Impact of Aging and Sex on the Expression and Function of Xenobiotic Transporters at the Blood-Cerebrospinal Fluid Barrier

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Abstract

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The blood-cerebrospinal fluid barrier (BCSFB), formed by the choroid plexuses (CP) in the brain ventricles, serves as a critical clearance organ in the brain by mediating bulk-flow CSF clearance and through the active removal of drugs and metabolites by transporters. The choroid plexuses epithelial (CPE) cells are understood to go through morphological and functional changes during aging, leading to a reduction in CSF secretion rate. Emerging evidence also indicate that drug transporter expression can exhibit sex differences in drug disposition organs such as the liver, potentially contributing to differences in drug response. However, currently little is known regarding the impact of aging and sex on transporter expression and function at the BCSFB, which is the focus of this master thesis. The mRNA expression of *Slc* transporters *(Slc22a6, Slc22a8, Slc15a2, Slc29a4, Slco1a1, Slco1a4, Slco1a5, Slco1a6, Slco1c1, Slco3a1*) and *Abc* transporters (*Abcb1a, Abcb1b, Abcg2, Abcc1, Abcc4*) were analyzed by RT-qPCR in young adult young adult (3 months) and old (20 months) mouse CP tissues. Aquaporin 1 (*Aqp1*) was used as a marker for CP epithelial cells, and *Gapdh* and *GusB*

were used as the housekeeping genes. The results showed that while the majority of analyzed genes showed no difference in expression between the two age groups, *Abcg2* (BCRP) mRNA levels increased in aged mice by 70% and 100% in male and female CP tissues, respectively. In contrast, the expression of *Slco3a1* (OATP3A1) was decreased in aged mice by 30% in males and 40% in females. In young adult mice, no significant sex differences were observed for all analyzed transporter genes. However, in old mice, *Slc22a8* (OAT3) CP expression in females was 40% lower compared to the male cohort. Using a quantitative real-time imaging method recently developed in our laboratory, the activity of OATP1A – MRP1/4 pathway was further investigated and the results showed that the activity of this main pathway for organic anions across the BCSFB is not influenced by aging or sex, consistent with minimal impact of aging on *Oatp1a* and *Mrp1/4* mRNA expression. As BCSFB transporters play a crucial role in removing drugs, toxins, and metabolic wastes from the brain, knowledge gained from this master thesis contributes to understandings of the effect of aging and sex on major BCSFB transporter expression and activity, which may further influence the CSF drug concentrations and the pharmacokinetics of CNS-targeted drugs in geriatric patients and between genders.

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List of abbreviation

CP	Choroid plexus
CSF	Cerebrospinal fluid
aCSF	Artificial cerebrospinal fluid
Blood-CSF barrier	Blood cerebrospinal fluid barrier
BCSFB	Blood cerebrospinal fluid barrier
CPE cell	Choroid plexus epithelial cell
BCRP	Breast cancer resistance protein
P-GP	P- glycoprotein
MRP1/4	Multi-drug resistance associated protein 1 and 4
OAT1/3	Organic anion transporter1/3
PEPT2	Peptide transporter 2
PMAT	Plasma membrane monoamine transporter
OATP1A1/4/5/6	Organic anion transporting polypeptide 1a1/4/5/6
OATP1C1	Organic anion transporting polypeptide 1c1
OATP3A1	Organic anion transporting polypeptide 3a1
MDR1a/1b	Multidrug resistance protein 1a/1b
AQP1	Aquaporin 1
GAPDH	Glycerealdehyde-3-phosphate dehydrogenase
GUSB	Glucuronidase beta
SR101	Sulforhodamine101
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
FL-MTX	Fluorescein-methotrexate
i.v.	Intravenous
PGE2	Prostaglandin E2
ALS	Amyotrophic lateral sclerosis
NaPi2	Na^+/Pi cotransporter isoform 2

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Dedication

To my parents, Xueping Chen and Zhen Shan for their constant support as I pursued my degree in a completely different country.

To my best friend, Yulu Zhang, for nine years of unwavering friendship and shared journeys.

Chapter 1: Introduction

1.1. Choroid Plexus and Blood Cerebrospinal Fluid Barrier

Choroid plexus (CP) tissues are located in each cerebrospinal fluid (CSF)-filled cavity within the brain ventricles (lateral, fourth, third brain ventricles). They exhibit frond-like network structures and are highly vascularized with blood capillary as a core in each frond (Spector & Johanson, 1989; Lun, Monuki, & Lehtinen, 2015) illustrated in Figure 1. The blood capillaries in the CP are separated from the cobblestone-like choroid plexus epithelial (CPE) cells by the stroma, with fenestrated leaky interendothelial junctions that facilitates rapid exchange of water and solutes between the blood circulation and interstitial fluids in the sub-epithelial region (Sun & Wang, 2021; Wolburg & Paulus, 2010; Redzic, 2013). The CPE cells are bound by tight junctions which prevents the free passage of molecules from the circulation to brain, forming a monolayer known as the blood-cerebrospinal fluid barrier (BCSFB) (Sun & Wang, 2021). However, this barrier does not impede every substance from entering the brain, e.g., entry of lipophilic compounds by passive diffusion, uptake of hydrophilic substances via transporter-mediated processes. Similar to epithelial cells found in the kidney, the CP tissues showed polarized structures with basolateral membrane side facing the blood and apical membrane side facing the CSF. On the apical membrane, well-developed microvilli into the CSF enlarges the surface area by 10-30-fold, facilitating the exchange of substances at this barrier (Sun & Wang, 2021; Kaur, Rathnasamy, & Ling, 2016).

Besides acting as a physical barrier, the blood-CSF barrier contributes to maintaining the homeostasis by several mechanisms, including generating and secreting CSF continuously which facilitates efficient removal of substance (metabolites, drugs, toxins) by active transport across the BCSFB, and inactivating the reactive or toxic substances by certain enzymes (Sun & Wang, 2021). The CP is the principal source of CSF production, accounting for approximately 90% of CSF found in the brain ventricles (Spector & Johanson, 1989; Lun, Monuki, & Lehtinen, 2015). With the bulk flow of the CSF generated by new CSF produced

by CPE cells through the ventricular systems, it facilitates the exchange of substances with other fluid compartment in the brain, eventually contributing to the clearance of drugs, metabolites, and macromolecules after draining into general circulations (Spector & Johanson, 1989). The CPE cells can also actively remove solutes from the CSF by transporting them across the BCSFB into the blood circulation. This process is coordinated by specific uptake and efflux transporters expressed on the apical and/or basolateral domain on the CPE cells (Sun & Wang, 2021). In addition, reactive and toxic substances can be inactivated by several intracellular phase I and phase II drug-metabolizing enzymes (CYP1A1, CYP2B1, UGTs, etc.) in the CPE cells. Other functions of the CP also include import of micronutrients and certain hormones (e.g., vitamin C, folate) into the brain (Sun & Wang, 2021), which can further play a role in nourishing internal brain cells, especially during early development (Spector & Johanson, 1989). The CP can also respond to neuroinflammation by recruiting immune cells to the CSF (Schwartz & Baruch, 2014).

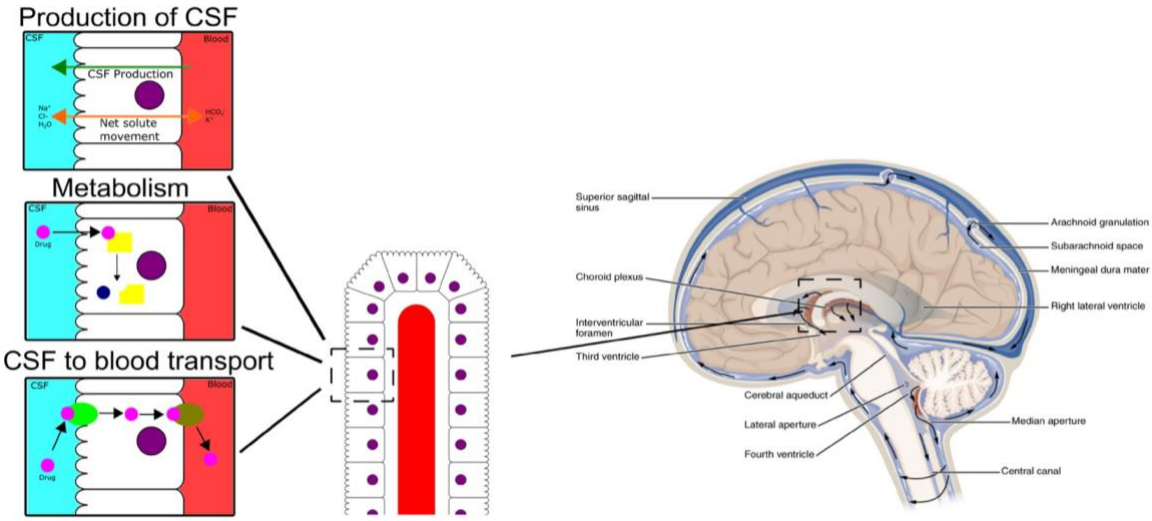

Figure 1. Illustration of the location, structure, and clearance pathways at the BCSFB. This figure is adapted from Sun and Wang 2021, AAPS J.

1.2. Transporters Expressed on Blood-CSF Barrier

Besides clearance by CSF bulk flow, several xenobiotic membrane transporters are expressed on the polarized structure of CPE cells to actively transport substrates from the CSF

side into the blood side to facilitate the removal of drugs and xenobiotics from the brain (Sun & Wang, 2021). A variety of xenobiotic transporters expressed at the BCSFB has been characterized at the mRNA level, such as organic anion transporter 3 (OAT3), organic anion-transporting polypeptide (OATPs), plasma membrane monoamine transporter (PMAT), peptide transporter 2 (PEPT2), which are involved in the uptake and disposition of a wide array of xenobiotics and endogenous substrates, as well as efflux transporters such as breast cancer resistance protein (BCRP), P-glycoprotein (P-GP), and multi-drug associated proteins (MRPs) (Ho, Dahlin, & Wang, 2012; Morris, Rodriguez-Cruz, & Felmlee, 2017; Duan & Wang, 2013; Hu, Zha, Sun, & Wang, 2022; Sun, Hagenbuch, Kelly, & Wang, 2023).

However, only a handful of these transporters have been studied at the functional level. OAT3 has been identified to be expressed at the apical membrane of rat choroid plexus epithelial cells by an immunofluorescence method (Kojima, et al., 2002). OAT3 handles a wide range of organic anions pool (e.g. PAH, benzylpenicillin, taurocholate) and drug substrates including diuretics and antibiotics (Sun & Wang, 2021; Sykes, et al., 2004). Confocal imaging studies indicated significant accumulation of fluorescein in wild-type CP, while accumulation in the *Oat3* knockout CP was significantly reduced (Sweet, et al., 2002; Sykes, et al., 2004).

PEPT2 is a secondary active uptake transporter driven by the proton gradient through proton co-transport. Di- and tri-peptides and antibiotics (e.g., Cefadroxil, Cefaclor, Amoxicillin) can be transported by PEPT2. Studies demonstrated its apical membrane localization on the CPE cells (Sun & Wang, 2021). In *Pept2* knockout mice, the CSF-to-blood concentration ratio of cefadroxil was 6-fold greater than wild-type cohorts (Shen, Ocheltree, Hu, Keep, & Smith, 2007), suggesting its contribution in regulating the clearance of drugs from the CSF.

PMAT is a polyspecific organic cation transporter for uptake of bioactive amines and xenobiotic cations (Wang, 2016). Our previous study demonstrated high mRNA expression

level of *PMAT* transporter and revealed the apical membrane localization in both human and mouse CP tissues (Duan & Wang, 2013; Wang, 2016). Hu et al. (2022) employed a quantitative live-tissue imaging method developed in our laboratory to uncover the transporter mechanism of PMAT, highlighting its major role in the uptake of organic cations from the CSF into the CPE cells.

Within the organic anion-transporting polypeptide (OATP) family, several OATPs are identified to be expressed at the mouse and human CP tissue at mRNA level. OATP1A4 and OATP1A5 have a substrate pool consisting of endogenous substances (e.g., DHEAS, estone-3-sulfate, taurocholate) and some drug compounds (e.g., fexofenadine, statins) (Sun & Wang, 2021). The functionality of OATP1A4 has not been characterized. OATP1A5 was recently shown to be a mouse analogue of OATP1A2 in humans at the BCSFB (Sun, Hagenbuch, Kelly, & Wang, 2023) with similar functionality. *Slco1c1* (OATP1C1) was characterized to be highly expressed and localized on both apical and basolateral side of the CPE cells in mouse CP and may play a role in thyroid hormone thyroxine (T4) uptake from the blood into the CSF (Ho, Dahlin, & Wang, 2012). *Slco3a1* (OATP3A1) is an uptake transporter expressed on both membranes of the BCSFB and may be involved in transport of various hormones (e.g., estrone-3-sulfate, prostaglandins E1 and E2 and thyroxine) (Bakos, et al., 2020). Further research is needed to provide evidence at the function level for OATPs at the BCSFB.

MRP1 and MRP4 are primary active ATP-dependent efflux transporters responsible for transporting endogenous substrates and xenobiotics. A study conducted by Leggas et al. (2004) identified the basolateral membrane domain location of MRP1/4, and our laboratory has suggested that MRP1/4 act coordinately with OATP1A at the BCSFB to mediate CSF-to-blood efflux transport of large organic anions (Hu, Zha, Sun, & Wang, 2022; Sun, Hagenbuch, Kelly, & Wang, 2023). Large organic anions are first taken up into the CPE cell space through OATPs at the apical membrane, followed by their efflux into the subepithelial regions via MRPs

located at the basolateral membrane of the CPE cells. Using fluorescence live-tissue imaging, the accumulation of a fluorescent anion probe, sulforhodamine 101 (SR101), was predominantly observed in the subepithelial region of wild-type mouse CP tissues (Sun, Hagenbuch, Kelly, & Wang, 2023). This accumulation was significantly reduced in *Oatp1a/1b* knockout mice compared with wild-type controls (Sun, Hagenbuch, Kelly, & Wang, 2023). Given that *Oatp1a5* is the only *Oatp1a* isoform expressed apically in the mouse CPE cells (Ohtsuki, et al., 2003), while *Oatp1b* isoforms are liver specific (Csanaky, et al., 2011), these findings suggest that SR101 is likely transported by OATP1A5 in mouse CP tissues (Sun, Hagenbuch, Kelly, & Wang, 2023).

P-GP and BCRP are primary active ATP-dependent efflux transporters with wide and considerably overlapping substrate pools (Sun & Wang, 2021). Both P-GP and BCRP are located at the apical membrane domain of CPE cells (Morris, Rodriguez-Cruz, & Felmlee, 2017). The recent study illustrated major efflux transport activity of BCRP and potential additional contribution of PGP at the apical membrane domains of the BCSFB (Sun & Wang, 2023). However, the function of the BCRP and PGP is still unknown.

Aquaporin 1 (AQP1), a water channel protein abundantly expressed in epithelial cells of the choroid plexus, is proposed to play an important role in maintaining CSF homeostasis (Trillo-Contreras, Toledo-Aral, Echevarría, & Villadiego, 2019). *AQP1* is specifically expressed in the apical membrane of the epithelial cells that line the choroid plexus, where it functions to facilitate water movement across the membrane. Due to its high expression in this tissue, AQP1 has been identified as a useful marker for the choroid plexus (Deffner, et al., 2022).

Similar to the xenobiotic excretory mechanisms found in the liver and kidney, the general pattern of CSF-to-blood drug transport is initiated by uptake into the CPE cells by an apical transporter followed by efflux into the blood circulation by an efflux transporter. This transport

process is believed to contribute to brain concentrations and dispositions of CNS drugs. Wijnholds et al. (2000) demonstrated that lack of MRP1 in mice contributes to an increased level of etoposide in the CSF by approximately 10-fold after i.v. administration in mice. Deletion of *Pmat* also reduces uptake of organic cations and biogenic mines in isolated mouse CP tissues (Duan & Wang, 2013; Hu, Zha, Sun, & Wang, 2022). Taken together, this evidence suggests that transporters at the BCSFB play a role in regulating the clearance of xenobiotics in the brain.

1.3. Methods to Study BCSFB Transport

Historically, both *in vitro* and *ex vivo* methods have been used to study blood-CSF barrier transport. *In vitro* methods include CP 2D cell culture models using primary human CP cells from embryos or post-mortem tissues and immortalized cell lines (Pellegrini & Lancaster, 2021). Cell-based systems have high throughput and can be used to study bidirectional transport (e.g., Transwell® system). However, it is very difficult to obtain primary cells from primary human CP cells from embryos or post-mortem tissues. The expression of xenobiotic transporters is not physiologically representative along with weakened formation of tight junction (Kläs, Wolburg, Terasaki, Fricker, & Reichel, 2010). Recently, a protocol for the high-yield isolation of primary epithelial cells from rat choroid plexus has been established by Majerova et al. (2024). Their model showed a monolayer displayed cuboidal shape polarized cell, formed and retained tight junctions, and active CSF secretion associated with functional expression of the pharmaceutically important influx/efflux transporters. Other *in vitro* models such as barrier- forming CP organoids producing CSF also serves as an alternative system for CP study (Pellegrini, et al., 2020). *Ex vivo* methods utilize fresh CP tissues harvested from preclinical species and they have been shown to retain *in vivo* physiology, barrier integrity and transporter expression. The CP isolated from these species freshly can be incubated with

fluorescent- or radio-labeled compounds, which allows characterization of the activity of specific transporters (Sun & Wang, 2022).

A live-tissue imaging approach to visualize the transepithelial transport processes of organic anions and cations in isolated mouse CP tissues was developed recently in our laboratory (Sun & Wang, 2022; Hu, Zha, Sun, & Wang, 2022). During the work conducted by Hu et al., the pivotal role of PMAT, OATP and MRP transporters at the BCSFB in organic cation and anion transport was shown. Using fluorescein-methotrexate (FL-MTX) as a fluorescent probe, a highly functional transepithelial transport process of large amphipathic organic anions (OAs) in isolated mouse CP tissue, mediated by OATP-MRPs (from the CSF side to the blood side), was revealed. FL-MTX is a known substrate of OATPs and MRPs. It rapidly moves across the CPE cells with little intracellular accumulation, and the majority of the FL-MTX is accumulated in the subepithelial region (Hu, Zha, Sun, & Wang, 2022). Another fluorescent anion, SR101, showed quantitative and real time accumulation trend similar to FL-MTX, and was identified as an OATP1A fluorescent probe at the BCSFB (Sun, Hagenbuch, Kelly, & Wang, 2023). For organic cations, the study conducted by Hu et al. also illustrated CSF-to-blood transport largely via the apical PMAT transporter, but accumulation of the organic cations occurs intracellularly due to the lack of an organic cation efflux system at the basolateral membrane domains of the CPE cells. Taken together, the quantitative live-tissue imaging approach can be used as a method to characterize transport mechanisms, rate-determining steps of transport, and compartment-specific tissue accumulation patterns, as well as to quantify the activity of different transport pathways across the BCSFB.

1.4. Research Gaps in Blood-CSF Barrier Transport Research Regarding Impact of Aging and Sex

The BCSFB serves as a major clearance organ in the brain by secreting the CSF and by removing drugs and metabolic wastes out of the brain actively via the xenobiotic transporters expressed in CPE cells. Approximately 90% of CSF in the brain is generated by the choroid plexus (Spector & Johanson, 1989). Previous studies have reported that with healthy aging, the CP epithelial cells undergo morphological changes (e.g., thickening of the basement membrane) and experience a reduction in CSF secretion rate (Silverberg, et al., 2001; Serot, Béné, & Faure, 2003; Chen, Chen, & Preston, 2010; May, et al., 1990; Matsumae, et al., 1996). Moreover, these morphological and functional changes were shown to be more significant in Alzheimer's disease patients (Serot J. M., Béné, Foliguet, & Faure, 2000). In rats with age range from 12 to 30 months, Chiu et al. (2012) studied the relationship between alteration of CSF dynamics with the accumulation of the major constituents of senile plaques of Alzheimer's disease, amyloid-β peptides (Aβ). Their results showed that CSF turnover rate decreased with aging whereas the accumulation of Aβ increased. It showed that age-related accumulation of brain amyloid plaques correlated with reduced CSF turnover rate (Chiu, et al., 2012). The same relationship of ventricular CSF clearance and the accumulation of Aβ was found in another study conducted by de Leon et al. (2017) as well. One of the clearance pathways at the BCSFB is through uptake and efflux processes at the CPE cells conducted by transporters expressed on the polarized membranes, which is widely believed to impact the clearance of drugs, metabolites, toxins and macromolecules from the brain. Given the morphological and functional changes observed at the BCSFB, along with increased risk in neurodegenerative disease development, it would be important to investigate whether the expression and function of the major BCSFB transporters would be altered with aging. Such changes could have potential implications for assessing the brain's ability to clear various xenobiotics at the BCSFB site during aging.

A systematic analysis for the Global Burden of neurological diseases found that the incidence and disability-adjusted life-years (DALY) rates in women were higher than those in men. Huang et al. (2023) identified DMET genes in the human liver that exhibit significant sex differences in expression and linked them to literature reported evidence on sex differences in drug response. Veiras et al. (2017) also identified sex differences in transporters (e.g., NHE3, Na^+/H^+ exchanger isoform 3) along the proximal nephron in rats, resulting in altered proximal tubule reabsorption of Na^+ in females than in males (Li, McDonough, Layton, & Layton, 2018). For aging-related neurodegenerative diseases, such as Alzheimer's disease, there is evidence that the clinical risk factor (e.g., depression) profile for the development of AD and neurodegeneration biomarkers (e.g. amyloid-beta, phosphorylated tau - P-tau, neurofilament light in the CSF and plasma) differ between men and women, suggesting sex may be an important factor involving in neurodegenerative disease progression (Mielke, et al., 2019; Aggarwal & Mielke, 2023; Mattsson, et al., 2016; Contador, et al., 2022). Currently, whether BCSFB transporters exhibit sexually dimorphic expression is unknown. As this could potentially alter transporter function, where the functional changes of transporters under the effect of aging and/or sex can potentially impact the clearance of xenobiotics from the CNS. This may, in turn, could affect the concentrations and pharmacokinetics of CNS-targeted drugs in the brain in geriatric patients and between males and females, thereby influencing the toxicity and efficacy in these specific populations. Thus, a comprehensive understanding of how aging and sex affect the expression and function of BCSFB transporters is essential.

1.5. Hypothesis and Aims

In this study, we investigated if the expression and activity of major xenobiotic transporters at the BCSFB can be impacted by aging and sex. We hypothesize that the expression of major xenobiotic transporters at the BCSFB can be impacted by aging and sex and may corresponds to the changes in BCSFB transport function.

This project involves two study aims described as follows:

Aim 1: To determine if mRNA expression of major xenobiotic transporters at the BCSFB is dependent on age and sex in young adult and aged C57BL/6 mice.

In this aim I will comprehensively analyze aging- and sex-dependent changes in the mRNA expression of major SLC and ABC transporters at the BCSFB using RT-qPCR. This aim would help us to characterize the changes of mRNA expression of each transporter with aging or between genders and may identify the transporter of interest to quantify the functional activity changes in future studies.

Aim 2: To examine if aging and sex have impacts on the activity of the Oatp1a-Mrp1/4 transport pathway at the BCSFB using a quantitative live-tissue imaging method.

While the OATP1A-MRP1/4 transport pathway has been characterized as a major large organic anion transport pathway by Sun et al. (2023), it remains important to investigate if this pathway activity would vary with aging and/or between genders. In this aim, I will quantify the differences of the activity of the OATP1A-MRP1/4 transport pathway at the BCSFB between two different age groups (young adult mice group and aged mice group) and between sex using the quantitative live-tissue imaging approach developed in our lab recently. SR101, an OATP1A specific fluorescence probe as mentioned in Section 1.3, was used in this study.

Chapter 2: Impact of Aging and Sex on the Expression and Function of Xenobiotic Transporters at the Blood-Cerebrospinal Fluid Barrier

2.1 Introduction

Brain disorders, such as Alzheimer's disease, Parkinson's disease and stroke, constitute a significant public health burden which continues to increase with the aging worldwide population. The need for new and more effective drugs to treat brain diseases is increasing. However, CNS drugs still face many hurdles in drug development. One of the challenges in CNS drug development is to enable new drug candidates with adequate brain delivery and proper CNS PK properties. The disposition of CNS drugs is determined by multiple and complex pathways including drug uptake and efflux processes at the blood-brain barrier and the blood-cerebrospinal fluid barrier (BCSFB).

Choroid plexus (CP) tissues are located within each of the brain's ventricles. The choroid plexus epithelial (CPE) cells are bound by tight junctions with a core of blood capillaries and form a monolayer called the BCSFB (Sun & Wang, 2021). The primary function of the CP is to generate and secrete cerebrospinal fluid (CSF), accounting for approximately 90% of CSF volume in the brain ventricles (Spector & Johanson, 1989; Lun, Monuki, & Lehtinen, 2015). The CP tissues show polarized structures with the basolateral membrane side facing the blood and the apical membrane side facing the CSF. Similar to the xenobiotic excretory mechanisms found in the liver and kidney, the general pattern of CSF-to-blood drug transport is initiated by uptake into the CPE cells by an apical transporter followed by efflux into the blood circulation by an efflux transporter. A variety of xenobiotic transporters expressed at the BCSFB, including *Oat3, Oatps, Pmat, Pept2, Bcrp, P-gp, and Mrps,* have been characterized at the mRNA level (Sun & Wang, 2023; Ho, Dahlin, & Wang, 2012; Morris, Rodriguez-Cruz, & Felmlee, 2017; Duan & Wang, 2013; Hu, Zha, Sun, & Wang, 2022). A few of them have been characterized at the functional level, while the rest still need further investigation. Confocal imaging studies in mice indicated a significant accumulation of fluorescein in wild-type CP, while accumulation in the *Oat3* knockout CP was significantly reduced (Sweet, et al., 2002;

Sykes, et al., 2004). Wijnholds et al. (2000) demonstrated that the lack of MRP1 in mice contributes to an increased level of etoposide in the CSF by approximately 10-fold after i.v. administration in mice. Deletion of *Pmat* also reduces uptake of organic cations and biogenic mines in isolated mouse CP tissues (Duan & Wang, 2013). Taken together, these studies demonstrated that the BCSFB transporters may play a role in mediating the clearance of xenobiotics at the BCSFB site.

During aging, morphological and functional changes have been observed in the CPE cells (Silverberg, et al., 2001; Serot, Béné, & Faure, 2003; Chen, et al., 2017; May, et al., 1990; Matsumae, et al., 1996). Transporter expressions were found to vary dynamically during early development (Liddelow, et al., 2012; Kratzer, et al., 2013). Emerging evidence also indicates that drug transporter expression can exhibit sex differences in drug disposition organs (e.g., liver, kidney), potentially contributing to differences in drug response (Huang, et al., 2023; Veiras, et al., 2017). For aging-related neurodegenerative diseases, such as Alzheimer's disease, there is evidence shown that the clinical risk factor (e.g., depression) profile for the development of AD and neurodegeneration biomarkers (e.g. amyloid-beta, phosphorylated tau - P-tau, and neurofilament light in the CSF and plasma) differ between men and women, suggesting sex may be an important factor involved in neurodegenerative disease progression (Mielke, et al., 2019; Aggarwal & Mielke, 2023; Mattsson, et al., 2016; Contador, et al., 2022). Currently, research on whether BCSFB transporters exhibit aging-related or sex-differential expression is limited.

A live-tissue imaging approach to visualize and quantify the transepithelial transport processes of organic anions and cations in freshly isolated intact mouse CP tissues was developed and validated recently in our laboratory (Sun & Wang, 2022; Hu, Zha, Sun, & Wang, 2022). This approach enables the study of real-time transport processes and quantification of the activity of different transport pathways across the BCSFB. In this study, we first

investigated if the expression of major xenobiotic transporters at the BCSFB can be impacted by aging and sex. We then determined if the transport activity of OATP1A-MRP1/4 pathway is affected by aging and sex. The impact of aging and sex on mRNA expression levels of major xenobiotic transporters at the BCSFB was investigated by RT-qPCR in young adult and aged C57BL/6 mice. The impact of aging and sex on trans-epithelial transport activity of large organic anions mediated by OATP1A-MRP1/4 was evaluated using an established fluorescent probe, sulforhodamine101 (SR101), in freshly isolated murine CP tissues (Sun, Hagenbuch, Kelly, & Wang, 2023).

2.2 Experimental – Methods and Materials

2.2.1 Materials

All chemicals purchased and used were of 95% or greater purity. Sulforhodamine101 was purchased from Sigma-Aldrich (St. Louis, MO). Unless otherwise specified, all other chemicals and reagents were from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ) or VWR (West Chester, PA). Animal handling supplies, confocal dishes, and other plastic wares were purchased from VWR (Radnor, PA). PCR plates and sealing film were purchased from USA Scientific (Ocala, FL).

2.2.2 Animals

Aged (20-23 months) and young adult (3 months) C57BL/6 wildtype male and female mice were obtained from National Institute on Aging (NIA) (Reno, NV) rodent resources program. After receiving the animals from NIA, they were acclimated to the specific pathogen free facility at the University of Washington for 1 week and maintained under standard conditions, with food and water available *ad libitum*.

2.2.3 Collection of Choroid Plexus from Mouse Brains

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, as established by the U.S. National Institutes of Health. All procedures

were approved by the Institutional Animal Care and Use Committee at the University of Washington. Lateral ventricle choroid plexuses (CPs) were isolated from mouse brains following the method described previously by Sun and Wang (2022). In brief, after euthanization with CO_2, C57BL/6 mice were decapitated. The mouse brains were harvested and maintained in ice-cold artificial cerebrospinal fluid (aCSF: 119 mM NaCl, 26.2 mM $NaHCO_3$, 2.5 mM KCl, 1 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose, pre-gassed with 95% O_2/5% CO_2) for CP tissue isolation. The isolation of intact lateral ventricle CPs was conducted under a dissecting microscope. An incision was made along midline of the sagittal plane between the hemispheres of the brain using surgical scissors or superfine forceps. Another incision was made by small forceps along the inner wall of the left cerebral hemisphere to reveal the cavity in the left lateral ventricle. After the cavity space was exposed, a set of superfine forceps was used to fix the open position of the left lateral ventricle. This revealed a floating, faintly vascularized, semi-transparent tissue inside the cavity, identified as the CP. The CP was removed using another set of superfine forceps. The same steps were repeated for the right lateral ventricle. The CP tissues were either snap-frozen for transporter mRNA expression analysis or transferred to pre-gassed aCSF for transporter-mediated uptake studies using a fluorescence live-tissue imaging method.

2.2.4 Quantification of Transporter mRNA Expression using RT-qPCR

Following CP dissection, tissues were then snap-frozen in liquid nitrogen and stored in a -80°C freezer until further processing. Frozen tissue was then homogenized by the Omni Bead Ruptor (Omni International, INC), and total RNA was extracted by RNeasy Mini Kit (Qiagen, Hilden, Germany). The concentration of the isolated RNA was determined using a BioTek Synergy HTX Multimode Reader (Gen5 3.03 software). Total RNA was reverse-transcribed to complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA) following conditions provided by the manufacturer's protocol

(25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min). The mRNA expression levels of major transporter genes were quantified with TaqMan real time PCR. The validated primer and probe assay sets for assayed genes were purchased from Applied Biosystems, Inc. The profile of TaqMan Gene Expression Assays is listed in Table 1. Taqman real-time PCRs were set up and run according to the manufacturer's protocols on the CFX96 real-time system-C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Each 20 µL reaction contained 10 µL of the TaqMan Universal PCR Master Mix, 1 µL of the 20× TaqMan Gene Expression Assay, and 9 µL of cDNA (approximately 5 ng of cDNA) diluted in RNase-free water. The thermocycling conditions were 30 seconds at 50 °C, 10 min at 95 °C, followed by 40 cycles for 15 s at 95 °C and 1 min at 60 °C. All samples were run in triplicate. The relative expression level of each transporter gene in the CP was normalized to the geometric mean of two housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and glucuronidase beta (*Gus B*). We also included *Aqp1* (Aquaporin 1) in this study to act as a marker for CP due to its high expression in the CP (Deffner, et al., 2022). The relative quantification of the mRNA expression in CP tissues from mice was determined using the comparative cycle threshold (Ct) method, i.e., $2^{-\Delta\Delta Ct}$, where ΔCt is calculated by the difference between the Ct values of the gene of interest and the geometric mean of the Ct values of *Gapdh* and *GusB*, and then $\Delta\Delta Ct$ is ΔCt of the aged group (or female group) subtracted by the mean ΔCt of the young adult group (or male group), respectively.

2.2.5 Confocal Imaging and Transport Studies in Freshly Isolated CP Tissues

Transport studies were carried out in freshly isolated mouse CP as previously described (Sun & Wang, 2022; Hu, Zha, Sun, & Wang, 2022). Isolated CP tissues have shown to maintain vitality and transport activity for up to 2-3 hours (Sun & Wang, 2022), and all transport studies were performed within 2 hours after tissue isolation. The CP tissues were then transferred to a 35mm sterile confocal dish (VWR, Radnor, PA) containing room temperature aCSF previously

gassed with 95% O_2/5% CO_2. Transport studies were initiated by adding a fluorescent substrate into the aCSF. Fluorescent signals were then recorded and quantified in the choroid plexus epithelial (CPE) cells and the adjacent sub-epithelial region. For real-time recording, fluorescent signals were recorded every minute for 20 minutes. Image acquisitions were performed using a Zeiss LSM 710 confocal microscope as previously described (Sun & Wang, 2022). Briefly, samples containing SR101 were illuminated using a 594nm fixed wavelength helium-neon laser. Appropriate corresponding dichroic and emission filters were used to detect the emission of SR101. Digital image analysis was conducted using Fiji ImageJ (1.53b) as described previously (Sun & Wang, 2022). Briefly, the CPE cell and sub-epithelial regions were defined and manually segmented by free hand drawing over the identified regions of interest. The mean fluorescence was then calculated by averaging the pixel intensity from three separate freehand segmentations.

2.2.6 Choroid Plexus Efflux Index (CPEI)

In a previous study conducted in our lab, we developed the CPEI to distinguish transport patterns across BCSFB between various fluorescent probes (Sun & Wang, 2022). The CPEI represents the percentage of a substrate taken up by CPE cells that is subsequently effluxed into the sub-epithelial region. Expressed as a percentage, the CPEI characterizes the ratio of the amount of fluorescent substrate crossing the CPE cells and entering the adjacent blood capillaries compared to the total uptake into the choroid plexus tissues. A higher CPEI suggests that a larger proportion of the substrate taken up by the CPE cells is transported into the sub-epithelial region and accumulates, indicating substantial CSF-to-blood flux across the BCSFB. Conversely, a lower CPEI indicates that the fluorescent substrate is taken up from the CSF but retained intracellularly within the CPE cell region, with little efflux into the blood capillary region. The CPEI is calculated as follows:

$$CPEI(\%) = \frac{\Sigma pixel\ intensity_{se}}{\Sigma pixel\ intensity_{cells+se}}$$

$\Sigma pixel\ intensity_{se}$ is the sum average pixel intensity in the sub-epithelial region from three segmentations, where $\Sigma pixel\ intensity_{cells+se}$ is the combined sum average pixel intensity in the intracellular CPE cell and adjacent sub-epithelial regions from three segmentations.

2.2.7 Data Analysis

Data points with error bars represent mean ± S.D. for independent biological replicates. All experiments were performed independently three times. Where applicable, p values were obtained through an unpaired Student's t test. Data groups are considered significantly different if $p < 0.05$. All data analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) and Microsoft Excel.

Table 1. Profile of genes analyzed in this study and their associated protein names and assay IDs used in RT-qPCR, including 15 SLC and ABC transporters, Aqp1 as a choroid plexus marker, and Gapdh and Gus B as housekeeping genes.

Gene	Protein	Assay IDs
Slc22a6	OAT1	Mm00456258_m1
Slc22a8	OAT3	Mm00459534_m1
Slc15a2	PEPT2	Mm00451610_m1
Slc29a4	PMAT	Mm00525578_m1
Slco1a1	OATP1A1	Mm01267415_m1
Slco1a4	OATP1A4	Mm01267407_m1
Slco1a5	OATP1A5	Mm00453720_m1
Slco1a6	OATP1A6	Mm01267368_m1
Slco1c1	OATP1C1	Mm00451845_m1
Slco3a1	OATP3A1	Mm00452449_m1
Abcb1a	MDR1a	Mm00440761_m1
Abcb1b	MDR1b	Mm00440736_m1
Abcg2	BCRP	Mm00496364_m1
Abcc1	MRP1	Mm00456156_m1
Abcc5	MRP4	Mm01226831_m1
Aqp1	AQP1	Mm01326466_m1
Gapdh	GAPDH	Mm99999915_g1
GusB	GUSB	Mm01197698_m1

2.3 Results

2.3.1 *Slc22a8* (OAT3) mRNA Expression in Aged C57BL/6 Mouse CP Tissues Showed Sex-dependent Difference

To evaluate the impact of gender on the mRNA expression levels of major SLC and ABC transporters, we isolated lateral ventricle choroid plexus (CP) tissues and quantified the relative mRNA expression levels of transporters studied as detailed in the Methods section. The young adult group and aged group were analyzed separately. As illustrated in Figure 2A, there were no significant sex differences in the expression of the analyzed transporter genes in young adult mice. In aged mice, there were no significant changes in the mRNA expression levels of the transporter studied except *Slc22a8* (OAT3), which showed 40% reduction in mRNA expression in female CP tissues compared to male CP tissues (Figure 2B).

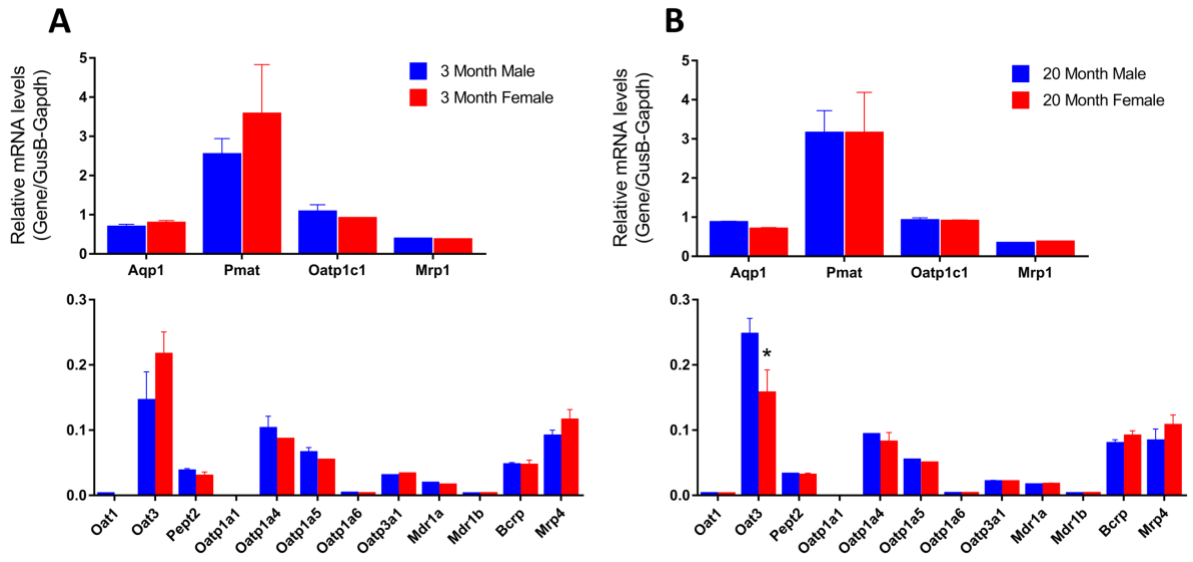

*Figure 2. Impact of sex on mRNA expression of major SLC and ABC Transporters at the BCSFB. Relative mRNA expression of major SLC and ABC Transporters in 3 month (A) and 20 month (B) old male and female mice. Total RNA was extracted from pooled choroid plexus tissues from wild –type mouse and reverse transcribed. TaqMan quantitative real-time PCR was used to determine the expression levels of the transporters. Glycerealdehyde-3-phosphate dehydrogenase (GAPDH) and glucuronidase beta (GUS B) were used as housekeeping genes. Data were plotted as mean ± S.D. in triplicate. An unpaired student's t-test, * P<0.05 was considered statistically significant.*

2.3.2 Aging Impacted *Abcg2* (BCRP), *Slco3a1* (OATP3A1) and *Abcb1b* (MDR1b) mRNA Expression

To determine the impact of aging on transporter mRNA expression, we compared mRNA expression in the two age groups and the fold changes relative to young adult group is shown Figure 3. Male and female cohorts were analyzed separately. The results showed that most analyzed genes showed no difference in mRNA expression between the two age groups. However, compared to young adults, a consistent trend was observed in *Abcg2* (BCRP) mRNA expression with 70% and 100% increase in older male and female CP tissues respectively (Figure 3d&h). In contrast, CP expression of *Slco3a1* (OATP3A1) decreased by 30% in males and 40% in females in the aged group (Figure 3c&d). In addition, in the male CP tissues, *Abcb1b* (MDR1b) expression increased by 30% in the aged group as compared to the young adult group (Figure 3d).

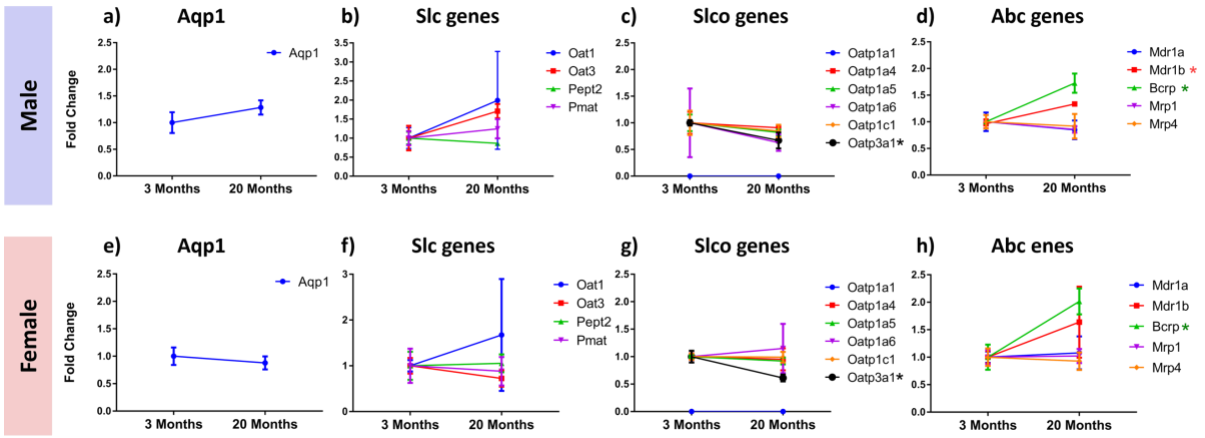

Figure 3. Impact of aging on mRNA expression of major SLC and ABC Transporters at the mouse BCSFB. Fold changes of relative mRNA expression of major SLC and ABC Transporters in 3-month and 20-month groups, both in male (upper row) and female (lower row) mice. (a) and (e) showed gene expression for Aqp1. (b) and (f) showed gene expression profiles for Slc genes. (c) and (g) showed gene expression profiles for Slco genes. Data were plotted as mean ± S.D. (N=3). T-test (two-tailed distribution, two-sample equal variance) was performed, $P<0.05$ was considered statistically significant.

2.3.3 OATP1A-MRP1/4-mediated SR101 Transport Activity at the BCSFB was Not Affected by Aging or Sex

Based on RT-qPCR results, we would like to conduct functional studies for transporters showing aging- and/or sex-dependent mRNA expression. However, the functional characterization of individual transporter activity at the BCSFB is not feasible for every transporter, since there is no well-defined specific fluorescent probe for these transporters. Our laboratory recently developed a live tissue imaging method that allows us to quantify OATP1A-MRP1/4 mediated transport of large organic anions at the BCSFB (Hu, Zha, Sun, & Wang, 2022; Sun, Hagenbuch, Kelly, & Wang, 2023). The work revealed a highly functional transepithelial transport processes of large amphipathic organic anions in isolated mouse CP tissue, mediated by OATP1A-MRP1/4, using SR101 as a fluorescent probe. SR101 rapidly across the CPE cells with little intracellular accumulation shown while majority of the SR101 accumulated in the subepithelial region, at where the blood capillaries locate (Sun, Hagenbuch, Kelly, & Wang, 2023). We thus applied this method to determine if aging and sex have an impact on the activity of the OATP1A-MRP1/4 pathway at the BCSFB. SR101 was used as the fluorescent probe substrate and images were taken every minute for 20 minutes to obtain the CPEI as described in Methods and Materials. Figure 4 shows the time courses of CPEI obtained from wild-type male and female mice. The overall trends of CPEI over time in different sex cohorts were superimposable, suggesting the activity of OATP1A-MRP1/4 pathway minimally affected by aging. We then compare the CPEI in the same age group but with different gender (Figure 5). Similar trends were shown of CPEI over time in different age cohorts, which suggests that sex had little effect on the OATP1A-MRP1/4 pathway activity. These data are consistent with no effect of aging and sex on *Oatp1a* and *Mrp1/4* mRNA expression (Figure 2, Figure 3), suggesting that the OATP1A-MRP1/4 axis at the BCSFB is unaffected by aging or sex.

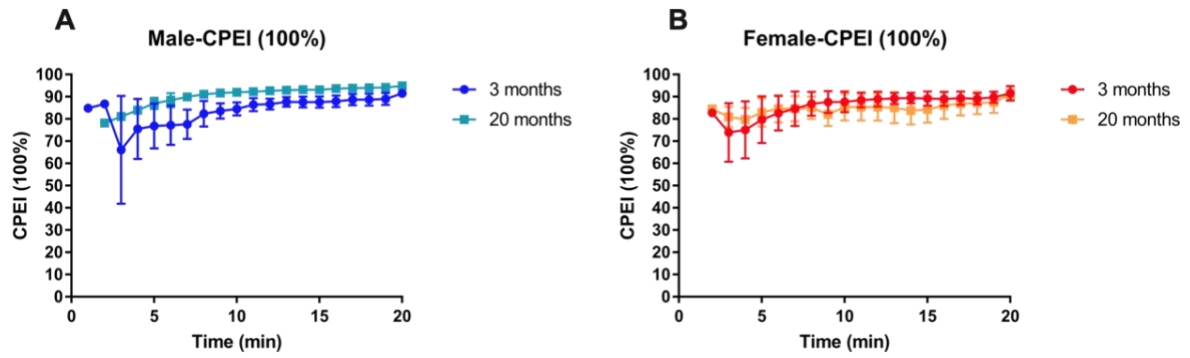

Figure 4. Effect of aging on the activity of time-dependent transport of SR101 mediated by OATP1A-MRP1/4 transport pathway. CPEI over time for 2 μM SR101 transport in (A) 3-month and 20-month male mice, and (B) 3-month and 20-month female mice. CPEI was calculated shown in the Method Section. The overall trend in different age groups was superimposable, both in male and female mouse choroid plexus tissues. Time course was performed independently three times. Data are plotted as mean ± S.D. from 3 animals.

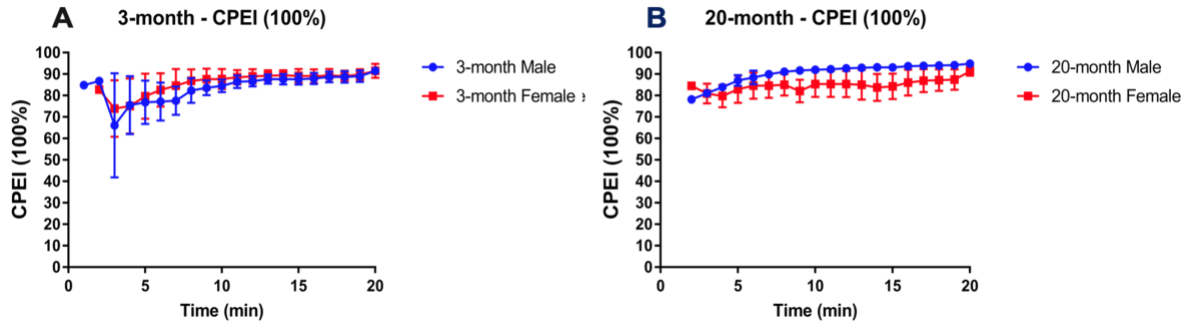

Figure 5. Effect of sex on the activity of time-dependent transport of SR101 mediated by OATP1A-MRP1/4 transport pathway. CPEI over time for 2 μM SR101 transport in (A) 3-month male and female mice, (B) 20-month male and female mice. The trends between genders were similar, both in 3-month and 20-month mice. Time course was performed independently three times. Data are plotted as mean ± S.D. from 3 animals.

2.4 Discussion

The BCSFB is an important, but understudied blood-brain interface with respect to drug, toxin, and metabolic waste clearance. The objective of this study is to characterize the age- and sex-related changes in transporter mRNA expression and function at the BCSFB using RT-qPCR and quantitative real-time fluorescence microscopy. The mice in this study are divided into two age groups: young adult mice group (3 months) and aged mice (20-23 months). We investigated a profile of transporter genes including *Slc* group (*Slc22a6, Slc22a8, Slc15a2, Slc29a4*), *Slco* group (*Slco1a1, Slco1a4, Slco1a5, Slco1a6, Slco1c1, Slco3a1*), *Abc* group (*Abcb1a, Abcb1b, Abcg2, Abcc1, Abcc4*), *Aqp1* as a marker for CP, and two housekeeping

genes (*Gapdh, GusB*) for data analysis. The results showed that while the majority of analyzed genes showed no difference in expression between the two age groups, *Abcg2* (BCRP) mRNA levels increased in aged mice by 70% and 100% in male and female CP tissues, respectively. In contrast, the expression of *Slco3a1* (OATP3A1) was decreased in aged mice by 30% in males and 40% in females. In young adult mice, no significant sex differences were observed for all analyzed transporter genes. However, in aged mice, *Slc22a8* (OAT3) CP expression in females was 40% lower compared to the male cohort.

Slco3a1 (OATP3A1) mRNA expression level was lower in aged mice in both male and female CP tissues. OATP3A1 is expressed on both apical and basolateral membranes of the BCSFB but primarily transports prostaglandins (Huber, et al., 2007; Bakos, et al., 2022). The efflux transport systems at the BCSFB serve as one of the primary cerebral clearance pathways for prostaglandins (PGE2 and PGD2) (Tachikawa, Hosoya, & Terasaki, 2014). The prostaglandins play an important role in brain inflammatory responses and might be involved in several pathological conditions (Barreiro-Iglesias, 2021). It has been reported that CSF levels of the pro-inflammatory PGE_2 are elevated in postmortem patients with ALS (Almer, et al., 2002). The decrease of *Slco3a1* (OATP3A1) expression in aged mice could suggest a decreased clearance of brain PGs, which may increase CSF PGE_2 levels and promote proinflammatory responses in the aging brain. For quantitative live-tissue imaging, it would be crucial to identify a transporter-specific fluorescent probe to visualize the change in OATP3A1 activity. Bakos et al. (2020) has demonstrated that P1, a fluorophore synthesized by Kele et al. and known as (E)-1-(3-azidopropyl)-4-(2-(7-(diethylamino)-2-oxo-2H-chromen-3-yl)vinyl)pyridine-1-ium-3-sulfonate, can serve as a robust fluorescent probe of OATP3A1. Another fluorescent probes, CBRD2-BAT and CBRD4-BAT were characterized to be OATP3A1 substrate by Németh et al. (2023). Whether these probes are substrates for other

transporters at the BCSFB remains to be determined. They may potentially be used in quantitative live-tissue imaging to characterize OATP3A1 activity.

BCRP and P-GP are two well-known efflux transporters for their role in multidrug resistance in cancer by limiting the cellular uptake of a wide range of substrates (drugs and toxicants), therefore regulating their concentrations in the brain (Agarwal, Hartz, Elmquist, & Bauer, 2011). Specifically, BCRP and P-GP are known to mediate drug efflux from the brain at the luminal membrane of the blood-brain barrier (Jani, et al., 2014; Uchida, et al., 2020). However, these efflux transporters are expressed at the apical (CSF-facing) membrane of CPE cells, suggesting an opposite mechanism compared with what we have known for BCRP and P-GP at the blood-brain barrier, where BCRP and P-GP function to limit the entry of drugs and xenobiotics into the brain. As *Bcrp* at the BCSFB showed a significant increase in mRNA expression level in aged groups compared with young adult group, along with its important role in drug efflux, further study on their functional roles at the BCSFB may help us understand the transport mechanisms of BCRP and P-GP at the BCSFB and their contribution in aging-related neurodegenerative disease development. Since BCRP and P-GP share a wide substrate pool, it would be difficult to identify a BCRP or P-GP specific fluorescent probe to utilize in live-tissue imaging method. Pheophorbide A, a BCRP specific fluorescent substrate has been indicated by Yasuda et al. (2018). This suggests a potential use of pheophorbide A as the BCRP -specific substrate to quantify the activity change of BCRP transporter at the BCSFB using live-tissue imaging method.

In aged mice, *Oat3* CP expression is sex-dependent with females exhibiting a lower expression than males (Figure 2). The CP can act as a sex hormone target, androgen receptors and estrogen receptors and a list of other sex hormone receptors were characterized to be expressed at the CP tissue (Santos, et al., 2017). In rat brain capillary endothelial cells, androgen receptors were found to play a role in inducing the mRNA expression of *Oat3* at the

blood-brain barrier with an increased activity found in uptake of benzylpenicillin (Ohtsuki, et al., 2005). However, the mechanism driving the sex-dependent *Oat3* CP expression needs further study. At the same time, several *Oatps* were found to be regulated by sex hormones, including *Oatp1a4* in mouse liver and *Oatp3a1* in mouse kidney (Cheng, Maher, Lu, & Klaassen, 2006). Recently, *Mrp4* expression at the mouse CP was shown to be sex-dependent with male mice displaying a significant reduction in *Mrp4* expression (Flores, Manautou, & Renfro, 2017). Although there was a trend of higher expression of Mrp4 in female mice CP tissue in both young adult mice group and aged mice group (Figure 2)., the difference was not statistically significant. Furthermore, functional data showed a slightly lower CPEI (%) in 20-month female cohort than the male cohort (Figure 5B). The reason for this discrepancy is unknown and whether there is sexual dimorphism of *Mrp4* expression and function at the BCSFB needs further investigation.

After comprehensively analyzing the changes in mRNA expression level with aging and between genders, it would be interesting to see if the aging and sex also similarly impact the activities of these BCSFB transporters. However, functional characterization of individual transporter activity at the BCSFB is not feasible for many transporters analyzed by RT-qPCR as there is no well-defined specific fluorescent probe for the transporter genes especially those that showed aging or sex-dependent changes. We hence decided to characterize if aging and sex have an impact on the activity of the OATP1A – MRP1/4 pathway at the BCSFB using quantitative live-tissue imaging method using SR101, a OATP1A specific fluorescent substrate shown in studies in our laboratory (Sun, Hagenbuch, Kelly, & Wang, 2023). OATP1A – MRP1/4 has been characterized as a major transport pathway for large organic anions at the BCSFB site (Sun, Hagenbuch, Kelly, & Wang, 2023; Hu, Zha, Sun, & Wang, 2022). The results showed that the activity of this main pathway for organic anions across the BCSFB is

not influenced by aging or sex, consistent with minimal change of *Oatp1a* and *Mrp1/4* mRNA expression.

AQP1 plays an important role in maintaining CSF homeostasis and the expression of this water channel protein was shown to be stable with aging in mice (Deffner, et al., 2022). As a possible cause, Trilo-Contreras et al. (2019) demonstrated that *AQP4* expressed on ependymal cells and glia limitans also facilitates the CSF formation with quantitative similar contribution to the process compared to AQP1. Other mechanisms including other water channel proteins such as AQP9 or decrease of Na/K-ATPase with aging Sprague-Dawley rat (Masseguin, LePanse, Corman, Verbavatz, & Gabrion, 2005) might also be involved in this process, which needs further research into the mechanism of decreased CSF production during aging.

In this study, we only studied mice aged 3 months and 20-23 months, we assumed the changes of mRNA expression and activity within this age range happen gradually. Transporter expressions were found to vary dynamically during early development (Liddelow, et al., 2012; Kratzer, et al., 2013). Relative mRNA expression of organic cation and organic anion transporters in mouse and human CP tissues have been compared in studies by Duang and Wang (2013) and Hu et al. (2022). Their data showed consistency and suggested that PMAT is the predominant poly-specific organic cation transporter in both human and mouse CP. On the other hand, OATP1A2, OATP1C1, OAT3, MRP1/4, and their equivalent mouse homologs are the main organic anion transporters at both human and mouse BCSFB. Human CP compared to CP in rodents is highly expanded, proportionally to brain size, with more abundant stromal tissue and distinct transporters on the epithelial membrane (Pellegrini & Lancaster, 2021). Although mice have four members in the OATP1A subfamily (OATP1A1/4/5/6), larger animals including humans only have one 1A member, OATP1A2. Some transporters expressed in mice (e.g., OATP1A5) but not in human, share similar functionality with human OATP1A2 and can mediate both endobiotic and xenobiotic clearance at the BCSFB (Sun, Hagenbuch,

Kelly, & Wang, 2023). Therefore, it would of great interest to compare if the aging- or sex-dependent changes in BCSFB transporter mRNA expression is similar between humans and mice.

A step further, we could also conduct *in vivo* studies. In vivo study for CSF concentrations would be difficult due to methodological challenges and the complexity of the brain environment. A microdialysis study was conducted *in vivo* in mice to study the influence of PEPT2 on the distribution of cefadroxil (Chen, et al., 2017). Another *in vivo* study in rabbits was conducted by Spector in 1986 to study the pharmacokinetics of ceftriaxone by intraventricular injection. Further studies on the BCSFB transporters could contribute to comprehensively understanding the dispositions of CNS drugs and optimizing dosing regimens of CNS drugs in geriatric patients and between genders.

In summary, we demonstrated that with aging, the expression of *Oatp3a1* showed a significant decrease in aged mice CP tissues, while the expression of *Bcrp* and *P-gp* significantly increased in the aged mice cohort. These changes are observed in both male and female cohorts. Sex did not affect BCSFB expression of most analyzed transporters, and only Oat3 mRNA expression in the aged mice group showed lower expression in female than in male CP tissues. As the change of mRNA expression is not always translatable to the change of transporter activity, for transporters (*Oatp3a1, Bcrp, Pgp, Oat3*) showing aging- and/or sex-dependent mRNA expression, further functional studies are needed to determine if these changes translate to differences in protein expression and transporter activity at the BCSFB.

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