Processing Mechanism of Guanidinoacetate in Choroid Plexus Epithelial Cells: Conversion of Guanidinoacetate to Creatine via Guanidinoacetate N-Methyltransferase and Monocarboxylate Transporter 12-Mediated Creatine Release into the CSF

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Abstract

Background: Guanidinoacetate (GAA) induces epileptogenesis and neurotoxicity in the brain. As epileptic animal models have been reported to show elevated cerebral GAA levels, the processing mechanism of GAA in the brain is important for maintaining brain homeostasis. Our previous study revealed that GAA in the cerebrospinal fluid (CSF) is removed by incorporation into the choroid plexus epithelial cells (CPxEpic), which form the blood-CSF barrier (BCSFB). However, the processing mechanism of GAA incorporated into CPxEpic remains unknown. Our previous studies have revealed that monocarboxylate transporter 12 (MCT12) functions as an efflux transporter of GAA and creatine (Cr), a metabolite of GAA, in the kidneys and liver. Therefore, we aimed to clarify the role of MCT12 in GAA dynamics in CPxEpic.

Methods: Protein expression and localization in CPxEpic were evaluated using immunohistochemistry. Metabolic analysis was performed using high-performance liquid chromatography (HPLC) 24 h after the addition of [^{14}C]GAA to TR-CSFB3 cells, which are conditionally immortalized rat CPxEpic. The efflux transport of [^{14}C]Cr was evaluated in TR-CSFB3 cells after transfection with MCT12 small interfering RNA (siRNA). The CSF-to-brain parenchyma transfer of Cr was measured after intracerebroventricular injection in rats.

Results: Immunohistochemical staining revealed that MCT12-derived signals merged with those of the marker protein at the apical membrane of CPxEpic, suggesting that MCT12 is localized on the apical membrane of CPxEpic. The expression levels of guanidinoacetate N-methyltransferase (GAMT), which catalyzes the conversion of GAA to Cr, in TR-CSFB3 cells was also indicated, and GAA was hypothesized to be metabolized to Cr after influx transport into CPxEpic, after which Cr was released into the CSF. Cr release from TR-CSFB3 cells decreased following MCT12 knockdown. The contribution ratio of MCT12 to the release of Cr was more than 50%. The clearance of CSF-to-brain parenchyma transfer of Cr was 4.65 $\mu\text{L}/(\text{min}\cdot\text{g brain})$, suggesting that biosynthesized Cr in CPxEpic is released into the CSF and supplied to the brain parenchyma.

Conclusions: In CPxEpic, GAA is metabolized to Cr via GAMT. Biosynthesized Cr is then released into the CSF via MCT12 and supplied to the brain parenchyma.

Background

Guanidino compounds, such as guanidinoacetate (GAA), γ -guanidinobutyrate (GBA), guanidinoethanesulfonate (GES), β -guanidinopropionate (GPA), guanidinosuccinate (GSA), α -guanidinoglutaric acid (GGA), N-acetylarginine, methylguanidine, homoarginine, taurocyamine, and creatinine (CRN), have been reported to induce seizures [1, 2]. In addition, animal models of epilepsy have been reported to exhibit elevated GAA, CRN, and methylguanidine levels in the brain after convulsions [1]. In contrast, GAA is known to be a precursor of creatine (Cr), which is metabolized to CRN and methylguanidine [3]. Cr is known to be essential for energy homeostasis in the brain as Cr deficiency induces cerebral Cr deficiency syndromes (CCDSs), which lead to mental retardation and language delay

[4, 5]. As Cr biosynthesis and metabolism occur in the brain [3, 4], strict control of Cr dynamics is needed to maintain the balance between Cr and guanidino compounds that induce convulsions. Therefore, elucidation of the mechanisms maintaining the levels of guanidino compounds in the brain can lead to an understanding of cerebral energy homeostasis, in addition to the pathology of epilepsy and the regulation mechanism of seizures.

Our previous studies revealed that GAA and CRN were eliminated from the brain via the blood-cerebrospinal fluid (CSF) barrier (BCSFB), although these compounds are hardly eliminated through the blood-brain barrier (BBB) [6–8]. BCSFB restricts the non-selective intercellular permeation of compounds between the CSF and circulating blood via tight junctions formed by the choroid plexus epithelial cells (CpxEpic) [2]. Transcellular transport occurs via various transport systems for the permeation of compounds between the CSF and circulating blood across the BCSFB [3]. Elimination of CRN across the BCSFB is reportedly mediated by the organic cation transporter 3 [OCT3/solute carrier (SLC) 22A3] [7]. Since CRN is the end product of Cr metabolism [5], CRN is considered to be circulated through the blood flow and then get excreted into urine after elimination from the brain. In contrast, GAA is known not only as a convulsant, but also as a biosynthetic precursor of Cr. Thus, GAA could be used as a source of Cr in CpxEpic. During the elimination process from the brain, GAA is taken up by CpxEpic via the creatine transporter (CRT/SLC6A8) and taurine transporter (TauT/SLC6A6) [3, 6, 9]. However, the processing mechanism after the incorporation of GAA into CpxEpic remains unclear. As GAA is a biosynthetic precursor of Cr, it is possible that CpxEpic play a role in maintaining GAA and Cr levels in the brain by Cr provision.

Efflux transporters of GAA and Cr are considered to be important to understand the dynamics of GAA and Cr in CpxEpic. GAA and Cr are reported substrates for SLC6A and SLC16A family transporters [3, 10]. Cr is a substrate for CRT, monocarboxylate transporter 9 (MCT9/SLC16A9), and MCT12 (SLC16A12) [3, 10, 11]. GAA is also recognized by CRT and MCT12 as a substrate [6, 10]. In addition, GAA is a substrate for TauT and γ -aminobutyric acid (GABA) transporters (GATs) [8]. SLC6A family transporters that recognize GAA and/or Cr as substrate(s) have been reported to be Na^+ - and Cl^- -coupled symporters [3]. Since the concentrations of Na^+ and Cl^- are much higher in the extracellular fluid than in the intracellular fluid, SLC6A transporters have been suggested to mediate the influx transport of substrates in non-excitabile cells, such as CpxEpic. On the other hand, MCT9 and MCT12 have been reported to exhibit different transport mechanisms than SLC6A transporters. MCT9 has been suggested to be an H^+ /Cr exchanger [12]. As the extracellular pH is higher than the intracellular pH, MCT9 seems to function as an influx transporter [11]. In contrast, MCT12 acts as a facilitative transporter [3]. Our previous studies revealed that MCT12 plays a role in the efflux transport of GAA and Cr from renal proximal tubular epithelial cells and hepatocytes, respectively, to the extracellular fluid of the blood side [10, 11]. Therefore, MCT12 may be a potential candidate for mediating the efflux transport of GAA or Cr from CpxEpic.

The purpose of this study was to elucidate the dynamics of GAA after incorporation into CpxEpic. To elucidate the possibility that MCT12 mediates efflux transport from CpxEpic, the localization of MCT12 protein in CpxEpic was determined by immunohistochemical analysis, which indicated that

MCT12 was localized on the CSF side membrane of CpxEpic. The expression levels and activity of guanidinoacetate N-methyltransferase (GAMT), which catalyzes the metabolism of GAA to Cr [13], were observed in CpxEpic, and it was conceived that GAA is metabolized to Cr and then Cr is released into the CSF. Cr transport was analyzed using TR-CSFB3 cells, which are conditionally immortalized rat CpxEpic [14]. This suggests that MCT12 contributes to the efflux of Cr from CpxEpic. After the *in vivo* intracerebroventricular injection of rats, transfer of Cr to the brain parenchyma was observed. Consequently, it is suggested that GAA is converted to Cr in CpxEpic, and then biosynthesized Cr is released into the CSF and supplied to the brain parenchyma.

Methods

Animals

Male Wistar/ST rats (6 weeks old, 150–180 g) were purchased from Japan SLC (Hamamatsu, Japan). The animals were maintained in a controlled environment, and all experiments were approved by the Animal Care Committee of the University of Toyama.

Reagents

Analytical-grade chemicals were used in this study. Creatine hydrate, [4-¹⁴C]-([¹⁴C]Cr, 57.0 mCi/mmol), and guanidinoacetic acid, [1-¹⁴C]-([¹⁴C]GAA, 55.0 mCi/mmol) were purchased from Moravек Biochemicals (Brea, CA, USA).

Immunohistochemical staining

Anesthetized rats were fixed by transcardial perfusion with 4% paraformaldehyde dissolved in 0.1 M sodium phosphate buffer (pH 7.4). The brains were isolated and immersed in paraformaldehyde solution for 3 h. Frozen sections (20 µm thick) were prepared using a cryostat (CM1900; Leica, Nussloch, Germany). The sections were immersed in 0.1% Triton-X100 dissolved in phosphate-buffered saline without Ca²⁺ and Mg²⁺ [PBS (-); 137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, and 1.5 mM KH₂PO₄] for 1 h at room temperature and then treated with 10% goat serum for 1 h at room temperature. The sections were incubated overnight with guinea pig anti-MCT12 (3 µg/mL) [10], guinea pig anti-GAMT (3 µg/mL) [15], and/or mouse anti-Na⁺, K⁺-ATPase α1 (2 µg/mL; clone: C464.6; Merck, Darmstadt, Germany) antibodies. For antigen absorption, anti-MCT12 antibodies were incubated with PBS (-) with or without the antigen peptide (4.36 µg/mL) for 6 h at 4°C before the primary antibody reaction. The sequence of antigen peptides for anti-MCT12 antibodies was KEDPSGPEKSHDRDAQRED, which is a 200–218 amino acid sequence of rat MCT12 [National Center for Biotechnology Information (NCBI) reference sequence: NM_001191637.1]. The sections or cells were incubated with species-specific secondary antibodies labeled with Alexa Fluor 488 or Alexa Fluor 568 (Thermo Fisher Scientific, Waltham, MA, USA) for 2 h at room temperature. Confocal images were obtained using a confocal laser-scanning microscope (LSM780; Carl Zeiss, Oberkochen, Germany).

Immunoblotting

The crude membrane fraction of TR-CSFB3 cells was collected by centrifugation after suspension in a hypotonic solution. TR-CSFB3 cells were rinsed with PBS (–) and treated with a hypotonic solution (10 mM Tris-HCl, 10 mM NaCl, and 1.5 mM MgCl₂, pH 7.4) for 1 h at 4°C. The solution was homogenized and centrifuged (10,000 × g for 15 min at 4°C). The supernatant was centrifuged again (100,000 × g for 60 min at 4°C). The pellets were suspended in a solution [1 mM EDTA, 1 mM EGTA, 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 320 mM sucrose, and 1% protease inhibitor cocktail, pH 7.4] and used as the crude membrane fraction. Protein concentrations of the fractions were determined using the Bio-Rad DC Protein Assay Kit II (Bio-Rad, Hercules, CA, USA).

The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on an acrylamide gel and electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Amersham Hybond P PVDF 0.45; GE Healthcare, Chalfont St. Giles, UK). The membranes were incubated with a blocking solution (125 mM NaCl, 0.1% Tween-20, 1% non-fat dry milk, and 25 mM Tris-HCl, pH 7.4) for 1 h at room temperature and then treated with primary antibodies (1 µg/mL anti-MCT12 or 0.1 µg/mL anti-Na⁺, K⁺-ATPase α1 antibodies) for 12 h at 4°C. PVDF membranes were then treated with horseradish peroxidase-conjugated anti-guinea pig or anti-mouse IgG antibodies for 2 h at room temperature. The signals were visualized using an ECL Prime Western Blotting Detection System (GE Healthcare). Signal intensity was quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). Protein expression levels of MCT12 were normalized to those of Na⁺, K⁺-ATPase α1.

Reverse transcription-polymerase chain reaction

Total RNA was isolated from the liver and TR-CSFB3 cells using the TRIzol reagent (Thermo Fisher Scientific) and an Rneasy Mini Kit (Qiagen, Hilden, Germany), respectively. cDNA was synthesized from 0.5 µg of total RNA as a template by reverse transcription using ReverTra Ace (TOYOBO, Osaka, Japan) and oligo dT primers. PCR was performed with a 96-Well Thermal Cycler (Thermo Fisher Scientific) using specific primers for 35 cycles: 98°C for 10 s, 60°C for 30 s, and 72°C for 1 min. The primer sequences used in this study are listed in Table 1. PCR amplicons were separated by electrophoresis on a 2–3% agarose gel and visualized by ethidium bromide staining. Sequences of the detected amplicons were confirmed by sequence analysis using a DNA sequencer (ABI PRISM 3130; Thermo Fisher Scientific).

Metabolic analysis

TR-CSFB3 cells were cultured in collagen type I-coated cell culture plates with high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (v/v) at 33°C. The cells reached confluence after two days of cultivation and were then incubated with high-glucose DMEM containing 1 µCi/mL [¹⁴C]GAA for 24 h at 37°C. After incubation, the medium was collected and centrifuged at 1000 × g for 1 min at 4°C. After washing the cells twice with PBS (–), TR-CSFB3 cells were resuspended in 70% methanol and centrifuged at 10,000 × g for 5 min at 4°C. The supernatant was used as the cell lysate for TR-CSFB3 cells. High-performance liquid chromatography (HPLC) was performed to

qualitatively detect biosynthesized [^{14}C]Cr from [^{14}C]GAA using an HPLC column (CAPCELL PAK C18 MG II S-5, 5 μm ; Shiseido, Tokyo, Japan). The mobile phase (5 mM 1-octanesulfonic acid, 30 mM K_2HPO_4 , and 0.5% methanol, pH 2.5) was passed through the column at a flow rate of 1 mL/min. Samples of the eluate were collected in vials every 2 min to 40 min. The radioactivity of the eluent fractions was measured using an AccuFLEX LSC-7400 instrument (Aloka, Tokyo, Japan). Typical chromatograms of [^{14}C]Cr and [^{14}C]GAA were obtained by HPLC analysis using intact [^{14}C]Cr and [^{14}C]GAA.

Transport analyses

TR-CSFB3 cells reached confluence after two days of cultivation and transport analyses were performed. For [^{14}C]Cr uptake, after washing TR-CSFB3 cells, the uptake reaction was initiated by replacing the medium with the transport solution (122 mM NaCl, 25 mM NaHCO_3 , 10 mM D-glucose, 3 mM KCl, 1.4 mM CaCl_2 , 1.2 mM MgSO_4 , 0.4 mM K_2HPO_4 , and 10 mM HEPES- NaOH , pH 7.4) containing [^{14}C]Cr (0.5 $\mu\text{Ci/mL}$). After incubation at 37°C for a designated period, the uptake reaction was terminated by removing the medium and washing the cells with ice-cold ECF buffer. TR-CSFB3 cells were solubilized in 1 N NaOH and neutralized. Radioactivity of the cell lysate was measured using an AccuFLEX LSC-7400 instrument. The cell-to-medium ratio [Eq. (1)] was used to express the uptake of [^{14}C]Cr.

$$\text{Cell/medium ratio } (\mu\text{L/mg protein}) = \frac{[\text{C}^{14}\text{Cr}] \text{ in the cells (dpm/mg protein)}}{[\text{C}^{14}\text{Cr}] \text{ in the medium (dpm}/\mu\text{L})} \quad (1)$$

For efflux transport, TR-CSFB3 cells were preincubated with high-glucose DMEM containing 0.5 $\mu\text{Ci/mL}$ [^{14}C]Cr for 20 min at 37°C and washed. Efflux transport was initiated by the addition of a transport solution. The cells were incubated at 37°C or 4°C for designated time periods. The transport was terminated by sampling the transport solution and washing the cells. TR-CSFB3 cells were solubilized in 1 N NaOH and neutralized. The radioactivity of each sample was measured using an AccuFLEX LSC-7400 instrument. The efflux ratio was calculated using Eq. (2).

$$\text{Efflux ratio } (\%) = \frac{[\text{C}^{14}\text{Cr}] \text{ in the medium (dpm)}}{[\text{C}^{14}\text{Cr}] \text{ in the medium and the cells (dpm)}} \times 100 \quad (2)$$

For knockdown analysis, TR-CSFB3 cells were transfected with MCT12 gene-specific small interfering RNAs (siRNAs) or Stealth RNAi Negative Control GC Duplexes (Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Thermo Fisher Scientific) dissolved in Opti-MEM I (Thermo Fisher Scientific), following the manufacturer's protocols. The MCT12 gene-specific siRNA sequence containing 3'-dTdT extensions was 5'-CAGGUCUUGGAUUUGCACUUUGUUA-3'. Immunoblotting and transport analyses were performed 48 h after transfection.

Lateral ventricular micro-injection

Anesthetized rats were placed in a stereotaxic frame (SR-5R; Narishige, Tokyo, Japan). The transport solution (10 µL/rat) consisting of [³H]D-mannitol (reference compound for diffusion; 0.3 µCi/rat) and [¹⁴C]Cr (0.05 µCi/rat) was microinjected into the left lateral ventricle. After the designated time period, CSF (50 µL) was collected from the cisterna magna, and the cerebrum, midbrain, and cerebellum were isolated. These isolated samples were dissolved in 2 N NaOH at 55°C for 3 h. The radioactivity of these samples was then measured using an AccuFLEX LSC-7400 instrument.

The concentration-time curves of [³H]D-mannitol and [¹⁴C]Cr were fitted to a one-compartment model [Eq. (3)] using a nonlinear least-squares regression analysis program (MULTI) [16]. C_{CSF} and k_{el} indicate the concentration in the CSF and elimination rate constant, respectively. The transfer of [³H]D-mannitol and [¹⁴C]Cr from the CSF to the brain is represented by Eq. (4). The brain-to-CSF concentration ratio [$X_{\text{Brain}}(t)/C_{\text{CSF}}(t)$] was calculated using Eq. (5), which is given by Eq. (4). The area under the CSF concentration-time curve (ACC) was obtained by integrating C_{CSF} from 0 min to the designated time period. Transfer clearance (CL_{transfer}) was determined using MULTI.

$$C_{\text{CSF}}(t) = C_{\text{CSF}}(0) \times \exp(-k_{\text{el}} \times t) \quad (3)$$

$$\frac{dX_{\text{Brain}}}{dt} = CL_{\text{transfer}} \times C_{\text{CSF}} \quad (4)$$

$$X_{\text{Brain}}(t)/C_{\text{CSF}}(t) = \frac{CL_{\text{transfer}} \times ACC_{0 \rightarrow t}}{C_{\text{CSF}}(t) + V_0} \quad (5)$$

Statistical analyses

Data are presented as the mean ± standard deviation (S.D.). Statistical analyses were performed using unpaired two-tailed Student's t-test. Differences were considered statistically significant at $P < 0.05$.

Results

Expression and localization of MCT12 in CpxEpic

Figures 1A-C show the double staining of MCT12 with Na⁺, K⁺-ATPase, which is a marker of the apical membrane of CpxEpic [17]. MCT12 immunoreactivity was observed in the choroid plexus and merged with that of Na⁺, K⁺-ATPase (Figures 1A-C). In addition, MCT12 immunoreactivity was abolished by pretreatment with antigen peptides of anti-MCT12 antibodies (Figures 1D-E). Immunoblotting of the crude membrane of TR-CSFB3 cells using anti-MCT12 antibodies detected a signal at 37 kDa (Figure 1F). This signal size was consistent with that detected in our previous study using MCT12-overexpressing cells [10]. These results confirmed the specific recognition of anti-MCT12 antibodies against MCT12 proteins. These results suggest that MCT12 is localized to the apical membrane of CpxEpic.

Expression and activation of GAMT in CpxEpic

Considering the localization of MCT12 in CpxEpic, it is hypothesized that GAA is converted to Cr in CpxEpic, and the biosynthesized Cr is provided to the CSF via MCT12. Thus, the expression of GAMT, which catalyzes Cr synthesis from GAA, was evaluated in CpxEpic. TR-CSFB3 cells expressed GAMT mRNA in addition to MCT12 mRNA (Figure 2A). Moreover, GAMT immunoreactivity was detected in CpxEpic (Figure 2B). Thus, GAMT expression is suggested in CpxEpic.

Figure 3 shows functional GAMT activity in TR-CSFB3 cells. Figures 3A and B are typical chromatograms of intact [^{14}C]Cr and [^{14}C]GAA, respectively. Figure 3C exhibits the typical HPLC chromatogram of the cell lysate of TR-CSFB3 cells after cultivation with the medium containing [^{14}C]GAA. In this chromatogram, a typical peak of [^{14}C]Cr was detected in addition to that of [^{14}C]GAA (Figure 3C). Therefore, functional expression of GAMT was observed in TR-CSFB3 cells. Based on these results, GAA is suggested to be converted to Cr via GAMT after incorporation into CpxEpic.

MCT12 contribution to the release of Cr from TR-CSFB3 cells

GAA is metabolized to Cr after its influx into CpxEpic. After this conversion, Cr release from CpxEpic into CSF via MCT12 is a considerable process. Thus, transport analyses were performed using TR-CSFB3 cells. Figures 4A and B show the time course of the influx and efflux transport of [^{14}C]Cr in TR-CSFB3 cells, respectively. The influx transport of [^{14}C]Cr into TR-CSFB3 cells exhibited a time-dependent increase (Figure 4A), confirming the results of our previous study [6]. In addition, TR-CSFB3 cells showed a time-dependent increase in the efflux transport of [^{14}C]Cr until at least 20 min after pre-incorporation of [^{14}C]Cr (Figure 4B). The efflux transport decreased at 4°C (Figure 4B, closed circles). To determine the involvement of MCT12 in Cr transport in CpxEpic, [^{14}C]Cr transport was evaluated after MCT12 knockdown using MCT12-specific siRNA. Reduction of MCT12 protein expression was confirmed 48 h after treatment with the negative control (N.C.) or MCT12 siRNA (Figure 4C). After comparing the signal intensities of the immunoblot after N.C. and MCT12 siRNA treatment, MCT12 protein expression levels were decreased by 57.8% in MCT12 siRNA-treated cells (Figure 4D). The influx transport of [^{14}C]Cr was barely altered by MCT12 knockdown (Figure 4E). In contrast, the efflux transport of [^{14}C]Cr from MCT12 siRNA-treated TR-CSFB3 cells decreased by 30.6% compared to that of N.C. siRNA-treated TR-CSFB3 cells (Figure 4F). In efflux transport of [^{14}C]Cr, the interception of the regression line in Figure 4B cannot be neglected for the efflux ratio of [^{14}C]Cr. As it has been reported that carrier-mediated transport, including facilitated transport, is almost abolished at 4°C, and Cr is hardly transported by passive diffusion [6, 8, 10, 18], the interception is considered to be increased by factors, such as the adsorption of [^{14}C]Cr on the cell culture plate and cell surface. To remove adsorption factors, the values of [^{14}C]Cr efflux ratio at 4°C were measured and subtracted from the values at 37°C (Figures 4G and H). Figure 4G shows the corrected values of the efflux ratio. The efflux ratio in MCT12 siRNA-treated cells decreased by 53.2% compared to that in the N.C. siRNA-treated cells (Figure 4H). Considering the reduction in MCT12 protein expression by the knockdown, the contribution ratio of MCT12 in Cr efflux transport from TR-CSFB3 cells was calculated as 92.0% ($= 53.2/57.8 \times 100$). Therefore, MCT12 suggestively contributes to the release of Cr from CPxEpic.

***In vivo* CSF-to-brain parenchyma transfer of Cr after intracerebroventricular injection**

Based on the results of MCT12 localization and contribution to Cr release, MCT12 is suggested to contribute to Cr release from CPxEpic into the CSF. After release from CPxEpic, it is hypothesized that biosynthesized Cr is supplied to the brain parenchyma, as it shows a high demand for Cr [4, 5]. To determine the CSF-to-brain transfer of Cr, rat brain and CSF were collected after the intracerebroventricular administration of Cr. After intracerebroventricular injection of [^{14}C]Cr, its concentration in the CSF was reduced at each time point for up to 10 min (Figure 5A). In contrast, the apparent brain/CSF concentration ratio of [^{14}C]Cr increased after intracerebroventricular administration, indicating the transfer of [^{14}C]Cr from the CSF to the brain parenchyma (Figure 5B). The apparent brain influx clearance of [^{14}C]Cr from the CSF per gram brain ($CL_{\text{app, CSF-to-brain transfer}}$) was determined to be $4.65 \pm 0.89 \mu\text{L}/(\text{min} \cdot \text{g brain})$. In addition, the $CL_{\text{app, CSF-to-brain transfer}}$ of [^3H]D-mannitol, which is used to estimate transfer through the intercellular space, was determined to be $5.18 \pm 1.91 \mu\text{L}/(\text{min} \cdot \text{g brain})$. Moreover, at the longest time point (Figure 5B), more than 50% of the total administered [^{14}C]Cr was detected in the brain sample.

Discussion

Based on the results of our study, the GAA dynamics in CPxEpic were as follows: First, GAMT catalyzes the conversion of GAA to Cr in CPxEpic (Figures 2 and 3). Second, Cr is released from CPxEpic into the CSF via MCT12 (Figures 1 and 4). Finally, released Cr is supplied to the brain parenchyma (Figure 5).

As GAA is known as an endogenous convulsant, the concentrations of GAA are maintained at a lower level in the human CSF (0.036–0.22 μM) than that in the plasma (0.35–3.5 μM) [6, 19]. Our previous study revealed that GAA is eliminated from the CSF by incorporation into CPxEpic [6]. In this study, along with the elimination of GAA, the choroid plexus was suggested to participate in the provision of Cr to the brain. Moreover, the protein expression and activity of GAMT were detected in CPxEpic (Figures 2 and 3). In addition, the majority of Cr was released via MCT12, which is predominantly localized on the CSF side membrane of CPxEpic (Figures 1 and 4). Cr in the CSF was transferred to the brain parenchyma (Figure 5). In the brain, astrocytes, oligodendrocytes, and olfactory ensheathing glia express GAMT and are considered to be Cr-supplying cells [15]. From the results of this study, CPxEpic are considered to be Cr supplying cell in the brain, as well as these glial cells. Furthermore, the Cr concentration in the brain parenchyma is lower in patients with choroid plexus tumor (1.2–1.3 mM) compared to the normal range (3.9–6.0 mM) [20–22]. Meanwhile, the GAA concentration in the brain parenchyma is higher in patients with choroid plexus tumor (3.0–3.1 mM) compared to the normal range (0.06–0.85 mM) [20, 22]. These reports support the importance of the choroid plexus in maintaining the GAA and Cr homeostasis in the brain.

Our study suggests that MCT12 functions in releasing Cr from CPxEpic, although it can recognize GAA as a substrate and has the ability of influx transport of Cr [10]. By biosynthesis of Cr in CPxEpic, its concentration is considered to be higher in CPxEpic than that in the CSF. Since MCT12 reportedly transports substrates along a concentration gradient [10], it is considered to function as an efflux

transporter of Cr in CPxEpic. Comparing MCT12-mediated transport of Cr with that of GAA, the affinity of MCT12 for Cr is approximately 10-fold greater than that for GAA [10]. Thus, MCT12 is suggested to predominantly recognize Cr and function as an efflux transporter of Cr in CPxEpic. Regarding the influx transport of GAA into CPxEpic, we found that GAA uptake by TR-CSFB3 cells and isolated choroid plexus was decreased by three-quarters in the absence of extracellular Na^+ in our previous study [6]. As MCT12-mediated GAA transport hardly changed in the absence of extracellular Na^+ [10], the contribution of MCT12 to GAA influx transport to CPxEpic is considered to be negligible. Taken together, MCT12 functions as an efflux transporter of the biosynthesized Cr in CPxEpic.

In vivo analysis revealed the transfer of [^{14}C]Cr from the CSF to the brain (Figure 5B). In addition, the $CL_{\text{app, CSF-to-brain transfer}}$ of Cr was determined to be $4.65 \mu\text{L}/(\text{min} \cdot \text{g brain})$. This value was consistent with that of D-mannitol [$5.18 \mu\text{L}/(\text{min} \cdot \text{g brain})$]. Since D-mannitol is a reference compound for diffusion into the brain interstitial space via the ependymal layer [23], Cr is considered to mainly pass through the interstitial space of the ependymal layer in the CSF-to-brain transfer process. Furthermore, the apparent brain influx clearance of Cr from circulating blood is $1.61 \mu\text{L}/(\text{min} \cdot \text{g brain})$. Comparing to the value of $CL_{\text{app, CSF-to-brain transfer}}$ of Cr with this value, the CSF-to-brain transfer of Cr is considered to be a potential route for Cr supplementation to the brain. In the brain parenchyma, Cr is concentrated in high-energy-demanding cells, such as the neurons [3]. Therefore, Cr concentration in the brain parenchyma ($3.9\text{--}6.0 \text{ mM}$) is much higher than that in the plasma ($10\text{--}200 \mu\text{M}$) and CSF ($17\text{--}90 \mu\text{M}$) [19, 20, 24]. Taken together, CSF-to-brain transfer of Cr is suggested to be one of the routes for Cr supplementation to the brain parenchyma.

In this study, the MCT12-mediated pathway was proposed as a route for Cr transfer from CPxEpic to the brain. Specifically, GAA is taken up by CPxEpic and converted to Cr. Subsequently, biosynthesized Cr is released into the CSF via MCT12. As the incorporation of GAA into CPxEpic is mediated by TauT in addition to CRT, the processes of GAA and Cr dynamics in CPxEpic can occur in a CRT-independent manner. Functional mutations in CRT induce CCDSs, which are known as neurodevelopmental disorders [4, 19]. CRT deficiency is reported to be the most likely cause of CCDS, accounting for 0.3–3.5% of males with intellectual disabilities [19]. As CRT is the main contributor that mediates Cr influx into the brain across the BBB [25], a lack of Cr in the brain is caused in the patients with CRT defects. Thus, an increase in the supply of Cr to the brain is important for the treatment of CRT deficiencies. In the Cr provision route proposed in this study, Cr transport is mediated by MCT12; thus, this route is independent of CRT. In addition, Cr concentration in the CSF is the same in patients with CRT deficiency and healthy people [19]. Therefore, this Cr delivery route to the brain/CSF mediated by MCT12 may be utilized for the treatment of CRT deficiency as a CRT-independent delivery route of Cr into the brain.

Conclusion

In this study, the choroid plexus was suggested to play a role in GAA elimination and Cr supplementation. In CPxEpic, GAA is metabolized to Cr, and MCT12 mediates the release of Cr into the CSF. Through these

processes, the choroid plexus may be associated with Cr homeostasis in the brain. In addition, our previous study suggested that CPxEpic plays a role in the elimination of D-serine, a co-agonist of N-methyl-D-aspartate (NMDA)-type glutamate receptors, from the brain by the incorporation and metabolism of D-serine [26]. Therefore, along with BCSFB constitution, the choroid plexus is proposed to have an important function in nutrient metabolism, thereby maintaining nutrient levels in the brain.

Since alternation of this MCT12-mediated Cr provision route is considered to disturb the balance of Cr and GAA levels in the brain and possibly cause epilepsy, further studies on this point can lead to understanding the pathology of epilepsy and regulating seizures. Furthermore, this MCT12-mediated pathway also functions as a Cr-supplementation system in the brain parenchyma. As Cr transport is mediated by MCT12 and occurs in a CRT-independent manner via the proposed route, it is possible that the proposed Cr supplementation route is beneficial for the development of novel treatment strategies for CRT deficiency.

Declarations

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Authors' contributions

RJ and SA designed this study. RJ performed the data collection and analyses. YK, MT, and KH coordinated the study. RJ drafted the manuscript, and SA and KH revised the manuscript. All authors have read and approved the final manuscript.

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Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Hiramatsu M. A role for guanidino compounds in the brain. *Mol Cell Biochem.* 2003; 244: 57-62.

2. Tachikawa M, Hosoya K. Transport characteristics of guanidino compounds at the blood-brain barrier and blood-cerebrospinal fluid barrier: relevance to neural disorders. *Fluids Barriers CNS*. 2011; 8: 13.
3. Jomura R, Akanuma S, Tachikawa M, Hosoya K. SLC6A and SLC16A family of transporters: contribution to transport of creatine and creatine precursors in creatine biosynthesis and distribution. *Biochim Biophys Acta Biomembr*. 2021; 1864: 183840.
4. Braissant O, Henry H, Béard E, Uldry J. Creatine deficiency syndromes and the importance of creatine synthesis in the brain. *Amino Acids*. 2011; 40: 1315-1324.
5. Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. *Physiol Rev*. 2000; 80: 1107-1213.
6. Tachikawa M, Fujinawa J, Takahashi M, Kasai Y, Fukaya M, Sakai K, Yamazaki M, Tomi M, Watanabe M, Sakimura K, Terasaki T, Hosoya K. Expression and possible role of creatine transporter in the brain and at the blood-cerebrospinal fluid barrier as a transporting protein of guanidinoacetate, an endogenous convulsant. *J Neurochem*. 2008; 107: 768-778.
7. Tachikawa M, Kasai Y, Takahashi M, Fujinawa J, Kitaichi K, Terasaki T, Hosoya K. The blood-cerebrospinal fluid barrier is a major pathway of cerebral creatinine clearance: involvement of transporter-mediated process. *J Neurochem*. 2008; 107: 432-442.
8. Tachikawa M, Kasai Y, Yokoyama R, Fujinawa J, Ganapathy V, Terasaki T, Hosoya K. The blood-brain barrier transport and cerebral distribution of guanidinoacetate in rats: involvement of creatine and taurine transporters. *J Neurochem*. 2009; 111: 499-509.
9. Braissant O. Creatine and guanidinoacetate transport at blood-brain and blood-cerebrospinal fluid barriers. *J Inherit Metab Dis*. 2012; 35: 655-664.
10. Jomura R, Tanno Y, Akanuma S, Kubo Y, Tachikawa M, Hosoya K. Monocarboxylate transporter 12 as a guanidinoacetate efflux transporter in renal proximal tubular epithelial cells. *Biochim Biophys Acta Biomembr*. 2020; 1862: 183434.
11. Jomura R, Tanno Y, Akanuma S, Kubo Y, Tachikawa M, Hosoya K. Contribution of monocarboxylate transporter 12 to blood supply of creatine on the sinusoidal membrane of the hepatocytes. *Am J Physiol Gastrointest Liver Physiol*. 2021; 321: G113-G122.
12. Futagi Y, Narumi K, Furugen A, Kobayashi M, Iseki K. Molecular characterization of the orphan transporter SLC16A9, an extracellular pH- and Na⁺-sensitive creatine transporter. *Biochem Biophys Res Commun*. 2020; 522: 539-544.
13. Braissant O, Béard E, Torrent C, Henry H. Dissociation of AGAT, GAMT and SLC6A8 in CNS: relevance to creatine deficiency syndromes. *Neurobiol Dis*. 2010; 37: 423-433.
14. Kitazawa T, Hosoya K, Watanabe M, Takashima T, Ohtsuki S, Takanaga H, Ueda M, Yanai N, Obinata M, Terasaki T. Characterization of the amino acid transport of new immortalized choroid plexus epithelial cell lines: a novel in vitro system for investigating transport functions at the blood-cerebrospinal fluid barrier. *Pharm Res*. 2001; 18: 16-22.
15. Tachikawa M, Fukaya M, Terasaki T, Ohtsuki S, Watanabe M. Distinct cellular expressions of creatine synthetic enzyme GAMT and creatine kinases uCK-Mi and CK-B suggest a novel neuron-glia

- relationship for brain energy homeostasis. *Eur J Neurosci.* 2004; 20: 144-160.
16. Yamaoka K, Tanigawara Y, Nakagawa T, Uno T. A pharmacokinetic analysis program (multi) for microcomputer. *J Pharmacobiodyn.* 1981; 4: 879-885.
 17. Tachikawa M, Tsuji K, Yokoyama R, Higuchi T, Ozeki G, Yashiki A, Akanuma S, Hayashi K, Nishiura A, Hosoya K. A clearance system for prostaglandin D2, a sleep-promoting factor, in cerebrospinal fluid: role of the blood-cerebrospinal barrier transporters. *J Pharmacol Exp Ther.* 2012; 343: 608-616.
 18. Kurosawa T, Tega Y, Sako D, Mochizuki T, Yamaguchi T, Kawabata K, Inoue K, Ito N, Kusuhara H, Deguchi Y. Transport characteristics of 6-mercaptopurine in brain microvascular endothelial cells derived from human induced pluripotent stem cells. *J Pharm Sci.* 2021; 110: 3484-3490.
 19. van de Kamp JM, Betsalel OT, Mercimek-Mahmutoglu S, Abulhoul L, Grünewald S, Anselm I, Azzouz H, Bratkovic D, de Brouwer A, Hamel B, Kleefstra T, Yntema H, Campistol J, Vilaseca MA, Cheillan D, D'Hooghe M, Diogo L, Garcia P, Valongo C, Fonseca M, Frints S, Wilcken B, von der Haar S, Meijers-Heijboer HE, Hofstede F, Johnson D, Kant SG, Lion-Francois L, Pitelet G, Longo N, Maat-Kievit JA, Monteiro JP, Munnich A, Muntau AC, Nassogne MC, Osaka H, Ounap K, Pinard JM, Quijano-Roy S, Poggenburg I, Poplawski N, Abdul-Rahman O, Ribes A, Arias A, Yaplito-Lee J, Schulze A, Schwartz CE, Schwenger S, Soares G, Sznajer Y, Valayannopoulos V, Van Esch H, Waltz S, Wamelink MM, Pouwels PJ, Errami A, van der Knaap MS, Jakobs C, Mancini GM, Salomons GS. Phenotype and genotype in 101 males with X-linked creatine transporter deficiency. *J Med Genet.* 2013; 50: 463-472.
 20. Panigrahy A, Krieger MD, Gonzalez-Gomez I, Liu X, McComb JG, Finlay JL, Nelson MD Jr, Gilles FH, Blüml S. Quantitative short echo time 1H-MR spectroscopy of untreated pediatric brain tumors: preoperative diagnosis and characterization. *AJNR Am J Neuroradiol.* 2006; 27: 560-572.
 21. Wellard RM, Briellmann RS, Jennings C, Jackson GD. Physiologic variability of single-voxel proton MR spectroscopic measurements at 3T. *AJNR Am J Neuroradiol.* 2005; 26: 585-590.
 22. Edvardson S, Korman SH, Livne A, Shaag A, Saada A, Nalbandian R, Allouche-Arnon H, Gomori JM, Katz-Brull R. L-Arginine:glycine amidinotransferase (AGAT) deficiency: clinical presentation and response to treatment in two patients with a novel mutation. *Mol Genet Metab.* 2010; 101: 228-232.
 23. Usui T, Nakazawa A, Okura T, Deguchi Y, Akanuma S, Kubo Y, Hosoya K. Histamine elimination from the cerebrospinal fluid across the blood-cerebrospinal fluid barrier: involvement of plasma membrane monoamine transporter (PMAT/SLC29A4). *J Neurochem.* 2016; 139: 408-418.
 24. Marescau B, De Deyn PP, Lowenthal A, Qureshi IA, Antonozzi I, Bachmann C, Cederbaum SD, Cerone R, Chamois N, Colombo JP, Hyland K, Gatti R, Kang SS, Letarte J, Lambert M, Mizutani N, Possemiers I, Rezvani I, Snyderman SE, Terheggen HG, Yoshino M. Guanidino compound analysis as a complementary diagnostic parameter for hyperargininemia: follow-up of guanidino compound levels during therapy. *Pediatr Res.* 1990; 27: 297-303.
 25. Ohtsuki S, Tachikawa M, Takanaga H, Shimizu H, Watanabe M, Hosoya K, Terasaki T. The blood-brain barrier creatine transporter is a major pathway for supplying creatine to the brain. *J Cereb Blood Flow Metab.* 2002; 22: 1327-1335.

26. Kasai Y, Tachikawa M, Hirose S, Akanuma S, Hosoya K. Transport systems of serine at the brain barriers and in brain parenchymal cells. *J Neurochem.* 2011; 118: 304-313.

Tables

Table 1. Primers used for gene expression analysis.

Genes	Gene Bank accession number	Orientation	Primer sequence (5'–3')	Product size
<i>MCT12</i>	NM_001191637.1	Forward	AGCCTTCCTTCTTTGTGG	179 bp
		Reverse	TCTGATCTAACTCCTTCGC	
<i>GAMT</i>	NM_012793.2	Forward	TCTGACACGCACCTGCAGATCC	584 bp
		Reverse	GCATAGTAGCGGCAGTCGGCTG	
<i>β-Actin</i>	NM_031144.3	Forward	TCATGAAGTGTGACGTTGACATCCGT	285 bp
		Reverse	CCTAGAAGCATTTGCGGTGCACGATG	

Figures

Figure 1

Localization of monocarboxylate transporter 12 (MCT12) in the rat choroid plexus. (A-C) Double immunohistochemical staining of MCT12 (red) and Na⁺, K⁺-ATPase (green) in the rat choroid plexus. Na⁺, K⁺-ATPase is used as a marker of the apical membrane of choroid plexus epithelial cells (CPxEpic). Arrowheads indicate the apical membrane of CPxEpic. (D and E) Immunohistochemical staining of anti-MCT12 antibodies after antigen absorption. Anti-MCT12 antibodies were incubated in PBS (–) with or without antigenic peptides of anti-MCT12 antibodies (4.36 µg/mL) for 6 h at 4°C and then used as the primary antibodies. Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, blue). Scale bar: 50 µm. (F) Protein expression of MCT12 in CPxEpic. The crude membrane fraction of TR-CSFB3 cells, conditionally immortalized rat CPxEpic, was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Figure 2

Expression of guanidinoacetate N-methyltransferase (GAMT) in CPxEpic. (A) mRNA expression of MCT12 and GAMT in TR-CSFB3 cells. Polymerase chain reaction was conducted with (+) or without (–) reverse

transcription. The rat liver was used as a positive control. (B) Immunohistochemical staining of GAMT in the rat choroid plexus. Arrowheads indicate CPxEpic. Nuclei were stained with DAPI (blue). Scale bar: 20 μm .

Figure 3

Functional expression of GAMT in TR-CSFB3 cells. Typical chromatograms of (A) [^{14}C]GAA and (B) [^{14}C]Cr. (C) A typical HPLC chromatogram of the cell lysate of TR-CSFB3 cells. TR-CSFB3 cells were incubated with the medium containing [^{14}C]GAA (1 $\mu\text{Ci/mL}$) for 24 h at 37°C and the cell lysate of these cells was separated by high-performance liquid chromatography (HPLC).

Figure 4

MCT12 contribution to Cr transport in TR-CSFB3 cells. (A) Time-course of [^{14}C]Cr uptake by TR-CSFB3 cells. The uptake was measured for the indicated time periods at 37°C. (B) Time-course of [^{14}C]Cr efflux from TR-CSFB3 cells. After pre-incubation of [^{14}C]Cr for 20 min at 37°C, the efflux transport was measured for the indicated time periods at 37°C (open circles) or 4°C (closed circles). (C) Immunoblotting of the crude membrane fractions of TR-CSFB3 cells after the transfection of negative control (N.C.) or MCT12-specific small interfering RNA (siRNA). (D) Protein expression levels of MCT12 in TR-CSFB3 cells after MCT12 specific-siRNA transfection. Intensity of the signal was evaluated using the ImageJ software and the signal levels of MCT12 were normalized to that of Na^+ , K^+ -ATPase $\alpha 1$. (E) [^{14}C]Cr uptake by TR-CSFB3 cells after MCT12 specific-siRNA transfection. The uptake was measured for 20 min at 37°C. (F and G) Efflux transport of [^{14}C]Cr from TR-CSFB3 cells after the knockdown of MCT12. After pre-incubation of [^{14}C]Cr for 20 min at 37°C, efflux was measured for 20 min at 37°C (F) or 4°C (G). (H) Subtraction of the efflux at 4°C (G) from that at 37°C (F). Each column represents the mean $\pm$ standard deviation (S.D.) ($n = 3$). Each open and closed circle represents an individual data point. * $p < 0.05$, ** $p < 0.01$, significantly different from the conditions of N.C. siRNA transfection.

Figure 5

***In vivo* cerebrospinal fluid (CSF)-to-brain parenchyma transfer of [^{14}C]Cr after intracerebroventricular injection.** (A) Time-course of [^{14}C]Cr concentration in the CSF after intracerebroventricular administration. The residual CSF concentration is the percentage of the dose remaining in 1 mL CSF. (B) CSF-to-brain parenchyma transfer of [^{14}C]Cr after intracerebroventricular injection. An extracellular fluid buffer containing [^{14}C]Cr (0.05 $\mu\text{Ci}/10 \mu\text{L}$) was directly injected into the CSF. The solid line was fitted using a

nonlinear least-squares regression analysis program. Each open circle represents an individual data point. Each central bar represents the mean $\pm$ S.D. ($n = 3$).