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Transcriptomic and Quantitative Proteomic Analysis of Transporters and Drug Metabolizing Enzymes in Freshly Isolated Human Brain Microvessels

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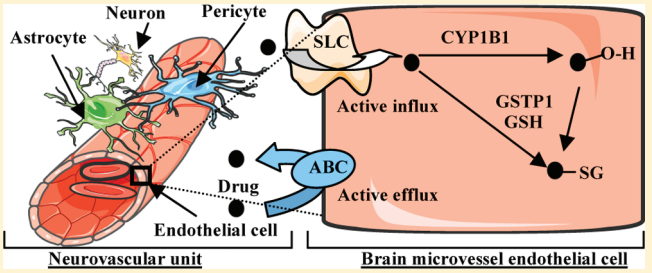
Supporting Information

ABSTRACT: We have investigated the transcriptomic and/or proteomic patterns of 71 solute carrier (SLC) and organic solute (OST) transporters, 34 ATP-binding cassette (ABC) transporters, and 51 metabolizing enzymes in human brain microvessels. We used quantitative RT-PCR and LC−MS/MS to examine isolated brain microvessels and cortex biopsies from 12 patients with epilepsia or glioma. SLC2A1/GLUT1, SLC1A3/EAAT1, and SLC1A2/EAAT2 were the main SLC proteins whereas ABCG2/BCRP, ABCB1/MDR1, ABCA2 and ABCA8 were the main ABC quantified in isolated brain microvessels; ABCG2/BCRP was 1.6-fold more expressed than ABCB1/MDR1, and ABCC4/MRP4 was 10 times less abundant than ABCB1/MDR1. CYP1B1 and CYP2U1 were the only quantifiable CYPs. Finally, GSTP1, COMT, GSTM3, GSTO1 and GSTM2 proteins were the main phase II enzymes quantified; UGTs and NATs were not detected. Our extensive investigation of gene and protein patterns of transporters and metabolizing enzymes provides new molecular information for understanding drug entry and metabolism in the human blood−brain barrier.



KEYWORDS: blood−brain barrier, transporters, drug-metabolizing enzymes, human, quantitative LC−MS/MS

INTRODUCTION

The blood−brain barrier (BBB) plays a critical role in the uptake and efflux of drugs from the blood to the brain, or vice versa, hence affecting their concentrations and effects in the central nervous system (CNS). The early concept of an anatomical barrier between the blood and the brain was supported by the finding of unique tight junctions between the brain endothelial cells so that they formed a continuous wall preventing the paracellular diffusion of solutes.[1] The BBB has more recently been defined as a pharmacological barrier since the endothelial cells were found to contain a range of metabolizing enzymes and transporters that control the rate and extent of drugs reaching the brain parenchyma via transcellular pathway.[2] Brain capillary endothelial cells express various transporters, or solute carriers (SLC) transporters, that facilitate brain uptake processes.[3] Some of the SLC transporters in the human BBB have been identified, but there is, as yet, no exhaustive absolute quantitative profile of SLC transporters. Solutes can also be extruded from the brain endothelium to blood by ATP binding cassette (ABC) efflux transporters.[4−6] The best example is the expression of P-glycoprotein (ABCB1/MDR1) by the luminal membrane, which limits the brain uptake of many CNS drug candidates.[7] Beside transporters, BBB expresses specific enzymes like monoamine oxidases (MAOs) and catechol *O*-methyltransferase (COMT).[8,9] Published reports on the presence of phase II enzymes at the BBB are conflicting[10] since metabolic activities were mostly investigated using brain homogenates instead of isolated brain microvessels.[11] For example, glucuronidation by UDP-glucuronosyl transferases (UGTs) was assumed to take place in the BBB while it was experimentally determined in the whole brain.[12−14] Similarly, little work has been done on the activities of other phase II drug metabolizing enzymes, particularly sulfotransferases (SULTs),

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glutathione *S*-transferases (GSTs), *N*-acetyltransferases (NATs), and methyltransferases (MTs) at the BBB.[15−17]

In a previous study, we reported the expression pattern of genes encoding 8 ABC transporters and 10 cytochrome P450 (CYPs) enzymes in human brain microvessels freshly isolated from 7 patients who underwent surgery for epilepsy or glioma.[18] We also reported qualitative protein detection of ABCB1/MDR1, ABCG2/BCRP and CYP1B1 without any absolute quantification.[18]

Transporter and enzyme systems at the BBB are important tools in mediating the transfer processes of essential nutrients and neuroactive agents. Currently there is a pressing need for detailed knowledge of the expression and functionality of these systems at the human BBB since the majority of the data came from either in vitro cell culture or animal studies, making in vitro to in vivo or interspecies scaling less reliable.

We have now used a combination of recently developed transcriptomic and quantitative proteomic analyses to determine the expression profiles of the genes encoding SLC transporters, ABC transporters, phase I and phase II metabolizing enzymes and the amounts of their protein in human brain microvessels. We used 7 human brain microvessel samples isolated from patients reported in our previous study[18] to determine the transcriptomics of SLC transporters, MAOs and phase II conjugation enzymes. In the current study, we determined the absolute protein quantities in 5 new brain microvessel samples. Our current investigation combining quantitative RT-PCR (qRT-PCR) and LC−MS/MS has enabled us to determine the transcriptomic and/or protein patterns of 71 SLC and organic solute (OST) transporters, 34 ABC transporters, and 51 phase I and phase II metabolizing enzymes.

Extensive knowledge of the transporter and enzyme system elements at the BBB provides insights in understanding drug entry and metabolism at the barrier, which may help in the identification of novel molecular targets for therapeutic agents.

METHODS

Microvessel Isolation. The brain specimens (patients 1−7, $n = 7$) have been described elsewhere.[18] The same protocol was followed for the new patient samples 8−12 ($n = 5$). Briefly, human brain tissues were collected from twelve patients ($N = 12$) who underwent surgery for epilepsy or glioma at the Department of Neurosurgery, Sainte-Anne Hospital (Paris, France). Each patient gave his/her informed consent, in accordance with the regulations set out by the ethics committee. The neurosurgeons provided brain cortex (gray matter) samples weighing between 200 and 1000 mg taken as far as possible from the tumor or the epileptogenic lesion.

Brain tissues were analyzed by neuroimaging, and those tissues considered healthy were used in the study. Patient information is given in Supplemental Table 1 in the Supporting Information. The cortex samples were kept in RPMI buffer at 4 °C for no longer than 1 h. Each specimen was weighed and divided in two. One portion was used to freshly isolate brain microvessels, and the other was homogenized and used for RNA extraction.[18]

BBB Cell Markers and Reference Gene. We used specific cell markers to determine the purity of the isolated microvessels.[18] The housekeeping gene encoding the TATA box-binding protein (*TBP*) was used as reference gene for qRT-PCR experiments (Supplemental Table 2 in the Supporting Information). Similarly, the amounts of endothelial, astrocyte, oligodendrocyte, pericyte, neuron and receptor mediated transcytosis marker proteins were determined by LC−MS/MS.

RNA Extraction, Reverse Transcription and qRT-PCR. RNA extraction, reverse transcription and quantitative real time PCR (qRT-PCR) were done as described previously[18] for patients 1−7 ($n = 7$). Briefly, total RNA was extracted from the human microvessel samples using the RNeasy micro kit (Qiagen GmbH, Hilden, Germany) following the supplier's recommendations. The basal membrane surrounding the brain microvessels was lysed with proteinase-K. The samples were then treated with DNase I (RNase-Free DNase Set, Qiagen SA) to remove genomic DNA. The quantities of total RNA extracted from the different microvessel samples varied between 100 and 400 ng depending on the sample size. The quantities of total RNA extracted from the corresponding cortex samples were above 1 μg. The concentration and purity of the RNA samples were assessed spectrophotometrically at 260 nm using the Nanodrop ND-1000 instrument (NanoDrop Technologies, Wilmington, DE).

For each patient the reverse transcription (RT) was performed on the whole RNA quantity extracted from microvessels and the corresponding brain cortex sample as described previously.[18]

All the primers used for qRT-PCR analysis of the genes encoding conjugation enzymes and solute carriers were checked on positive controls (liver and/or commercial brain cortex). All the primers were tested on an ABI Prism 7900 HT sequence detection system (Applied Biosystems, Foster City, CA) using SYBR Green fluorescence detection in a revalidation step before they were used to analyze the brain microvessels (Supplemental Table 2 in the Supporting Information).

Relative Expression. The relative expression of each gene was determined using the second derivative method.[18] Briefly, the background was proportionally adjusted and the cycle at which the log−linear signal was distinguishable from the background was taken as the crossing-threshold value (C_t) for each sample. The target gene was considered to be quantifiable when the C_t of the middle diluted cDNA (1/80) sample was below 33. Similarly, this C_t was also taken as the detection limit. The relative expression of each target gene in the isolated microvessels compared to the corresponding cortex was determined by the $\Delta\Delta C_t$ method, which endorsed the normalization of the C_t value of each target gene with that of the *TBP* (*TBP*), since the expressions of the *TBP* gene in the isolated microvessels and the corresponding cortex were not statistically different. The calculation was based on samples for which the reverse transcription was carried out with equal amounts of starting mRNA using the following equation:

$$\Delta\Delta C_t = (C_t \text{ target gene} - C_t \text{ TBP})_{\text{isolated microvessels}} - (C_t \text{ target gene} - C_t \text{ TBP})_{\text{cortex}}$$

The difference between the amounts of mRNA in the isolated microvessels and the corresponding cortex was determined from the $2^{-\Delta\Delta C_t}$ values, when the cortex value was arbitrarily defined as 1.

The expression profiles of the genes encoding SLC transporters and metabolizing enzymes were established using the ΔC_t method, according to the following equation:

$$\Delta C_t = (C_t \text{ target gene} - C_t \text{ the least expressed gene})$$

Expressions of SLC transporters and metabolizing enzymes were set to 100% each. The expression percentage of each gene was determined from the $2^{-\Delta C_t}$ values.

Probe Peptides. All probe peptides for the target molecules were chosen using the in silico selection criteria described

previously[19] and synthesized by Thermoelectron Corporation (Sedanstrabe, Germany) with >95% peptide purity. The concentrations of dissolved peptides were determined by quantitative amino acid analysis (Lachrom Elite, Hitachi, Tokyo, Japan). The amino acid sequences of the probe peptides are shown in Supplemental Table 3 in the Supporting Information.

LC−MS/MS Analysis. The protein expression amounts of the target molecules were simultaneously determined by multiplexed multiple reaction monitoring (MRM) in HPLC−MS/MS or nanoLC−MS/MS as described previously.[19,20] Briefly, quantification of human transporters and receptors was based on the MRM conditions previously developed in the Uchida et al. study;[20] whereas quantification of human CYPs and UGTs was based on the MRM conditions developed in the Kawakami et al.[21] and Sakamoto et al.[22] studies, respectively. Lysates of isolated brain microvessels (50 μg of protein) were dissolved in 500 mM Tris−HCl (pH 8.5), 7 M guanidine hydrochloride, 10 mM EDTA, and the proteins were S-carbamoylmethylated. The alkylated proteins were precipitated in a mixture of methanol and chloroform. The precipitates were dissolved in 6 M urea in 100 mM Tris−HCl (pH 8.5), diluted 5-fold with 100 mM Tris−HCl (pH 8.5) and incubated with TPCK-treated trypsin (Promega, Madison, WI) at 37 °C for 16 h (enzyme/substrate ratio: 1/100). Each tryptic digest was mixed with internal standard peptides and formic acid and centrifuged at 17360 *g* for 5 min at 4 °C prior to analysis of the supernatant by high performance HPLC−MS/MS, or for 15 min prior to analysis of the supernatant by nanoLC−MS/MS.

LC−MS/MS analyses were performed on an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) coupled to a triple quadrupole mass spectrometer (API5000; Applied Biosystems, Foster City, CA) equipped with a Turbo V ion source (Applied Biosystems). Samples equivalent to 3.33 to 33.3 μg of protein were injected onto either an Agilent 300SB-C18 (0.5 × 150 mm, 5.0 μm) column or a Waters XBridge BEH130 C18 (1.0 × 100 mm, 3.5 μm) column together with 500 fmol of internal standard peptides. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The peptides were eluted at room temperature using a linear gradient (total time, 120 min; flow rate, 50 μL/min). The sequence was (A:B) 99:1 for 5 min after injection, 50:50 at 55 min, 0:100 at 56 min and up to 58 min, 99:1 at 60 min and up to 120 min.

NanoLC−MS/MS analyses were performed on a nanoLC system (LC Assist, Tokyo, Japan) coupled to a triple quadrupole mass spectrometer (4000QTRAP; Applied Biosystems) equipped with a NanoSpray source (Applied Biosystems) and AD-H4 (AMR, Tokyo, Japan). Samples equivalent to 0.50 to 5.0 μg of protein, plus 50 fmol of internal standard peptides, were injected onto a direct nano flow spray tip reversed-phase column (0.15 × 50 mm) packed with Mightysil RP-18GP (3 μm particles; Kanto Chemicals, Tokyo, Japan) that was connected through an electric column-switching valve to an automated solvent desalting device. Mobile phase A was 0.1% formic acid in water, and B was 0.1% formic acid in acetonitrile. Peptides were eluted at room temperature using a linear gradient of 5−45% B for 50 min (flow rate: 100 nL/min).

Eluted peptides were detected by electrospray ionization in multiplexed MRM mode; this method can quantify many molecules simultaneously using a maximum of 300 MRM transitions (Q1/Q3). The dwell time was 10 ms per MRM transition. Each molecule was monitored with four sets of MRM transitions (Q1/Q3-1, Q1/Q3-2, Q1/Q3-3, Q1/Q3-4) derived from one set of standard and internal standard peptides (Supplemental Table 3 in the Supporting Information). Chromatogram ion counts were determined using the data acquisition

Table 1. Absolute Quantities of SLC Transporter Proteins, ABC Transporters, CYPs, Phase II Enzymes, Receptors, Endothelial Markers, Oligodendrocyte Marker, Pericyte Marker, Neuron Marker, Membrane Marker, and Others (fmol/μg of protein) in Human Brain Microvessel Samples from Patients 8−12 (n = 5)[a]

family	protein	mean	SD
SLC transporters	SLC1A2/EAAT2	5.7	1.9
	SLC1A3/EAAT1	30.72	13.07
	SLC1A4/ASCT1	1.81	0.53
	SLC2A1/GLUT1	78.5	23
	SLC2A3,14/GLUT3,14	2.53	0.73
	SLC3A2/4F2hc	3.56	1.11
	SLC6A12/BGT1	2.89	0.81
	SLC7A1/CAT1	0.99	0.34
	SLC7A5/LAT1	0.8	0.25
	SLC16A1/MCT1	1.46	0.39
	SLC16A2/MCT8	1.31	0.37
	SLC19A1/RFC	0.55	0.18
	SLC27A1/FATP1	2.08	0.38
	SLC29A1/ENT1	0.86	0.13
ABC transporters	ABCA2/ABC2	2.11	0.78
	ABCA8	0.67	0.23
	ABCB1/MDR1	3.98	0.88
	ABCC4/MRP4	0.31	0.11
	ABCG2/BCRP	6.15	1.41
CYPs	CYP1B1	0.45	0.13
	CYP2U1	0.44	0.18
phase II enzymes	GSTM2	2.23	0.7
	GSTM3	3.85	1.77
	GSTM5	1.9	0.61
	GSTO1	2.98	1.41
	GSTP1	23.42	6.7
	COMT	4.45	1.29
receptors	INSR	1.15	0.6
	LRP1	1.76	0.76
	TfR1	1.97	0.49
endothelial markers	γ-gtp	2.09	0.69
	PECAM1	1.57	0.21
	von Willebrand factor	1.41	0.47
	Na^+/K^+ ATPase	20.8	6.46
	claudin-5	3.62	0.96
astrocyte markers	GFAP	503.2	174.09
oligodendrocyte marker	CNPase	12.96	4.12
pericyte marker	NG2 proteoglycan	1.07	0.41
neuron marker	synaptophysin	1.45	0.37
others	JAM-A	3.56	0.61
	VE-cadherin	1.61	0.43
	NADPH	0.95	0.16
	MTTP	0.16	0.02

[a] Results are means (± SD).

procedures provided by Analyst software, version 1.4.2 (Applied Biosystems). Signal peaks of over 5000 counts detected at the same retention time as an internal standard peptide were defined as positive. A molecule was considered to be present in brain microvessels if the positive peaks were observed in three or four sets of MRM transitions. The amount of protein was determined as the average of three or four quantitative values. The lower limit of quantification (LQ) was calculated assuming a minimum signal peak of 5000 counts as described previously.[20] The amino acid sequences and MRM transitions of the probe peptides are shown in Supplemental Table 3 in the Supporting Information.

Statistical Analysis. Gene expression profiles in the different donor cortex and the commercially available cortex (Clontech, BD Biosciences, Woburn, MA). Samples were analyzed using a linear mixed model with Holm−Bonferroni correction. Gene expression in donor microvessels and the corresponding cerebral cortex samples was compared using an unpaired two-tailed *t* test after normalization to the expression of *TBP*. Similarly, gene expression in patients with epilepsy and oligodendroglioma was compared using an unpaired two-tailed *t* test. The statistical significance was considered when the *p* value was <0.05.

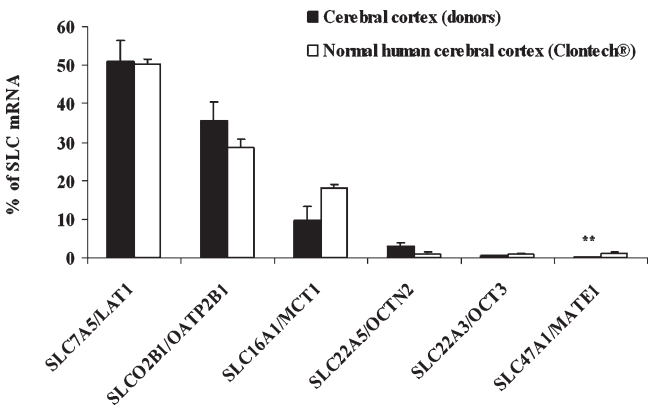

Figure 1. Relative amounts of mRNAs in donor and normal cortex samples (Clontech) of SLC transporters after setting the expression of each to 100%. Results are means (± SD) for patient samples 1−7 ($n = 7$) and means (± SD) from 3 independent Clontech experiments. Results were tested using a linear mixed model with Holm−Bonferroni correction. The statistical significance was considered when the *p* value was * <0.05; ** <0.01; *** <0.001.

RESULTS

Integrity of cDNA and Sample Enrichment in Brain Microvessels. The integrity of the cryopreserved cDNA obtained from brain microvessels and corresponding cortex from patients 1−7 ($n = 7$) was reevaluated by qRT-PCR amplification of BBB cell-specific marker genes as described elsewhere.[18] The details of the patients are presented in Supplemental Table 1 in the Supporting Information. These results were very similar to those reported previously.[18]

The enrichment in endothelial markers and the impoverishment in astrocyte and neuron markers have been already reported for samples 1−7 ($n = 7$).[18] The great abundance of Na^+/K^+ ATPase and SLC2A1/GLUT-1 proteins in the new samples 8−12 ($n = 5$) confirmed their enrichment in microvessels (Table 1).

Effect of Epilepsy and Glioma on the Expression of Genes of Interest. The effects of a disease state and drug therapy on the expression of genes encoding SLC transporters and metabolizing enzymes were evaluated by comparing the gene expression in the cortex samples obtained from patients 1−7 with a sample of commercially available cortex (Clontech) and, similarly, between patients with epilepsy and glioma. The human brain cortex RNA was from pooled brain samples from 10 males/females who died suddenly from reasons other than brain disease. With the exception of *SLC47A1/MATE1*, the gene expression patterns of SLC transporters (Figure 1) and metabolizing enzymes (Figure 2) in the patient cortex samples seem to be similar to that in the commercial cortex sample. When gene expression patterns of SLC transporters and metabolizing enzymes were compared between epilepsy and glioma samples, interestingly, gene expression was not statistically significant for all transporters and enzymes compared. We already reported similar results for ABC transporters and CYPs,[18] suggesting that the overall patterns of the majority of these transporters and all enzymes in the brains of these patients were essentially unaffected by a diseased state or presurgery drug therapy.

SLC and OST Transporters in Brain Microvessels. Transcripts encoding 6 SLC transporters were detected in brain microvessel samples from patients 1−7 (Figure 3). *SLC7A5/LAT1* was the most expressed gene in microvessels (relative expression 61.2%), followed by *SLCO2B1/OATP2B1* (relative expression 29.2%). The relative expressions of the rest of SLC transporters (*SLC16A1/MCT1*, *SLC22A3/OCT3*, *SLC22A5/OCTN2*, and *SLC47A1/MATE1*) were less than 10%. Conversely, transcripts of *OSTalpha*,

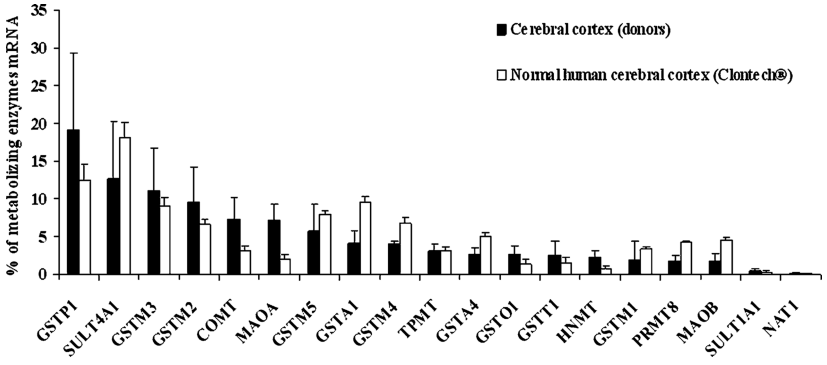

Figure 2. Relative amounts of mRNAs in donor and normal cortex samples (Clontech) of metabolizing enzymes after setting the expression of each to 100%. Results are means (± SD) for patient samples 1−7 ($n = 7$) and means (± SD) from 3 independent Clontech experiments. Results were tested using a linear mixed model with Holm−Bonferroni correction. The statistical significance was considered when the *p* value was * <0.05; ** <0.01; *** <0.001.

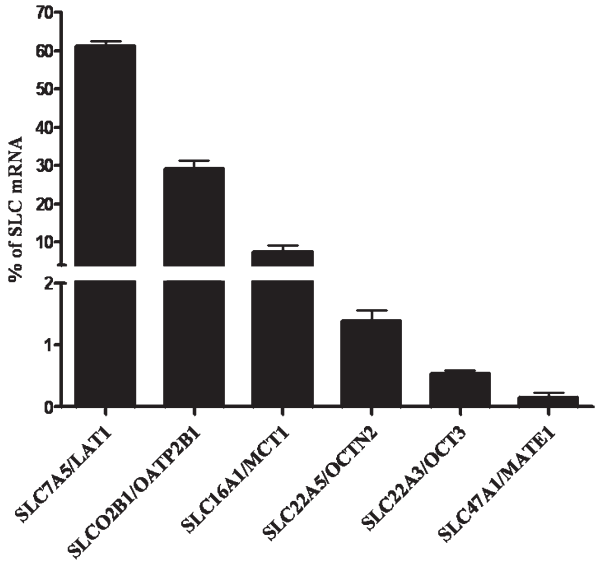

Figure 3. Relative amounts of SLC transporter mRNAs in brain microvessels after setting the expression to 100%. Percentage expression was determined using the ΔC_t method comparative to the least expressed gene in all patients. Results are means (± SD) for patient samples 1−7 ($n = 7$).

OSTbeta, *SLC10A1/NTCP*, *SLC22A1/OCT1*, and *SLCO1B1/OATP1B1* were not detected; however, their transcripts were readily detected in the positive control (liver) (Table 2).

A similar hierarchy was found in the corresponding cortex (Figure 1). *SLC7A5/LAT1* was the most expressed gene in the cortex samples (relative expression 50.9%), followed by *SLCO2B1/OATP2B1* (relative expression 35.7%). The transcripts of the vast majority of SLC transporters tested were much more abundant in brain microvessels than in total cortex (Figure 4); their relative expression ranged from 2.5- to 13.4-fold, suggesting that they are mainly expressed in brain microvessels rather than in the brain parenchyma.

The absolute amounts of 14 SLC transporter proteins were also readily quantified in brain microvessels from patients 8−12 using LC−MS/MS as shown in Table 1. SLC2A1/GLUT1 and SLC1A3/EAAT1 proteins were more abundant in brain microvessels than other SLC transporters; their absolute amounts were 78.5 (SLC2A1/GLUT1) and 30.7 fmol/μg of protein (SLC1A3/EAAT1). Although transcripts of *SLC16A1* were less abundant than the *SLC7A5* transcripts, the quantity of SLC16A1/MCT1 protein was almost 2-fold that of the SLC7A5/LAT1. The amounts of 57 other SLC and OST transporters were below the limit of quantification (Supplemental Table 4 in the Supporting Information).

ABC Transporters in Brain Microvessels. The gene expression pattern of ABC transporters in microvessel samples has been reported.[18] This study investigates the amounts of 34 ABC transporter proteins in brain microvessels using LC−MS/MS. Of these, only 5 were readily quantified (Table 1). ABCG2/BCRP was the most abundant ABC transporter; it was 1.6-fold more abundant than ABCB1/MDR1. ABCC4/MRP4 was also present but at a much lower concentration than ABCG2/BCRP (20-fold) or ABCB1/MDR1 (12.8-fold). The lipid transporters ABCA2 and ABCA8 were also detected, but the amounts of the other ABC transporters were below the detection limit (Supplemental Table 4 in the Supporting Information).

Metabolizing Enzymes in Brain Microvessels. *Brain Microvessels.* We investigated the expression patterns of the *MAOA* and *MAOB* genes in human brain microvessels and in the corresponding cerebral cortex samples from each patient by qRT-PCR. *MAOA* transcripts were almost 5.7-fold more abundant than *MAOB* transcripts (Figure 5). The expression patterns of CYP genes in brain microvessels have been reported.[18] This study investigates the amounts of their protein in microvessel samples using LC−MS/MS. Only two of the 21 CYPs investigated, CYP1B1 and CYP2U1, were readily quantified (Table 1). The amounts of the other enzymes were under the limit of quantification (Supplemental Table 4 in the Supporting Information).

We investigated the mRNA and/or proteins of 28 phase II metabolizing enzymes in the brain microvessels. Of these, 13 were readily detected by qRT-PCR (Table 2 and Figure 5). Gene expression profiles, along with those of the *MAOA* and *MAOB* genes, are shown in Figure 5. We confirmed these findings by analyzing the amounts of 15 phase II enzyme proteins by LC−MS/MS. Only 5 GSTs and the COMT proteins were quantified (Table 1). The gene expressions and the amounts of protein were generally in agreement, except for GSTM4 and GSTT1, whose concentrations were below the limit of quantification (Supplemental Table 4 in the Supporting Information).

The gene encoding *GSTP1* was the most highly expressed (relative expression 33.2%). Similarly, GSTP1 was the most abundant protein among phase II enzymes detected by LC−MS/MS. The GSTs were the most abundant enzyme family in microvessels (overall relative expression 65.8%). *COMT*, *HNMT* and *TPMT* transcripts were the only methyltransferases detected in the microvessels, among which *COMT* was the most expressed (relative expression 7.4%) (Figure 5). *SULT1A1* was the only sulfotransferase transcript detected in microvessels; its gene was also the least well expressed in microvessels (relative expression less than 1%).

In contrast, transcripts of *NAT1*, *NAT2*, *SULT2A1*, *SULT4A1*, *SULT1E1*, *UGT1A1*, *UGT1A4*, *UGT1A6*, *UGT2B7*, *GSTA2*, *GSTM1*, and *PRMT8* were not detected in brain microvessels, whereas their mRNAs were readily detected in the liver and/or in the commercial cortex sample (Table 2). The results of the protein analyses were also consistent. Neither UGT transcripts nor proteins were detected in brain microvessel samples (Table 2 and Supplemental Table 4 in the Supporting Information).

Whole Cortex. In addition to the genes detected in brain microvessel samples, transcripts for *GSTM1*, *NAT1*, *SULT4A1*, and *PRMT8* were detected in the cerebral cortex samples (Table 2). *GSTP1* transcripts were the most abundant in cortex as well as in the microvessels (relative expression 19.2%). Similar to microvessels, GSTs were the most abundant enzyme in the cortex (overall relative expression 63.4%). The cortex samples contained relatively high *SULT1A4* transcripts (relative expression 12.6%). *NAT1* transcripts were detected in the cortex samples; in contrast, transcripts for *NAT1* and *NAT2* were not detected in the brain microvessels. Interestingly, the genes encoding *GSTA1*, *GSTA4* and *GSTM3* were more expressed in cerebral cortex than in brain microvessels, in contrast to the vast majority of the SLC transporters. However, the gene encoding *GSTT1* was more expressed in the brain microvessels than in the cerebral cortex samples (Figure 6).

■ DISCUSSION

We have quantified the expression of the genes encoding SLC transporters, ABC transporters, phase I and phase II metabolizing enzymes and the amounts of their proteins in brain microvessels isolated from 12 patients suffering from epilepsy or glioma. We used a mechanical isolation method developed and

Table 2. Expressions of Genes Encoding SLC Transporters and Metabolizing Enzymes in Commercially Available Liver, Commercially Available Brain Cortex (Clontech), Donor Brain Cerebral Cortex from Patients 1−7 (n = 7), and Brain Microvessel Samples from Patients 1−7 (n = 7)[a]

family	gene	liver	commercial brain cortex (Clontech)	donor cortex	brain microvessels
SLC Transporters					
SLC	SLC7A5/LAT1	+	+	+	+
	SLC10A1/NTCP	+	nd	nd	nd
	SLC16A1/MCT1	+	+	+	+
	SLC22A1/OCT1	+	nd	nd	nd
	SLC22A3/OCT3	+	+	+	+
	SLC22A5/OCTN2	+	+	+	+
	SLC47A1/MATE1	+	+	+	+
	SLCO1B1/OATP1B1	+	nd	nd	nd
	SLCO2B1/OATP2B1	+	+	+	+
Phase I Metabolizing Enzymes					
MAO	MAOA	+	+	+	+
	MAOB	+	+	+	+
Phase II Metabolizing Enzymes					
UGT	UGT1A1	+	nd	nd	nd
	UGT1A4	+	nd	nd	nd
	UGT1A6	+	nd	nd	nd
	UGT2B7	+	nd	nd	nd
GST	GSTA1	+	+	+	+
	GSTA2	+	nd	nd	nd
	GSTA4	+	+	+	+
	GSTM1	+	+	+	nd
	GSTM2	+	+	+	+
	GSTM3	+	+	+	+
	GSTM4	+	+	+	+
	GSTM5	+	+	+	+
	GSTO1	+	+	+	+
	GSTP1	+	+	+	+
	GSTT1	+	+	+	+
NAT	NAT1	+	+	+	nd
	NAT2	+	nd	nd	nd
SULT	SULT1A1	+	+	+	+
	SULT2A1	+	nd	nd	nd
	SULT4A1	nd	+	+	nd
	SULT1E1	+	nd	nd	nd
MT	COMT	+	+	+	+
	HNMT	+	+	+	+
	PRMT8	nd	+	+	nd
	TPMT	+	+	+	+
Others					
	OSTalpha	+	nd	nd	nd
	OSTbeta	+	nd	nd	nd

[a] SLC, solute carrier; MAO, monoamine oxidase; UGT, UDP-glucuronosyltransferase; GST, glutathione *S*-transferase; NAT, *N*-acetyltransferase; SULT, sulfotransferase; MT, methyltransferase; nd, not detected.

validated in our laboratory.[18,23] The purity and integrity of the cDNAs (patients 1−7) and proteins (patients 8−12) of the isolated microvessels were critically analyzed using brain endothelial markers. However, contamination with astrocyte and neuron proteins was observed. When we used the same protocol to isolate microvessels from rat brains, we did not detect the neuron marker synaptophysin by Western blotting, whereas the concentration of the astrocyte marker Gfap was higher in microvessels than in the whole cortex; in the present study, a considerable amount of GFAP has been detected.[23] The abundance of astrocyte protein in isolated microvessels is probably due to the presence of the plasma membranes of astrocyte foot

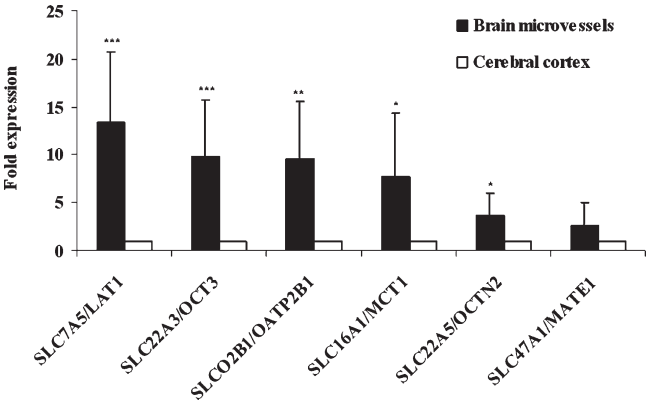

Figure 4. Fold amounts of mRNAs in donor brain microvessels and corresponding cerebral cortex samples of SLC transporters. The relative expression of each target gene was determined using the $\Delta\Delta C_t$ method from samples for which the reverse transcription was carried out with equal amounts of starting mRNA. The difference between the amounts of mRNA in the isolated microvessels and the corresponding cortex was determined by the $2^{-\Delta\Delta C_t}$ values, when the cortex value was arbitrarily defined as 1. Gene expression in donor microvessels and corresponding cortex samples was compared using an unpaired two-tailed *t* test. The statistical significance was considered when the *p* value was * <0.05; ** <0.01; *** <0.001.

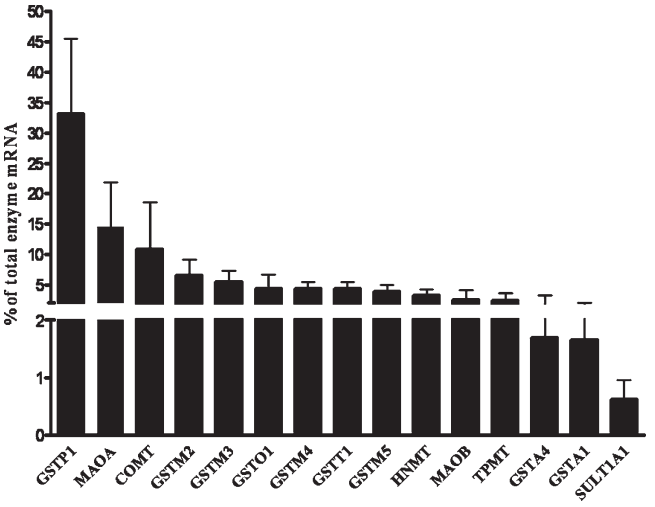

Figure 5. Relative amounts of metabolizing enzymes mRNAs in brain microvessels after setting the expression to 100%. Results are means (± SD) for patients 1−7). Percentage expression was determined using the ΔC_t method comparative to the least expressed gene in all patients. Results are means (± SD) for patient samples 1−7 (n = 7).

processes, which are tightly bound to the basal membrane of the neurovascular unit, these membranes could not be removed during mechanical brain disruption. This is the major limitation of our study since we cannot guarantee that the quantified mRNA and proteins are exclusively from endothelial cells but may also come from astrocytes as well. However, the BBB is considered as a neurovascular unit made of different cell types (endothelial cells, pericytes, and processes from astrocytes and neurons); transporters and enzymes expressed at this level may control brain disposition of drugs regardless of their localization in the neurovascular unit.

This discussion focuses on the transporters and enzymes involved in drug transport and metabolism. We found more

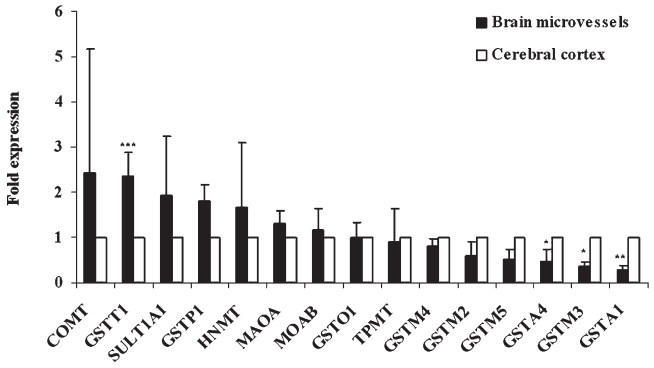

Figure 6. Fold amounts of mRNAs in donor brain microvessels and corresponding cerebral cortex samples of metabolizing enzymes. The relative expression of each target gene was determined using the $\Delta\Delta C_t$ method from samples for which the reverse transcription was carried out with equal amounts of starting mRNA. The difference between the amounts of mRNA in the isolated microvessels and the corresponding cortex was determined by the $2^{-\Delta\Delta C_t}$ values, when the cortex value was arbitrarily defined as 1. Gene expression in donor microvessels and corresponding cortex samples was compared using an unpaired two-tailed *t* test. The statistical significance was considered when the *p* value was * <0.05; ** <0.01; *** <0.001.

SLC transporter transcripts in microvessels than in the cerebral cortex. Protein analyses revealed high concentrations of SLC2A1/GLUT1 and the two glutamate transporters SLC1A2/EAAT2 and SLC1A3/EAAT1 in brain microvessels. Absolute protein amounts may help to further clarify the contribution of each transporter to facilitating the entry of endogenous substances and nutrients like glucose, glutamate, folate, amino and fatty acids into the brain, in addition to the xenobiotics that exploit these mechanisms. The system L, consisting of SLC7A5/LAT1 and SLC3A2/4F2hc, supplies the brain with large neutral amino acids like phenylalanine, tryptophan and leucine; previous investigations showed that it is used by drugs like levodopa[24] and melphalan[25] to penetrate the BBB in rodents. SLC16A1/MCT1 and SLC16A2/MCT8 have been involved in the transport of γ-hydroxybutyrate (GHB) and valproic acid at the rodent BBB.[26] SLC29A1/ENT1 protein was also detected; the transporter is probably a key component in the uptake of some anticancer drugs.[27]

The protein analyses showed that there was almost twice as much ABCG2/BCRP as ABCB1/MDR1 in the microvessels, confirming our previous findings of 8 times more ABCG2/BCRP mRNA than ABCB1/MDR1 mRNA.[18] In contrast, using the same methodology with isolated mouse brain microvessels, Kamiie et al. showed that there was three times more Mdr1a/P-gp than Bcrp.[19] This interspecies difference through post-transcriptional and/or post-translational regulation of *Mdr1a/P-gp* and *Abcg2/Bcrp* questions the reliability of extrapolating animal data for predicting brain distribution of *ABCG2/BCRP* and *ABCB1/ MDR1*substrates in human. ABCG2/BCRP is a "half-transporter" that functions as a homodimer to transport many substrates including anticancer agents like mitoxantrone, topotecan, and methotrexate. It has been postulated that ABCG2/BCRP protects the brain against toxicants, xenobiotics, and their metabolites.[28] The published literature on the role of the transporters ABCA2 and ABCA8 at the human BBB is inconclusive. However, Kim et al. have proposed that they are involved in intracellular lipid trafficking rather than trans-plasma membrane transport.[29]

In a recent study, Uchida et al.[20] quantified some SLC transporters, ABC transporter and receptor proteins in brain microvessels isolated from human brain samples using a similar isolation technique. Brain samples were taken and cryopreserved 1.5−6 h postmortem from 6 Caucasians and 1 Asian who died from peripheral diseases. Considering the interindividual variation in protein amounts, which was up to 4.24-fold, our results were essentially similar, suggesting that transporter and receptor proteins in our patients were essentially unaffected by a diseased state or presurgery drug therapy, in accordance with results at the mRNA level.

Our previous study investigated the expression of the genes encoding CYPs in isolated brain microvessels as well as qualitative protein expression of P-gp, BCRP, and CYP1B1 by Western blotting.[18] CYP1B1 and CYP2U1 transcripts were the main CYPs detected in brain microvessels, in agreement with the present study, which now quantifies the proteins (Table 2), whereas none of the other CYP proteins were detected, in agreement with the gene expression profile. CYP2U1 has an established role in the metabolism of fatty acids like arachidonic acid. CYP1B1 metabolizes several endogenous compounds like retinol, prostanoids, estradiol, and melatonin in the CNS,[30] but its role at the BBB remains unknown.

The present study reveals that UGTs are almost absent from the human BBB. We found no mRNA transcripts, and the protein concentrations were under the limit of quantification. These findings are in line with those of King et al., who did not detect transcripts or activities for UGT1A6 and UGT2B7 in human brain cortex, although morphine glucuronidation activity was detected in homogenates of the human cerebellum.[13] Previous investigations have shown transcripts encoding UGT2B18 and UGT2B19 in homogenates of monkey brains and cerebellum.[31,32] Moreover, Ghersi-Egea et al. reported substantially higher UGT activity in homogenates of rat brain microvessels than in rat brain homogenates,[10] however, suggesting differences in the UGT equipment of human, rodent, and other primate brain microvessels.

Our results indicate that the concentration of GST in human brain microvessels is comparatively high. Several studies have reported GST in the brain, but there has been little good evidence for their presence at the BBB. GSTP and GSTM were evidenced by immunohistochemistry using brain specimens from epileptic patients.[11] GSTP and GSTM were detected in the cortex, endothelial and glial cells, in agreement with our findings. A study on postmortem human tissues concluded that the brain is rich in GSTP1, GSTM2 and GSTM3, while GSTA is more abundant in the pituitary than in the cerebral cortex.[33] Liu and colleagues reported higher concentrations of GSTA4 mRNA and protein in adult and fetal brains than in the liver.[34] Bauer and colleagues found GSTp in isolated rat brain microvessels[17] which colocalized with Abcc2/Mrp2, whose gene expression was coordinated by the pregnane-X-receptor (PXR). In agreement, we found large amounts of GSTP1 transcripts and proteins. In contrast, we found no ABCC2/MRP2 transcripts or proteins in human brain microvessels. The GSTs at the BBB are believed to contribute to the poor intraparenchymal accumulation of antiepileptic drugs. The metabolic reactions mandated by GSTs could prevent antiepileptic drugs from reaching seizure foci since the majority of these foci are in the cortex.[11]

Other phase II enzymes have been quantified. COMT has been identified in the dorsolateral prefrontal cortex of the human brain by RT-PCR and confirmed by Western blotting, which is consistent with our results.[8] In addition, the amounts of COMT mRNA were not correlated with age or diseased state. COMT may be important in the dopamine flux in the prefrontal cortex.[8] PRMT8 was detected only in the cerebral cortex; PRMT8 is believed to be involved in neuron differentiation, but this is not yet fully understood.[35] Boukouvala et al. found NAT1 and NAT2 transcripts in the human brain.[36] We, however, found only NAT1 transcripts in the cerebral cortex. Low concentrations of NAT2 mRNA have also been reported in the cortex and cerebellum.[37] SULT1A1 was the only SULT detected in brain microvessels and was more abundant in the cerebral cortex than in brain microvessels. These findings are consistent with those of a previous study[38] that mapped the distribution of SULT1A1 and SULT1A4 in different regions of the human brain.

The expression of ABC transporters and enzymes is known to be coordinately regulated by several transcriptional factors like PXR, CAR and AhR in the liver.[39,40] Expression and functionality of these ligand-activated receptors at the human BBB are still vague. ABC transporters and CYPs have been shown to be regulated by PXR, CAR and AhR at the rodent BBB.[41−43] In our previous study *CAR* transcripts were not detected while *PXR* were barely detected.[18] In the present study CAR protein was under the limit of quantification (LOQ), suggesting that CAR is not expressed in human brain microvessels. PXR protein was also not detected, but its high LOQ did not allow us to conclude that this protein is not present in brain microvessels. Moreover, PXR has been shown to be functional in hCMEC/D3 cells.[44] *AhR* transcripts were detected in our previous study,[18] but we did not detect AhR protein. This discrepancy can be attributed to its high LOQ since large amounts of *AhR* transcripts were found in human brain microvessels.[18] Further work is needed to identify the AhR and PXR-regulation pathway at the human BBB. Similarly, the high LOQ for some proteins made it difficult to be sure that these transporters and enzymes were absent, especially when they are present at critically minute amounts. Although we cannot rule out the possibility that some molecules may have been insufficiently digested with trypsin, nevertheless, our previous studies have shown the efficient digestion of Glut1 and Bcrp in mouse brain microvessels, liver and kidney and the human P-gp in MDR1-overexpressed cells when we compared LC−MS/MS quantification values with binding assays, including quantitative Western blot.[19] Although our results suggest that trypsin digestion proceeded efficiently, it does not always result in complete digestion of all molecules. Biological samples digested by trypsin contain huge mixture of peptides; nontarget peptides produce noise which results in an increased LOQ. Quantification by the LC−MS/MS method is limited by peptide probe sensitivity, which is usually lower than that of a specific antibody. In routine analysis, a target protein can be quantified when several peptide probes give intensity above the background noise. The quantification can be improved by changing the peptide probe which elutes to lower noise area, hence improving the LOQ.

Here, we reported gene expression of *SLC22A5/OCTN2* and *SLCO2B1/OATP2B1* in human brain microvessels. Grube et al. showed that SLC22A5/OCTN2 transports L-carnitine through the BBB[45] but is also able to transport drugs such as verapamil in Madin−Darby canine kidney (MDCKII) cells.[46] Nozawa et al. also showed that SLCO2B1/OATP2B1 mediates the hepatic uptake of many xenobiotics including fexofenadine.[47] However, the presence of OATP in the BBB is still controversial. SLCO1A2/OATP1A2 was reported to be expressed in human brain microvessels.[48] The high LOQ for this OATP did not allow us to quantify it in human brain microvessels.

SLC16A1/MCT1, SLC16A2/MCT8, SLC29A1/ENT1, ABCG2/BCRP, ABCB1/MDR1, GSTP1, COMT and GSTM3

are the key elements controlling disposition of CNS drugs at the human BBB. Our findings should provide insight into contribution of these transporters and enzymes to the modulation of drug penetration, metabolism and efflux at the human BBB. These new quantitative data may be useful to build an appropriate PBPK model able to predict CNS distribution of new chemical entities during early drug development by scaling in vitro to in vivo data. However, there is still a need for further information on intrinsic transport and metabolism activities before we can fully understand the cerebral uptake, efflux and metabolism of drugs within the BBB. Functional activity assays showing the possible influence on drug disposition in a model system mimicking human brain microvessels can substantiate and further validate the data already presented in this report. Similarly, immunohistochemistry may be used to identify, correlate and show observed differences with the cell surface localization of uptake and/or efflux transporters. These additional investigations should enhance the existing knowledge about possible role of these transporters in brain microvessels.

■ ASSOCIATED CONTENT

Supporting Information. Supplemental Table 1 listing sample donor information like gender, age, disorder, the brain region sampled, medications intake and smoking status; Supplemental Table 2 listing the sequences of primers used for qRT-PCR and expression of genes encoding metabolizing enzymes, and SLC transporters in the human liver, brain cortex and isolated brain microvessels; Supplemental Table 3 listing probe sequences and MRM transitions for peptides used for LC−MS/MS absolute protein quantification; Supplemental Table 4 listing proteins of undetected SLC transporters, ABC transporters, CYPs, phase II enzymes, receptors, microglia and others in brain microvessels and their limit of quantification. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

§These two authors contributed equally to this work

■ ABBREVIATIONS USED

SLC transporter, solute carrier transporter; OST transporter, organic solute transporter; ABC transporter, ATP-binding cassette transporter; CYP, cytochrome P450; BBB, blood−brain barrier; CNS, central nervous system; MAO, monoamine oxidase; COMT, catechol *O*-methyltransferase; UGT, UDP-glucuronosyl transferase; SULT, sulfotransferase; GST, glutathione *S*-transferase; NAT, *N*-acetyltransferase; MT, methyltransferase; qRT-PCR, quantitative RT-PCR; TBP, TATA box-binding protein; GFAP, glial fibrillary acidic protein; GHB, γ-hydroxybutyrate; PXR, pregnane-X-receptor; CAR, constitutive androstane receptor; AhR, aryl-hydrocarbon receptor; LOQ, limit of quantification

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