ABCB1	Yes	(Ekins et al., 2002; Mahar Doan et al., 2002; Rao et al., 1999; Renaud et al., 1996; Saunders et al., 2015; Yasuda et al., 2002)
	Pergolide			(Brooks, 2000)
	Lisuride			(Brooks, 2000)
	Cabergoline			(Brooks, 2000)
		ABCB1	Yes	(Athanasoulia et al., 2012; Rao et al., 1999; Saunders et al., 2015)
	Ropinirole			(Brooks, 2000)
	Pramipexole			(Brooks, 2000)
		SLC22A2	Yes	(Diao et al., 2010; Ishiguro et al., 2005; Knop et al., 2015; Saunders et al., 2015; Sweet et al., 2001)
		SLC22A1	Yes	(Antonini & Calandrella, 2011; Choudhuri et al.,

				2003; Diao et al., 2010; Ishiguro et al., 2005)
		SLC22A3	Yes	(Choudhuri et al., 2003; Diao et al., 2010)
	Entacapone			(Kaakkola, 2000)
	Tolcapone			(Kaakkola, 2000)
	Amantadine			(Brocks, 1999; Rascol et al., 2021)
		SLC22A2	Yes	(Busch et al., 1998; Goralski et al., 2002; Ishiguro et al., 2005; Saunders et al., 2015; Sweet et al., 2001; Urakami et al., 2002)
		SLC22A1	Yes	(Choudhuri et al., 2003; Goralski et al., 2002; Harrach et al., 2016; Li et al., 2014)
	Biperiden			(Brocks, 1999; Rascol et al., 2021)
	Orphenadrine			(Brocks, 1999; Rascol et al., 2021)
	Diphenydramine			(Brocks, 1999; Rascol et al., 2021)
		SLC22A2	Yes	(Saunders et al., 2015; Sweet et al., 2001; Urakami et al., 2001)
		SLC22A5	Yes	(Kratzer et al., 2013; Ohashi et al., 2001)
	Procyclidine			(Brocks, 1999; Rascol et al., 2021)
	Trihexyphenidyl			(Brocks, 1999; Rascol et al., 2021)
	Benztropine			(Brocks, 1999; Rascol et al., 2021)
Epilepsy	Valproic acid			(Kohrman, 2007)
		SLC22A6	Yes	(Alebouyeh et al., 2003; Sekine et al., 1997)
		SLC22A8	Yes	(Alebouyeh et al., 2003; Cha et al., 2001; Nagata et al., 2002; Ohtsuki et al., 2002; Roberts et al., 2008; Sykes et al., 2004)
		SLC22A5	Yes	(Kratzer et al., 2013; Ohashi et al., 1999)
		SLC16A1	Yes	(Tamai et al., 1999; Ulloa et al., 2019)
		SLC22A7		(Kobayashi et al., 2002)
		SLCO2B1	Yes	(Karlgren, Vildhede, et al., 2012; Roberts et al., 2008)

	Phenobarbital			(Kohrman, 2007)
		ABCB1	Yes	(Luna-Tortós et al., 2008; Rao et al., 1999; Saunders et al., 2015; Schuetz et al., 1996)
		ABCC3	Yes	(Bernd et al., 2015; Cherrington et al., 2003; Kiuchi et al., 1998; Ogawa et al., 2000; Slitt et al., 2003)
		ABCB11		(Kast et al., 2002)
		ABCC1	Yes	(Bernd et al., 2015; Gazzin et al., 2008; Kiuchi et al., 1998; Ohtsuki et al., 2003; Roberts et al., 2008)
		SLCO2A1	Yes	(Hagenbuch et al., 2001; Kratzer et al., 2013)
		ABCC2	Yes	(Bernd et al., 2015; Courtois et al., 2002; Johnson et al., 2002; Kast et al., 2002; Kauffmann & Schrenk, 1998; Schrenk et al., 2001)
	Phenytoin			(Kohrman, 2007)
		SLCO1C1	Yes	(Berman et al., 2000; Ho et al., 2012; Westholm et al., 2009)
		ABCB1	Yes	(Baltes et al., 2007; Luna-Tortós et al., 2008; Rao et al., 1999; Saunders et al., 2015; Simon et al., 2007; Thorn et al., 2012)
		ABCC2	Yes	(Baltes et al., 2007; Bernd et al., 2015; Potschka et al., 2003b; Simon et al., 2007)
	Carbamazepine			(Kohrman, 2007)
		RALBP1		(Awasthi et al., 2005)
		ABCC2	Yes	(Bernd et al., 2015; Potschka et al., 2003a; Ufer et al., 2009)
	Lamotrigine			(Kohrman, 2007)
		ABCB1	Yes	(Luna-Tortós et al., 2008; Rao et al., 1999; Saunders et al., 2015; Weiss et al., 2003)
		SLC22A2	Yes	(Dos Santos Pereira et al., 2014; Saunders et al., 2015;

				Sweet et al., 2001)
		SLC22A1	Yes	(Choudhuri et al., 2003; Dickens et al., 2012; Wagner et al., 2016)
	Benzodiazepines			(Kohrman, 2007)
	Ethosuximide			(Kohrman, 2007)
	Felbamate			(Kohrman, 2007)
	Gabapentin			(Kohrman, 2007)
		SLC7A5	Yes	(Bockbrader et al., 2010; Dickens et al., 2013; Roberts et al., 2008; Xiang et al., 2003)
	Vigabatrin			(Kohrman, 2007)
		SLC36A1	Yes	(Abbot et al., 2006; Agulhon et al., 2003)
	Topiramate			(Kohrman, 2007)
		ABCB1	Yes	(Luna-Tortós et al., 2009; Rao et al., 1999; Saunders et al., 2015; Sills et al., 2002)
	Tiagabine			(Kohrman, 2007)
	Levetiracetam			(Kohrman, 2007)
		ABCB1	Yes	(Baltes et al., 2007; Luna-Tortós et al., 2008; Rao et al., 1999; Saunders et al., 2015)
	Oxcarbazepine			(Kohrman, 2007)
		ABCB1	Yes	(Antunes et al., 2017; Rao et al., 1999; Saunders et al., 2015; Zhang et al., 2011)
	Zonisamide			(Kohrman, 2007)
		ABCB1	Yes	(Chan et al., 2014; Rao et al., 1999; Saunders et al., 2015)
	Pregabalin			(Kohrman, 2007)
		SLC1A1	Yes	(Akanuma et al., 2015; Dahlin et al., 2009; Ryu et al., 2012)
		SLC7A5	Yes	(Roberts et al., 2008; Takahashi et al., 2018; Xiang et al., 2003)
Stroke	Tissue Plasminogen Activator			(Yepes, 2023)
CNS Infection	**Brain Abscess** Penicillin G			(Mathisen & Johnson, 1997)
		SLC22A5	Yes	(Ganapathy et al., 2000; Kratzer et al., 2013)
		SLC15A1	Yes	(Luckner & Brandsch, 2005; Morris et al., 2017; Tsuji, 2002)

		SLC15A2	Yes	(Luckner & Brandsch, 2005; Shen et al., 2005)
		SLC22A6	Yes	(Alebouyeh et al., 2003; Hasegawa et al., 2002; Hosoyamada et al., 1999; Jariyawat et al., 1999; Jung et al., 2001; Lu et al., 1999; Takeda, Khamdang, Narikawa, Kimura, Hosoyamada, et al., 2002)
		SLC22A8	Yes	(Alebouyeh et al., 2003; Cha et al., 2001; Hasegawa et al., 2002; Jung et al., 2001; Kusuhara et al., 1999; Nagata et al., 2002; Ohtsuki et al., 2004; Roberts et al., 2008; Sykes et al., 2004; Takeda, Khamdang, Narikawa, Kimura, Hosoyamada, et al., 2002)
		SLC22A4	Yes	(Sweet et al., 2001; Yabuuchi et al., 1999)
		SLC22A11		(Babu et al., 2002; Takeda, Khamdang, Narikawa, Kimura, Hosoyamada, et al., 2002)
		SLCO2B1	Yes	(Roberts et al., 2008; Tamai et al., 2000)
		SLCO4A1	Yes	(Tamai et al., 2000; Zhang et al., 2010)
		SLCO3A1	Yes	(Kratzer et al., 2013; Tamai et al., 2000)
		SLCO1B1		(Tamai et al., 2000)
	Metronidazole			(Mathisen & Johnson, 1997)
		ABCB1	Yes	(Rao et al., 1999; Saunders et al., 2015; Tan et al., 2011)
	Cephalosporin			(Mathisen & Johnson, 1997)
	Vancomycin			(Mathisen & Johnson, 1997)
	Oxacillin			(Mathisen & Johnson, 1997)
		SLC15A1	Yes	(Luckner & Brandsch, 2005; Morris et al., 2017)
		SLC15A2	Yes	(Luckner & Brandsch, 2005; Shen et al., 2005)

	Nafcillin			(Mathisen & Johnson, 1997)
		SLC22A6	Yes	(Alebouyeh et al., 2003; Jariyawat et al., 1999)
	Chloranphenicol			(Mathisen & Johnson, 1997)
		SLC22A6	Yes	(Alebouyeh et al., 2003; Jariyawat et al., 1999)
	Cefotaxime			(Mathisen & Johnson, 1997)
		SLC15A1	Yes	(Luckner & Brandsch, 2005; Morris et al., 2017)
		SLC15A2	Yes	(Luckner & Brandsch, 2005; Shen et al., 2005)
		SLC22A6	Yes	(Alebouyeh et al., 2003; Jariyawat et al., 1999; Jung et al., 2002; Takeda, Babu, et al., 2002)
		SLC22A8	Yes	(Alebouyeh et al., 2003; Jung et al., 2002; Nagata et al., 2002; Roberts et al., 2008; Sykes et al., 2004; Takeda, Babu, et al., 2002)
		SLC22A11		(Takeda, Babu, et al., 2002)
	Latamoxef			(Mathisen & Johnson, 1997)
	Ceftazidime			(Mathisen & Johnson, 1997)
		SLC22A6	Yes	(Alebouyeh et al., 2003; Jariyawat et al., 1999)
	Imipenem			(Mathisen & Johnson, 1997)
	Ciprofloxacin			(Mathisen & Johnson, 1997)
		ABCB1	Yes	(Rao et al., 1999; Saunders et al., 2015; Wang et al., 2001; Yamaguchi et al., 2004)
		SLC47A1	Yes	(Ohta et al., 2009; Tanihara et al., 2007; Uchida et al., 2015)
		SLC47A2	Yes	(Ek et al., 2015; Tanihara et al., 2007)
Bacterial Meningitis	Ampicillin			(Brouwer et al., 2010; van de Beek et al., 2006)
		SLC22A5	Yes	(Ganapathy et al., 2000; Kratzer et al., 2013)
		SLC15A1	Yes	(Covitz et al., 1996; Guo et al., 1999; Luckner & Brandsch, 2005; Morris et al., 2017; Sala-Rabanal et al., 2006; Terada et al., 1997)

	SLC15A2	Yes	(Luckner & Brandsch, 2005; Shen et al., 2005; Terada et al., 1997)
	SLC16A1	Yes	(Li et al., 1999; Ulloa et al., 2019)
Vancomycin			(Brouwer et al., 2010; van de Beek et al., 2006)
Ceftriaxone			(Brouwer et al., 2010; van de Beek et al., 2006)
	SLC15A1	Yes	(Luckner & Brandsch, 2005; Morris et al., 2017)
	SLC15A2	Yes	(Luckner & Brandsch, 2005; Shen et al., 2005)
	SLC22A6	Yes	(Alebouyeh et al., 2003; Jariyawat et al., 1999; Jung et al., 2002; Takeda, Babu, et al., 2002)
	SLC22A8	Yes	(Alebouyeh et al., 2003; Jung et al., 2002; Nagata et al., 2002; Roberts et al., 2008; Sykes et al., 2004; Takeda, Babu, et al., 2002)
	SLC22A2	Yes	(Saunders et al., 2015; Sweet et al., 2001; Takeda, Babu, et al., 2002)
	ABCB1	Yes	(Hankø et al., 2003; Rao et al., 1999; Saunders et al., 2015)
Cefotaxime			(Brouwer et al., 2010; van de Beek et al., 2006)
	SLC15A1	Yes	(Luckner & Brandsch, 2005; Morris et al., 2017)
	SLC15A2	Yes	(Luckner & Brandsch, 2005; Shen et al., 2005)
	SLC22A6	Yes	(Alebouyeh et al., 2003; Jariyawat et al., 1999; Jung et al., 2002; Takeda, Babu, et al., 2002)
	SLC22A8	Yes	(Alebouyeh et al., 2003; Jung et al., 2002; Nagata et al., 2002; Roberts et al., 2008; Sykes et al., 2004; Takeda, Babu, et al., 2002)
	SLC22A2	Yes	(Saunders et al., 2015; Sweet et al., 2001; Takeda, Babu, et al., 2002)

		SLC22A7		(Khamdang et al., 2003)
	Penicillin G			(Brouwer et al., 2010; van de Beek et al., 2006)
		SLC22A5	Yes	(Ganapathy et al., 2000; Kratzer et al., 2013)
		SLC15A1	Yes	(Luckner & Brandsch, 2005; Morris et al., 2017; Tsuji, 2002)
		SLC15A2	Yes	(Luckner & Brandsch, 2005; Shen et al., 2005)
		SLC22A6	Yes	(Alebouyeh et al., 2003; Hasegawa et al., 2002; Hosoyamada et al., 1999; Jariyawat et al., 1999; Jung et al., 2001; Lu et al., 1999; Takeda, Khamdang, Narikawa, Kimura, Hosoyamada, et al., 2002)
		SLC22A8	Yes	(Alebouyeh et al., 2003; Cha et al., 2001; Hasegawa et al., 2002; Jung et al., 2001; Kusuhara et al., 1999; Nagata et al., 2002; Ohtsuki et al., 2004; Roberts et al., 2008; Sykes et al., 2004; Takeda, Khamdang, Narikawa, Kimura, Hosoyamada, et al., 2002)
		SLC22A4	Yes	(Sweet et al., 2001; Yabuuchi et al., 1999)
		SLC22A11		(Babu et al., 2002; Takeda, Khamdang, Narikawa, Kimura, Hosoyamada, et al., 2002)
		SLCO2B1	Yes	(Roberts et al., 2008; Tamai et al., 2000)
		SLCO4A1	Yes	(Tamai et al., 2000; Zhang et al., 2010)
		SLCO3A1	Yes	(Kratzer et al., 2013; Tamai et al., 2000)
		SLCO1B1		(Tamai et al., 2000)
	Amoxicillin			(Brouwer et al., 2010; van de Beek et al., 2006)
		SLC15A1	Yes	(Li et al., 2006; Luckner & Brandsch, 2005; Morris et al., 2017; Sala-

				Rabanal et al., 2006; Terada et al., 1997; Wenzel et al., 1996)
		SLC15A2	Yes	(Li et al., 2006; Luckner & Brandsch, 2005; Shen et al., 2005; Terada et al., 1997)
		SLC22A6	Yes	(Alebouyeh et al., 2003; Jariyawat et al., 1999; Li et al., 2006)
Viral Meningitis and Encephalitis	Acyclovir			(Bergström & Alestig, 1990)
		SLC22A1	Yes	(Boxberger et al., 2014; Choudhuri et al., 2003; Shugarts & Benet, 2009; Takeda, Khamdang, Narikawa, Kimura, Kobayashi, et al., 2002; Wagner et al., 2016)
		SLC22A6	Yes	(Alebouyeh et al., 2003; Takeda, Khamdang, Narikawa, Kimura, Kobayashi, et al., 2002; Wada et al., 2000)
		SLC22A8	Yes	(Alebouyeh et al., 2003; Cha et al., 2001; Nagata et al., 2002; Ohtsuki et al., 2002; Roberts et al., 2008; Sykes et al., 2004; Takeda, Khamdang, Narikawa, Kimura, Kobayashi, et al., 2002)
		SLC10A2		(Tolle-Sander et al., 2004)
		SLC47A1	Yes	(Nies et al., 2012; Uchida et al., 2015; Xu et al., 2015)
		SLC47A2	Yes	(Ek et al., 2015; Nies et al., 2012; Yonezawa & Inui, 2011)
Migraine	Acetaminophen			(Becker, 2015)
		ABCB1		(Faassen et al., 2003; Manov et al., 2006; Novak et al., 2013; Wang et al., 2001)
	Acetylsalicylic Acid			(Becker, 2015)
		SLC22A6		(Apiwattanakul et al., 1999; Parvez et al., 2017)
		ABCB1	Yes	(Faassen et al., 2003; Flescher et al., 2000; Kugai et al., 2013; Li et al., 2015; Oh et al., 2014; Rao et al.,

				1999; Saunders et al., 2015)
		SLC22A8	Yes	(Alebouyeh et al., 2003; Nagata et al., 2002; Parvez et al., 2017; Roberts et al., 2008; Sykes et al., 2004; Wang et al., 2014)
	Ibuprofen			(Becker, 2015)
		SLCO2B1	Yes	(Roberts et al., 2008; Satoh et al., 2005)
		ABCB1	Yes	(Faassen et al., 2003; Rao et al., 1999; Saunders et al., 2015)
		ABCC4	Yes	(Bernd et al., 2015; Reid et al., 2003; Roberts et al., 2008)
		ABCC1	Yes	(Bernd et al., 2015; Gazzin et al., 2008; Ohtsuki et al., 2003; Reid et al., 2003; Roberts et al., 2008)
		SLCO1A2		(Shitara et al., 2002)
		SLC22A6	Yes	(Alebouyeh et al., 2003; Apiwattanakul et al., 1999; Mulato et al., 2000; Takeda, Khamdang, Narikawa, Kimura, Hosoyamada, et al., 2002; Uwai et al., 2000)
		SLC22A8	Yes	(Alebouyeh et al., 2003; Cha et al., 2001; Kobayashi et al., 2004; Nagata et al., 2002; Roberts et al., 2008; Sykes et al., 2004; Takeda, Khamdang, Narikawa, Kimura, Hosoyamada, et al., 2002)
		SLC22A11		(Cha et al., 2001; Takeda, Khamdang, Narikawa, Kimura, Hosoyamada, et al., 2002)
	Naproxen			(Becker, 2015)
		SLCO1A2		(Shitara et al., 2002)
		SLC22A6	Yes	(Alebouyeh et al., 2003; Apiwattanakul et al., 1999; Mulato et al., 2000)
		ABCB11		(Pedersen et al., 2013)

		ABCB1	Yes	(Frost et al., 2014; Rao et al., 1999; Saunders et al., 2015)
		SLC22A8	Yes	(Alebouyeh et al., 2003; Iwaki et al., 2017; Nagata et al., 2002; Roberts et al., 2008; Sykes et al., 2004)
	Diclofenac Potassium			(Becker, 2015)
		SLC22A6	Yes	(Alebouyeh et al., 2003; Apiwattanakul et al., 1999; Kuze et al., 1999; Mulato et al., 2000)
		ABCC4	Yes	(Bernd et al., 2015; Reid et al., 2003; Roberts et al., 2008)
		ABCC1	Yes	(Bernd et al., 2015; Gazzin et al., 2008; Ohtsuki et al., 2003; Reid et al., 2003; Roberts et al., 2008)
		SLC22A8	Yes	(Alebouyeh et al., 2003; Cha et al., 2001; Nagata et al., 2002; Roberts et al., 2008; Sykes et al., 2004)
		SLC22A11		(Cha et al., 2000)
		SLCO1C1	Yes	(Berman et al., 2000; Ho et al., 2012; Sugiyama et al., 2003; Westholm et al., 2009)
		SLCO1B1		(Karlgren, Ahlin, et al., 2012)
		ABCB11		(Pedersen et al., 2013)
		SCN4A		(Jones et al., 2004; Voilley, 2004; Yang & Kuo, 2005)
		ASIC1	Yes	(Bassilana et al., 1997; Voilley et al., 2001)
		KCNQ2		(Peretz et al., 2005; Xiong et al., 2008)
		KCNQ3	Yes	(Klinger et al., 2011; Peretz et al., 2005; Xiong et al., 2008)
		ABCB1	Yes	(Rao et al., 1999; Sanchez-Covarrubias et al., 2014; Saunders et al., 2015)
	Almotriptan			(Becker, 2015)
	Eletriptan			(Becker, 2015)
		ABCB1	Yes	(Evans et al., 2003; Rao et al., 1999; Saunders et al., 2015)
	Frovatriptan			(Becker, 2015)

	Naratriptan			(Becker, 2015)
	Rizatriptan			(Becker, 2015)
	Sumatriptan			(Becker, 2015)
		SLCO1A2		(Lu et al., 1996)
		ABCB1	Yes	(Mahar Doan et al., 2002; Pontier et al., 2001; Rao et al., 1999; Saunders et al., 2015)
		ABCG2	Yes	(Janvilisri et al., 2003; Tomioka et al., 2016)
		SLCO1B1		(Suzuki & Sugiyama, 2000)
	Zolmitriptan			(Becker, 2015)
		ABCB1	Yes	(Rao et al., 1999; Saunders et al., 2015; Yu & Zeng, 2007)
Traumatic Brain Injury	None has been approved by the competent agencies.			(Hiskens, 2022)

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