ATP-binding Cassette Subfamily C Member 5 (ABCC5) Functions as an Efflux Transporter of Glutamate Conjugates and Analogs*

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The ubiquitous efflux transporter ABCC5 (ATP-binding cassette subfamily C member 5), also known as MRP5 (multidrug resistance-associated protein 5), is a member of the C branch of the superfamily of ATP-binding cassette transporters, which use the energy provided by the hydrolysis of ATP to transport substrates across the plasma membrane. ABCC5 is ubiquitously expressed, but levels in brain and muscle are especially high (3–5). In the brain, ABCC5 is present in brain capillary endothelial cells, neurons, and glia (6–8). In most cells, ABCC5 routes to the basolateral plasma membrane (1), but in the brain capillary endothelial cells that form the blood-brain barrier, it appears to reside in the apical membrane (7, 9, 10). Based on this location, ABCC5 is considered to be a part of the blood-brain barrier by many (11), but its actual function has remained a mystery. In terms of drug resistance, ABCC5 is best known for its ability to transport nucleotide analogs (12, 13) and antifolates like methotrexate (14) but elevated ABCC5 levels have not been linked to clinical drug resistance (1). Although ABCC5 transports endogenous metabolites like cyclic nucleotides (15), folic acid (14), and the recently identified N-lactoyl-amino acids (16) in vitro, the physiological relevance of this transport is unclear. Abcc5−/− mice do not have an overt phenotype and do not provide clues about the in vivo substrates of ABCC5 either (12). ABCC5 has recently been associated with primary angle closure glaucoma (17), osteoclast formation in bone metastases (18), and heme homostasis (19), but the ABCC5 substrates responsible for these phenotypes are not known. Because the substrates and function of ABCC5 are still largely unknown, we have exploited the Abcc5−/− mice to find additional endogenous substrates of ABCC5, as we have previously done for Abcc2−/− (20), Abcc3−/− (21), and Abcg2−/− (22) mice. Here we present the results of an untargeted metabolic screen detecting metabolites that accumulate in tissues of Abcc5−/− mice.

Experimental Procedures

**Chemicals**—β-Citryl glutamate was kindly provided by the group of van Schaftingen (23), whereas succinylaminoimidazolecarboxamide riboside (SAICAr)² was obtained from Maria Zikánová (24). The peptides Asp-Gly-Glu, NAAG₂, and Val-Asp-Gly-Glu were from Thermo Fisher Scientific, whereas (20)-(α)-kainic acid was from Cayman Chemical, and domoic acid was from Abcam Biochemicals. NAAG was obtained from Sigma or Bachem. Radiolabeled L-[2,3,4-3H]glutamic acid and L-[2,3-3H]aspartic acid were from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals and reagents were from Sigma, unless stated otherwise. A library containing 20 N-acetylated dipeptides containing a C-terminal glutamate (see Fig. 7) was synthesized by the peptide facility of the Netherlands Cancer Institute using Fmoc (N-(9-fluorenly)methoxycarbonyl) chemistry with triethylsilane as scavenger (solid phase peptide synthesis, preloaded Wang resin). After lyophilization, the crude peptide library was used without further purification.

**Animals**—Abcc5−/− mice on a FVB background (25) and wild type FVB mice were housed in constant temperature rooms with a 12-hour light/12-hour dark cycle and received food and water *ad libitum*. All experiments were reviewed and

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*The authors declare that they have no conflicts of interest with the contents of this article.

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²The abbreviations used are: SAICAr, succinylaminoimidazolecarboxamide riboside; NAA, N-acetyl aspartate; NAAG, N-acetylaspartylglutamate; NAAG₂, N-acetylaspartylglutamate; BCG, β-citrylglutamate; BCG₂, β-citryldiglutamate; GCP2, glutamate carboxypeptidase 2.
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Approved by our Institutional Animal Care and Use Committee. Approximately 16-week-old male mice (n = 5 per genotype) were sacrificed by CO₂-asphyxiation after which the various tissues were collected and frozen on dry ice. Whole blood was collected by cardiac puncture and kept on ice until separation of plasma and erythrocytes by centrifugation (10 min, 5,000 × g, 4 °C). The plasma layer was collected, and the buffy coat containing peripheral blood mononuclear cells was discarded from the erythrocyte pellet, which was subsequently washed with 1 ml of cold PBS. All samples were stored at −20 °C until further processing.

Automated Home Cage Monitoring—Automated home cage monitoring was performed by Sylics (Amsterdam, the Netherlands) on six wild type and six Abcc5−/− mice, as described (26).

Phencyclidine-induced Locomotor Activation—Mice were housed in Phenotyper cages (Noldus, Wageningen, The Netherlands) to automatically track animal behavior. The first 2 days, the mice were allowed to adapt to their new environment. On the third day, spontaneous behavior was determined (dark phase only). 5, 8, and 11 days after being placed in the Phenotyper cages, mice got 5, 15, and 0 (saline) mg of PCP/kg of body weight, respectively, all administered intraperitoneally. Locomotor activity was subsequently followed automatically and presented as distance moved/5 min.

Cell Lines and Culture Conditions—Cells were cultured in DMEM as described (27). The generation of the hABCC5-overexpressing cell line HEK293/5i has been described elsewhere (13). HEK293 cells were transfected with pEF6Myc-His constructs encoding ribosomal modification proteins RIMKLA and RIMKLB (rimK-like family members A and B) (kindly provided by van Schaftingen and co-workers (23) using calcium phosphate precipitation. Clones resistant to blasticidin (Invitrogen) were selected and tested for expression of GFP or hABCC5. Clones that were resistant to puromycin (2 μg/ml; Invitrogen) were selected and tested for GFP and hABCC5 expression on Western blot. Cells were seeded in 6-well plates (Corning Costar, Lowell, MA) at a density of 7.5 × 10⁵ cells/well and allowed to grow overnight. At this point, the medium was replaced with 2 ml of fresh medium, and the cells were incubated for 3 days. Culture medium was collected on ice, whereas cultured cells were washed with cold PBS and lysed in 1 ml of 70% cold MeOH.

Sample Processing—Collected tissues were weighed, transferred to 2 ml of Eppendorf vials on ice containing 70% methanol, and lysed for 10 min at 50 Hz using the TissueLyser LT (Qiagen). For plasma, erythrocytes and culture medium a volume of 200 μl of sample was mixed with 600 μl of cold methanol, vortexed, and left on ice for 30 min. After centrifugation (10 min, 20,800 × g, 4 °C) of the extracts and cell lysates, the supernatant was transferred to a second Eppendorf vial and evaporated to dryness in a SpeedVac at room temperature. The dried extracts were reconstituted in mobile phase A (5 mM N,N-dimethylhexylamine in water:methanol 95:5 (v/v) adjusted to pH 7.0), centrifuged (10 min, 20,800 × g, 4 °C), and analyzed by untargeted LC-MS metabolomics.

Untargeted Metabolomics—Untargeted metabolomics was performed using ion pair liquid chromatography coupled to an LTQ-Orbitrap Discovery (Thermo Fisher Scientific) operated in the negative ionization mode, as previously described (27), with a scan range of m/z 50–1500. Global metabolite profiles of wild type and Abcc5−/− mice were first compared per organ using XCMS (28). From these data we selected the features that were significantly (p < 0.05) higher (>2-fold) in Abcc5−/− mice in at least two tissue types using metaXCMS (29). The retention time and peak shape of each feature were compared to manually filter out features associated with the same metabolite. The accurate mass of the differentially present compounds was used for searches in the METLIN (30), HMDB (31), and MassBank (32) databases. Additionally, high resolution fragmentation spectra were obtained as described (27). The fragmentation spectra and isotope distribution pattern were used to determine the elemental composition with Sirius2 software (33, 34).

Vesicular Uptake—Inside-out membrane vesicles were prepared from HEK293 cells transiently transfected with hABCC5 or GFP (control) as described (35). Vesicular uptake of radiolabeled substrates was assessed in samples containing 16 μg of protein using a rapid filtration technique, followed by liquid scintillation counting (36). Transport of nonradiolabeled substrates was assessed in samples containing 75 μg of protein as described (20). Filtered vesicles were lysed, evaporated to dryness, and reconstituted in 50 μl of mobile phase A before analysis by LC-MS (see “Untargeted Metabolomics”). Extracted ion chromatograms corresponding to the tested substrates, their sodium adduct (NAAG), or in-source fragment (domoic acid, m/z 222.15) were manually integrated using XCalibur software (Thermo Fisher Scientific).

Gene Expression Profiling—Whole brains were collected from CO₂-asphyxiated wild type and Abcc5−/− mice and immediately frozen on dry ice. After homogenization in TRIzol (Invitrogen), RNA was extracted according to the manufacturer’s protocol. Sequencing libraries were generated according to the manufacturer’s instructions (TruSeq RNA Sample Preparation v2 Guide; Illumina; catalog no. 15026495 Rev. F). Briefly, polyadenylated RNA from intact total RNA was purified using oligo(dT) beads. Following purification the RNA was fragmented, random primed, and reverse transcribed using SuperScript II reverse transcriptase (Invitrogen, catalog no. 18064-014). Second strand synthesis was performed using polymerase I and RNaseH. The resulting cDNA was converted to blunt end fragments using an end repair mix (Illumina), followed by 3’ end adenylation and ligated to Illumina paired end sequencing adapters and subsequently amplified by 16 cycles of PCR. The libraries were analyzed on a 2100 Bioanalyzer (Agilent) using a 7500 chip (Agilent) and diluted to 10 nM. The samples were single-end sequenced with a length of 51 bp on a HiSeq2000 machine. The reads were aligned using Tophat (Version 2.0.6) against the Mouse reference (build 37). Tophat was supplied with a known set of gene models using a GTF file (Ensembl version 66). HTSeq-count was used to generate a list with the number of uniquely mapped reads for each gene. All samples were combined into one data set, and genes that have no expression across all samples in the data set were removed. To determine the differentially expressed genes between groups,
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TABLE 1
Summary of metabolites that are more than 2-fold higher in multiple Abcc5−/− tissues compared to wild type tissues

| m/z     | Retention time | Elemental composition | Identity                                                                 |
|---------|----------------|-----------------------|--------------------------------------------------------------------------|
| 320.062 | 17.4           | C₇H₁₂N₂O₁₀            | β-Citrylglutamic acid (β-citryl-Glu; BCG)                                  |
| 449.105 | 21.0           | C₈H₁₅N₂O₁₃            | β-Citrylglutamic acid (β-citryl-Glu; BCG)                                 |
| 303.083 | 11.9           | C₈H₁₄N₂O₈             | N-Acetylaspartylglutamic acid ((Ac)Asp-Glu; NAAG)                         |
| 432.125 | 17.3           | C₈H₁₅N₂O₁₁            | N-Acetylaspartylglutamic acid ((Ac)Asp-Glu; NAAG)                        |
| 318.095 | 5.0            | C₇H₁₅N₂O₈             | Asp-Gly-Glu                                                              |
| 373.010 | 9.0            | C₈H₁₅N₂O₁₀            | Succinylaminomidazolecarboxamide riboside (SAICAr)                       |
| 289.086 | 3.7            | C₇H₁₄N₂O₇             | Unknown; contains sulfate moiety                                         |
| 421.055 | 18.5           | Unknown                | Unknown                                                                  |
| 456.175 | 13.5           | Unknown                | Unknown                                                                  |

* Putative identification; reference standard is not available.

The R package EdgeR and Limma were used. The voom function was used to transform the count data to log2 counts per million and estimate the variance across the samples. Different groups were defined, and the differentially expressed genes were determined using lmFit and eBayes.

Results

Endogenous Glutamate Conjugates Accumulate in Abcc5−/− Tissues—Using LC-MS untargeted metabolomics, we generated global metabolite profiles of various tissues collected from wild type and Abcc5−/− mice (accessible via the MetaboLights repository (37), MTBLS197). We compared these profiles using (meta)XCMS (28, 38) to detect metabolites that were more abundant in Abcc5−/− tissue. Nine metabolites were significantly increased in multiple Abcc5−/− tissues, which made them potential ABCC5 substrates (Table 1). Using accurate mass-based metabolite database searches, MS-MS fragmentation spectra, and reference standards (Fig. 1), we were able to identify six of these metabolites (Table 1). Two of the identified accumulating metabolites, β-citrylglutamylglutamate (BCG) and Asp-Gly-Glu, have never been described as endogenous metabolites before. Strikingly, five of the identified metabolites contain a C-terminal glutamate moiety, whereas the sixth contains a C-terminal aspartate moiety, very similar to glutamate (Fig. 2A).

The tissue levels of the accumulating metabolites are represented on a logarithmic scale in Fig. 3. Although each metabolite accumulated differently, all metabolites strongly accumulated in brain, as depicted on a linear scale in Fig. 4. Compared with other tissues, the concentrations of the accumulating glutamate conjugates were particularly high in brain as well. These results point to a prominent role for ABCC5 in the brain. It was unclear, however, whether the metabolite changes were directly or indirectly related to ABCC5 expression.

Endogenous Glutamate Conjugates Are Effluxed from HEK293/ABCC5 Cells—To confirm our in vivo results, we determined the levels of the putative ABCC5 substrates in cell lysate and culture medium of parental HEK293 cells and of HEK293 cells overexpressing human ABCC5 (hABCC5). Although some of the putative substrates were expected to be present in parental HEK293 cells, we also expressed hABCC5 in HEK293 cells that stably overexpressed ribosomal modification proteins RIMKLA and RIMKLB, the enzymes that synthesize NAAG, N-acetylaspartylglutamate (NAAG₂), and β-citrylglutamate (BCG) (23, 39).

As expected, RIMKLA and RIMKLB overexpression resulted in increased levels of NAAG, NAAG₂, and BCG in the cells (Fig. 5). The concentration of the newly discovered metabolite BCG₂ was also strongly increased in RIMKLA-overexpressing cells, indicating that RIMKLA ligates a second glutamate not only to NAAG (39), but also to BCG.

In line with the in vivo results, overexpression of hABCC5 resulted in a strong decrease in intracellular levels of the metabolites that accumulated in Abcc5−/− mice. As anticipated for ABCC5 substrates, the levels of BCG and BCG₂ in medium increased upon hABCC5 overexpression. It was unclear whether the same holds true for NAAG₂, SAICAr, and Asp-Gly-Glu, because their levels were below the limit of detection in culture medium.

Unexpectedly, the extracellular levels of NAAG were not affected by hABCC5 overexpression. A potential mechanism that explains the reduced intracellular NAAG and NAAG₂ levels without the expected extracellular increase is that their precursor N-acetylaspartate (NAA) is an ABCC5 substrate. NAA levels were indeed strongly decreased in cell lysates of hABCC5-overexpressing cells, suggesting that NAA is also an ABCC5 substrate (Fig. 5). Medium NAA levels were not altered, however, and the tissue levels of NAA were not significantly different between wild type and Abcc5−/− mice. Apparently NAA levels are also affected by mechanisms other than ABCC5. The three unidentified metabolites were not detected in lysate or medium samples. Taken together, these results show that the putative mouse ABC5 (mABCC5) substrates identified through our metabolomics screen in vivo are also transported by human ABCC5 in cultured cells in vitro.

ABCC5 Is a General Glutamate Conjugate and Analog Transporter—To verify whether the differentially present metabolites are genuine ABCC5 substrates, we determined the transport of available metabolites into ABCC5-containing inside-out membrane vesicles. NAA, NAAG, NAAG₂, and SAICAr were transported into inside-out membrane vesicles in an ABCC5- and ATP-dependent fashion, confirming that these metabolites are bona fide ABCC5 substrates (Fig. 6). BCG was also transported into ABCC5-containing vesicles in an ATP-dependent fashion, but the background levels of BCG were much higher in control vesicles than in ABCC5-containing vesicles. This difference in background levels must be due to the difference in intracellular BCG levels in the HEK293 cells transduced with control or ABCC5 virus that were used to prepare the
### Metabolite MS² fragmentation spectrum

| Metabolite       | MS² fragmentation spectrum |
|------------------|----------------------------|
|                  | Unknown                    | Reference standard |
| C₁₁H₁₈N₂O₅S     | ![Graph](chart1.png)       | Not available      |
| (Ac)Asp-Glu      | ![Graph](chart2.png)       | ![](chart3.png)   |
| (Ac)Asp-Glu-Glu  | ![Graph](chart4.png)       | ![](chart5.png)   |
| β-Citryl-Glu     | ![Graph](chart6.png)       | ![](chart7.png)   |
| β-Citryl-Glu-Glu | ![Graph](chart8.png)       | Not available      |
| Asp-Gly-Glu      | ![Graph](chart9.png)       | ![](chart10.png)  |
| SAICAr           | ![Graph](chart11.png)      | ![](chart12.png)  |
inside-out membrane vesicles. Vesicular transport of Asp-Gly-Glu could not be verified because the levels of this metabolite inside the vesicles were below our LC-MS detection limits. Reference BCG2 was not available.

A striking feature of the chemical structure of all novel substrates identified here, and the previously identified ABCC5 substrates folate, methotrexate, and their polyglutamylated metabolites (14), is the presence of a glutamate or aspartate moiety (Fig. 2A). To test whether other compounds with glutamate or aspartate moieties (Fig. 2B) are also ABCC5 substrates, we tested these potential ABCC5 substrates in vesicular transport assays. We found that N-acetylglutamate; the tetrapeptide Val-Asp-Gly-Glu; the excitotoxic glutamate analogs NMDA, kainic acid, and domoic acid; and the therapeutic glutamate analog ZJ43 were all transported into vesicles in an ABCC5- and ATP-dependent fashion (Fig. 6). Additionally, we synthesized a library containing 20 N-acetylated dipeptides, all containing a C-terminal glutamate. When incubated with membrane vesicles, these dipeptides were transported into the membrane vesicles by ABCC5 (Fig. 7). Glutamic acid and aspartic acid were not transported themselves, however (data not shown). Note that the relative transport of the dipeptides in Fig. 7 more likely reflects the relative affinity of the peptides for the transporter than the maximal rate of transport of each peptide, because there will be competition for the transporter by the dipeptide mix.

For the endogenous glutamate conjugate NAAG and the therapeutic glutamate analog ZJ43, we investigated the

FIGURE 1. MS2 fragmentation spectra of unknowns and available reference standards resulted in the identification of most of the accumulating metabolites. The neutral loss of 79.957 in the spectrum of C11H18N2O5S indicates the presence of a sulfate moiety. The spectrum of putative BCG2 contains fragments (m/z 128.035 and 240.051) that are identical to that of BCG, strengthening its putative identification. The peak at m/z 196.919 in the spectrum of Asp-Gly-Glu is a nonspecific background peak that we commonly observe in low abundant MS2 spectra. The unknowns with m/z 421.0557 and 456.1745 did not yield any database hits, nor could we accurately determine the elemental composition because of the relatively high mass and low abundance.

FIGURE 2. Chemical structures of glutamate conjugates and analogs found to be transported by ABCC5. A, in vivo substrates found through comparative metabolomics of wild type and Abcc5−/− mouse tissues. B, additional ABCC5 substrates identified through in vitro vesicular transport studies. MTX transport was shown by Wielinga et al. (14). Glutamate moieties are highlighted in red, whereas aspartate moieties are highlighted in blue.
ABCC5-mediated transport in more detail. Both NAAG and ZJ43 were taken up into ABCC5-containing vesicles in a time- and ATP-dependent fashion (Fig. 8A). The concentration-dependent transport fits Michaelis-Menten kinetics with a comparable $K_m$ for NAAG (3.5 mM) and ZJ43 (1.9 mM) (Fig. 8B).

Immediate Early Genes Are Down-regulated in Abcc5$^{-/-}$ Mouse Brain—To assess the potential effects of the altered metabolite distribution, we performed RNA sequencing on brains of wild type and Abcc5$^{-/-}$ mice (GEO accession number GSE68628) after CO$_2$ asphyxiation. Interestingly, many of the most differentially transcribed genes (Fig. 9) were imme-
mediate early genes that are known to be activated through glutamate receptors (40, 41). Immediate early genes represent markers for neuronal activity (40) that can be induced rapidly in response to diverse stimuli like stress or ischemia (42, 43). The decreased induction of these genes in Abcc5/H11002/ mice might indicate attenuated glutamate excitotoxicity in response to acute ischemia.

**Discussion**

Thus far only a handful of ABCC5 substrates were known. Using untargeted metabolomics, we have now identified over 30 additional substrates, demonstrating that ABCC5 is a general glutamate conjugate and analog transporter. Glutamate is the principal excitatory neurotransmitter in the brain. It is essential for everyday brain functions like learning and memory but causes excitotoxic damage in traumatic brain injury and stroke (44). Glutamate levels in the brain are therefore highly regulated, and glutamate-like compounds can strongly affect glutamate neurotransmission (45).

The endogenous glutamate conjugate NAAG, for example, attenuates glutamate neurotransmission and has been implicated in schizophrenia and inhibition of glutamate excitotoxicity (46–48). Several exogenous glutamate analogs like NMDA, domoic acid and kainic acid can, in contrast, act as potent excitotoxins. To protect the brain against such neurotoxic glutamate analogs, the blood-brain barrier strongly limits their brain penetration (49, 50). Interestingly, we found that ABCC5 transports NAAG, as well as NMDA, domoic acid, and kainic acid. Although structurally similar, the functions of the metabolites we identified are diverse, and only some have known functions in the brain, as described below.

NAAG is mainly present in peripheral and central nervous tissue where it is the third most prevalent neurotransmitter, reaching millimolar levels (51). NAAG is transported into synaptic vesicles by sialin (SLC17A5) (52), and these vesicles are released into the synaptic cleft upon depolarization (53). Our data indicate that NAAG is also constitutively released from cells by ABCC5, a completely different mechanism of release. NAAG is reported to be an NMDA receptor-ligand and an agonist at the metabotropic glutamate receptor 3 (46, 47). Activated metabotropic glutamate receptor 3 inhibits subsequent glutamate and glycine release (47).

Extracellular NAAG is hydrolyzed to NAA and glutamate by glutamate carboxypeptidase 2 (GCP2) (54, 55). Pharmacological GCP2 inhibition increases NAAG levels while at the same time reducing glutamate levels. GCP2 inhibitors, like ZJ43,
FIGURE 6. Vesicular transport assays show that ABCC5 transports glutamate conjugates and analogs. Inside-out membrane vesicles without (control) or with ABCC5 (ABCC5) were incubated (10 min, 37 °C) with test substrates (100 μM) in the presence and absence of 5 mM ATP. The relative amount of test substrate in the vesicles was determined using LC-MS. The data are presented as means plus S.D. (n = 3). AU, arbitrary units; ND, not detected.

FIGURE 7. Vesicular transport of an N-acetylated dipeptide library shows that ABCC5 is a general C-terminal glutamate dipeptide transporter. Inside-out membrane vesicles with or without ABCC5 were incubated (10 min, 37 °C) with a library of 20 N-acetylated, C-terminal glutamate dipeptides (∼10 μM per peptide) in the presence and absence of 5 mM ATP. The relative amount of each peptide in the vesicles was determined using LC-MS and normalized on the peptide signal in the original peptide library. Poor LC-MS sensitivity for (Ac)Lys-Glu resulted in a low signal in the input library and undetectable levels in vesicles. We were unable to discriminate (Ac)Ile-Glu and (Ac)Leu-Glu because of their identical mass. Hence the uptakes of both of these dipeptides are labeled (Ac)(iso)Leu-Glu. The data are presented as means plus S.D. (n = 4). AU, arbitrary units.

FIGURE 8. NAAG and ZJ43 are transported into inside-out membrane vesicles by ABCC5. A and B, 100 μM NAAG (A) or 100 μM ZJ43 (B) was incubated with control vesicles (squares) and ABCC5-containing vesicles (circles) at 37 °C, in the presence (solid line) and absence (dashed line) of 5 mM ATP. At the indicated time points, samples containing 75 μg of protein were taken. After washing over a filter, the vesicular content was analyzed by LC-MS (n = 3). C and D, various concentrations of NAAG (C) or ZJ43 (D) were incubated with control vesicles and ABCC5-containing vesicles for 10 min at 37 °C, in the presence of 5 mM ATP (n = 4). ABCC5-dependent transport was calculated and fitted to Michaelis-Menten kinetics (solid line) using GraphPad Prism. The data are presented as means ± S.E. because rare outliers resulted in a high S.D. that required a scale on which the data were not interpretable. AU, arbitrary units.
have initially been tested as neuroprotective agents in ischemic brain injury (56) but were also shown to be effective in animal models for traumatic brain injury, schizophrenia, and neuropathic pain (57). NAAG is formed from NAA and glutamate by RIMKLA, which is also known as NAAG synthase 2. It was recently shown that the same enzyme can also ligate a second glutamate to NAAG, forming NAAG2 (39). NAAG2 also accumulated in Abcc5<sup>−−</sup> brain, but its function, if any, is currently not known.

Analogous to NAAG, BCG is formed by RIMKLB and hydrolyzed back to citrate and glutamate by GCP3 (58), which is ubiquitously expressed in mouse brain (59). We now show that RIMKLA can also ligate a second glutamate to BCG, forming the previously unknown BCG<sub>2</sub>. The intriguing metabolite BCG was first identified in newborn rat brain, where it is present up to low millimolar levels (60). It is present at particularly high levels in the developing brain of many species but decreases to low millimolar levels (60). It is present at particularly high levels in the developing brain of many species but decreases to low millimolar levels (60). Despite these extremely high brain concentrations, the function of BCG is elusive. Thus far, BCG has been suggested to be related to cell differentiation (62–65) and neuronal protection by metal chelation (66–68). Because we are the first to report the existence of BCG<sub>2</sub>, its function, if any, is unclear. Likewise, Asp-Gly-Glu was not known to be present in animals, and the source and function of this tripeptide remain to be determined.

Finally, SAICAr is a dephosphorylated purine synthesis intermediate that reaches high brain concentrations in patients lacking adenylosuccinate lyase, who show severe psychomotor delay (69). SAICAr can induce neuronal damage, but at levels that are much higher than the 2.6-fold increase found in the brains of our Abcc5<sup>−−</sup> mice (70).

Regardless of their diverse functions, all metabolites were transported into ABCC5-containing vesicles. Vesicular transport of NAAG and ZJ43 shows that ABCC5 has a relatively low affinity for glutamate conjugates, which is comparable to the $K_m$ reported for folic acid (1.3 mM) (14) and $N$-lactoyl-phenylalanine (≈1 mM) (16). As the physiological levels of NAAG and BCG can reach millimolar concentrations in the brain, these substrates can still be transported by ABCC5 at considerable rates.

Unexpectedly, metabolites from known endogenous ABCC5 substrate classes (cyclic nucleotides, folates, and $N$-lactoyl-amino acids) were not found in this screen. The basal levels of the cyclic nucleotides were likely too low to be detected reproducibly. Moreover, cyclic nucleotide transport by mouse ABCC5 is doubtful, because the transporter did not contribute to cGMP transport in mouse erythrocyte vesicles (25). Folate accumulated in Abcc5<sup>−−</sup> tissues, but this accumulation was nonsignificant in all tissues except brain. The lack of difference in levels of $N$-lactoyl-amino acids can be explained by the fact that it is in intracellular equilibrium with the levels of lactate and amino acids (16).

Although Abcc5<sup>−−</sup> mice were found to be healthy and fertile (13), one would expect the observed changes in metabolite levels to result in a (subtle) phenotype. The accumulation of glutamate conjugates and altered gene expression in Abcc5<sup>−−</sup> brains indicate that this phenotype could be of neurological nature. We did not find major differences in the spontaneous behavior of wild type and Abcc5<sup>−−</sup> mice using an automated home cage monitoring system that monitors various aspects of behavior (26), however. Zuo et al. (71) previously showed that pharmacological inhibition of GCP2 results in increased NAAG levels and reduced phencyclidine-induced locomotor activity in an animal model for schizophrenia. Such a difference was not found between our wild type and Abcc5<sup>−−</sup> mice, however (data not shown). Dedicated behavior assays should be performed to find more subtle neurological phenotypes, such as the reduced social interaction found in GCP2<sup>−−</sup> mice (72) and the reduced susceptibility to traumatic brain injury found in GCP2<sup>−−</sup> mice (73). Behavioral phenotypes would likely be more obvious in humans, but ABCC5-deficient humans have never been described, despite the likely existence of null alleles (74).

Many drugs that target glutamate neurotransmission are glutamate analogs and therefore potential ABCC5 substrates. Interestingly, one of the major problems in the development of GCP2 inhibitors is that they do not reach the brain (75). Our results indicate that part of the poor brain penetration of these glutamate analogs might be due to the presence of ABCC5 in the blood-brain barrier.

Our data suggest that ABCC5 can also protect the brain against the prototypical neurotoxins kainate and NMDA. When administered outside the brain, kainate only becomes toxic at a relatively high dose, underlining the important role of the blood-brain barrier in the protection against toxic glutamate analogs (49). Many similar excitotoxic glutamate analogs, like domoic acid, can be present in food and represent potential ABCC5 substrates (76–78). Further studies are required to delineate the role of ABCC5 in the protection of the brain against domoic acid and other toxic glutamate analogs. In conclusion, our results show that ABCC5 is a general glutamate conjugate and analog transporter that can limit the brain levels of endogenous metabolites, drugs, and toxins.
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Author Contributions—R. S. J. designed and performed most of the experiments, analyzed the data, and drafted the paper. S. M. performed vesicular transport experiments, and M. D. H. made DNA constructs. K. V. D. W. handled the laboratory animals, designed the research, supervised the project, and wrote the paper with P. B. All authors reviewed the results and approved the final version of the manuscript.

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