Involvement of Mlc1 in the white matter development and maintenance

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Summary

Astrocytes are one of the major glial cells in the CNS and maintain brain milieu through the blood brain barrier formation, uptake of neurotransmitters and supply of nutrients to neurons. In addition to this well-known function, astrocytes may be involved in the white matter development and /or maintenance. This idea came from the analysis of several glial specific gene deficient mice and human diseases. Glial specific genes, connexin and inwardly rectifying potassium channel (Kir4.1), are involved in the homeostasis of potassium concentration. It has been reported that deletion of connexin or Kir4.1 in glial cells results in the leukodystrophy in mice, (Lutz et al, 2009 ; Mognotti et al, 2011 ; Odermatt et al, 2003 ; Menichella et al, 2006). Moreover, a human disease, Alexander disease, is caused by missense mutations in the GFAP gene, leading to infantile onset leukodystrophy. These previous studies support the idea that astrocyte dysfunction could cause leukodystrophy. However, studies on the relationship between astrocyte and the white matter development/maintenance are still rare. One of the limitations is a lack of mice recapitulating leukodystrophy phenotype through astrocyte dysfunction. In this study, I generated a mouse model for a human disease, Megalencephalic leukoencephalopathy with subcortical cysts (MLC, OMIM604004), to study whether astrocyte dysfunction can lead to leukodystrophy. MLC (OMIM604004) is a rare autosomal recessive neurological disorder with infantile onset characterized by chronic white matter edema, macrocephaly, slowly progressive deterioration in motor function, cerebellar ataxia, spasticity, and mental decline. Two genes, encoding Mlc1 and GlialCAM, were identified to be responsible for MLC (Leegwater et al, 2001; López-Hernández et al,

2011). Mutations in the Mlc1 gene were reported in about two-thirds of MLC patients (Leegwater et al, 2002; van der Knaap et al, 2012). Various mutations in the Mlc1 gene (missense mutations, frame shift and splice site mutations) have been reported in human MLC patients. However, correlation between types of Mlc1 mutations and the severity of MLC clinical symptoms has not been clarified (Leegwater et al, 2002).

Human Mlc1 and murine Mlc1 genes encode a membranous protein with eight transmembrane domains, whose function is unknown. Northern blot analyses demonstrated that both human Mlc1 and murine Mlc1 genes were predominantly expressed in the brain and in situ hybridization demonstrated that Mlc1 mRNA is present in astrocyte lineage cells including astrocytes, ependymal cells and Bergmann glia, but not in oligodendrocytes (Schmitt et al, 2003; Teijido et al, 2004). Thereby, astrocytic abnormalities should be involved in the leukodystrophy of MLC patients. Studies using the heterologous expression of Mlc1 gene demonstrated that Mlc1 mutants failed to be transported to the plasma membrane (Duarri et al, 2008; Teijido et al, 2004). In addition, biopsy samples from a MLC patient harboring missense mutation displayed low expression levels of Mlc1 protein (Teijido et al, 2004). These data suggested that Mlc1 mutants show a loss-of-function effect, and the model animal could be established by generating Mlc1 null mouse.

More importantly, whether MLC1 mutations lead to loss-of-function or gain-of-function was not well addressed so far. Interestingly, some of glia specific gene-related diseases that are inherited in a Mendelian manner were modeled by wild type gene overexpression in mice. Thereby, I generated Mlc1 over expressing mouse. I took advantages of tetracycline-controlled gene induction system and confirmed Mlc1 overexpression (Mlc1 OE) was achieved

in astrocyte lineage cells including astrocytes, ependymal cells and Bergmann glia. Embryonic malformation was not observed in Mlc1 OE mouse even when the Mlc1 overexpression is induced from mid-embryo. At birth the size and the body weight were indistinguishable between Mlc1 OE and the control groups. Mlc1 OE mouse showed growth retardation and mild ataxic gait transiently appeared around postnatal two weeks but these symptoms caught up to the control by three month of age. Moreover, Mlc1 OE mouse was viable at least till 18 month of age, and reproduction was not affected. I then performed histological analysis and found that leukodystrophy is present in the white matter, especially in the corpus callosum and the globus pallidus. Leukodystrophy in the CC appeared from postnatal one to two weeks, and persisted for the whole life. Histological analysis indicated that area with leukodystrophy contained numerous vacuole-like structures by the light microscopic observation. This result indicated that leukodystrophy as seen in human MLC was recapitulated in the mouse when Mlc1 overexpression was induced in astrocytes. By using Mlc1 overexpressing mouse, I studied the process of leukodystrophy formation.

I then examined the ultrastructural feature of the leukodystrophy formation by electron microscopy, and compared them with those of MLC patients. Vacuole-like structure as observed in the light microscopic study was composed of both astrocytic swelling and oligodendrocyte-associating vacuoles. These ultrastructural findings were relevant to those of human MLC. By manipulating the timing of tTA-mediated Mlc1 overexpression from P28 to P38 by DOX administration ($OE^{P28\text{-}P38}$), I found that the astrocytic swelling, but not oligodendrocyte-associated vacuolus, correlated with the extent of tTA-mediated Mlc1 overexpression.

Moreover, astrocytic swelling and oligodendrocyte-associated vacuoles were dramatically improved when Mlc1 overexpression was cancelled after completion of the leukodystrophy formation ($OE^{embryo\text{-}P28}$), resulting in the regression of leukodystrophy lesions. Interestingly, Mlc1 overexpression induced only during young adult stage ($OE^{P28\text{-}P90}$), astrocytic swelling occurred in accordance with the induction of tTA-mediated Mlc1 OE, but niether oligodendrocyte-associated vacuoles nor leukodystrophy phenotype were observed. These results indicated that Mlc1 overexpression primarily caused astrocytic swelling and affected the white matter formation in infantile mouse. However, leukodystrophy lesions did not appear when Mlc1 overexpression was induced after young adult stage even though the astrocytic swelling was present in the white matter, suggesting astrocytes are involved in the white matter development and/or maintenance at the critical period.

Finally, to validate the cell autonomous effect of overexpressed Mlc1, I searched for Mlc1 interacting proteins. Because of the lack of immunoprecipitation compatible anti-Mlc1 antibody, I generated primary antibodies recognizing mouse Mlc1 N-terminus and C-terminus, respectively. By using newly developed anti-Mlc1 antibodies, We screened for Mlc1 interacting proteins by immunoprecipitaton and mass spectrometry analysis, and identified Na^+/K^+ ATPase α subunits, a member of P-type ATPase, as a Mlc1 interacting protein. Na^+/K^+ ATPase α subunit is a component of sodium pump, which is essential for generating electrochemical gradients across the cell membrane. Interestingly, pharmacological and genetic studies indicated that mice with reduced sodium pump activity displayed astrocytic swelling relevant to Mlc1 OE mouse. Thus, I examined whether overexpressed Mlc1 affects sodium pump property using cultured astrocytes. In vitro studies indicated that

sodium pump activity was reduced in Mlc1 overexpressing astrocytes without alterating the amount of Na^+/K^+ ATPase α subunits. Reduced sodium pump activity could be reflected by the decreased cell surface expression of sodium pump, and I measured the amount of cell surface Na^+/K^+ ATPase α subunits by ^3H-ouabain binding assay. Unexpectedly, the amount of cell surface Na^+/K^+ ATPase α subunits increased in the Mlc1 overexpressing astrocyte. Since oigomerization of Na^+/K^+ ATPase α subunits and β subunits on the endoplasmic reticulum is essential for stabilization of the α subunit, trafficking of the α subunit to the plasma membrane and functional maturation as a pump may have been affected in the Mlc1 overexpressed astrocytes. However, Mlc1 overexpression did not alter the amount of Na^+/K^+ ATPase α subunits, thereby disturbance of α-β oligomerization in the endoplasmic reticulum is unlikely to be involved in the reduction of sodium pump activity in the Mlc1 overexpressing astrocyte. Moreover, no evidence for Mlc1 interaction with Na^+/K^+ ATPase ß2 subunit was obtained. From these findings, I propose that 1) overexpressed Mlc1 formed "Mlc1-α subunit complex", resulting in the increased amount of the cell surface Na^+/K^+ ATPase α subunits, 2) increased "Mlc1-α complex" formation disturbed "α-β subunits complex" formation, resulting in lowered Na^+/K^+ ATPase pump activity.

In this study, I generated Mlc1 over expression mouse to examine relationship between the astrocytes and the white matter development, which showed leukodystrophy phenotype relevant to MLC, and found that astrocytes dysfunction is involved in the leukodystrophy formation via impairment of the sodium pump activity. My study will open a new insight into the relationship between astrocytes and the white matter abnormality.

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1. Introduction

Astrocytes are one of the major glial cells in the CNS and maintain brain milieu though the blood brain barrier formation, uptake of neurotransmitters and supply of nutrients to neurons. In addition to this well known function, astrocytes may be involved in the white matter development and /or maintenance. This idea is provided from the analysis of several glial specific gene deficient mice and human diseases. Glial specific genes, connexin and inwardly rectifying potassium channel (Kir4.1) were involved in the homeostasis of potassium concentration. It has been reported that deletion of connexin and Kir4.1 in glial cells results in the leukodystrophy in mice, (Lutz et al, 2009 ; Mognotti et al, 2011 ; Odermatt et al, 2003 ; Menichella et al, 2006). Moreover, a human disease, Alexander disease, is caused by missense mutations in the GFAP gene and leads to infantile onset leukodystrophy. These previous studies support the idea that astrocyte dysfunction results in leukodystrophy. However, studies on the relationship between astrocyte and the white matter development/maintenance are still rare. One of the limitations is a lack of mice recapitulating leukodystrophy phenotype after astrocyte dysfunction. In this study, I generated a model for a human disease, Megalencephalic leukoencephalopathy with subcortical cysts (MLC, OMIM604004), to study whether astrocyte dysfunction can lead to leukodystrophy.

MLC is a rare autosomal recessive neurological disorder with infantile onset characterized by chronic white matter abnormality leading to macrocephaly, slowly progressive deterioration in motor function, cerebellar ataxia, spasticity, and mental decline (van der Knaap et al, 1995 and 2012). Magnetic resonance imaging (MRI) diagnostically shows swollen brain with diffuse edematous white

matter and subcortical cysts mainly in the anterior-temporal region (van der Knaap et al., 1996). Indeed, electron microscopic examination of brain biopsy from a MLC patient shows the presence of several vacuoles in the myelin sheath and in astrocytic endfeet (van der Knaap et al., 1995; Duarri et al, 2011).

Two genes encoding Mlc1 and GlialCAM, were identified to be responsible for MLC (Leegwater et al., 2001; López-Hernández et al., 2011; Jeworutzki et al., 2012). Mutations in the Mlc1 gene were reported in about two-thirds of MLC patients and those in the GlialCAM were seen in about one-third of MLC (Leegwater et al., 2002; van der Knaap et al., 2012). Various mutations in Mlc1 (missense mutations, frame shift and splice site mutations) have reported in human MLC patients. However, correlation between the type of Mlc1 mutations and the severity of MLC clinical symptoms has not been clarified (Leegwater et al., 2002).

Human Mlc1 and murine Mlc1 genes encode a membranous protein with eight transmembrane domains, whose function is unknown. Northern blot analyses demonstrated that both human Mlc1 and murine Mlc1 genes were predominantly expressed in the brain (Leegwater et al., 2001; Steinke et al., 2003) and *in situ* hybridization demonstrated that Mlc1 mRNA is present in astrocyte lineage cells including astrocytes, ependymal cells and Bergmann glia, but not in oligodendrocytes (Schmitt et al, 2003, Glia., Teijido et al. 2007). Thereby, astrocytic abnormalities should be involved in the leukodystrophy observed in human MLC. More importantly, whether Mlc1 mutations lead to loss-of-function or gain-of-function was not addressed so far. Studies using the heterologous expression of Mlc1 gene demonstrated that Mlc1 mutants failed to be transported to the plasma membrane (Duarri et al, 2008; Teijido et al, 2004). In addition, biopsy samples from a MLC patient harboring missense mutation

displayed low expression levels of Mlc1 protein (Teijido et al, 2004). These data suggested that Mlc1 mutants show a loss-of-function effect and the model animal could be established by generating Mlc1 knockout mouse.

Moreover, leukodystrophy is a unique phenotype and several studies have been reported the model mouse showing leukodystrophy phenotype that lack the glial specific genes; connexin 30, 43 and 47 (Lutz et al, 2009; Mognotti et al, 2011; Odermatt et al, 2003), inwardly rectifying potassium channel (Kir4.1)(Menichella et al, 2006), and chloride channel (ClC-2) (Blanz et al, 2007), respectively. These molecules are important for the formation of glial syncytium or maintenance of ion homeostasis. These previous studies suggested that the disturbance of glial function leads to leukodystrophy. However, precise studies focusing on the relationship between astrocytes and the white matter development/maintenance have not been performed. Thereby, establishment of MLC model mouse may provide us with the insight of MLC pathophysiology and the relationship between astrocytes and the white matter development/maintenance.

In this study, I generated a mouse line, in which Mlc1 gene expression can be turned off or over expressed in astrocytes, and found that Mlc1 gene knock down mouse was normal while over expression mouse showed astrocyte swelling and white matter abnormality. I investigated and clarified the mechanism by which Mlc1 over expression can lead to astrocytic swelling and discuss how this astrocyte abnormality can lead to white matter abnormality.

Material and Method

2-1. Aniamal ethics

All experimental procedures using animals were approved by the National Institute for Physiological Sciences Animal Care and Use Committee and were in accordance with NIH guidelines.

2-2. Genotyping

Mlc1 STOP-tetO knock-in homozygote, Mlc1 tetO knock-in homozygote, and Mlc1-tTA BAC transgenic mouse were generated as previously described (Tanaka et al, 2010). Genotype of each mouse was diagnosed by PCR using following sets of primers: Mlc-657U (5'-AAATTCAGGAAGCTGTGTGCCTGC-3') and mtTAL24 (5'-CGGAGTTGATCACCTTGGACTTGT-3') for Mlc1-tTA, the size of PCR product is approximately 680bp: Mlc-80U (5'-AGGGAATGGTGGTCTGAGTCTGTT-3') and Mlc-159L (5'-GAGAACACCCATGTCTTGTAGCTG-3') for the tetO knock-in allele, sizes of PCR products are approximately 240bp from the wild type allele, 850bp from the Mlc1 tet0 knock-in allele and no bands from the Mlc1 STOP-tetO knock-in allele : Mlc-657U and PGKproL1 (5'-GTTGGCGCCTACCGGTGGATGTGGAATGTG-3') for the STOP-tetO knock-in allele, the size of PCR product is approximately 800bp from the MLC1 STOP-tetO knock-in allele and no bands from the wild type and Mlc1 tet0 knock-in alleles.

2-3. Doxycycline administration

Doxycycline (Sigma, Japan) was administered by feeding chow containing 100 mg/kg doxycycline (Nosan Co., Japan).

2-4. Tissue preparation

For cryosection, mice were deeply anesthetized with pentobarbital (50mg/kg) and intracardially perfused with 0.1M phosphate-buffer (PB, pH7.4) containing 4%(g/v) paraformaldehyde (4%PFA/PB). Brains were removed from the skull and post-fixed with the same fixative for overnight at 4℃. After cryoprotection, brains were embedded in OCT compound (Sakura Fine Technical Co, Japan), and used for in situ hybridization and immunohistochemistry. For paraffin sectioning, mice deeply anesthetized with pentobarbital were sacrificed by decapitation and brains were removed from the skull, then immersed into Bodian Ⅱ fixative [5%(g/v) paraformaldehyde and 5%(v/v) acetic acid in 80% ethanol]. After graded dehydration, they were embedded in paraffin wax (Fisher Scientific, USA), and used for hematoxyline eosin staining.

2-5. Transmission electron microscope (Collaboration with Prof. K. Tohyama in Iwate Medical University)

Mice were perfused intracardially through the left ventricle with a fixative containing 2.5% (v/v) glutaraldehyde and 2.0% (w/v) PFA in PB. Brains were dissected and postfixed by immersion into the same fixative overnight at 4℃, then brain slices at 50μm thickness were prepared. After osmification in a 1% (w/v) osmium tetroxide solution for 1h at room temperature, the specimens were dehydrated through a graded alcohol series and embedded in Epon 812 (TAAB Laboratories, Aldermasto, UK). Semithin sections at 1μm thickness were collected and stained with 0.5% (w/v) toluidine blue in PB. Ultrathin sections were cut using an Ultracut microtome (EM-UC6, Leica, Germany) and stained with uranyl acetate and lead citrate for contrast. The sections were examined with transmission electron microscope (H-7100 and H-7650, Hitachi, Japan).

2-6. In situ hybridization (ISH)

I used a method previously described (Ma et al, 2006) with minor modifications. Cryosections were fixed with 4%PFA (g/v) in phosphate-buffered saline (PBS, pH7.4) for 20min, followed by two washing with PBS for 5min each, and treated with proteinase K (40μg/ml) for 30min at RT. Sections were then washed with PBS and immerged into 4%PFA for 15min to inactivate proteinase K. After washing with PBS, sections were immersed into the 0.1M triethanolamine-HCl containing 0.25% acetic anhydride for 15min to prevent the electrostatic binding of cRNA probes. Pre-hybridization was carried out for 2-3 hours at 60℃ in hybridization buffer [10× Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% ficoll and 0.1% bovine serum albumin: Sigma, Japan), 50%(v/v) formamide, 0.2mg/ml yeast tRNA, 200μg/ml heparin, 1%(v/v) Tween-20 in 5×SSC (0.75M sodium chloride, 75mM sodium citrate, pH7.0)]. After removing pre-hybridization buffer, hybridization buffer containing appropriately diluted digoxigenin (DIG)-labeled cRNA probe was added and hybridized for overnight at 60℃. For colorimetric ISH, after serial stringency washing with 5×SSC, 2×SSC, 0.2×SSC containing 50%(v/v) formamide, and 0.2×SSC for 5min each at 65℃, the sections were immerged into a blocking buffer [100mM Tris-Cl, 150mM NaCl, pH7.5, 1%(w/v) blocking reagent (Roche, Japan)] for 60 min at room temperature, then incubated with diluted alkaline-phasphatase conjugated anti DIG antibody (1:5000, Roche, Japan) for 90 min at room temperature. Following washing with MABT buffer (100mM maleic acid, 150mM NaCl, 0.1% Tween-20, pH7.4), sections were pre-immersed in coloring buffer (100mM Tris-HCl, 100mM NaCl, 5mM $MgCl_2$, pH9.5) and cRNA probes were visualized with freshly prepared colorimetric substrate [0.0001% (v/v) nitro blue tetrazolium chloride (NBT) and 0.0035% (v/v)

5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt (BCIP) in coloring buffer, Roche, Japan] at room temperature. Colorimetric developments were stopped by immersion into PBS. Nuclear fast red (Vector Lab, Canada) was used for counter staining.

For fluorescence ISH, after hybridization and serial stringency washing, the sections were immersed into a blocking buffer [100mM Tris-Cl, 150mM NaCl, pH7.5, 0.05% (v/v) Tween20, pH 7.4] for 60 min at room temperature. After blocking with 1% (v/v) normal goat serum, sections were incubated with diluted peroxidase conjugated anti DIG antibody (1:1000, Roche, Japan) for overnight at 4℃. Following washing with blocking buffer, cRNA probes were visualized with fluorescein- conjugated tyramide in accordance with manufacture's protocol (TSA system: Perkin Elmer, Japan). cRNA probe for mouse Mlc1 was kindly gifted from Dr. Klaus-Peter Lesch (Schmitt et al, 2003).

2-7. Hematoxylin - Eosin staining

Paraffin sections were hydrated by sequential immersion into xylene and degraded alcohol series, and rinsed with distilled water (DW), then sections were stained into Mayer's hematoxyln solution for 10min. After rinsing with DW, sections ware counter stained with eosin solution for 5min. After washing with DW, sections were dehydrated through graded alcohol series and xylene.

2-8. Immunohistochemistry

Cryosections were hydrated with PBS and boiled in 0.01M citrate buffer (pH6.0) for 5min as an antigen retrieval. After blocking with 10% (v/v) normal goat serum, sections were incubated with diluted primary antibodies for overnight at 4℃. For the fluorescence microscopy, after washing with PBS, the sections were incubated

with diluted Alexa-conjugated secondary antibodies (1:5000, Invitrogen, USA) for 90 min at room temperature to visualize primary antibodies. The sections were mounted and examined by a fluorescence microscope (BX-50, Olympus, Japan), or a confocal microscope (LSM510, Zeiss, Germany). For the light microscopy, after washing with PBS, sections were incubated with diluted biotinylated secondary antibodies. After washing and intensification with avidin-biotin complex, sections were incubated with 3.3-diaminobenzidine (DAB) to visualize primary antibodies. The sections were dehydrated though graded alcohol series and mounted, then examined by a light microscope (BX-50, Olympus, Japan). Following antibodies were used: anti GFAP (1:1000, Dako, Denmark), anti glutamine synthase (1:500, kindly gifted from Prof. M. Watanabe in Hokkaido University).

2-9. Cultured astrocyte

Cultured astrocyte was obtained from whole brain of neonatal mouse as previously described (Lee et al, 2013) with minor modification. A newborn mouse (postnatal day 0 to 2) was sacrificed by decapitation, and whole brain was aseptically dissected out from the skull. After meninges had been carefully removed, brains were placed into chilled PBS. Brains were dissociated in papain (10U/mL: Worthington Biochemical Corporation, Lakewood, NJ) dissolved in Earle's balanced salt solution (Invitrogen, Karlsruhe, Germany) for 20 min at 37℃. The supernatant was removed and resuspended in fresh medium and cells were triturated in fire-polished Pasteur pipettes. Cells were then passed through a 70-lm nylon filter, plated in culture flasks (Iwaki, Scitech DIV, Ashahi Techno Glass, Tokyo, Japan), and cultured in a 95/5% (v/v) mixture of atmospheric air and CO_2. The culture medium was renewed after 3 days and subsequently

2 times per week. After 2 weeks, the cultures reached to more than 98% confluency and, there were oligodendrocyte precursors (OPCs) and microglia on the layer sheet of astrocyte. To strip OPCs and microglia, cultures were rinsed with PBS at 2 times and vigorously shaken by hand in the presence of PBS. After washing out, remaining cells on a culture plate were treated with 0.25% trypsin and incubated for 10min at 37℃. To stop enzymatic reaction, 10% FBS/DMEM was added. The dissociated cells were collected to 50ml Falcon tubes and pelleted by centrifugation for 5min at 800rpm [(LC-121, TOMY, Japan), (Rotor; TS-7, TOMY, Japan)]. Cell pellets were suspended into 1ml of 10% FBS/DMEM, and were plated on to 4 well-dishes (Thermo Scientific, Denmark) at a density of 0.25×10^5 per a well. Two days after plating on to 4well-dishes, cell cultures reached to more than 95% confluency, and more than 90% cells were positive for GFAP by immunocytochemistry (ICH). These cultures were used for tritiated-ouabain binding assay and rubidium-86 uptake assay.

2-10. Tritiated-ouabain (^{3}H-ouabain) binding assay

I used the method previously described (Imura et al., 2007; Munzer et al., 1994) with miner modifications. Cells were washed with Ringer solution (135mM NaCl, 5mM KCl, 1.5mM $MgCl_2$, 1.5mM $CaCl_2$, 5mM Glucose, 10mM HEPES-Na, pH7.4) and incubated for 30min at 37℃ in Ringer solution containing 0.1μM ^{3}H-ouabain (0.55-1.11TBq/mmol, Perkin Elmer, Japan) with or without 1mM non-RI ouabain (TOKYO CHEMIC, Japan) as a negative control. Following incubation, cells were washed for 3 times for 3 min with Ringer solution, total washing procedure did not exceed 10min, and were lysed with 130μl of lysis buffer (CelLytic™ M, Sigma, USA). After centrifugation for 15min at 12000rpm, 90 μl supernatants were

mixed with liquid scintillation cocktail (Ultima Gold™ XR, Perkin Elmer, Japan) and radioactivity was measured by a liquid scintillation counter (LSC-5100, Hitachi-Aloka Medical, Japan). Remaining cell lysates were used to measure the protein concentration by the method of Bradfold (Bio Rad protein assay kit, Bio Rad, USA). The non-RI insensitive component was defined as cell-surface expression of Na^+/K^+ ATPase α subunit. The results were represented as count per minute per milligram protein (cpm / mg protein).

2-11. Rubidium-86 (^{86}Rb) uptake assay

I used the method previously described (Imura et al., 2007; Bernstein et al., 1970) with miner modifications. Cells were washed with Ringer solution and were incubated in modified Ringer solution (135mM NaCl, 5mM RbCl, 1.5mM $MgCl_2$, 1.5mM $CaCl_2$, 5mM Glucose, 10mM HEPES-Na, pH7.4) containing 2μCi/well of ^{86}Rb (1Ci/g, Perkin Elmer, Japan) for 20min at 37℃. To define the sodium pump specific uptake of ^{86}Rb ion, specimens preincubated with 1mM non-RI ouabain for 5min at 37℃ were used as a negative control for each experiment. ^{86}Rb incorporated cells were washed 3 times for 1 min with Ringer solution and were lyses with 200μl of lysis buffer (CelLytic™ M, Sigma, USA). After centrifugation for 15min at 12000rpm, 130μl of lysate was mixed with liquid scintillation cocktail (Ultima Gold™ XR, Perkin Elmer, Japan) and the radioactivity was measured by a liquid scintillation counter (LSC-6100, Hitachi-Aloka Medical, Japan). Remaining cell lysates were used to measure the protein concentration by the method of Bradfold. The ouabain insensitive component was defined as a sodium pump activity and represented as the relative ratio to that in wild type.

2-12. Magnetic resonance imaging (Collaboration with Dr. K. Fujiyoshi in Keio University School of Medicine)

MRI was performed using a 7 tesla Biospec 70/16 MRI scanner (Bruker Biospin GmbH; Ettlingen, Germany) equipped with actively shielded gradients at a maximum strength of 700 mT/m and transmitting/receiving volume coil of inner diameter of 22 mm. (We used a post-mortem MR image of brain specimens. Under deep anesthesia (pentobarbital sodium, 100 mg/kg, iv), each animal was transcardially perfused with PBS and sequentially with 4% PFA. Brain specimens were firmly fixed into an acrylic tube filled with fluorinert (Sumitomo 3M Limited, Tokyo, Japan) to minimize the signal intensity attributable to the medium surrounding the specimen. T2-weighted images were acquired by using relaxation enhancement (RARE) with the following parameters: effective echo time (TEeff) = 15 ms, repetition time (TR) = 4000 ms; number of averages (NA) = 12, RARE factor = 2, field of view (FOV) = 7 × 14 $(mm)_2$, matrix = 64 × 128 and slice thickness = 0.5 mm. Each scan took less than 30 min.

2-13. Development of anti MLC1 antibody (Collaboration with Prof. M. Watanabe in Hokkaido University)

Antibody production. The method for production of antibodies was described previously (Uchigashima et al, 2007). cDNA fragments preceded by a *Bam*HI site, encoding N-terminal 50 amino acid (aa) (amino acid residues 1-50; MTREGQFREE LGYDRMPTLE RGRQDAGRQD PGSYTPDSKP KDLQLSKRLP) and C-terminal 52 aa of mouse Mlc1 (amino acid residues 331-382; CVSFKVSARL QAASWDPQSC PQERPAGEVV RGPLKEFDKE KAWRAVVVQM AQ) were obtained by PCR using single-stranded mouse brain cDNA library. After the TA cloning using a pGEM-T Easy Vector System I

kit (Promega, Madison, WI), cDNA fragments were sequenced and excised by *Bam*HI and *Eco*RI digestion. These fragments were subcloned into pGEX4T-2 plasmid (GE Healthcare, Piscataway, NJ) for the expression of glutathione S-transferase (GST) fusion proteins. GST fusion proteins were purified using glutathione–Sepharose 4B (GE Healthcare), according to the manufacturer's instructions. GST fusion proteins were emulsified with Freund's complete or incomplete adjuvant (DIFCO, Detroit, MI) and immunized subcutaneously to New Zealand White rabbits and Hartley guinea pigs at intervals of 2 weeks. After the sixth injection, Igs specific to antigens were affinity-purified using GST-free peptides coupled to CNBr-activated Sepharose 4B (GE Healthcare). GST-free peptides were prepared by in-column thrombin digestion of GST fusion proteins bound to glutathione-Sepharose 4B media. The specificity of antibody and immunohistochemistry for Mlc1 was confirmed by the lack of characteristic immunolabelings in the brains of Mlc1 null mice (Fig. 3).

2-14. Sample preparation for biochemistry (Collaboration with Prof. M.Fukata, NIPS)

A mouse brain was taken into the homogenate buffer (320 mM sucrose, 20mM Tris-Cl, pH 8.0, 2mM EDTA, and 200μg/ml PMSF), and homogenized by Potter homogenizer. Homogenate was centrifuged at 20,000 g for 1 h at 4℃ [(L-80, Beckman, USA), (Rotor; SW55Ti, Beckman, USA)], then pellet was resuspended in solubilizing buffer (20mM Tris-Cl, 1 mM EDTA, 1.3% Triton X-100, pH 8.0). After additional homogenization, homogenate was solubilized by incubation in solubilizing buffer for 1hr at 4℃, and lysate was centrifuged again at 100,000 g for 1 h at 4℃. The supernatant was collected as the P2 soluble fragment, and used for

immunoprecipitation and western blotting.

2-15. Immunoprecipitation (Collaboration with Prof. M.Fukata, NIPS)

P2 soluble fractions derived from wild type and Mlc1 null mouse were incubated with the protein A sepharose beads (CL-4B, Amersham Bioscience, Sweden) to reduce the proteins that bind to protein A sepharose. After centrifugation at 100,00 g for 1h at 4℃, pre-cleared lysates (5mg protein) were incubated with 5μg of the N-terminal or C-terminal recognizing rabbit anti Mlc1 antibodies for 1h at 4℃. Following incubation with 80μl of protein A sepharose beads for 1h at 4℃, proteins unbound to protein A sepharose beads were removed by washing, and eluted into an elution buffer [2%(w/v) SDS, 10%(v/v) glycerol, 0.075M Tris-HCl (pH6.8), 2%(v/v) 2-mercaptoethanol]. Samples were denatured at 37℃ for 30min to detect P-type ATPase or at 100℃ for 5min to detect others.

2-16. Mass spectrometry (Collaboration with Prof. M.Fukata, NIPS)

For the in-gel digestion, specific protein bands were excised, reduced with 10 mM dithiothreitol and alkylated with iodoacetamide. Band slices were digested with trypsin (12 μg/ml) overnight and desalted with ZipTip C18 (Millipore). The extracted peptides were then separated via nano flow liquid chromatography (LC) (Paradigm MS4, AMR) using a reverse phase C18 column (Magic C18). The LC eluent was coupled to a micro-ion spray source attached to a LCQ Advantage MAX mass spectrometer (Thermo Electron Corporation). For protein identification and semiquantification, we used the score of the SEQUEST algorithm from BioWorks soft ware (Thermo Scinectific). The specificity was determined by subtracting the

results obtained from wild-type mice. The proteins fitted following both criteria, 1) Probability: less than 1.0E-04 and 2) Score: more than 10, were listed in table 1.

2-17. Western blotting

Samples were separated by SDS-PAGE and blotted onto PVDF membranes (Millipore, USA). Following blocking with 0.5% skim milk in 0.1%(v/v) Tween20 in PBS (PBS-T, pH7.4), each PVDF membranes were incubated with diluted primary antibodies for overnight at 4℃. After washing with PBS-T buffer, primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies (MP Biomedicals, USA) and visualized by chemiluminescence systems (ECL or ECLplus, GE Healthcare, USA) and luminometer (LAS-3000, FUJIFILM, Japan). Following antibodies were used: anti Na^+/K^+ ATPase α2 subunit (1:1000, Millipore, USA), anti Na^+/K^+ ATPase α1 subunit (1:1000, 464.6, Novus Biologicals, USA), anti pan PMCA (1:1000, 5F10, Abcam, UK), anti pan SERCA (1:1000, Abcam, UK), anti Na^+/K^+ ATPase ß2 subunit (1:200, 35, Santa Cruz, USA), anti GAPDH (1:2000, 6G5, Biogenesis, UK), rabbit anti Mlc1-N (1:4000, 0.5μg/ml), rabbit anti Mlc1-C (1: 500, 0.5μg/ml) and guinea pig anti Mlc1-N (1:3000, 0.5μg/ml).

2-18. Statistical analysis

Data were analyzed by Student's t-test. Probability values less than 0.05 were considered statistically different.

3. Results

3-1. Mlc1 null mice developed normally and did not result in leukodystrophy

To examine whether Mlc1 deficiency exhibits leukodystrophy phenotype as seen in human MLC, I developed Mlc1 null mice in which STOP-tetO cassette was inserted before the translation initiation site of Mlc1 gene (Tanaka et al, 2010) (Fig. 1A-a). STOP sequence terminates the endogenous transcription of a targeted gene (Lakso et al, 1992), and STOP-tetO knock-in homozygotes ($Mlc1^{STOP/STOP}$) are expected to behave as Mlc1 null mice (Guy et al, 2007; Tanaka et al, 2010). In fact, I did not detect Mlc1 mRNA or protein in $Mlc1^{STOP/STOP}$ mice by in situ hybridization (Fig. 1B-a, b, c) or western blotting (data not shown), respectively (Tanaka et al, 2010).

$Mlc1^{STOP/STOP}$ mice did not show any behavioral abnormalities, such as ataxic gait or shivering, and developed normally characterized by body weight (Fig. 2A). Both male and female $Mlc1^{STOP/STOP}$ mice were fertile and they were able to survive at least for 18 months. Hereafter, we referred $Mlc1^{STOP/STOP}$ to Mlc1 null mouse. In addition, I did not see any abnormalities at the MRI (Fig. 3B-a), light microscopic (Fig. 4A-a) and electron microscopic (Fig. 5A) levels. These results indicated that Mlc1 deficiency did not recapitulate leukodystrophy phenotype, which is contradictory to my initial idea.

Interestingly, some of glia specific gene-related disease that are inherited in a Mendelian manner were modeled by wild type gene overexpression in mice; Alexander disease can be caused by the GFAP (glial fibrillary acidic protein) gene mutation or by the wild type GFAP overexpression reproducing the Rosenthal fiber formation, the

hallmark of pathology (Messing et al., 1998); Pelizaeus-Merzbacher disease is caused by the PLP (proteolipid protein) gene mutation or by the wild type PLP overexpression (Kagawa et al.,1994), reproducing the leukodystrophy phenotype. Thus, I hypothesized that wild type Mlc1 overexpression could recapitulate MLC phenotype in mice.

3-2. Mlc1 overexpression resulted in the vacuole formation in the white matter

To generate Mlc1 overexpressing animals, we took advantages of tetracycline-controlled gene induction system. I first generated tetO knockin mice ($Mlc1^{tetO/+}$) by crossing $Mlc1^{STOP/+}$ mouse with ROSA-Flpe mouse; flippase excised FRT-flanking STOP sequence from the $Mlc1^{STOP}$ allele, but tetO site, consisting of tTA-binding element and CMV minimal promoter remained at the same position (Fig. 1A-b) (Tanaka et al, 2010). I then crossed $Mlc1^{tetO/+}$ with Mlc1-tTA (tetracycline transactivator) line to obtain $Mlc1^{tetO/tetO}$ homozygotes with or without Mlc1-tTA (Mlc1-tTA:: $Mlc1^{tetO/tetO}$ or $Mlc1^{tetO/tetO}$). In Mlc1-tTA:: $Mlc1^{tetO/tetO}$ mice, tTA was expressed under the control of Mlc1 promoter, and tethered tetO site in a doxycycline (DOX) dependent manner. tTA-mediated Mlc1 mRNA transcription initiated from the CMV minimal promoter in addition to the endogenous Mlc1 mRNA transcription, yielding Mlc1 overexpressing animals (Fig. 1A-c). $Mlc1^{tetO/tetO}$ mouse was used as a control.

I examined the onset and the localization of tTA-mediated Mlc1 overexpression by in situ hybridization. The robust Mlc1 mRNA signals were detected at least from embryonic days 14 in Mlc1-tTA:: $Mlc1^{tetO/tetO}$ mice (Fig. 1B-g, h), whereas wild type ($Mlc1^{+/+}$) (data not

shown) and the control ($Mlc1^{tetO/tetO}$) weakly expressed Mlc1 mRNA at the same age (Fig. 1B-d, e). In the postnatal period, sparsely distributed Mlc1 mRNA signals were detected in both genotypes but the signal intensity in Mlc1-tTA:: $Mlc1^{tetO/tetO}$ was much higher than that in $Mlc1^{tetO/tetO}$. Co-labeling of cell type specific markers revealed that Mlc1 mRNA signals co-localized with astrocyte-specific markers such as GFAP (Fig. 1B-f, i) and glutamine synthase (GS) (Fig. 2B) but not with oligodendrocyte- or neuron-specific markers (data not shown). Mlc1 mRNA overexpression was observed in both the gray and the white matter. These data indicated that the overexpression coincided with endogenous expression. Hereafter, we referred Mlc1-tTA:: $Mlc1^{tetO/tetO}$ to OE.

I first examined how the Mlc1 overexpression affected the development. At birth, the size and the weight were indistinguishable between OE and control groups, but the growth retardation characterized by the body weight loss was apparent in OE after postnatal days 14 (Fig.2A). Body weight caught up to the control level by three months of age. Macrocephaly was not evident. Moreover, OE mice were viable at least till 18-months of age and the reproduction was not affected. Ataxic gait was transiently exhibited around postnatal days 21 to 28, but improved with age (data not shown). Other behavioral abnormalities were not apparent.

I next examined the gross anatomy of OE mice. We found empty spaces in the corpus callosum (CC) at postnatal days 7 and later period (Fig. 3A). Magnetic resonance images (MRI) showed that the increased signals by T2-weighted mode in CC of ex vivo OE brains (Fig.3 B-c) compared with that of the control brain (Fig. 3B-b), indicating the existence of vacuole formation or swelling in the white matter of OE mice.

I further examined the pathology in the OE white matter by

microscopy. Paraffin sections displayed that white matter regions examined contained vacuole-like structures more or less and the vacuole formation was evident at CC (Fig. 4A-c), globus pallidus (GP) (Fig. 4A-f), internal capsule (IC), fimbria of hippocampus, and cerebellar peduncles (data not shown). Lesion area containing vacuole-like structure did not expand after postnatal days 28 and rather reduced with age (Fig.4B). I did not observe vacuole formation in the gray matter of OE mice though Mlc1 was overexpressed by the gray matter astrocytes as well. These observations indicate that Mlc1 overexpression results in the vacuole formation in the white matter, but the resultant pathology is not progressive and lethal.

3-3. Astrocytic swelling and myelin splitting are found in vacuolating lesions of the white matter

To reveal the ultrastructure of the vacuole in OE mice, we performed electron microscopic (EM) analyses of OE mice at three months of age. We initially observed CC and realized that the destruction accompanied by sectioning was too severe to preserve ultrastructural architectures in the vacuolating lesion. Thus we selected GP for EM analyses. In accordance with the observation of vacuoles by light-microscopy, numerous vacuoles with an electron dens-boundary were observed in EM images (Fig. 5C). In addition, enlarged electron-lucent areas were easy to find and some areas were connected with blood vessels (Fig. 6A). We identified enlarged electron-lucent areas as an astrocytic cytoplasm because of the close apposition to blood vessels and the presence of intermediate filaments (arrows in Fig. 6B-a, b, c, d). Swollen endfeet of astrocytes were a specific feature of OE mice; 158 out of 161 vessels were associated with swollen endfeet of astrocytes, in which the swelling

was defined as longer thickness of endfoot than the radial of the lumen of the vessel: 18 out out of 132 vessels in the control. Even with the structural change of astrocytes, the distance between astrocytic endfeet and the basal lamina of vessels was not altered (arrow heads in Fig. 6B-c).

Higher magnified images demonstrated the precise localization of the vacuole within oligodendrocytes. Less than 10% of myelinated axons contained myelin-associated vacuoles, but others (more than 90%) exhibited normal structure (Fig. 5C). I divided the myein-associated vacuoles structurally into three types according to the following criteria that previously been used to characterize myelin-associated vacuoles (Odermatt et al., 2003; Menichella DM et al., 2006; Lutz SE et al. 2009). 1) Intra-myelin vacuole; they are surrounded by thin myelin sheath and contain aberrant myelin debris (Fig. 7a, b). 2) Inner loop vacuole; they are present between compact myelin and inner loop. Axonal tracts are retained by the inner loop vacuole (Fig. 7c, d). 3) Outer aspect vacuole; they are associated with an outer aspect of myelin sheaths (data not shown). However, there were no positional preferences of vacuoles in examined sections (208 out of 570 myelin-associated vacuoles were identified as intra-myelin vacuole, 303 out of 570 were inner loop vacuole, and 59 out of 570 were unidentified). Moreover, aberrant oligodendrocytes, such as those with apoptotic feature, were not observed. These results indicated that astrocytic swelling and myelin splitting compose vacuolating lesions of the white matter.

3-4. Astrocytic swelling closely correlated with the extent of tTA-mediated Mlc1 overexpression

Given that the occurrence of the structural abnormality of CC

preceded the onset of myelination, I hypothesized that astrocytic structural changes precede those of oligodendrocyte after the Mlc1 overexpression. To prove this idea, we manipulated the timing of tTA-mediated Mlc1 overexpression by DOX administration; feeding with Dox-containing chow (100 mg DOX/kg) enabled us to suppress tTA-mediated Mlc1 gene induction in OE mice (Fig. 8A). From the conception to postnatal days 28, OE mice were fed with DOX chows, and then fed with normal chows for additional 10 days, yielding tTA-mediated Mlc1 overexpression only from postnatal days 28 to 38 ($OE^{P28\text{-}P38}$) (Fig. 8B).

We examined CC and GP of $OE^{P28\text{-}P38}$ mice electron-microscopically and found the expanded electron-lucent areas surrounding vessels (Fig. 9A-b, c), which was relevant to astrocytic swelling as seen in the case of OE mice without DOX during the whole life (OE^{whole}). On the other hand, we observed neither oligodendrocytic swelling nor the vacuole formation associated with myelin in $OE^{P28\text{-}P38}$ mice in examined sections (Fig. 9B-a, b). These findings strongly suggested that Mlc1 over expression in astrocytes primarily caused astrocytic swelling and the oligodendrocytic vacuole formation subsequently followed.

3-5. Cancellation of Mlc1 overexpression alleviated the white matter lesion

I then examined whether leukodystropy phenotype in Mlc1 overexpressing mouse is reversible. I cancelled the Mlc1 overexpression for a long period after postnatal days 28, when formation of the white matter lesion is almost completed, and examined the histology at postnatal days 90. About 23% area of CC was occupied by vacuole-like structure at postnatal days 90 in Mlc1

OE mouse as examined by light microscope (OE^{whole}; Fig. 10A-a,b). Interestingly, area with vacuole-like structure in CC dramatically decreased to 7.2 ± 2.0% (Mean ± SEM, $p<0.0010$) when Mlc1 overexpression was cancelled after one-month of age by feeding DOX-containing chow ($OE^{Embryo\text{-}P28}$: Fig. 10B-a,b, 10C). $OE^{Embryo\text{-}P28}$ mice displayed astrocytic swelling, but areas containing swollen astrocytes were less than those from OE^{whole} specimen as examined by EM (Fig. 10B-c). Importantly obvious structural abnormalities in oligodendrocyte, such as myelin-associated vacuoles as seen in OE^{whole} mice (Fig. 5C), were not observed (Fig.10B-d). This result indicated that astrocytic swelling and myelin-associating vacuole in OE mouse was reversibly improved by cancellation of Mlc1 overexpression.

We further examined whether leukodystrophy phenotype appear when Mlc1 overexpression is induced after young adult stage in mice. Mlc1 OE mouse was maintained with DOX containing chow till one month of age and then Mlc1 overexpression was induced until three month of age ($OE^{P28\text{-}P90}$, Fig. 11B-a). No histological abnormalities were observed during DOX feeding (OE^{DOX}, Fig. 11A-a). In $OE^{P28\text{-}P90}$ mouse, light-lucent area in CC was statistically increased compared with OE^{DOX}, but clear vacuole-like structure was not observed in LM study (Fig. 11B-b, C). Moreover, EM images displayed the astrocytic swelling that is structurally relevant to OE^{whole} and $OE^{P28\text{-}P38}$ (Fig. 11B-c, Fig. 6, Fig. 9A-b, c). In contrast, no obvious structural abnormalities in oligodendrocytes were observed even though astrocytic swelling is present in $OE^{P28\text{-}P90}$ (Fig. 11B-d). This result indicated that long-term induction of Mlc1 overexpression after young adult stage is sufficient to induce the astrocytic swelling but insufficient to cause oligodendrocytic abnormalities.

3-6. Mlc1 interacted with P-type ATPases

Histological evidences so far indicated that gain-of-function but not loss-of-function of Mlc1 caused MLC-like pathology in mouse. One plausible explanation regarding genotype-phenotype correlation in OE mice was that Mlc1 interacted with cell volume regulating molecules and Mlc1 overexpression exerted a dominant negative effect. However, the lack of Mlc1 antibody that can be used for immunoprecipitation made the biochemical analyses difficult.

We thus generated mouse Mlc1 primary antibodies usable for immunoprecipitation. We immunized rabbits or guinea pigs with mouse Mlc1 N-terminal 50 amino acids or C-terminal 52 amino acids peptides (Fig. 12A), and generated four types of primary antibodies: rabbit Mlc1-N, rabbit Mlc1-C, guinea pig Mlc1-N, guinea pig Mlc1-C. Western blotting (Fig. 12B; using Mlc1-N antibody) and immunohistochemical analysis (Fig. 12C; using Mlc1-N antibody) using negative control samples obtained from Mlc1 null mice confirmed a specific immunoreactivity of newly developed antibodies.

We performed an immunoprecipitation of Mlc1 using rabbit Mlc1-N and Mlc1-C antibodies from brain extracts of Mlc1 null and wild type mice. Gel electrophoresis patterns demonstrated the presence of potential interacting molecules, whose amounts were distinguishable from those in negative controls, indicating that mouse Mlc1 antibodies developed would be usable for immunoprecipitation (Fig. 13A).

To identify Mlc1 interacting molecules, we excised pieces as indicated in Fig.13 (blackets 1-13) and proceeded to analyses with a mass spectrometry (table 1). Interestingly, members of plasma membrane type (P-type) ATPases, Na^+/K^+ ATPase alpha subunit (ATP1α1, 1α2, 1α3), plasma membrane Ca^{2+} ATPase (ATP2β1, 2β2, 2 β 3, 2 β 4), sarco/endoplasmic reticulum Ca^{2+}-ATPase (ATP2α1, 2 α

2, 2α 3) were identified as molecules interacting with Mlc1. To confirm specific interaction of members of P-type ATPase with Mlc1, I performed western blotting in the same sets of immunoprecipitates. Bands recognized by isoform specific antibody against Na^+/K^+ ATPase α2 and α1 subunit were detected in the immunoprecipitate from wild type brain extract but not from that of Mlc1 null or no brain extract control, which corresponded to the molecular weight of previously determined Na^+/K^+ ATPase α subunits (Peng et al., 1997; Watts et al., 1991). Moreover, other members of P-type ATPase, PMCA and SERCA, were also detectable only in the wild type immunoprecipitate (Fig. 13B). This result demonstrates that members of P-type ATPase interact with Mlc1.

Na^+/K^+ ATPase α subunits is a component of sodium pump. They form complex with Na^+/K^+ ATPase ß subunit and α–ß oligomerization is essential for maturation of sodium pump and trafficking to the plasma membrane (Geering et al., 1996). Interestingly, pharmacological and genetic studies suggested that mice with reduced sodium pump activity displays astrocytic swelling (Cornog et al, 1967; Magyar et al, 1994). This phenotype resembles our findings of Mlc1 OE mouse. Thereby, we hypothesized that overexpressed Mlc1 affects the sodium pump property.

3-7. Overexpressed Mlc1 reduces sodium pump activity and increases the cell surface expression of Na^+/K^+ ATPase α subunits

To study whether over expressed Mlc1 alter sodium pump property, first I measured sodium pump activity by rubidium-86 (^{86}Rb) uptake assay. Primary cultured astrocyte was prepared from the whole brain of Mlc1 overexpressing and wild type neonates. More

than 90% cells in prepared culture were positive for GFAP as examined by immunocytochemistry (data not shown). ^{86}Rb is incorporated into the cell through the same machinery as potassium, and radioactivity that is incorporated into cultured astrocytes is equivalent to the sodium pump activity. I defined non-RI ouabain (1mM) insensitive ^{86}Rb uptake as sodium pump activity in this study. The sodium pump activity in Mlc1 overexpressing astrocyte decreased by 77.0±0.5% (Mean±SEM, compared with that from wild type) (Fig. 14A). To exclude the possibility that Mlc1 overexpression resulted in the lowered amount of Na^+/K^+ ATPase α subunits, I prepared membrane extract from the whole brain of wild type, Mlc1 overexpressing and Mlc1 null mice, performed western blotting for Na^+/K^+ ATPase α subunits and compared the band intensity among them after normalization against those of GAPDH (Fig. 14B). However, significant differences in the amount of Na^+/K^+ ATPase α1 and α2 subunit were not detected (Fig. 14C), indicating that overexpressed Mlc1 did not alter the amount of Na^+/K^+ ATPase α subunits. These results indicated that Mlc1 overexpression coincided with lowered function of Na^+/K^+ ATPase pump activity in astrocytes, but did not affect the bulk expression levels.

To confirm whether the level of Na^+/K^+ ATPase molecules on the plasma membrane decreased in accordance with lowered pump activity, I performed ^3H-ouabain biding assay using cultured astrocytes. ^3H-ouabain is a tritiated inhibitor of sodium pump that binds to extracellular domain of Na^+/K^+ ATPase α subunits. In other words, ^3H-Ouabain should label cell surface Na^+/K^+ ATPase α subunits. Unexpectedly, I found that radioactivity derived from the cell surface bound ^3H-ouabain significantly increased in Mlc1 overexpressing astrocyte compared with that of wild type, whereas no differences between wild type and Mlc1 null astrocytes were detected

(Fig. 15A). This result indicated that the amount of cell surface Na^+/K^+ ATPase α subunits increased in Mlc1 overexpressing astrocyte, whereas depletion of Mlc1 did not affect the cell surface-expression of Na^+/K^+ ATPase α subunits.

Since, α-ß oligomerization is essential for the functional maturation of sodium pump and its transport to the plasma membrane (Laughery et al, 2004; Geering et al, 1996), it was expected that the over produced Mlc1 could reduce α-ß complex. We validated whether Mlc1 interact with Na^+/K^+ ATPase ß subunits (astrocyte specific isoform) by using immunoprecipitation with anti Mlc1 antibody (the same set as those used in Fig. 13B). However, no clear evidence showing Mlc1 to interact with Na^+/K^+ ATPase ß2 subunit was obtained in my experiment (Fig. 15B). This result indicated that "Mlc1-α subunit complex" is preferred and "Mlc1-ß subunit complex" or "Mlc1- α - ß complex" formation are unlikely to occur in Mlc1 overexpressing astrocyte.

Taken together these experiments indicate that 1) overexpressed Mlc1 formed "Mlc1-α subunit complex", resulting in higher amount of ^3H-ouabain biding, 2) increased "Mlc1-α complex" formation may have depleted α subunits and disturbed formation of "α-β subunits complex", resulting in lowered Na^+/K^+ ATPase pump activity.

4. Discussion

4-1. MLC model mouse

MLC is a rare autosomal recessive neurological disorder with infantile onset, characterized by chronic white matter abnormality, which is mainly caused by mutations in the Mlc1 gene. It has been suggested that functional loss or trafficking defects of Mlc1 is associated with MLC. However, the involvement of Mlc1 deficiency in the MLC disease has been controversial. Therefore, I first generated Mlc1 null mouse, then validated whether MLC-like phenotype is recapitulated or not. In Mlc1 null mouse, in which STOP-tetO cassette is inserted in Mlc1 loci, endogenous Mlc1 expression becomes below the detection level, but no behavioral and histological abnormalities were observed. Thus loss-of-function mutation in the Mlc1 gene does not cause MLC at least in mouse. It has been shown that some of glia specific gene-related diseases that are inherited in a Mendelian manner can be modeled by wild type gene overexpression in mice. Thereby, I next generated Mlc1 overexpressing mouse through mating tTA-expressing mouse under the control of Mlc1 promoter with tetO cassette knocked in mouse. tTA-mediated Mlc1 overexpression was achieved in both the gray matter and the white matter astrocytes.

Mlc1 overexpressing mouse displayed leukodystrophy phenotype with edematous white matter. Leukodystrophy phenotype appeared from postnatal 1 to 2 weeks and was almost completed around one month of age, and then persisted for whole life. The onset of phenotype and the pathological feature reproduced that of human MLC. Therefore, I referred Mlc1 overexpressing mouse as MLC model mouse and examined the process of leukodystrophy.

Moreover, Mlc1 was overexpressed in both the white matter

and the gray matter astrocytes, but no obvious abnormalities were found in the gray matter. This region-restricted effect might be caused by the different milieu surrounding astrocytes, by the different nature between the gray matter and the white matter astorocytes.

4-2. Leukodystrophy in Mlc1 overexpressing mouse

EM studies indicated that the leukodystrophy with astrocytic swelling and myelin-associating vacuoles in Mlc1 overexpressing mouse, was relevant to an ultrastructural description of MLC lesions. Although massive vacuoles were present in oligodendrocytes, its occurrence was less then 10% in examined myelinated-axon; while the occurrence was 98% in astrocytes. Moreover, astrocytic swelling occurred when Mlc1 was overexpressed after the young adult stage ($OE^{P28\text{-}P38}$ and $OE^{P28\text{-}P90}$), but no remarkable abnormalities in oligodendrocyte were observed. Therefore, it is likely that Mlc1 overexpression primarily leads to astrocytic swelling, which is followed by the formation of myelin-associated vacuoles. Myelinogenesis is a hallmark of oligodendrocyte differentiation and many transcription factors play important role in the differentiation of the oligodendrocyte lineage cells (Xin et al., 2005; Chen et al., 2009). Indeed, oligodendrocyte differentiation program could be affected by the Mlc1 overexpression but I did not address this question because Mlc1 overexpression was restricted to astrocyte-lineage cells and more than 90% of myelinated axons showed normal morphology.

In addition, when Mlc1 overexpression was cancelled after one month of age ($OE^{embryo\text{-}P28}$), leukodystrophy area dramatically decreased in accordance with improved astrocytic swelling and oligodendrocyte-associated vacuoles. Therefore, leukodystrophy in

Mlc1 overexpressing mouse was reversible. Because astrocytic swelling tightly correlated with Mlc1 expression level, alleviation of leukodystrophy is attributable to the improvement of astrocytic swelling. Interestingly, Mlc1 overexpression induced only during young adult stage ($OE^{P28\text{-}P90}$), astrocytic swelling did not lead to oligodendrocyte-associated vacuoles or leukodystrophy. Therefore, astrocytes should be play an important role in the white matter development and maintenance at the critical period. These results also provide a possibility that the leukodystrophy as seen in human MLC could be a target for therapeutic intervention.

4-3. Comparison of leukodystrophy in Mlc1 OE mouse with previously described leukodystrophy in mouse

Myelin associated vacuole is a unique phenotype but has also been reported in mice lacking glial specific genes; connexins (Lutz et al, 2009; Mognotti et al, 2011; Odermatt et al, 2003) and inwardly rectifying potassium channel (Kir4.1) (Menichella et al, 2006), respectively. Connexin (Cx) is a component of gap junction and includes several isoforms. Oligodendrocytic connexin, connexin 47 (Cx47), knock-out resulted in inner loop vacuole. Connexin30 and 43 (Cx30 and Cx43) are expressed by astrocyte and are involved in intracellular exchange of ion. Moreover, astrocytic connexin, Cx30/43, double knock out resulted in the appearance of edematous astrocyte and the vacuole formation at the outer aspect of myelin sheath (Luts et al, 2009). Gap junctions allow the intercellular exchange of ions and small molecules. The formation of heterotypic gap junctions between astrocytes and oligodendrocytes is described previously (Kamasawa et al, 2005). This previous report suggested that functional coupling between astrocytes and oligodendrocytes forms the molecular basis for a panglial syncytium important in

redistribution of potassium ion released into the periaxonal space during neuronal activation (Kettenmann and Ransom, 1988; Luts et al, 2009). Indeed, astrocytic connexin, Cx40/43, double knock out mouse displayed an elevation of extracellular potassium concentration in hippocampus accompanied by the vacuole formation (Wallraff et al, 2006; Luts et al, 2009). In addition, Kir4.1 mediates potassium uptake into glial cells, and is found in astrocytes and oligodendrocytes. Kir4.1 conditional knock out in glial cells led to myelin associated vacuoles preferred at the outer aspect of myelin (Meninchella et al, 2006). Moreover, it is also suggested that Kir channel may siphon extracellular potassium toward sinks such as capillaries and is involved in the setting of resting membrane potential in cultured astrocytes. Indeed, Kir4.1 knock out in glial cells displayed leukodystrophy accompanying the impaired potassium uptake (Djukic et al, 2007), and the resting membrane potentional in cultured astrocytes shifted to the depolarizing direction when Kir4.1 was knocked-down (Kucheryavykh et al, 2007).

Comparing these phenotypes, myelin-associating vacuoles as seen in Mlc1 OE mouse was relevant to those of Cx47 knock out mouse, and astrocytic swelling was relevant to those of Cx30/43 double knock out mouse. These similar appearance lead us to a hypothesize that Mlc1 could participate in the potassium homeostasis. Actually, we indicated that Mlc1 interacted with Na+/K+ ATPase α subunits and the sodium pump activity was reduced in cultured astrocytes from Mlc1 OE mouse. Therefore, disturbance of ion gradients caused by sodium pump dysfunction may induce astrocytic swelling and leukodystrophy.

4-4. Screening and identification of Mlc1 interacting proteins

Histological analysis of Mlc1 null mouse and Mlc1 overexpressing mouse suggested that the astrocytic swelling is caused cell autonomously by Mlc1 overexpression. Hence, I hypothesized that excess Mlc1 might have dominant negative effects on other molecules involved in the cell volume regulation. Thereby, I searched for Mlc1 interacting proteins. Because of the lack of immunoprecipitation compatible anti Mlc1 antibody, I generated primary antibodies recognizing mouse Mlc1 N-terminus and C-terminus, respectively. By using newly developed anti Mlc1 antibodies, I screened for Mlc1 interacting proteins by immunoprecipitaton and mass spectrometry analysis, and identified P-type ATPases as interacting proteins. P-type ATPase family consists of Na^+/K^+ ATPase α subunits, PMCA and SERCA . In particular, Na^+/K^+ ATPase α subunit is the major component of sodium pump and necessary for maintaining the sodium and potassium ion gradients. Moreover, it has been reported that Mlc1 is enriched in the sodium pump-containing fraction when purified on ouabain affinity column (Brignone et al, 2011). Na^+/K^+ ATPase α subunit includes several isoforms. Na^+/K^+ ATPase α1 subunit is ubiquitously expressed in all cell types; in contrast, α2 and α3 subunit are predominantly expressed in astrocytes and neurons in the adult CNS (Watts et al, 1991; Tokhtaeva et al, 2012). Since pharmacological and genetic studies indicated that mice with reduced sodium pump activity display astrocytic swelling relevant to Mlc1 OE mouse (Cornog et al., 1967; Magyar et al., 1994), I focused on the sodium pump property in this study. However, possibility that Mlc1 is involved in the function of other interacting peoteins, such as PMCA and SERCA, remains an open question (F resu et al., 1999;

Simpson et al., 1998).

4-5. Overexpressed Mlc1 alters sodium pump property

To address whether overexpressed Mlc1 affects sodium pump property, I performed ^{86}Rb uptake assay and found that the sodium pump activity was reduced in the Mlc1 overexpressing astrocyte without alteration of the amount of Na^+/K^+ ATPase α subunits. Because the oligomerization of Na^+/K^+ ATPase α subunit with Na+/K+ ATPase β subunit is essential for the sodium pump function, we hypothesized that overexpressed Mlc1 reduces the α–β complex resulting in a reduced sodium pump activity. In our biochemical studies, Mlc1 interacted with Na^+/K^+ ATPase α subunits, but no clear evidence for Mlc1 to interact with Na^+/K^+ ATPase ß2 subunit was obtained. This result indicated that the "α- ß subunit complex" and "α-Mlc1 complex" exist but "ß-Mlc1 complex" or "α-ß-Mlc1 complex" do not. From these findings, I propose that overexpressed Mlc1 may disturb α-ß oligomerization or remove α subunit from α-ß complex, resulting in the reduced sodium pump activity. In ^3H-ouabain binding assay, the amount of cell surface Na^+/K^+ ATPase α subunits increased in Mlc1 overexpressing astrocytes even though the sodium pump activity was reduced. Assembly of Na^+/K^+ ATPase α and ß subunit on the endoplasmic reticulum is also necessary for the stable expression of α subunit (Geering et al, 1996; Gatto et al., 2001). Because the amount of Na^+/K^+ ATPase α subunits was not altered in Mlc1 overexpressing astrocytes, it is unlikely that overexpressed Mlc1 impaired α-ß oligomerization on the endoplasmic reticulum. As other possibilities, 1) α subunit interacting with Mlc1 obtained stability and transported to the plasma membrane via unknown rote, 2) overexpressed Mlc1 removes α subunit from α-ß complex on the plasma membrane and trafficking of α-ß complex from the

endoplasmic reticulum to the plasma membrane may compensatory be accelerated, leading to the reduced sodium pump activity and increased amount of cell surface Na^+/K^+ ATPase α subunit. However, I was not able to address these possibilities in this study.

4-6. Conclusion

In this study, I generated Mlc1 overexpressing mouse to examine relationship between the astrocytes and the white matter development, which showed leukodystrophy phenotype relevant to MLC, and found that astrocytes dysfunction is involved in the leukodystrophy formation via impairment of sodium pump activity. My study will open a new insight of the relationship between astrocytes and the white matter development/ maintenance and MLC pathophysiology.

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6. References

Bernstein JC, Israel Y. (1970) Active transport of Rb^{86} in human red cells and rat brain slices. J Pharmacol Exp Ther. 174(2): 323-9

Blanz J, Schweizer M, Auberson M, Maier H, Muenscher A, Hübner CA, Jentsch TJ. (2007) Leukoencephalopathy upon disruption of the chloride channel ClC-2. J Neurosci. 27(24): 6581-9

Brignone MS, Lanciotti A, Macioce P, Macchia G, Gaetani M, Aloisi F, Petrucci TC, Ambrosini E. (2011) The beta-1 subunit of Na+/K+ ATPase pump interacts with megalencephalic leukoencephalopathy with subcortical cysts protein1 (MLC1) in brain astrocytes: new insights into MLC pathogenesis. Hum Mol Genet. 20(1): 90-103

Chen Y, Wu H, Wang S, Koito H, Li J, Ye F, Hoang J, Escobar SS, Gow A, Arnett HA, Trapp BD, Karandikar NJ, Hsieh J, Lu QR. (2009) The oligodendrocyte-specific G protein-coupled receptor GPR17 is a cell-intrinsic timer of myelination. Nat Neurosci. 12(11): 1398-406

Cornog JL, Gonatas NK, Feierman JR. (1967) Effects of intracerebral injection of ouabain on the fine structure of rat cerebral cortex. Am J Pathol. 51(4): 573-90

Djukic B, Casper KB, Philpot BD, Chin LS, McCarthy KD. (2007) Conditional knock-out of Kir4.1 leads to glial membrane

depolarization, inhibition of potassium and glutamate uptake, and enhanced short-term synaptic potentiation. J Neurosci. 27(42): 11354-65

Duarri A, Lopez de Heredia M, Capdevila-Nortes X, Ridder MC, Montolio M, López-Hernández T, Boor I, Lien CF, Hagemann T, Messing A, Gorecki DC, Scheper GC, Martínez A, Nunes V, van der Knaap MS, Estévez R. (2011) Knockdown of MLC1 in primary astrocytes causes cell vacuolation: a MLC disease cell model. Neurobiol Dis. 43(1): 228-38

Duarri A, Teijido O, López-Hernández T, Scheper GC, Barriere H, Boor I, Aguado F, Zorzano A, Palacín M, Martínez A, Lukacs GL, van der Knaap MS, Nunes V, Estévez R. (2008) Molecular pathogenesis of megalencephalic leukoencephalopathy with subcortical cysts. Hum Mol Genet. 17(23): 3728-39

Fresu L, Dehpour A, Genazzani AA, Carafoli E, Guerini D. (1999) Plasma membrane calcium ATPase isoforms in astrocytes. Glia. 28(2): 150-5

Gatto C, McLoud SM, Kaplan JH. (2001) Heterologous expression of Na(+)-K(+)-ATPase in insect cells: intracellular distribution of pump subunits. Am J Physiol Cell Physiol. 281(3): C982-92

Geering K, Beggah A, Good P, Girardet S, Roy S, Schaer D, Jaunin P. (1996) Oligomerization and maturation of Na,K-ATPase: functional interaction of the cytoplasmic NH2 terminus of the beta subunit with the alpha subunit. J Cell Biol. 133(6): 1193-204

Guy J, Gan J, Selfridge J, Cobb S, Bird A. (2007) Reversal of neurological defects in a mouse model of Rett syndrome. Science. 315(5815): 1143-7

Imura A, Tsuji Y, Murata M, Maeda R, Kubota K, Iwano A, Obuse C, Togashi K, Tominaga M, Kita N, Tomiyama K, Iijima J, Nabeshima Y, Fujioka M, Asato R, Tanaka S, Kojima K, Ito J, Nozaki K, Hashimoto N, Ito T, Nishio T, Uchiyama T, Fujimori T, Nabeshima Y. (2007) alpha-Klotho as a regulator of calcium homeostasis. Science. 316(5831): 1615-8

Jeworutzki E, López-Hernández T, Capdevila-Nortes X, Sirisi S, Bengtsson L, Montolio M, Zifarelli G, Arnedo T, Müller CS, Schulte U, Nunes V, Martínez A, Jentsch TJ, Gasull X, Pusch M, Estévez R. (2012) GlialCAM, a Protein Defective in a Leukodystrophy, Serves as a CLC-2 Cl- Channel Auxiliary Subunit. Neuron. 73(5): 951-61

Kagawa T, Ikenaka K, Inoue Y, Kuriyama S, Tsujii T, Nakao J, Nakajima K, Aruga J, Okano H, Mikoshiba K. (1994) Glial Cell Degeneration and Hypomyelination Caused by Overexpression of Myelin Proteolipid Protein Gene. Neuron. 13(2): 427-42

Kamasawa N, Sik A, Morita M, Yasumura T, Davidson KG, Nagy JI, Rash JE. (2005) Connexin-47 and connexin-32 in gap junctions of oligodendrocyte somata, myelin sheaths, paranodal loops and Schmidt-Lanterman incisures: implications for ionic homeostasis and potassium siphoning. Neuroscience. 136(1): 65-86

Kettenmann H and Ransom BR. (1988) Electrical coupling between astrocytes and between oligodendrocytes studied in mammalian cell

cultures. Glia. 1(1): 64-73

Kucheryavykh YV, Kucheryavykh LY, Nichols CG, Maldonado HM, Baksi K, Reichenbach A, Skatchkov SN, Eaton MJ. (2007) Downregulation of Kir4.1 inward rectifying potassium channel subunits by RNAi impairs potassium transfer and glutamate uptake by cultured cortical astrocytes. Glia. 55(3): 274-81

Lakso M, Sauer B, Mosinger B Jr, Lee EJ, Manning RW, Yu SH, Mulder KL, Westphal H. (1992) Targeted oncogene activation by site-specific recombination in transgenic mice. Proc Natl Acad Sci U S A. 89(14): 6232-6

Laughery M, Todd M, Kaplan JH. (2004) Oligomerization of the Na,K-ATPase in cell membranes. J Biol Chem. 279(35): 36339-48

Lee HU, Yamazaki Y, Tanaka KF, Furuya K, Sokabe M, Hida H, Takao K, Miyakawa T, Fujii S, Ikenaka K. (2013) Increased astrocytic ATP release results in enhanced excitability of the hippocampus. Glia. 61(2): 210-24

Leegwater PA, Boor PK, Yuan BQ, van der Steen J, Visser A, Könst AA, Oudejans CB, Schutgens RB, Pronk JC, van der Knaap MS. (2002) Identification of novel Mutations in MLC1 responsible for megalencephalic leukoencephalopathy with subcortical cysts. Hum Genet. 110(3): 279-83

Leegwater PA, Yuan BQ, van der Steen J, Mulders J, Könst AA, Boor PK, Mejaski-Bosnjak V, van der Maarel SM, Frants RR, Oudejans CB, Schutgens RB, Pronk JC, (2001) van der Knaap MS. Mutations of

MLC1 (KIAA0027), Encording a Putative Membrane Protein, Cause Megalencephalic Leukoencephalopathy with Subcortical Cysts. Am J Hum Genet. 68(4): 831-8

López-Hernández T, Ridder MC, Montolio M, Capdevila-Nortes X, Polder E, Sirisi S, Duarri A, Schulte U, Fakler B, Nunes V, Scheper GC, Martínez A, Estévez R, van der Knaap MS. (2011) Mutant GlialCAM Causes Megalencephalic Leukoencephalopathy with sub cortical cysts, Benign Familial Macrocephaly, and Macrocephaly with retardation and Autism. Am J Hum Genet. 88(4): 422-32

Lutz SE, Zhao Y, Gulinello M, Lee SC, Raine CS, Brosnan CF. (2009) Deletion of Asterocyte Connexin 43 and 30 Leads to a Dysmyelinating Phenotype and Hippocampal CA1 vaculation. J Neurosci. 29(24): 7743-52

Ma J, Tanaka KF, Shimizu T, Bernard CC, Kakita A, Takahashi H, Pfeiffer SE, Ikenaka K. (2011) Microglial cystatin F expression is a sensitive indicator for ongoing demyelination with concurrent remyelination. J Neurosci Res. 89(5): 639-49

Magyar JP, Bartsch U, Wang ZQ, Howells N, Aguzzi A, Wagner EF, Schachner M. (1994) Degeneration of Neural Cells in the Central Nervous System of Mice Degicient in the Gene for the Adhesion Molecule on Glia, the ß2 Subunit of Murine Na,K-ATPase. J Cell Biol. 127(3): 835-45

Menichella DM, Majdan M, Awatramani R, Goodenough DA, Sirkowski E, Scherer SS, Paul DL. (2006) Genetic and Physiological Evidence That Oligodendrocyte Gap Junctions Contribute to Spatial

Buffering of Potassium Released during Neuronal Activity. J Neurosci. 26(43): 10984-91

Messing A, Head MW, Galles K, Galbreath EJ, Goldman JE, Brenner M. (1998) Fetal encephalopathy with astrocyte inclusion in GFAP transgenic mice. Am J Pathol. 152(2): 391-8

Munzer JS, Daly SE, Jewell-Motz EA, Lingrel JB, Blostein R. (1994) Tissue- and isoform-specific kinetic behavior of the Na,K-ATPase. J Biol Chem. 269(24): 16668-76

Odermatt B, Wellershaus K, Wallraff A, Seifert G, Degen J, Euwens C, Fuss B, Büssow H, Schilling K, Steinhäuser C, Willecke K. (2003) Connexin 47 (Cx47)-deficient Mice with Enhanced Green Fluorescent Peotein Reporter Gene Receal Predominat Oligodendrocyte Expression of Cx47 and Display Vacuolized Myelin in the CNS. J Neurosci. 23(11): 4549-59

Peng L, Martin-Vasallo P, Sweadner KJ. (1997) Isoforms of Na,K-ATPase alpha and beta subunits in the rat cerebellum and in granule cell cultures. 17(10): 3488-502

Schmitt A, Gofferje V, Weber M, Meyer J, Mössner R, Lesch KP. (2003) The Brain-Specific Protein MLC1 Implicated in Megalencephalic Leukoencephalipathy With Subcortical Cysts Is Expressed in Glial Cells in the Murine Brain. Glia. 44(3): 283-95

Simpson PB, Russell JT. (1998) Role of mitochondrial Ca2+ regulation in neuronal and glial cell signalling. Brain Res Brain Res Rev. 26(1): 72-81

Steinke V, Meyer J, Syagailo YV, Ortega G, Hameister H, Mössner R, Schmitt A, Lesch KP. (2003) The genetic organization of the murine Mlc1 (Wkl1, KIAA0027) gene. J Neural Transm. 110(4): 333-43

Tanaka KF, Ahmari SE, Leonardo ED, Richardson-Jones JW, Budreck EC, Scheiffele P, Sugio S, Inamura N, Ikenaka K, Hen R. (2010) Flexible accelerated STOP tetracycline operator-knockin(FAST):A versatile and efficient new gene modulating system. Biol Psychiatry. 67:770-3

Teijido O, Casaroli-Marano R, Kharkovets T, Aguado F, Zorzano A, Palacín M, Soriano E, Martínez A, Estévez R. (2007) Expression patterns of MLC1 protein in central and peripheral neruvous systems. Neurobiol Dis. 26(3): 532-45

Teijido O, Martínez A, Pusch M, Zorzano A, Soriano E, Del Río JA, Palacín M, Estévez R. (2004) Localization and functional analyses of the MLC1 protein involved in megalencephalic leukoencephalopathy with subcortical cysts. Hum Mol Genet. 13(21): 2581-94

Tokhtaeva E, Clifford RJ, Kaplan JH, Sachs G, Vagin O. (2012) Subunit isoform selectivity in assembly of Na,K-ATPase α-β heterodimers. J Biol Chem. 287(31): 26115-25

van der Knaap SM, Boor I, Estévez R. (2012) Megalencephalic leukoencephalopathy with subcortical cysts : chronic white matter oedema due to a defect in brain ion and water homeostasis. Lancet Neurol. 11(11): 973-85

van der Knaap SM, Barth GP, Stroink H, van Nieuwenhuizen O, Arts

MFW, Hoogenraad F, Valk J. (1996) Leukoencephalopathy with Swelling and a Discrepansy Mild Clinical Course in Eight Children. Annals of Neurology. 37(3): 324-34

van der Knaap SM, Barth GP, Vrensen FG, Valk J. (1995) Histopathology of an infantile-onset spongiform leukoencephalopaty with a oliscrepantly mild clinical course. Acta Neuropathol. 92: 206-12

Watts AG, Sanchez-Watts G, Emanuel JR, Levenson R. (1991) Cell-specific expression of mRNAs encoding Na(+),K(+)-ATPase alpha- and beta-subunit isoforms within the rat central nervous system. Proc Natl Acad Sci U S A. 88(16): 7425-9

Xin M, Yue T, Ma Z, Wu FF, Gow A, Lu QR. (2005) Myelinogenesis and axonal recognition by oligodendrocytes in brain are uncoupled in Olig1-null mice. J Neurosci. 25(6): 1354-65