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The distribution and density of monocarboxylate transporter 2 in cerebral cortex, hippocampus and cerebellum of wild-type mice

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Abstract

Monocarboxylates cannot cross the blood-brain barrier freely to participate in brain energy metabolism. Specific monocarboxylate transporters (MCTs) are needed to cross cellular membranes. Monocarboxylate transporter 2 (MCT2) is a major monocarboxylate transporter encoded by the SLC16A7 gene. Recent studies reported that neurodegenerative diseases of the CNS, such as Alzheimer's disease (AD) and Parkinson's disease (PD), were related to energy metabolic impairment. MCT2 also plays an important role in energy metabolism in the CNS. To provide experimental evidence for future research on the role of MCT2 in the pathological process of CNS degenerative diseases, the distribution and density of MCT2 in different subregions of wild-type mouse brain was examined using immunohistochemistry, western blot and immunogold post-embedding electron microscopic techniques. The amount of MCT2 was higher in cerebellum than in cortex and hippocampus on western blots, and there was no statistical difference between cortex and hippocampus. Immunohistochemistry assay revealed the highest density of MCT2 in the CA3 of the hippocampus. The granular cell layer of the cerebellum contained more MCT2 than the molecular layer. The MCT2 density on the end feet of astrocytes of molecular layer was lower than in hippocampus, but the postsynaptic densities (PSDs) of asymmetric synapses in the molecular layer exhibited a high density using immunogold post-embedding electron microscopic techniques.

Key words: cerebellum; cortex; hippocampus; monocarboxylate transporter 2.

Introduction

Monocarboxylate substrates for cerebral metabolism include lactate and ketone bodies. Lactate is primarily taken up from the blood (Chen et al. 2000a,b), but the brain itself also produces lactate from glycogen stored in astrocytes via glycolysis, which may provide neighbouring neurons with lactate (Hanu et al. 2000). This transfer of energy substrates is referred to as the astrocyte-neuron lactate shuttle (Pellerin et al. 1998). Lactate is continuously formed in the cytosol from pyruvate in the last step of glycolysis via the enzyme lactate dehydrogenase (LDH). The conversion of pyruvate to lactate is essential for cellular ATP production during anoxic periods because this reaction also forms nicotinamide adenine dinucleotide (NAD^+) from NADH, a step that is necessary to maintain a high rate of glycolysis (Tekkok et al. 2005; Suzuki et al. 2011). Ketone bodies, i.e. acetoacetate and beta-hydroxybutyrate, are formed in the liver from fatty acids and are interconverted by 3-hydroxybutyrate dehydrogenase with NADH as the coenzyme and may be oxidized in many tissues, including the brain (Morris, 2005). Relevant reports demonstrated that lactate, ketones and other monocarboxylates maintain the homeostasis of the brain (Gallagher et al. 2009) and provide energy to the brain through glycolysis during degenerative diseases of the central nervous system, including hypoxic or mitochondrial dysfunction. Monocarboxylates also play various roles in the brain, such as ketone bodies and lactate. The presence of ketone bodies in the brain may increase the brain energy stores, stabilize neuronal membrane potential, enhance GABA-mediated inhibition (Gerhart et al. 1997; Pierre & Pellerin, 2005), inhibit the transport of glutamate into synaptic

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vesicles and attenuate the release of vesicular glutamate from neurons (Juge et al. 2010). Glucose transported from blood is the dominant energy source for the enhancement of neuronal activity, but recent studies have demonstrated that lactate was used as an energy substrate and neuromodulatory factor in the hippocampus to enhance memory formation (Suzuki et al. 2011). Therefore, we speculate that monocarboxylic acids are critical for the maintenance of normal brain function.

Brain monocarboxylates are transferred to neurons via monocarboxylate transporters (MCTs) (Merezhinskaya & Fishbein, 2009). The MCT family is encoded by solute carrier family 16 (SLC16), which consists of 14 members (Halestrap & Price, 1999; Halestrap, 2012). The monocarboxylate transporters family of proton-linked transporters are involved in the uptake and release of lactate, pyruvate and ketone bodies. Of the 14 members described so far, MCT1, MCT2 and MCT4 are clearly expressed in the central nervous system, and these transporters are the primary research hotspot (Pierre et al. 2000; Pierre & Pellerin, 2005). MCT1 is predominantly expressed by astrocytes and the endothelial cells forming blood vessels (Chiry et al. 2006). MCT4 expression was observed on Bergmann glial cells in the cerebellum and hippocampal astrocytes (Debernardi et al. 2003; Pellerin et al. 2005).

Unlike MCT1 and MCT4, MCT2 was encoded by the SLC16A7 gene and described predominantly in neuronal and astrocyte end feet (Rafiki et al. 2003). MCT2 is the main neuronal monocarboxylate transporter when lactate is used as an additional energy substrate. Previous studies demonstrated that MCT2 which was expressed in neurons and astrocytes plays a role in lactate uptake into neurons (Pierre & Pellerin, 2005). Monocarboxylates must cross the blood-brain barrier to enter the brain. Brain endothelial cells in the blood-brain barrier are surrounded by the basal lamina and closely opposed to several other cell types, including perivascular astrocyte end foot processes, pericytes and neurons (Fig. 1). These cells constitute a neurovascular unit that is essential for the health and function of the central nervous system (Hawkins & Davis, 2005). Transporter proteins, such as MCT2, on astrocyte end feet and neurons become a part of the blood-brain barrier (Fig. 1). However, there are few studies on the location and density of MCT2 in the subregions of wild-type mouse brain. The present study discovered MCT2 expression in the cerebral cortex, hippocampus and cerebellum using immunohistochemistry, western blot and immunogold post-embedding electron microscopic techniques.

Materials and methods

Animals and chemicals

Adult male wild-type mice [3 months of age; C57BL/6, SCXK (JING) 2016-0011] were used in this study (n = 15). Each

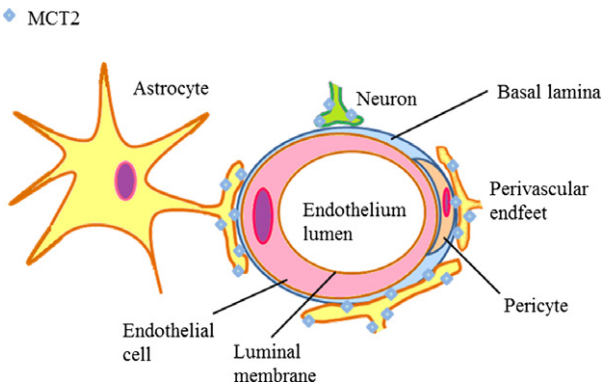

Fig. 1 Localization of MCT2 in the neurovascular system. MCT2 is primarily expressed on the luminal membrane of endothelial cells and the perivascular end feet of astrocytes.

mouse was housed in a plastic cage in a controlled environment (22–25 °C; 50 ± 10% relative humidity and automatic 12-h light/dark cycle) with access to food and water *ad libitum*. The Committee of Zhengzhou University approved the experiments. Primary antibodies: Mouse anti-MCT2 was obtained from Santa Cruz Biotechnology (cat. no. sc-166925, US). A rabbit anti-MCT2 antibody was obtained from Professor A. P. Halestrap, University of Bristol, UK. Secondary antibodies: Goat anti-mouse antibody for immunohistochemistry was purchased from Santa Cruz Biotechnology (cat. no. sc-2010, US). Goat anti-rabbit immunoglobulin conjugated with 10-nm diameter colloidal gold was obtained from BB Internal (cat. no. EMGFAR10, UK).

Tissue preparation

Mice were anaesthetized using isoflurane and perfused transcardially with saline followed by 4% paraformaldehyde (PFA) with 0.1% glutaraldehyde in phosphate buffer (PB; 0.1 M, pH 7.4). The brains were removed from the skulls and stored in 4% PFA with 0.1% glutaraldehyde PB for 24 h at 4 °C. Small rectangular pieces (0.5 × 0.5 × 1 mm) from the CA1 region of hippocampus and cerebellar cortex were prepared for electron microscopy. Mice were anaesthetized and perfused transcardially with saline followed by 4% PFA in PB (0.01 M, pH 7.4). The brains were removed from the skulls and stored in 4% PFA with 0.1% glutaraldehyde PB for 24 h at 4 °C. Tissue was dehydrated in 20% and 30% sucrose in PB gradients (0.01 M, pH 7.4). Slices (25 μm) for immunohistochemistry were obtained on a freezing microtome. Mice were anaesthetized and brains were removed from the skulls. Separation of the hippocampus and cortex for western blots was achieved using a stereoscopic microscope in brain slices. Separation of the granular and molecular layers of the cerebellum was not realized because of technical problems.

Western blot

Brain tissue was removed and placed into a precooled brain trough ($n = 5$). Sections were obtained from the brain, cortex, cerebellum and hippocampus. A BCA kit (cat. no. CW0014S, CWBIO, Beijing, China) was used to measure protein concentrations. Proteins were separated using a 3% concentration gel (80 V, 30 min) and 10% separation gel (120 V, 60 min) electrophoresis as identified in a literature search and comparison. A ladder of 10–245 kD molecular weight standard protein (cat. no. PR1920, Solarbio, Beijing, China) markers were used for electrophoresis. A PAGE gel was selected to perform transmembrane based on the relative molecular weight of MCT2, and proteins were transferred to PVDF membranes (280 V, 90 min). The PVDF membrane was incubated for 2 h at room temperature and with primary antibodies (MCT2, diluted 1:200, SANTA CRUZ) at 4 °C overnight. Membranes were incubated with secondary antibodies (goat anti-mouse, diluted 1 : 1000 CST) for 2 h at room temperature. The protein bands were visualized using enhanced chemiluminescence and imaged with the Bio-Image Analysis system (Bio-Rad Laboratories, USA). The ratios of protein band intensities to β-actin were determined.

Immunohistochemistry

Coronal slices (25 μm) of brain tissue ($n = 5$) were obtained on a freezing microtome for immunohistochemistry. H_2O_2 3% (endogenous peroxidase inhibitor) was added to each well to incubate the slices at room temperature for 30 min. Sections were incubated with primary anti-MCT2 antibody (diluted 1 : 400; SANTA CRUZ, USA) at 4 °C overnight followed by incubation with a secondary antibody (diluted 1 : 500; Santa Cruz, USA). MCT2-positive cells were stained brown, and haematoxylin was used to visualize the nuclei of all cells. Images of MCT2 deposition in the cortex, hippocampus and cerebellum were captured using an optical microscope (DMI4000; Leica Microsystems, Germany). The average labelled positive area (positive area/total area of field) from three slices for each mouse with three fields of view/slice was used for statistical analysis.

Post-embedding immunogold electron microscopy

The procedure for post-embedding immunogold electron microscopy was adapted from Bergersen and colleagues. Ultrathin sections were incubated overnight with primary antibodies diluted in TBST containing 2% HSA (albumin from human) (MCT2, diluted 1 : 400, Professor A. P. Halestrap). Bound antibodies were visualized via incubation for 2 h with secondary immunoglobulin conjugated with 10-nm-diameter colloidal gold (goat anti-rabbit, diluted 1 : 20, Abcam, UK). Sections were observed using a Tecnai 12 electron microscope (FEN, USA). Please refer to the detailed operation steps (Bergersen et al. 2008). Photographs were taken randomly from microvessels and neurons in hippocampus and granular and molecular cell layers of the cerebellum.

Quantitative immunogold analysis

Immunohistochemistry staining results were analysed using one-way ANOVA, and comparisons between groups were performed in SPSS. A quantitative analysis was performed of electron microscopic immunogold micrographs taken randomly from the sample. For each antibody solution and animal, 20 microvessels from the hippocampus and cerebellum (10 in the hippocampus, five in the granular cell layer and five in the molecular layer) were analysed. For each micrograph, luminal, abluminal membrane or astrocyte end feet were marked using ImageJ software with the Point density plug-in. The density of immunogold particles including 25 nm on each side of the membranes was calculated using the software.

Forty neuron micrographs with MCT2-labelled synapses from the hippocampus and cerebellum (20 in the hippocampus and 20 in the molecular layer of the cerebellum) were analysed for each animal. The percentage of labelled MCT2 synapses within each micrograph was calculated, and the difference between the hippocampus area and molecular layer was compared. The density of labelled MCT2 in the postsynaptic area (PSD) was analysed by calculating 30 synapses (15 in the hippocampus and 15 in the molecular layer) of each animal.

Statistical analysis

All western blot, immunohistochemistry and quantitative electron microscopic data are represented as the mean ± standard error of the mean ($n = 5$). The statistical significance of the western blot and immunohistochemistry data was determined using one-way ANOVA, and the statistical significance was determined using unpaired two-tailed Student's *t*-tests unless otherwise stated. The null hypothesis was rejected at the 0.05 level.

Results

MCT2 expression

MCT2 expression was detected in the cortex, hippocampus regions and cerebellum of the brain. The protein level in the cerebellum was the highest of the three areas, but no significant difference between the cortex and hippocampus was observed (Fig. 2A,B).

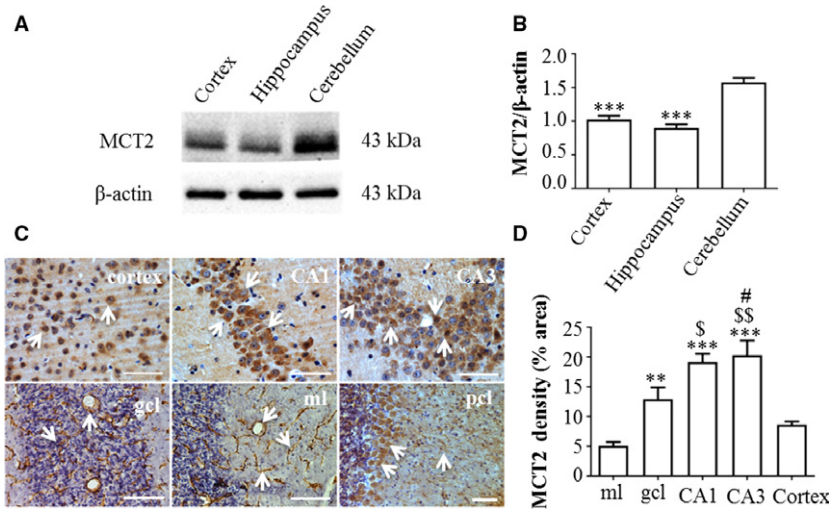

Fig. 2 MCT2 expression in subregions of the brain. (A) MCT2 was detected in wild-type mice, and its level in different regions of the brain was measured using western blots. β-Actin was used as the control. (B) The expression of MCT2 in the cortex, hippocampus and cerebellum (n = 6) was analysed using SPSS 21.0 software (SPSS IBM, Armonk, NY, USA). (***$P < 0.001$ compared with the CB). (C) MCT2-positive areas present on neurons in the hippocampus, cortex, molecular layer, granular cell layer and Purkinje cell layer. Scale: 100 μm (400×: hippocampus, cortex, molecular layer, granular cell layer; 200×: Purkinje cell layer). (D) Average density of MCT2-positive areas (mean ± SEM) in the cerebellum, hippocampus and cortex (n = 6). (**$P < 0.01$, ***$P < 0.001$ compared with the ml; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the cortex; $^{\$}P < 0.05$ compared with the gcl). CA1, CA3, subfields of hippocampus; ml, molecular layer of cerebellum; gcl, granular cell layer of cerebellum.

MCT2 immunohistochemistry

Immunohistochemistry revealed a widespread network of MCT2-positive labelling in brain regions. MCT2 was clearly visible on the cell membrane of neurons in the CA1 and CA3 hippocampal formation, cortex and cerebellum (Fig. 2C). In particular, the labelled densities of MCT2 in CA1 and CA3 were significantly higher than that in the cortex ($^{\$}P < 0.05$, $^{\$}P < 0.05$, Fig. 2D), but there was no statistical difference between the two areas (Fig. 2D). MCT2 immunoreactivity in the cerebellum tissue was found in Purkinje neurons (Fig. 2C) and mossy fibres of the molecular layer and granular cell layer (Fig. 2C) and end feet. The densities of MCT2 in the granular cell layer and CA1 and CA3 were dramatically higher than in the molecular layer (**$P < 0.01$, ***$P < 0.001$, Fig. 2D). The density in CA3 was higher than that in the granular cell layer ($^{\#}P < 0.05$, Fig. 2D). However, no statistical difference between the cortex and granular cell layer or molecular layer was found (Fig. 2D).

Density of MCT2 on astrocytic end feet around microvessels and synapses

Microvessel photographs of the hippocampus and cerebellum revealed MCT2 labelling by immunogold on the end feet of astrocytes (Fig. 3A). MCT2 density was lower on end feet of astrocytes in the molecular layer of cerebellum than on end feet of astrocytes in the hippocampus or in the granular cell layer of the cerebellum (**$P < 0.01$, Fig. 3B). However, the percentage of labelled MCT2 asymmetric synapses in the molecular layer of the cerebellum was over 80% and was < 10% in the hippocampus (***$P < 0.01$, Fig. 4A,B). The density of MCT2 in the PSD area from the molecular layer was higher than that in the hippocampus (***$P < 0.01$, Fig. 4A,C).

Discussion

A continuous and adequate fuel supply is greatly needed for the brain to maintain normal functioning and intracerebral metabolism homeostasis. This viewpoint is emphasized in the observation that the brain is responsible for 20% of the total body resting metabolism but only constitutes 2% of the total bodyweight (Rolfe & Brown, 1997). Therefore, it is not surprising that metabolic maladjustment may have detrimental consequences on physiological brain activity, and improvements of brain performance depend on efficient metabolic substrates (Attwell & Laughlin, 2001; Aubert et al. 2005). The present research focused on monocarboxylates and its transporters for profound investigation.

Many *in vitro* and *in vivo* studies demonstrated that monocarboxylates were sometimes used by neurons as fuel for aerobic brain metabolism (Ivanov et al. 2011; Wyss et al. 2011). Monocarboxylates include ketone bodies and lactate, and many experiments demonstrate that these substances are critical for normal brain function (Hassel & Brathe, 2000). First, ketone bodies, i.e. acetoacetate and beta-hydroxybutyrate, may be necessary to maintain normal mitochondrial function (Bough et al. 2006). Secondly,

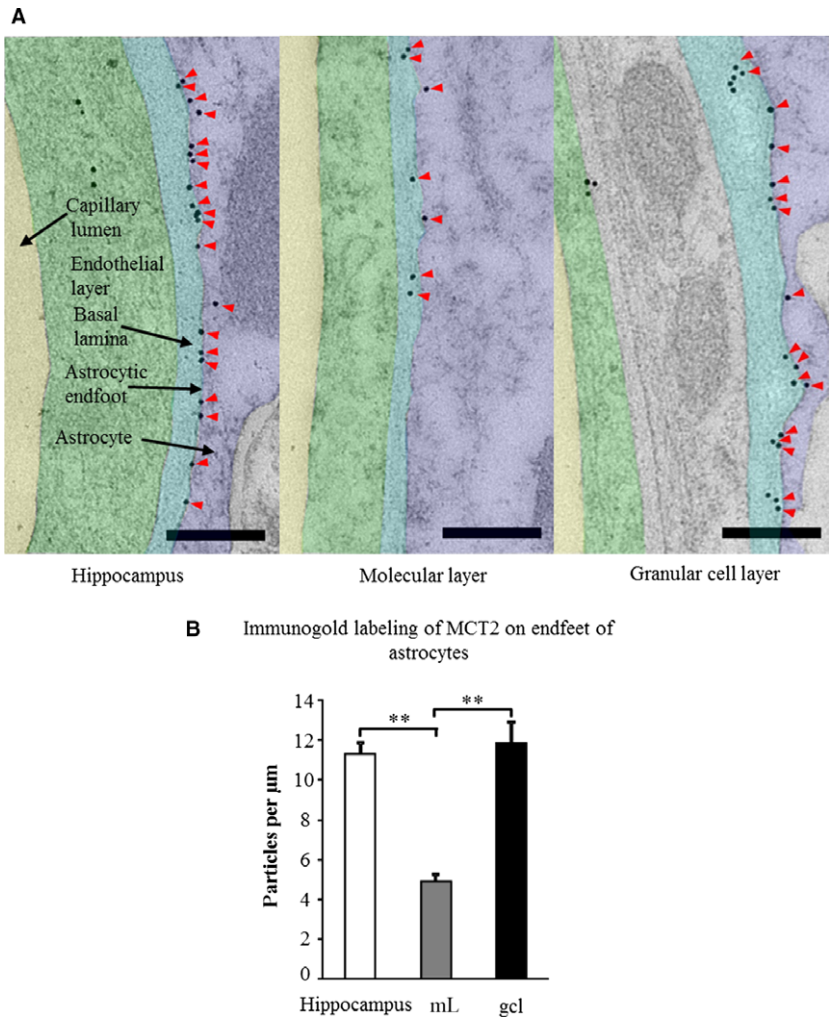

Fig. 3 Immunogold labelling of MCT2 in CA1 of the hippocampus and cerebellum. (A) Electron micrographs illustrate the distribution of MCT2 labelled by immunogold particles (red arrowheads) on astrocytic end feet around microvessels. Scale bar: 200 nm. Yellow, capillary lumen. Green, endothelial layer. Blue, basal lamina. Purple, astrocyte. (B) The density of immunogold labelling of MCT2 on astrocyte end feet in molecular layer is lower than that in the hippocampus and granular cell layer. (**$P < 0.01$ compared with the ml). ml, molecular layer of cerebellum; gcl, granular cell layer of cerebellum.

acetoacetate adjusts vesicular glutamate release (Juge et al. 2010). Thirdly, beta-hydroxybutyrate may regulate the transmission of excitatory neurotransmitters directly (Chmiel-Perzynska et al. 2011). Finally, ketone bodies protect the activity of neurons (Yudkoff et al. 2004). Relative to ketone bodies, lactate is an important carbon source for other brain cells, especially neurons. The astrocyte-neuron lactate shuttle model suggests that neurons use the lactate released from astrocytes for oxidative metabolism (Pellerin & Magistretti, 1994), and the increased production of lactate from glucose decomposition may regulate MCT1 and MCT2 synthesis via specific feedback mechanisms.

Several studies have demonstrated that the monocarboxylate transporter MCT2 is strongly expressed in the central nervous system (Koehler-Stec et al. 1998; Bergersen et al. 2002; Pierre et al. 2002), and many studies reported that MCT2 protein expression was strong and widespread in different cortical areas of the human brain (Chiry et al. 2008). MCT2 expression is also common in the rodent brain (Tekkok et al. 2005; Cheng et al. 2012). Previous articles have shown that MCT2 is a prominent neuronal and astrocyte transporter at the cellular level (Price et al. 1998), and it is consistent with current research results. The author found that MCT2 was mainly expressed in neurons and astrocyte end feet in the cortex, hippocampus and cerebellum. Most interestingly, the expression of MCT2 is highest in the cerebellum, and further study found that the density of MCT2 in the CA1 and CA3 of hippocampus was higher than that in the molecular layer or the granular cell layer of cerebellum. At the subcellular level, colocalization with the postsynaptic protein PSD95 indicates that MCT2 is enriched postsynaptically in the human brain (Pierre et al. 2002) as in

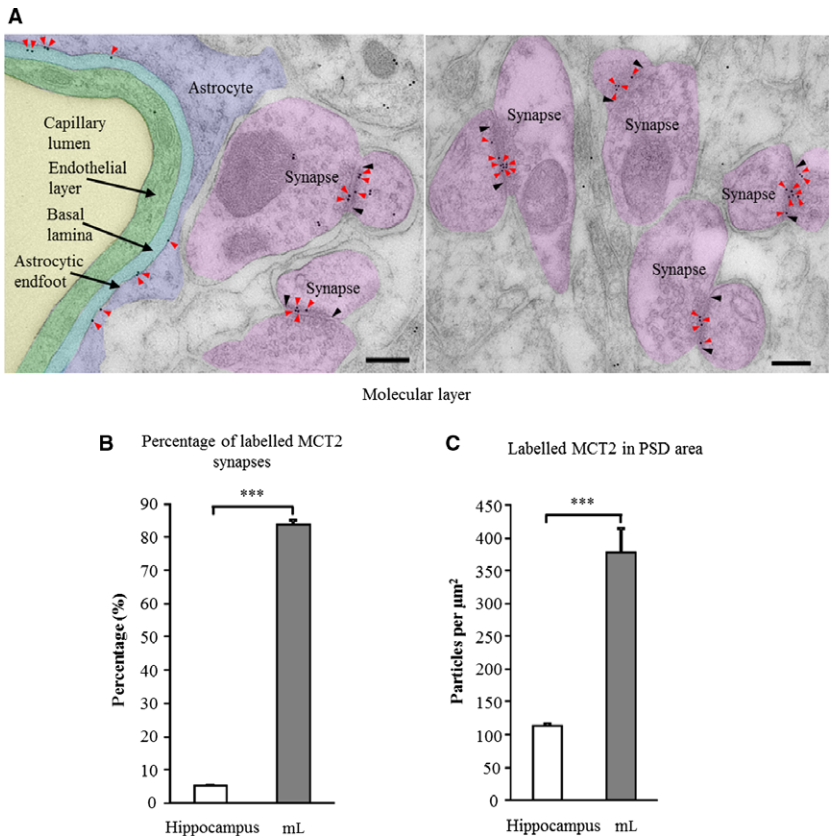

Fig. 4 Electron micrographs show MCT2 labelling in the end feet of astrocytes and postsynaptic densities in the molecular layer of cerebellum. (A) In the molecular layer of the cerebellum, low expression of MCT2 labelling (red arrowheads) is seen on the end feet of astrocytes around microvessels and high expression in PSD (between black arrowheads) of asymmetric synapses. Scale bar: 200 nm. Yellow: capillary lumen. Green, endothelial layer. Blue, Basal lamina. Purple, astrocyte. Pink, neuron. (B) Percentage of labelled MCT2 asymmetric synapses in the molecular layer is much higher than that in the CA1 of hippocampus. (***$P < 0.001$ compared with the ml). (C) Labelled asymmetric synapses in the molecular layer have higher MCT2 density in postsynaptic area than in CA1 of hippocampus (***$P < 0.001$ compared with the ml). ml, molecular layer of cerebellum; gcl, granular cell layer of cerebellum.

rodent models (Bergersen et al. 2001, 2002; Bergersen, 2015). Electron microscopy confirmed this specific localization at asymmetric synapses, and research results revealed that there were MCT2 labels in the astrocyte end feet of the microvascular periphery in hippocampus, molecular layer and granular cell layer. Simultaneously, the author also found that MCT2 existed in the asymmetric synapses and postsynaptic membrane of hippocampus and molecular layer. Although the density of MCT2 was the lowest in the astrocyte end feet of molecular layer, the highest MCT2 density was found in the asymmetric synapses and postsynaptic membrane of molecular layer.

MCT2 is expressed in structures where lactate may be produced in the brain, and its concentration and density may be transferred to the absorption and utilization of lactate. The current study demonstrated the expression of MCT2 in different brain regions, including cortex, hippocampus and cerebellum. The authors further found MCT2 expression in astrocyte end feet, asymmetric synapses and postsynaptic membranes of different tissues. These findings in this article provide a basis for the study of MCT2-related neurodegenerative diseases. MCT2 was associated with the severity of metabolic alterations and it may be a potential biomarker under pathological conditions. However, we only performed relevant studies on wild-type mice. Little is known about the completed mechanism of these phenomena, but our results provide a basis for further research on lactate and related protein molecules.

Conclusions

The present study demonstrated that the density of MCT2 was greatest in CA3 among the various subregional areas in the brain. The expression of MCT2 around microvessels in hippocampus and the granular cell layer of cerebellum was stronger than expression in the molecular layer, and MCT2-labelled synapses in the molecular layer exhibited a higher quantity than the hippocampus.

Acknowledgements

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Conflict of interest

The authors declare no competing financial interests.

Authors' contributions

Cheng Chang and Xiaoqun Gao conceived the study, and Rui Qi Pang, Xiao Fan Wang conducted the experiments. Rui Qi Pang, Zhi Qiang Du performed the data analysis. Zhi Yue Li, Li Bing Sun, Shu Ying Wang, Ying Nan Peng, Xu Peng Lu performed the mouse brain dissections. Rui Qi Pang, Xiao Fan Wang, Fei Fei Pei wrote the article.

Data availability statement

Datasets are centralized in the authors' data folder and are publicly visible if necessary.

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