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**Live Tissue Imaging Reveals Distinct Transcellular Pathways for
Organic Cations and Anions at the Blood-CSF Barrier**

Tao Hu, Weibin Zha, Austin Sun, and Joanne Wang

Department of Pharmaceutics (T.H., W.Z., A.S., J.W.)

University of Washington, Seattle, WA 98195, United States

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Correspondence:

Joanne Wang, Ph.D.

University of Washington

Department of Pharmaceutics

H272 Health Sciences Building

Seattle WA 98195-7610

Phone: 206-221-6561

Fax: 206-543-3204

Email: jowang@uw.edu

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Abbreviations:

aCSF, artificial cerebrospinal fluid; BCSFB, blood-cerebrospinal fluid barrier; CP, choroid plexus; CPE cells, choroid plexus epithelial cells; CSF, cerebrospinal fluid;

FL-MTX, fluorescein methotrexate; MATE, multidrug and toxin extrusion protein;
MRP, multidrug resistance associated protein; OAT, organic anion transporter; OATP,
organic anion transporting polypeptide; OCT, organic cation transporter; PMAT,
plasma membrane monoamine transporter

ABSTRACT

Formed by the choroid plexus epithelial (CPE) cells, the blood-cerebrospinal fluid (CSF) barrier plays an active role in removing drugs, toxins and metabolic wastes from the brain. Several organic cation and anion transporters are expressed in the CPE cells, but how they functionally mediate transepithelial transport of organic cations and anions remain unclear. In this study, we visualized the transcellular transport of fluorescent organic cation and organic anion probes using live tissue imaging in freshly isolated mouse choroid plexuses (CPs). The cationic probe IDT307 was transported into CPE cells at the apical membrane and highly accumulated in mitochondria. Consistent with the lack of expression of organic cation efflux transporters, there was little efflux of IDT307 into the blood capillary space. Furthermore, IDT307 uptake and intracellular accumulation was attenuated by approximately 70% in CP tissues from mice with targeted deletion of the plasma membrane monoamine transporter (Pmat). In contrast, the anionic probe fluorescein-methotrexate (FL-MTX) was rapidly transported across the CPE cells into the capillary space with little intracellular accumulation. Rifampicin, an inhibitor of organic anion transporting polypeptides (OATPs), completely blocked FL-MTX uptake into the CPE cells whereas MK571, a pan-inhibitor of multidrug resistance associated proteins (MRPs), abolished basolateral efflux of FL-MTX. In summary, our results suggest distinct transcellular transport pathways for organic cations and anions at the blood-CSF barrier and reveal a pivotal role of PMAT, OATP and MRP transporters in organic cation and anion transport at the blood-CSF interface.

SIGNIFICANCE STATEMENT

Live tissue imaging of the murine blood-CSF barrier revealed that while organic cations are transported from the CSF side into the CP epithelial cells by PMAT without efflux into the blood, amphipathic anions in the CSF are efficiently transported across the blood-CSF barrier through the collaborated function of apical OATPs and basolateral MRPs. These findings contribute to a mechanistic understanding of the molecular and cellular pathways for CP clearance of cationic and anionic xenobiotics and endobiotics from the brain.

INTRODUCTION

The choroid plexus (CP) is a highly vascularized tissue in the brain ventricles and acts as the blood-cerebrospinal fluid (CSF) barrier (BCSFB). The CP consists of a monolayer of polarized epithelial cells joined together by tight junctions with a network of capillaries embedded in its core (Figure S1). The primary function of the CP is to produce and secrete CSF, which is imperative for the development, neuroprotection and neurochemical homeostasis of the brain (Johanson et al., 2011; Lun et al., 2015). As the CSF flows through the ventricular and subarachnoid spaces, metabolic wastes and harmful substances are removed from the brain. The CP epithelial (CPE) cells also actively remove endogenous and exogenous solutes from the CSF by directly transporting them across the BCSFB into the blood circulation. This function is carried out by the coordinated function of uptake and efflux transporters expressed at the apical (CSF-facing) and the basolateral (blood-facing) membranes of the CPE cells (Keep and Smith, 2011; Morris et al., 2017; Sun and Wang, 2021). By transporting potentially harmful substances out of the brain, the CP plays an essential role in maintaining the homeostasis and normal function of the brain. Indeed, impaired CP function and CSF production is implicated in various neurological disorders such as ischemia, Alzheimer's disease, infection and inflammation of the CNS (Kaur et al., 2016; Lun et al., 2015; Marques et al., 2017).

The BCSFB is also of pharmacological and toxicological significance as it can regulate the distribution of therapeutic drugs and neurotoxic substances in the CSF (Keep and Smith, 2011; Smith et al., 2011; Sun and Wang, 2021). Previous gene

profiling studies have revealed the expression of a number of multispecific drug transporters in the CPE cells (Ho et al., 2012; Morris et al., 2017; Sun and Wang, 2021). However, only a handful of these transporters have been studied at the functional level. In drug elimination organs such as the kidney and liver, two distinct transport systems are involved in transepithelial transport of cationic and anionic drugs (International Transporter et al., 2010; Pelis and Wright, 2011; Stieger and Hagenbuch, 2014). Pioneer work by Miller and coworkers suggested that, as in hepatocytes and renal proximal tubule cells, CPE cells may also possess transport systems for organic cations and organic anions (Breen et al., 2002; Miller, 2004). In particular, the fluorescent organic anions, fluorescein and fluorescein-methotrexate (FL-MTX), were shown to be transported across the CPE cells in CP tissues isolated from rats and dogfish sharks (Baehr et al., 2006; Breen et al., 2002). Mechanistic studies in organic anion transporter 3 (Oat3) knockout mice showed that the uptake of the smaller anion, fluorescein, from the CSF, the first step in its CSF-to-blood transport, is mediated by Oat3 at the apical membrane (Sweet et al., 2002; Sykes et al., 2004). On the other hand, CP uptake of the larger, amphipathic anion FL-MTX is Oat3-independent (Sweet et al., 2002). The efflux of organic anions from CPE cells into the blood capillaries involves the multidrug resistance-associated proteins (MRPs), especially MRP1 and 4, at the basolateral membrane of CPE cells (Breen et al., 2004; Reichel et al., 2010; Wijnholds et al., 2000).

Compared to organic anions, much less is known about the molecular mechanisms underlying organic cation transport at the BCSFB. We previously

reported that the plasma membrane monoamine transporter (PMAT), a polyspecific organic cation transporter first cloned in our laboratory (Engel et al., 2004; Wang, 2016), is the predominant transporter responsible for organic cation uptake at the apical membrane of the BCSFB (Duan and Wang, 2013). Using radiotracer uptake studies in intact mouse CP tissues, we showed that CP uptake of organic cations is impaired in *Pmat* (*Slc29a4*) knockout mice (Duan and Wang, 2013). However, radiotracer uptake studies only measure total tissue accumulation without providing information on transcellular transport. Thus, the subcellular fate of organic cations after their initial uptake into the CPE cells remains unclear.

IDT307 is a structural analog of 1-methyl-4-phenylpyridinium (MPP⁺), a prototypical organic cation transported by all known organic cation transporters (Figure 1) (Duan et al., 2015; Koepsell, 2020; Wagner et al., 2016). In this study, we used IDT307 to image transepithelial transport of organic cations in live CP tissues isolated from the mouse brain. The role of *Pmat* at the BCSFB was further evaluated by imaging studies in CP tissues from *Pmat* null mice. The molecular mechanism of amphipathic organic anion transport was also explored using FL-MTX and compared with IDT307.

MATERIALS AND METHODS

Materials. IDT307 (4-[4-(dimethylamino)phenyl]-1-methylpyridinium iodide) was purchased from Sigma-Aldrich (St. Louis, MO). FL-MTX was obtained from Molecular Probes (Eugene, OR). MK571 was obtained from EMD Millipore

(Darmstadt, Germany). Poly-D-lysine (PDL) was obtained from MP Biomedicals (Solon, OH). Cell culture media and reagents were from Life Technologies (Carlsbad, CA). Cell culture plastic wares were purchased from BD Biosciences (San Jose, CA). Unless otherwise specified, all other chemicals and reagents were from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

Cell Lines and Cell Culture. Flp-In human embryonic kidney 293 (HEK293) cell lines stably expressing human and mouse PMAT were previously generated in our laboratory (Duan and Wang, 2010; Duan and Wang, 2013). The cells were maintained in DMEM medium, supplemented with 10% FBS, 150 µg/mL hygromycin B, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO₂. Before seeding the cells, the plastic surfaces of cell culture wares were coated with 0.1 mg/mL PDL in PBS for a better attachment.

Animals and CP Tissue Collection. *Pmat*^{+/+} and *Pmat*^{-/-} C57BL/6J mice were housed in the specific pathogen free facility at the University of Washington. All the experiments were carried out in accordance with the animal protocols approved by the Institutional Animal Care and Use Committee at the University of Washington. Three month-old male mice were used in this study. After euthanasia with CO₂, intact CP tissues were isolated from the lateral ventricles of mouse brain under a dissecting microscope as described previously (Duan and Wang, 2013). Freshly isolated tissues were maintained in ice cold artificial CSF (aCSF: 119 mM NaCl, 26.2 mM NaHCO₃, 2.5 mM KCl, 1 mM NaH₂PO₄, 1.3 mM MgCl₂, 2.5 mM CaCl₂, 10 mM glucose, previously gassed with 95% O₂/5% CO₂) and used for

imaging studies within 2 hours of isolation.

Reverse Transcription and Quantitative Real-time PCR. Quantitative real-time PCR was performed to determine the relative mRNA levels of major organic cation and organic anion transporters in mouse and human CP. Total RNA was extracted from mouse and human CP tissues using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The human CP tissue was obtained from a donor at 5.1 hours postmortem (donor information: 74 years old, Hispanic, male, diagnosed with bladder cancer as the cause of death) from the National Disease Research Interchange (Philadelphia, PA). Total RNA (2 µg) was used to synthesize cDNA by reverse transcription using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Expression of Oct1, Oct2, Oct3, Pmat, Mate1, Mate2, Oat1, Oat3, Oatp1a4, Oatp1a5, Oatp1c1, Oatp3a1, Mrp1, Mrp4 in mouse CP and expression of OCT1, OCT2, OCT3, PMAT, MATE1, MATE2, OAT1, OAT3, OATP1A2, OATP1C1, OATP3A1, MRP1, MRP4 in human CP were quantified using TaqMan Real-Time PCR Master Mix (Applied Biosystems, Foster City, CA) as described previously (Duan and Wang, 2013). The relative mRNA levels of these transporters in CP were normalized to GAPDH.

Uptake of IDT307 in PMAT-transfected Cells. pcDNA5, human PMAT (hPMAT) and mouse Pmat (mPmat) transfected HEK293 cells were seeded in 96-well plate at a density of 40,000 cells/well and allowed to grow overnight. Measurement of IDT307 uptake was performed using a procedure as previously described (Duan et al., 2015). Briefly, cells were washed once with uptake buffer (Hank's Balanced Salt

Solution with 20 mM HEPES, pH 7.4). Uptake was then initiated by adding 100 μ L uptake buffer containing IDT307 in the absence or presence of quinine, a known PMAT inhibitor. Relative fluorescence unit (RFU) was recorded immediately after adding IDT307 (time 0) and at the end of the uptake period using a Perkin Elmer Wallac 1420 Multilabel Counter capable of precise temperature control and kinetic measurements. The excitation and emission wavelengths of IDT307 were 440 nm and 520 nm, respectively. Specific uptake of IDT307 was calculated by subtracting the fluorescence readings at time 0 from the end point ($\text{RFU}_{\text{end}} - \text{RFU}_{\text{time0}}$).

Fluorescence Microscopy of IDT307 in PMAT-transfected Cells.

Fluorescent imaging was used to visualize the cellular uptake of IDT307 in hPMAT and mPmat transfected HEK293 cells. Cells were seeded onto Nunc Glass Bottom Dishes (Thermo Scientific, Rochester, NY) at a density of 1.0×10^5 cells/mL and allowed to grow overnight. Cells were washed once with uptake buffer and then incubated with IDT307 in the absence or presence quinine for 10 min. The fluorescent signals were detected using a Zeiss LSM 510 META confocal microscope (Jena, Germany) using 488 nm excitation and 520 nm emission wavelengths. To further study the intracellular distribution of IDT307, the HEK293 cells were also incubated with IDT307 and Mitotracker Deep Red (Life Technologies, Eugene, OR) simultaneously and subject to the same confocal imaging protocol.

Live Tissue Imaging in Freshly Isolated Mouse CP. Breen et al. previously analyzed FL-MTX transport in rat CP using confocal microscopy (Breen et al., 2004). A modified protocol was used in our study to image IDT307 and FL-MTX transport

in mouse CP. Freshly isolated intact CP tissues from *Pmat*^{+/+} and *Pmat*^{-/-} mice were transferred into Nunc glass bottom dishes (Thermo Scientific, Rochester, NY) containing artificial CSF (aCSF) buffer pre-gassed with 95% O₂/5% CO₂ at room temperature. The dish was mounted on the stage of Zeiss LSM 510 META confocal microscope and viewed through a 40× oil immersion objective. Uptake was initiated by adding a fluorescent compound with or without inhibitors into the aCSF. Confocal images were acquired using 488-nm argon ion laser excitation with appropriate dichroic and long-pass emission filters. With the settings used, tissue auto fluorescence was undetectable, and fluorescence bleaching was minimal. For each CP tissue under investigation, 4–8 areas including CPE cells and the adjacent blood capillary region were selected, and the fluorescent signals were recorded. The integrity of the CP tissue was verified by visual inspection under phase-contrast light microscopy. For quantification of IDT307 uptake, the fluorescence intensity of IDT307 (Fluorescence_{end} - Fluorescence_{time0}) for each area was calculated using ImageJ (NIH, Bethesda, MD), and the fluorescence value for each CP was the mean of all selected areas.

To record the real-time transport of IDT307 or FL-MTX in mouse CP, a specific observation area containing CPE cells and the adjacent blood capillary was selected and immobilized in aCSF. Fluorescent compounds were then added and the fluorescent signals were recorded every one minute for 20 minutes. The movie clips were obtained by combining the fluorescent images at different time points using Imaris analysis software (Keck Microscopy Center, University of Washington).

Data Analysis. Statistical analysis of the data was carried out using Prism 5.0 (GraphPad Software, CA, USA). All the data were expressed as mean $\pm$ standard deviation (SD) from at least three independent experiments. The kinetics data were fitted to the Michaelis-Menten equation to obtain kinetic parameters by nonlinear regression using Prism software (GraphPad Software Inc., La Jolla, CA). The significance of difference between groups was estimated by an unpaired Student's *t* test. A *P* value less than 0.05 was considered as a statistically significant difference.

RESULTS

Expression of Transporters for Organic Cations and Anions in Mouse and Human CP

To compare the relative mRNA expression of organic cation and organic anion transporters in mouse and human CP tissues, real-time PCR analysis of major uptake and efflux transporters for organic cations and anions was performed. Consistent with previous reports (Dahlin et al., 2009; Duan and Wang, 2013), Pmat is highly expressed in mouse CP whereas expression of organic cation transporters 1-3 (Oct1-3) was not detected (Figure 2A). There was also no detectable expression of the mouse multidrug and toxin extrusion (Mate) 1 and 2, two known efflux transporters for organic cations (Koepsell, 2020; Wagner et al., 2016). In the human CP, the expression pattern of organic cation transporters is similar to that of the mouse CP, although very low expression of OCT3 and MATE1/2 was detected by real-time PCR (Figure 2B). Among the organic anion transporters tested, Oatp1c1/OATP1C1 and Mrp1/MRP1 showed high expression followed by Oat3/OAT3 and Mrp4/MRP4 in both mouse and human CP tissues (Figure 2). In addition, Oatp1a4/5, OATP1A2 and OATP3A1 also showed expression in mouse or human CP. Taken together, our data suggest that PMAT is the predominant polyspecific organic cation transporter in both human and mouse CP. On the other hand, OATP1C1, OAT3, MRP1/4 and their equivalent mouse homologs, are the main organic anion transporters at both human and mouse BCSFB.

Distinct Transcellular Transport Pathways of IDT307 and FL-MTX in Live Mouse CP Tissue

To probe the functional characteristics of CSF-to-blood transport of organic cations and anions at the BCSFB, the transport processes of IDT307 (a fluorescent cation) and FL-MTX (a fluorescent anion conjugate) were investigated using live tissue imaging in intact CP tissues isolated from wild type mice. IDT307 is a fluorescent analog of MPP⁺, a prototypical substrate transported by all known organic cation transporters. FL-MTX is a larger conjugated anion (Mwt 925 Da) previously shown to be transported into rodent CP tissues by an Oat3-independent mechanism (Sweet et al., 2002). As shown in Figure 3A and movie clip (supplemental video 1), IDT307 (2 μ M) added to the aCSF solution accumulated in CPE cells in a time-dependent manner. The fluorescence signal primarily resides within CPE cells with little accumulation in the subepithelial capillary region. The Z-stack of confocal slices of mouse CP after incubation with IDT307 further showed that there was no fluorescent signal of IDT307 in the blood capillary areas even after 60 min of incubation (Figure 3A). Together, these data suggest that IDT307 is transported into in CPE at the apical side with little efflux at the basolateral membrane. Further, co-incubation with Mitotracker Deep Red revealed that IDT307 was mainly accumulated in mitochondria in the CPE cells (Figure 3B). In contrast, imaging of the organic anion FL-MTX in mouse CP showed a completely different pattern. When added to the aCSF solution, FL-MTX (2 μ M) rapidly accumulated in the capillary areas in mouse CP, demonstrating that the anion was first transported into CPE cells and then

effluxed across the basolateral membrane (Figure 3C, supplemental video 2). The transepithelial transport of FL-MTX across the CPE cells was very fast as the fluorescent signal was detected in the blood capillary areas shortly after adding it to the incubating aCSF and reached steady state within 10-20 min (Figure 3C, supplemental video 2). There is minimal FL-MTX accumulation inside the CPE cells, suggesting that apical uptake is the rate-limiting step in the two-step transcellular transport process.

Transport of IDT307 by Mouse and Human PMAT Transporters

Based on the high expression of PMAT in human and mouse CP tissues (Figure 2) and its known localization at the apical membrane of CPE cells (Duan and Wang, 2013), we hypothesized that apical uptake of IDT307 into the CPE cells is mediated by PMAT. We previously showed that IDT307 is a substrate of hPMAT (Duan et al., 2015). To confirm that IDT307 is a substrate for mPmat and to determine IDT307 transport kinetics, time- and concentration-dependent uptake of IDT307 was performed in HEK cells stably expressing hPMAT or mPmat (Figure 4). The results showed that uptake of IDT307 was much greater in hPMAT and mPmat transfected HEK293 cells when compared with the pcDNA5 vector cells (Figure 4A), demonstrating that IDT307 is transported by both hPMAT and mPmat. For kinetic analysis, specific uptake by PMAT was obtained by subtracting background uptake in pcDNA5 cells, and the data were fitted to the Michaelis-Menten equation to obtain kinetic parameters (Figure 4B). The obtained K_m values (mean $\pm$ SD) are $3.63 \pm$

1.02 μ M for hPMAT and 6.12 ± 2.44 μ M for mPmat. The V_{max} values (mean $\pm$ SD) are $7,600 \pm 955.9$ RFU/min for hPMAT and $13,874 \pm 2,950$ RFU/min for mPmat.

In addition, intracellular accumulation of IDT307 was also visualized by fluorescent imaging. When compared with the control cells, the fluorescent signal of IDT307 was greatly enhanced in hPMAT and mPmat transfected HEK293 cells (Figure 5A and 5B), which was diminished in the presence of quinine (200 μ M), a known inhibitor of PMAT (Duan et al., 2015). Of note, concurrent imaging of IDT307 and Mitotracker also revealed substantial colocalization suggesting that mitochondria are the major site of IDT307 intracellular accumulation in HEK cells (Figure 5C).

Pmat Mediates the Uptake of IDT307 into Mouse CPE Cells

To determine the role of PMAT in organic cation transport at the BCSFB, the transport of IDT307 in CP tissues from *Pmat*^{+/+} and *Pmat*^{-/-} mice was further evaluated using real-time tissue imaging. Quantification of fluorescence signal showed that IDT307 fluorescence increased in a time-dependent manner in *Pmat*^{+/+} CP and started to plateau after 40 min. IDT307 fluorescence is lower in *Pmat*^{-/-} CP; and at steady state, the fluorescent intensity of IDT307 in *Pmat*^{-/-} CP was only about 30% of that in *Pmat*^{+/+} CP (Figure 6A). The effect of quinine on the intracellular accumulation of IDT307 in CP cells was also evaluated. As shown in Figure 6B&C, the fluorescent intensity of IDT307 was greatly reduced in *Pmat*^{+/+} CP in the presence of quinine. In contrast, quinine had no effect on the uptake of IDT307 in

Pmat^{-/-} CP. Collectively, these findings demonstrate that *Pmat* plays a major role in the uptake of IDT307 from the CSF into CPE cells.

Oatps and Mrps Mediate the Transport of FL-MTX across BCSFB

To further explore the transporters mediating the transport of FL-MTX at CP, the transport of FL-MTX (2 μ M) was imaged in wild type (*Pmat*^{+/+}) CP tissues in the presence of pharmacological inhibitors of uptake and efflux transporters. As shown in Figure 7, there was no difference between *Pmat*^{+/+} and *Pmat*^{-/-} CP tissues in terms of FL-MTX distribution into the blood capillary areas, demonstrating that *Pmat* was not involved in the transport of FL-MTX across the BCSFB. Importantly, in the presence of MK571 (100 μ M), a pan-inhibitor of MRPs (Liu et al., 2018), the fluorescent intensity of FL-MTX was reduced in the blood capillary areas but greatly increased within CPE cells (Figure 7 & supplemental video 3), demonstrating that the efflux of FL-MTX through the basolateral membrane of CP epithelium was likely mediated by Mrps. In the presence of rifampicin (100 μ M), an inhibitor of OATPs (Hagenbuch and Stieger, 2013; Vavricka et al., 2002), the CP showed almost no fluorescent signal of FL-MTX (Figure 7), suggesting that one or more OATP transporters are responsible for the apical uptake of this organic anion from CSF into CPE cells.

DISCUSSION

A large portion of drugs on market exists as organic cations or anions at physiological pH. In addition, endogenous monoamine neurotransmitters (e.g. serotonin, dopamine, and norepinephrine) and some neurotoxins (e.g. MPP+, paraquat) are organic cations. Certain neurohormones (e.g. DHEAS) and cellular metabolites are organic anions (Grube et al., 2018). Hence, knowledge of the transport mechanisms of organic cations and anions at the BCSFB has important implications in understanding the disposition and action of drugs, neurotoxins and metabolites in the CNS.

In hepatocytes and renal proximal tubule cells, apical and basolateral transporters often collaboratively mediate transepithelial transport of cationic or anionic drugs. For instance, secretion of organic cations in the human kidney is initiated by basolateral uptake via OCT2 followed by apical efflux via MATE1 and 2-K (International Transporter et al., 2010; Yin et al., 2019; Yin and Wang, 2016). On the other hand, organic anions are secreted by the sequential action of basolateral OAT1/3 and apical efflux transporters such as MRP2/4 (International Transporter et al., 2010; Pelis and Wright, 2011; Yin and Wang, 2016). Interestingly, our imaging studies in live mouse CP tissues revealed different transport patterns for organic cations and anions (Figure 3, supplemental videos 1 and 2). The anionic FL-MTX is rapidly transported across the CPE cells into blood capillary space, suggesting the existence of both functional uptake and efflux transporters in CPE cells for organic anions. Similar patterns of transepithelial transport of organic anions (e.g. fluorescein,

FL-MTX) were previously observed in CP tissues isolated from dogfish sharks and rats (Baehr et al., 2006; Breen et al., 2002; Villalobos et al., 2002). In contrast, IDT307, an analog of the prototypical cation MPP⁺, is transported into CPE cells but is not effluxed at the basolateral membrane. The lack of IDT307 efflux across the basolateral membrane corroborates with the lack of expression of organic cation efflux transporters Mate1 and 2 in mouse CP (Figure 2). Taken together, our data suggest that organic anions and cations in the CSF are handled differently by the CPE cells. Organic anions in the CSF are efficiently extracted by the CP into the blood circulation whereas organic cations are likely to accumulate in CPE cells after their uptake via an apical transporter.

Previously, OCT2 was proposed to mediate CP uptake of organic cations based on RT-PCR analysis and the apical localization of an GFP-tagged OCT2 after transfection into rat CP (Sweet et al., 2001). However, multiple studies have failed to confirm endogenous OCT2 expression and function in CP tissues from several species (Choudhuri et al., 2003; Duan and Wang, 2013; Uchida et al., 2015). We previously showed that PMAT is highly expressed in CP and localized to the apical membrane in human and mouse CP tissues (Duan and Wang, 2013; Wang, 2016). Radiotracer uptake studies in CP tissues from *Pmat*^{-/-} mice suggested that PMAT is the predominant transporter responsible for organic cation uptake (Duan and Wang, 2013). In the current study, we further confirmed robust expression of PMAT in mouse and human CPs (Figure 2). Consistent with previous findings, the expression of other organic cation uptake transporters (e.g. OCT1/2/3), was either undetectable or

extremely low (Figure 2). Live tissue imaging studies further showed that IDT307 uptake into CP tissue is reduced by ~ 70% in *Pmat*^{-/-} mice at steady state (Figure 6). Quinine, which inhibits both Pmat and Octs (Duan et al., 2015; Engel and Wang, 2005), reduced IDT307 uptake in *Pmat*^{+/+} CP but had no effect in *Pmat*^{-/-} CP (Figure 6). These findings demonstrated a major role of PMAT in mediating apical uptake of organic cations from the CSF into CPE cells. The mechanism underlying the residual IDT307 uptake in *Pmat*^{-/-} CP tissue is unclear and possibly due to passive diffusion.

PMAT substrates include brain monoamine neurotransmitters (e.g. serotonin, dopamine, norepinephrine) and neurotoxins (e.g. MPP⁺ and its analogs) (Engel and Wang, 2005; Wang, 2016). As the CP apparently lacks an efficient organic cation efflux system at the basolateral membrane, several cellular fates are possible for organic cations after their entry into the CPE cell. CPE cells are known to express several drug metabolizing enzymes (Gherssi-Egea and Strazielle, 2001; Kratzer et al., 2018; Sun and Wang, 2021). In particular, monoamine oxidases, which catalyze the oxidation of monoamines, are highly active at the BCSFB (Sun and Wang, 2021; Vitalis et al., 2002). The CP also possesses high activities for UDP-glucuronosyltransferases and sulfotransferases (Gherssi-Egea and Strazielle, 2001; Kratzer et al., 2018). The presence of these enzymes in CPE cells provides a microenvironment where bioactive amines can be converted into anionic metabolites and conjugates (e.g. homovanillic acid for dopamine). Once the anionic metabolites are formed, they can be further transported into the blood circulation by organic anion

efflux transporters at the basolateral membrane. However, non-metabolizable organic cations, such as MPP⁺ and IDT307, can accumulate in CPE cells. MPP⁺ is neurotoxin that acts by interfering with oxidative phosphorylation in mitochondria (Singer et al., 1988). Interestingly, our imaging studies revealed that IDT307 also co-localizes with mitochondria in CPE and HEK cells (Figures 3B and 5C). The mechanism by which IDT307 accumulates in mitochondria in CPE cells is currently unknown. Nevertheless, the accumulation of organic cations in mitochondria of CPE cells may lead to mitochondrial toxicity, which can compromise the normal physiological functions of CP such as CSF secretion, transport and barrier function at the blood-CSF interface.

FL-MTX is a larger amphipathic anion with a molecular mass of 925 Da. Our real-time imaging studies in mouse CPs revealed that FL-MTX is rapidly transported across the CPE cells from the CSF side to the blood capillary side (Figure 3, supplemental video 2). There was little FL-MTX signal within the CPE cells, suggesting that apical uptake is the rate-limiting step. Although OAT3 is expressed in CPE cells, FL-MTX is not a transportable substrate of OAT3 (data not shown). Previous study in CP tissues from Oat3 knockout mice demonstrated that Oat3 does not play a role in FL-MTX transport at the BCSFB (Sweet et al., 2002). In the body, larger amphipathic anions (Mwt > 400-500 Da) are often eliminated by the liver through the coordinated action of OATPs and MRPs (Hagenbuch and Stieger, 2013; Kovacsics et al., 2017; Stieger and Hagenbuch, 2014). Real-time PCR analysis revealed the expression of several OATP isoforms in human and mouse CP tissues

(Figure 2). FL-MTX is a known substrate of several OATP transporters. Importantly, rifampicin, an OATP inhibitor, abolished apical uptake whereas MK571, a pan-MRP inhibitor, impaired basolateral efflux (Fig. 2C). These data suggest that similar to hepatocytes, OATP and MRP transporters at the BCSFB can act coordinately to actively transport structurally diverse OAs from the CSF into the blood. This transport system may represent a major clearance pathway for brain removal of OA neurotoxins, therapeutic drugs and endogenous metabolites. In addition, several other transporters, including the breast-cancer resistance protein (BCRP) and the reduced folate carrier (RFC), are expressed in the CP and accept unconjugated methotrexate as a substrate (Grapp et al., 2013; Li et al., 2013; Morris et al., 2017). These transporters may also mediate OA transport at the BCSFB. Given the broad presence of organic anion metabolites (e.g. acids, conjugates) from brain metabolism, the existence of a multispecific detoxification system for anionic metabolites and waste products at the BCSFB are of special significance for maintaining the homeostasis of CNS. Altered transporter expression and function at the BCSFB may lead to a compromised detoxification function and exacerbate brain disorders such as Alzheimer's disease, where the buildup of waste products and protein debris is implicated in disease etiology.

In summary, using live CP tissue imaging and fluorescent probes, we demonstrated distinct transcellular pathways for organic cations and anions at the BCSFB. Our results suggest that organic cations in the CSF are transported into CPE cells largely via the apical PMAT transporter and accumulate in CPE cells as the

CPE cells lack an apparent organic cation efflux system at the basolateral membrane. In contrast, organic anions are efficiently transported across the BCSFB via the collaborated function of apical OATPs (and OAT3 as previously shown (Sweet et al., 2002)) and basolateral MRPs. These findings provide novel insights into the molecular and cellular mechanisms governing CP clearance of xenobiotics and endobiotics. Our work also suggests an important role of PMAT, OATP and MRP transporters in the transport and detoxification function of the BCSFB. Further validation of these transport mechanisms at the human BCSFB may benefit mechanism-based prediction of the disposition and action of drugs and neurotoxins in the CNS.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Hu, Wang

Conducted experiments: Hu, Zha

Performed data analysis: Hu, Sun, Wang

Wrote or contributed to writing of the manuscript: Hu, Sun, Wang

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Chemical structures of (A) MPP⁺ and IDT307 (B) Fluorescein-methotrexate (FL-MTX).

Figure 2. Relative mRNA expression of major organic cation and organic anion transporters in mouse (A) and human (B) CP tissues. Total RNA was extracted from pooled CP tissues from 4 wild type mouse (A) or human CP (B) and reverse transcribed. TaqMan quantitative real-time PCR was used to determine the expression levels of the transporters. GAPDH was used as the internal standard. Data were expressed as mean $\pm$ SD (N = 4).

Figure 3. Distinct transcellular pathways of IDT307 and FL-MTX at mouse CP by live tissue imaging. (A) Fluorescence imaging of IDT307 (2 μ M) in CP tissues from wild type mice over time and z-stack of confocal slices after incubation with IDT307 for 60 min. Scale bar: 20 μ m. (B) Confocal imaging of IDT307 (2 μ M) and Mitotracker Deep Red (100 nM) in CP from wild type mouse after 40 min incubation. Scale bar: 10 μ m. (C) Fluorescent imaging of FL-MTX (2 μ M) in CP tissues from wild type mice. CPE Cell, choroid plexus epithelial cells; BC, blood capillary. Scale bar: 20 μ m.

Figure 4. IDT307 is a fluorescent substrate of both hPMAT and mPmat. (A) Time course of IDT307 (1 μ M) uptake in hPMAT and mPmat transfected HEK293 cells.

(B) Concentration response of IDT307 uptake in hPMAT and mPmat transfected HEK293 cells. Data are presented as the means $\pm$ SD from three independent experiments.

Figure 5. Uptake of IDT307 and its co-localization with mitochondria in hPMAT and mPmat transfected HEK293 cells. (A) and (B) Fluorescent imaging of IDT307 in hPMAT and mPmat transfected HEK293 cells. Cells were incubated with IDT307 (1 μ M) in the absence or presence of quinine (200 μ M) in HBSS buffer for 10 min. Scale bar: 20 μ m. (C) Fluorescent imaging of IDT307 (1 μ M) and Mitotracker Deep Red (20 nM) in hPMAT and mPmat transfected HEK293 cells after 10 min incubation. Scale bar: 10 μ m.

Figure 6. PMAT mediates the uptake of IDT307 into mouse CP epithelial cells. (A) Fluorescence intensity of IDT307 in CP tissues from *Pmat*^{+/+} and *Pmat*^{-/-} mice over time. (B) Fluorescent imaging of IDT307 (with or without quinine) in CP tissues from *Pmat*^{+/+} and *Pmat*^{-/-} mice. Scale bar: 20 μ m. (C) Fluorescence intensity of IDT307 (with or without quinine) in CP tissues from *Pmat*^{+/+} and *Pmat*^{-/-} mice. Tissues were incubated with IDT307 (2 μ M) alone or with IDT307 (2 μ M) and quinine (200 μ M) in aCSF for 40 min. For each CP tissue, 4–8 adjacent cellular and capillary areas were selected. The average fluorescence intensity (Fluorescence_{end} - Fluorescence_{time0}) for each area was calculated and the value for each CP was the

mean of all selected areas. Data shown in A and C were presented as means $\pm$ SD of individual mean values in CP tissues obtained from different animals (4 animals per group). Statistical significance was determined by an unpaired Student's *t* test, compared with each control group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) with no correction for multiple comparisons.

Figure 7. OATPs and MRPs mediate the transport of FL-MTX across CP tissues. Fluorescent imaging of FL-MTX was recorded in CP tissues from *Pmat*^{+/+} and *Pmat*^{-/-} mice in the presence of MRP inhibitor MK571 or OATP inhibitor rifampicin. Tissues were incubated with FL-MTX (2 μ M) alone or in the presence of MK571 (100 μ M) or rifampicin (100 μ M) in aCSF. Scale bar: 20 μ m.

Figure 1

A

MPP+

IDT307

B

**Fluorescein-methotrexate
(FL-MTX)**

Figure 2

A

B

Figure 3

Figure 4

Figure 5

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A

pcDNA5

hPMAT

Vehicle control

Vehicle control

Quinine

IDT307

B

pcDNA5

mPmat

Vehicle control

Vehicle control

Quinine

IDT307

C

hPMAT

mPmat

IDT307

Mitotracker

Merged

Figure 6

A

B

C

Figure 7