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Postnatal alteration of monocarboxylate transporter 1 expression in the rat corpus callosum

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Short title: Postnatal MCT1 expression in corpus callosum

Summary In the central nervous system (CNS), monocarboxylate transporter 1 (MCT1) is expressed in astrocytes and endothelial cells but also in oligodendroglia. Oligodendroglia support neurons and axons through lactate transportation by MCT1. Limited information is available on the MCT1 expression changes in candidate cells in the developing rat brain, especially in corpus callosum which is the most vulnerable area in demyelinating diseases. In the present study, we investigated the expression pattern of MCT1 during postnatal development in the rat corpus callosum using immunofluorescene staining, western blotting analysis and RT-PCR. We reported that MCT1 gene and protein were consistently expressed in the rat corpus callosum from birth to adult. MCT1/CNPase and MCT1/GFAP immunofluorescence staining demonstrated that most of MCT1 positive cells were co-labeled with cyclic nucleotide 3′ phosphodiesterase (CNPase) in rat corpus callosum from P7 to adult, whereas $MCT1^+/GFAP^+$ cells preserve the dominate position before P7. Moreover, There were significant associations between the expression of MCT1 protein and the expression of myelin basic protein (MBP) protein (correlation coefficient: r=0.962, P=0.009) from P7 to adult. Similarly, the MCT1 mRNA expression was also significantly associated with MBP mRNA expression (r=0.976, P=0.005). Our results are proposing that in the developing brain white matter, MCT1 is predominately expressed in oligodendrocyte though it mainly expressed in astrocyte in early postnatal, which indicate that MCT1 may involve in the oligodendrocyte development and myelination.

Key words MCT1 · Corpus callosum · Oligodendrocyte · Astrocyte· Rat

Introduction

Monocarboxylate transporters (MCTs) are transmembrane proteins that transport short chain monocarboxylates such as lactate, pyruvate and butyrate, along with protons, down their concentration gradient across membranes (Pierre and Pellerin 2005). Currently, fourteen members of this transporter family have been identified by sequence homology, of which only the first four members (MCT1-MCT4) have been shown to mediate the proton-linked transport of monocarboxylates. MCT1, along with MCT2 and MCT4, is the predominant transporter among the MCT isoforms and is present in almost all tissues (de Araujo *et al.* 2015), including the central nervous system (CNS) (Vijay and Morris 2014, Pierre and Pellerin 2005), whereas MCT3 is exclusively expressed in the retinal pigment epithelium (Philp *et al.* 1998, Philp *et al.* 2001). Consequently, MCT1 is important for the regulation of metabolic homeostasis in the CNS.

MCT1 is expressed in oligodendroglia and a few specific neuronal populations (Rinholm *et al.* 2011, Lee *et al.* 2012), although it may be present in much smaller amounts in astrocytes and endothelial cells (Pellerin *et al.* 1998b, Hanu *et al.* 2000). Experiments have shown that MCT1 expression and lactate transport is considerably greater in oligodendroglia than astrocytes (Lee *et al.* 2012). MCT2 is expressed primarily in neurons (Rafiki *et al.* 2003, Pellerin *et al.* 1998a, Bergersen *et al.* 2002) and MCT4 in astrocytes (Pellerin *et al.* 1998a, Bergersen *et al.* 2002). Oligodendroglia cells are considered as a significant site of MCT1 expression in the CNS and are the principal metabolic supplier of lactate to axons and neurons (Lee *et al.* 2012). In cultured oligodendrocytes, lactate is not only used to fuel the mitochondria but also for lipid synthesis, presumably to make myelin (Sanchez-Abarca *et al.* 2001).

Before and immediately after birth, lactate which is transported across cell membranes by MCTs from the blood is also an important energy source (Nehlig and Pereira de Vasconcelos 1993, Erecinska *et al.* 2004). There has some evidence suggesting that MCT expression may vary during development (Pellerin *et al.* 1998b, Leino *et al.* 1999). Both mRNA and protein expression of MCT1 were increased 25-fold in 17-day-old rat pups compared with adults (Leino *et al.* 1999). In addition, mRNA levels for both MCT1 and MCT2 in mice brain were at least seven times higher at postnatal day 15 than in the adult (Pellerin *et al.* 1998b). During the first postnatal week, MCT1 immunoreactivity extended massively to the vessel walls and moderately to the developing astrocytes in the cortex, which suggesting that expression of MCTs throughout the perinatal period has a potential relationship with the maturation of the blood-brain barrier (Baud *et al.* 2003). However, little is known about MCT1 expression is associated with the development of what kinds of cells in the developing CNS. Characterization of the cellular type of MCT1 expression during development would provide insight into the neonatal cerebral metabolism and the clinical disorders related to hypoxic-ischemic insults or energy deprivation.

In the current study, we used RT-PCR and western blotting techniques to investigate the gene and protein expression of MCT1 in the developing rat brain corpus callosum from birthday to adult. Double-labeling and confocal microscopy helped us to identify the cellular type in which MCT1 is expressed and to elucidate the time course of its expression during development.

Methods

Experimental animals

Pregnant Sprague-Dawley rats obtained from the center of experiment animal of Xuzhou Medical University were housed in Plexiglas cages individually and checked daily for delivery. The pregnant rats were kept on a 12-h light/dark cycle, with food and water freely available. After delivery, pups of P0 (the day of birth), P7, P14, P21 and P28 and P42 were sacrificed at the corresponding time point, respectively. There were 15 rats in every time point. All experimental procedures were conducted in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China. The animal protocol was approved by the Animal Use Committee of Xuzhou Medical University.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was used to detect the expression of MCT1, MBP, GFAP and vWF mRNA in the rat corpus callosum at different postnatal time points. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) following instructions from the manufacturer. One microgram of total RNA was first reverse-transcribed into cDNA using PrimeScript^{TM} RT Master Mix Kit (TAKARA BIO INC., Cat. #RR036A). Subsequently, PCR was carried out by 2×Taq PCR MasterMix Kit (TIANGEN Biotech, Beijing, China, Cat.KT201). GAPDH was considered to be an internal control. Specific primers were available from Integrated DNA Technologies (Sangon Biotech, Shanghai, China). The sequences of specific primers were given as follows, MCT1: sense 5’-GTGACTGGTCGGTCGTGTAG-3’, antisense 5’-GCAGGTGGCATCTTAGGTGT-3’, product length 195 bp; MBP: sense 5’-AGGCGTAGAGGAACTATGGGT-3’, antisense 5’-CAGAGTGAC ACCCAATCGCA-3’, product length 234 bp; GFAP: sense 5’-TCTCGAATGACGCCTCCACT-3’, antisense 5’-GAAGCGGACCTTCTCGATGT-3’, product length 150 bp; vWF: sense 5’-AGTCC TGATGTCAGACAGACA-3’, antisense 5’-GAGGAGCTGGTGCAGTTAGT-3’, product length 201 bp; GAPDH: sense 5’-CCATTCTTCCACCTTTGATGCT-3’, product length 98 bp, antisense 5’-TGTTGCTGTAGCCATATTCATTGT-3’. PCR products were resolved by 2% agarose gel electrophoresis, stained by GelRed (BIOTIUM, USA) and visualized under ultraviolet light. Images were analyzed by imageJ software, and the results were given in relative fold of MCT1/GAPDH, MBP/GAPDH, GFAP/GAPDH and vWF/GAPDH.

Western Blotting

To study the expression change pattern of MCT1, MBP, GFAP and vWF in postnatal rat corpus callosum, the expression levels were quantified by western blotting. Tissue samples were micro-dissected and homogenized in RIPA lysis buffer with protease inhibitor cocktail which containing 0.1 % Nonidet P-40, 1 mM dithiothreitol, 10 mM EDTA, 40 mM Tris-HCl (pH 7.4), 120 mM NaCl for 30 min on ice to promote lysis, and then spun down at 12,000rpm for 10 min at 4 ℃. Protein concentrations were quantified using BCA assay (Beyotime Institute of Biotechnology, China). Equal amounts of protein (20 µg/lane) were separated on 10% SDS-PAGE gels, and the protein was transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in PBS for 1 h and then incubated overnight at 4 ℃with the following primary antibodies: anti-MCT1 (abcam 1:1,000), anti-MBP (chemicon 1:800), anti-GFAP (sigma 1:2,000) and anti-vWF (abcam 1:1,000). After three washes with Tris-Tween buffered saline, membranes were subsequently incubated with fluorescent secondary antibodies (IRDye 700 or IRDye 800) for 2 h at R/T on a shaker. Lastly, membranes were detected by Odyssey Infrared Imaging System (LI-CON). Band size and density measurements from each sample were collected using ImageJ. Values were normalized by the levels of β-actin.

Immunofluorescence

Rats were anesthetized and perfused transcardially with 0.9% saline, followed by 0.1 M phosphate buffer containing 4% paraformaldehyde (PFA) at each time point. Then, brains were removed then post-fixed in 4% PFA at 4℃for 12 hours. The fixed tissue was embedded in paraffin after dehydrated and 6μm sections were cut on a microtome (Leica, Germany), and then mounted on slides. To investigate the expression changes of MCT1 on the separate cell type in postnatal rat cc, immunofluorescence was performed. After rehydrated sections, antigen retrieval was achieved by microwave using the sodium citrate solution with pH 6.0. After incubation with a blocking reagent (10% goat serum diluted in 0.3% PBS) for 1 h at room temperature, all sections were incubated with mouse anti-MCT1 (1:200) and rabbit anti-MBP (1:500) /anti-GFAP (1:1000), respectively, overnight at 4℃. Stains with PBS served as negative controls. Then sections were incubated with secondary antibodies Alex488-conjugated goat anti-mouse and CY3-conjugated goat anti-rabbit IgG (all 1:200; all from Amersham Biosciences Ltd. GE Healthcare, USA) for 0.5 h at 37℃. Fluorescence signals were visualized by the confocal laser scanning microscope system (FV10i, Olympus, Japan). Images were analyzed using Image-Pro Plus software (Media Cybernatics, Inc., Silver Spring, MD, USA).

Statistical analyses

Significance was calculated using SPSS17.0 software (SPSS Inc., Chicago, IL, USA).All of the date was represented as the mean ± SD. Statistical analysis was carried out with a one-way analysis of variance (ANOVA). The Pearson correlation analysis was performed on RT-PCR and Western blotting results for the correlation of MCT1 expression between MBP/GFAP/vWF expressions. $P<0.05$ was considered statistically significant.

Results

MCT1 is predominately co-located with CNPase in developing corpus callosum

We detected the MCT1 expression in main candidate cells by double immunofluorescence staining. The results showed that minority cells in the corpus callosum were MCT1$^+$ cells at P0. At P14 the number of MCT1$^+$ cell sharply increased and up to the summit at P21, and then retained a relatively stable standard till adulthood (Fig. 1, 2). CNPase positive cells at P0 were relatively poor (Fig. 1), at this time, about 20% MCT1$^+$ cells co-labeled with CNPase (Fig. 3). At P7, amount of MCT1$^+$/CNPase$^+$ cells ascend to about 39%. Since then, the proportion of MCT1$^+$/CNPase$^+$ cells began to sharply increase, and remained at around 70% of all MCT1$^+$ cells in the corpus callosum to adulthood (Fig. 3). GFAP, a marker of astrocyte, was expressed relatively abundant at P0 (Fig. 2) compared with CNPase, there was 70% MCT1$^+$ cells co-labeled with GFAP (Fig. 3). After that time point, the amount of double positive cells began to decline. At P14, the proportion of MCT1$^+$/GFAP$^+$ cells fell to 30% and then continued to decrease to about 19% at P21 and remained to adulthood (Fig. 3).

The protein expressions of MCT1 and MBP were linear correlation

We used the western blotting to detect the protein changes of MCT1 at different time points after P7 (Fig. 4A). Results revealed that there was a gradual upward trend after P7, followed by a slight decrease at P21, and then slowly increased to P28 and maintain a stable level (Fig. 4B). In order to detect the development alteration of candidate cells which could express MCT1 in the corpus callosum, we also detected the MBP (another marker of oligodendrocyte), GFAP and vWF (a marker of endothelial cell) protein expression, respectively (Fig. 4A). Results showed that MBP protein had a relatively low abundance at P7. It began to gradually increase with the development and then peaked at P28 and holding a relatively steady state into adulthood. The expression of GFAP protein at P7 was also relatively lack, and then gradually rose to the peak at P42. The vWF protein expression was relatively more at P7, followed by a decrease at P14, and then ascended once again and reached the highest level at P42 (Fig. 4B). Based on the statistical analysis, it was found that there was a statistically significant linear correlation between MCT1 protein expression and MBP protein expression (r=0.962, P=0.009), whereas the correlation coefficients of MCT1 protein expression between GFAP and vWF protein expression were 0.814 (P=0.093) and 0.790 (P=0.112), respectively (Fig. 4C).

The mRNA expressions of MCT1 and MBP were linear correlation

For observing the situation MCT1 expression in the corpus callosum at gene level at different time points after P7, RT-PCR was carried out (Fig. 5A). The results showed that MCT1 gene expressed relatively few at P7, followed by a gradual upward tendency. It had its climax at P28 and then relatively decreased at P42 (Fig. 5B). To explore the developmental changes of MCT1-expressed cells in the corpus callosum, we also detect the gene expression changes of MBP, GFAP and vWF in different time point, respectively (Fig. 5A). The results showed that from the P7, MBP gene expression was a relatively steady increase trend then reached the highest at P28, also followed by a faint decline at P42. GFAP gene expression was relatively abundant at P7, and then it was reduced at P14. Subsequently, it gradually increased and reached the peak at P21 and after that it kept a stabilized state until P42. From P7 to P42, the expression of vWF gene remained relatively ascendant stable, except that a slight reduction at P14 (Fig. 5B). Statistical analysis showed that coefficient of correlation of MCT1 gene expression between MBP, GFAP and vWF gene expression was 0.976 (P=0.005), 0.834 (P=0.079) and 0.512 (P=0.378), respectively. It can be seen that the correlation between MCT1 gene expression and MBP gene expression was statistically significant (Fig. 5C).

Discussion

Most of the brain's energy demand is supplied by glucose. Additionally, monocarboxylates such as lactate, pyruvate and ketone bodies have been known for some time to represent substantial energy substrates for the brain (Vannucci and Vannucci 2000) through rapidly transport hydrophilic monocarboxylates across the plasma membrane of cells and the blood-brain barrier (Pierre and Pellerin 2005). Monocarboxylates, especially lactate, which has long been regarded as a waste of cellular activity, plays a significant role in brain energy metabolism (Smith et al., 2013). Recently, lactate has been proposed to act also both as a neurotransmitter through specific receptors (Tang *et al.* 2014) and as a modulator of NMDA receptor-mediated synaptic plasticity via a redox effect (Yang *et al.* 2014).

MCT1 expression, both at the mRNA and protein levels, is homogenously distributed throughout the whole rodent brain. Some studies report that the MCT1 mRNA was found to be abundant in the cortex, the hippocampus and the cerebellum of the 15-day-old and adult rodent brain (Vannucci and Simpson 2003, Pellerin *et al.* 1998b). However, in the current literature, the characterization of MCT1 expression in the postnatal rat corpus callosum is little reported. Moreover, there are not any reports regarding the correlation between MCT1 expression and candidate cell development, particularly in the CNS white matter. Therefore, our present studies is aimed at elucidating the time course of MCT1 gene and protein expression and then identify the cellular type in which MCT1 is expressed in the developing rat brain corpus callosum from birthday to adult.

Lactate covers more than 40% of the energy needs of the brain at P14 (Dombrowski *et al.* 1989). Thus, lactate oxidase is a major pathway to obtain the energy for newborn mammalian nervous system. The MCT family, also called SLC 16 gene family, is the essential transmembrane protein which transported monocarboxylic acid out of or in mammalian cells. Interestingly, MCT1 could transport lactate bi-directionally, being dependent upon the relative intracellular and extracellular concentrations of substrates (i.e., lactate and hydrogen ions) (Pierre and Pellerin 2005). It is extremely vital for cellular metabolism and homeostasis. Thus, MCT1 had proven to be widely distributed in almost all tissues, including brain, heart, muscle (Pierre and Pellerin 2005, Halestrap and Price 1999) , lymph node (Zheng *et al.* 2014), and so on.

In the present study, as shown in previous reports (Pellerin *et al.* 1998b, Vannucci and Simpson 2003), our results showed that MCT1 is largely expressed throughout the brain of rats, both in the neonatal period and adulthood. Previous papers have shown that MCT1 protein was abundantly expressed in microvessels endothelial cells and astrocytes not only in young but also in the adult rodent brain (Pierre *et al.* 2000, Gerhart *et al.* 1997). Thus, the temporal and spatial MCT1 expression was considered a potential relationship with the maturation of the blood-brain barrier in the brain (Baud *et al.* 2003). Recent studies have demonstrated that MCT1 is highly enriched within oligodendrocytes (Lee *et al.* 2012) in the CNS, and suggested that oligodendroglia are critical intermediaries for lactate transport to neurons (Funfschilling *et al.* 2012, Lee *et al.* 2012, Morrison *et al.* 2013). Oligodendrocytes develop from oligodendrocyte precursor cells (OPCs) which, during the early postnatal (suckling) period, develop into immature oligodendrocytes and then into mature oligodendrocytes (Rinholm *et al.* 2011). Lactate can support oligodendrocyte development and myelination. In CNS diseases involving energy deprivation at times of myelination or remyelination, such as periventricular leukomalacia and multiple sclerosis, lactate transporters in oligodendrocytes may play an important role (Rinholm *et al.* 2011). Oligodendrocyte-specific down regulation of MCT1 in both the optic nerve and corpus callosum was capable of producing axon degeneration (Lee *et al.* 2012). In addition, new experiments suggested that MCT1 also present in myelinated Schwann cells in peripheral nervous system and they can participates in the regulation of the Schwann cells myelination program and mediate the axon-glial metabolic interactions (Domenech-Estevez *et al.* 2015).

Consequently, we set out to investigate whether the MCT1 expression was related to the development of oligodendrocytes in the rat corpus callosum from birthday to adult. Oligodendroglial markers CNPase (Rasband *et al.* 2005) or astrocytes marker GFAP double labeling showed that in rat corpus callosum, double positive cells of MCT1^{+}/CNPase^{+} had a high percentage than the MCT1^{+}/GFAP^{+} positive cells' after P7 to adult, whereas it has a exactly opposite expression from P0 to P7. We speculated that in the early postnatal period (birthday to P7) when the glucose transporters had not yet matured completely, astrocytes may use monocarboxylates to provide energy through MCT1, and thus play its physiological function. Before P7, oligodendrocytes had not yet fully matured and consequently they can't effectively wrapped axon to form the myelin sheath. Oligodendrocytes can uptake monocarboxylates by Cx43/47 (Morrison *et al.* 2013) or MCT1 (Rinholm *et al.* 2011) released from astrocytes, although there was evidence that oligodendroglia MCT1 transporters are generally exporters, not importers, of lactate (Lee *et al.* 2012), and then converted into lipids through a series of biochemical processes and eventually involved in the myelination (Funfschilling *et al.* 2012). After P7, with the number of matured oligodendrocytes and myelinated axons are gradually increased, lactate (or pyruvate when NADH is oxidized in oligodendroglial mitochondria) can be transferred via MCT1 which reside in internodal myelin (Rinholm *et al.* 2011) into the neuron axonal compartment, such that lactate can rapidly provide energy for them.

In our study, to further confirm that the relationship between MCT1 expression and oligodendrocyte development, RT-PCR and western blotting were used to detect the mRNA and protein expression alteration of MCT1, MBP, GFAP and vWF (a marker of endothelial cell) (Gluhovschi *et al.* 2010) at different time point from P7 to adult, respectively. These results indicate that expression patterns of MCT1 and MBP were most linearly correlated, as demonstrated by our data. Our results demonstrated that in the developing rat corpus callosum, in addition to was expressed in astrocytes, MCT1 mainly expressed in oligodendrocytes. In our future research, we will use genetic technology to study the role of MCT1 in different stages of oligodendrocyte development, and further explore the involved mechanisms.

Our results provide the first evidence that there is a direct correlation between MCT1 expression changes and development of oligodendrocyte in the rat corpus callosum from birth to adulthood; in this way, we speculated that MCT1 may be involved in the postnatal formation of myelin in the CNS. Our results offered a certain amount of experimental evidence to further understand the biology of oligodendrocytes. Moreover, MCT1 may be a novel target for treatment of myelin-based disorders in the future.

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Conflict of interest The authors declare no conflict of interest.

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Figures:

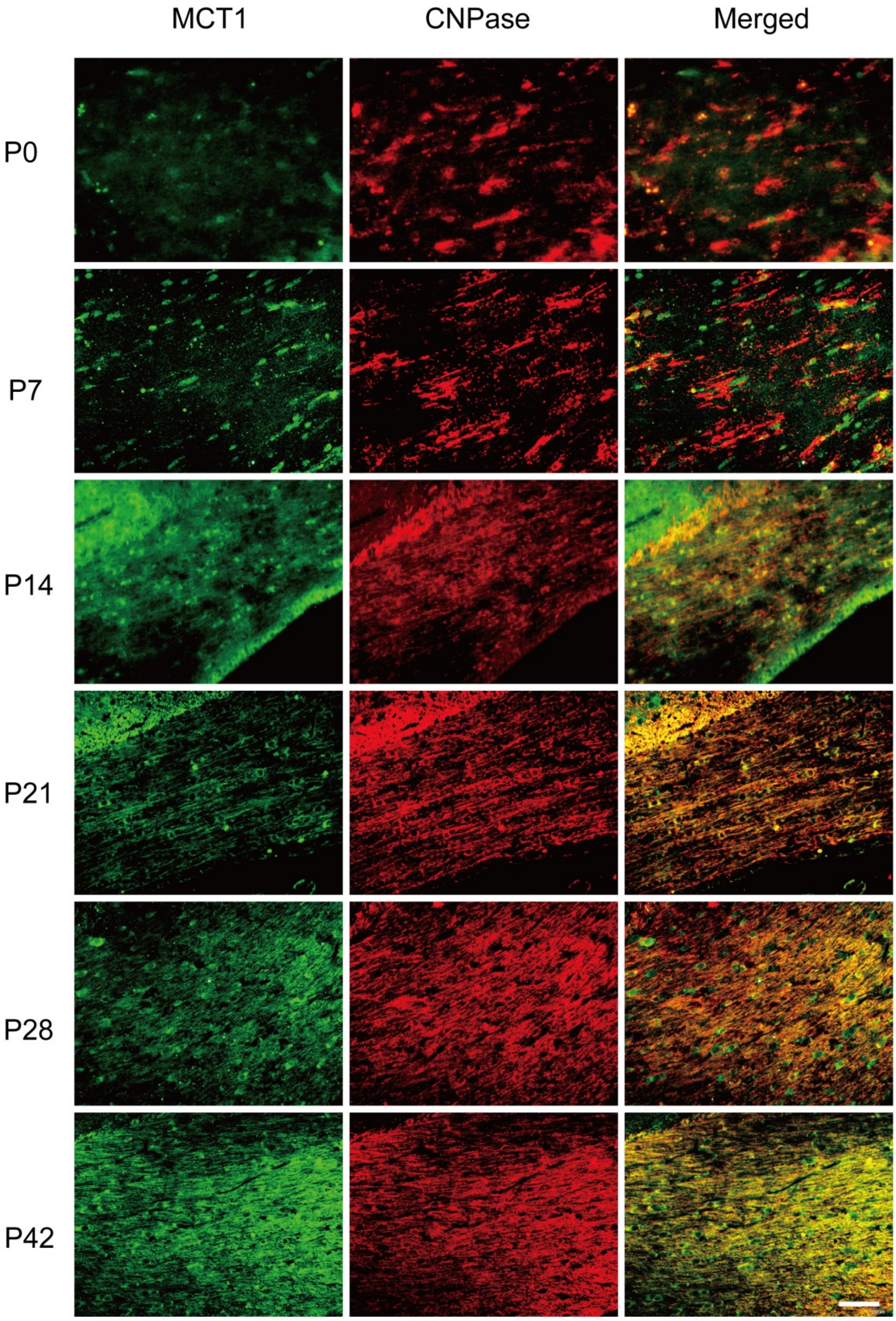

Fig.1. Double Immunostaining for MCT1 and CNPase in the rat corpus callosum after birth to adulthood. Confocal microscopy showing MCT1 immunoreactivity (green) alteration in CNPase (red) positive oligodendrocytes. Scale bars: 20μm.

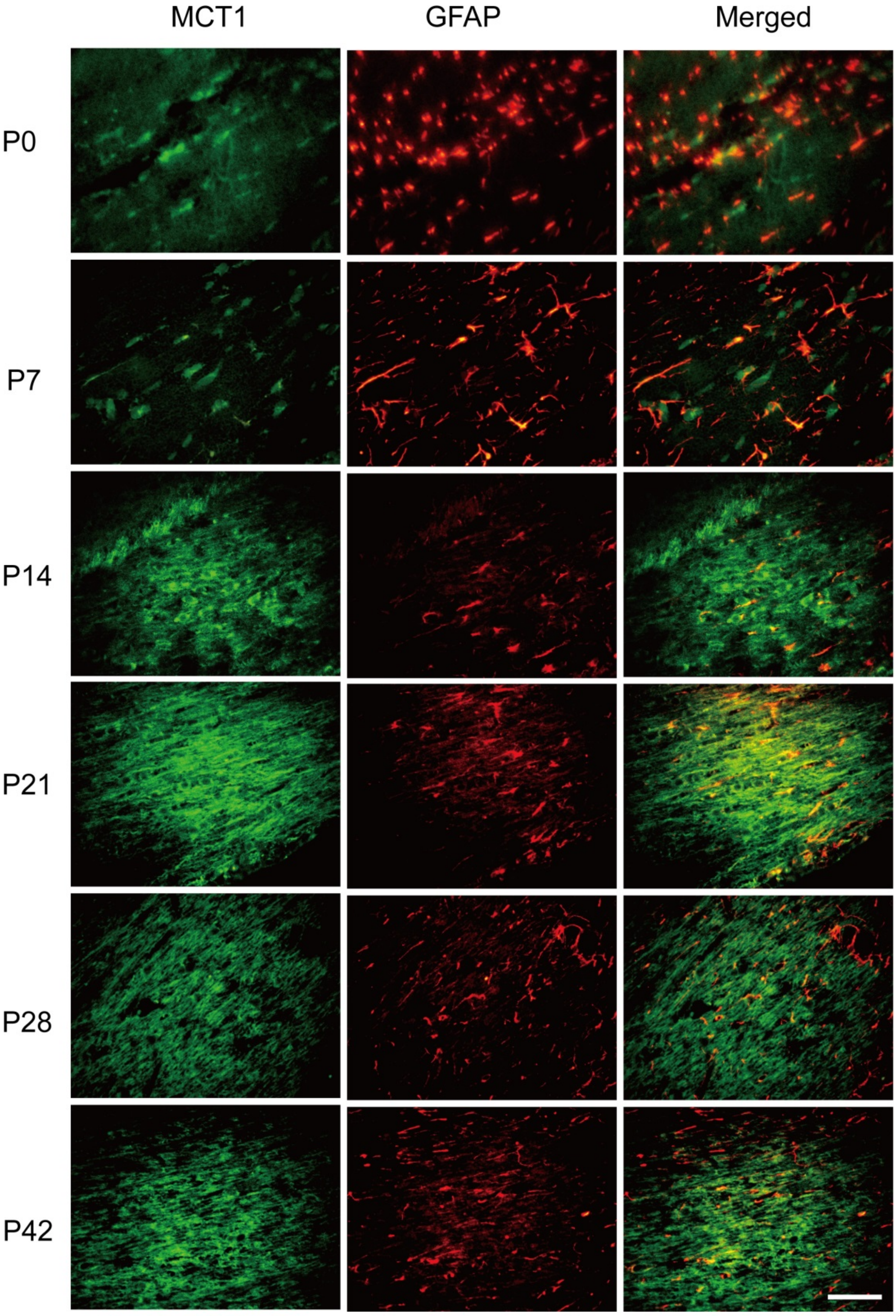

Fig.2. Double Immunostaining for MCT1 and GFAP in the rat corpus callosum after birth to adulthood. Confocal microscopy results showing MCT1 immunoreactivity (green) alteration in GFAP (red) positived astrocytes. Scale bars: 20μm.

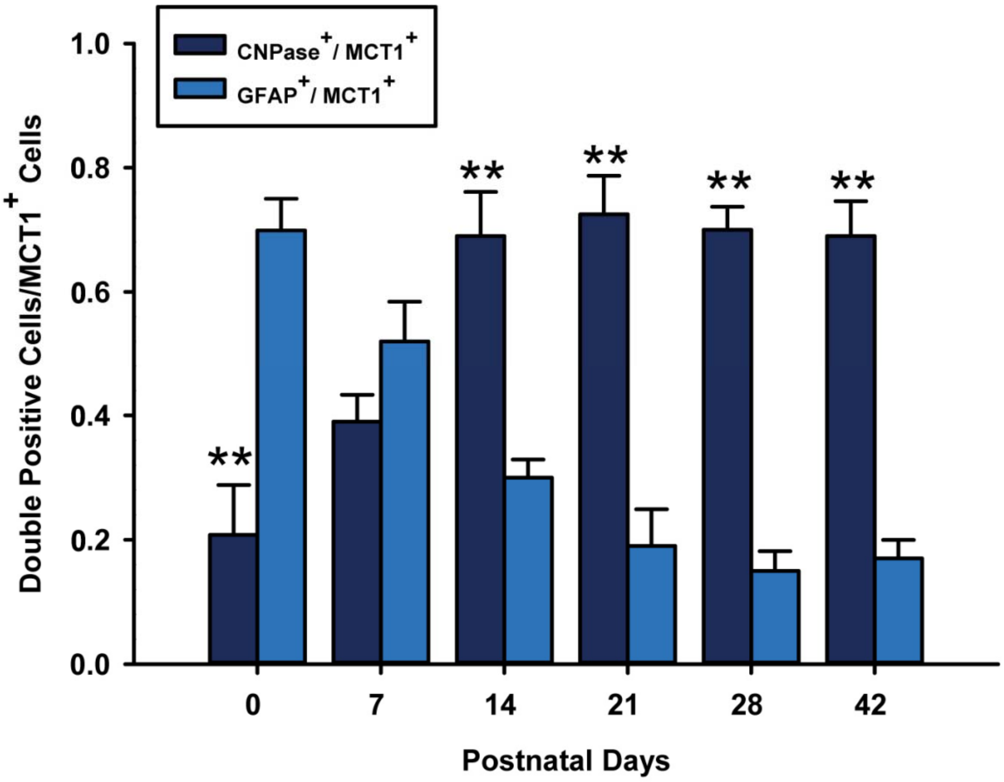

Fig.3. Quantitative evaluations of CNPase$^+$/MCT1$^+$ cells and GFAP$^+$/MCT1$^+$ cells number. Double-label confocal microscopy showing MCT1 was mainly expressed in oligodendrocyte but not in astrocyte of rat corpus callosum after birth to adulthood. The expression levels of double positive cells were normalized to MCT1$^+$ cells. n=5. Values are mean ± SD. $*P<0.05$, $**P<0.01$ versus GFAP$^+$/MCT1$^+$ group.

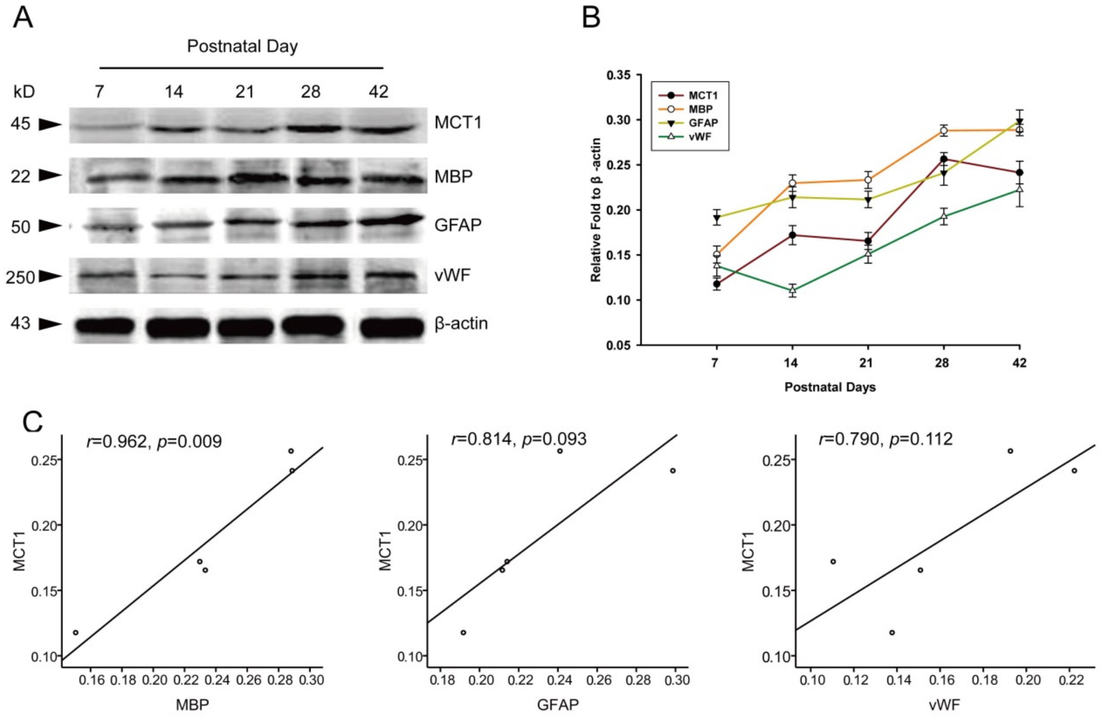

Fig.4. MCT1 protein expression alteration was highly correlated to oligodendrocyte development in the rat corpus callosum after birth to adulthood. **A.** By western blotting, we detected protein expression alteration of MCT1 and MBP, GFAP as well as vWF at P7, P14, P21, P28 and P42. **B.** The bands were quantified by densitometry, normalized to β-actin levels, and expressed as relative fold activation (n=5). Values are mean ± SD. **C.** We studied the correlation MCT1 protein expression alteration between MBP, GFAP and vWF's, respectively. The result indicates that the correlation between MCT1 and MBP is significant (*r*=0.962, *P*<0.01).

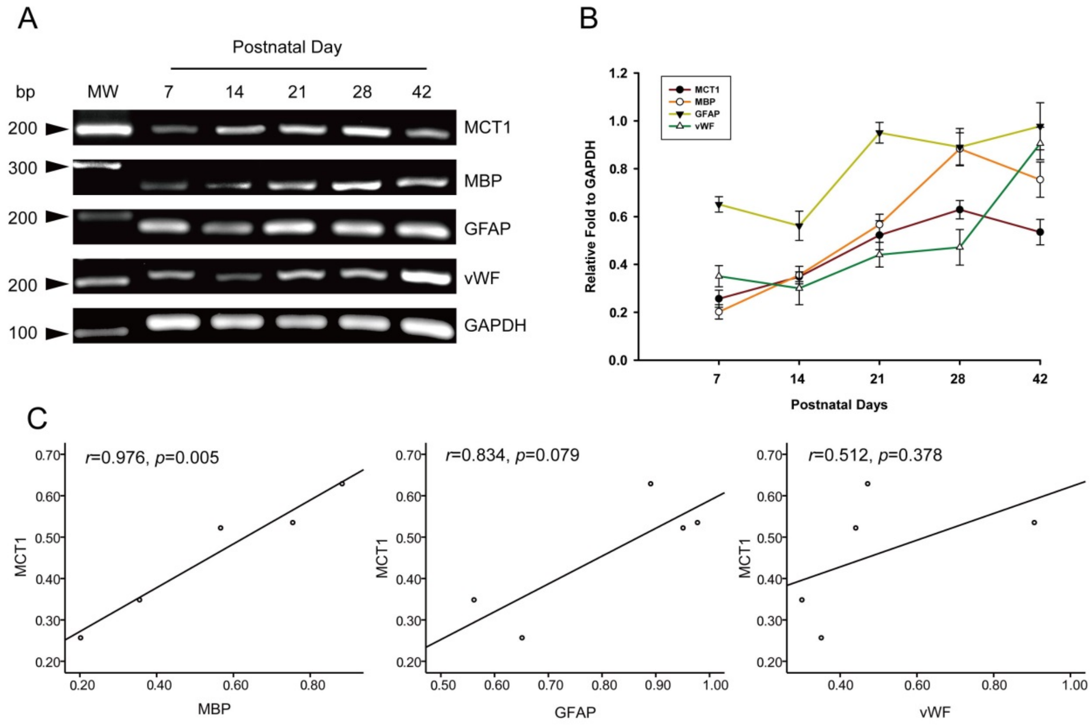

Fig.5. MCT1 mRNA expression alteration was highly correlated to oligodendrocyte development in the rat corpus callosum after birth to adulthood. **A.** By RT-PCR, we detected mRNA expression alteration of MCT1 and MBP, GFAP as well as Vwf at P7, P14, P21, P28 and P42. **B.** The bands were quantified by densitometry, normalized to GAPDH levels, and expressed as relative fold activation (n=5). Values are mean ± SD. **C.** We studied the correlation between MCT1 mRNA expression alteration between MBP, GFAP and vWF's, respectively. The result indicates that the correlation between MCT1 and MBP is significant (r=0.976, P<0.05).