Monocarboxylate transporters in temporal lobe epilepsy

by

Fredrik Lauritzen

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Department of Anatomy and Centre for Molecular Biology and Neuroscience

Institute of Basic Medical Sciences

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Contents

Abbreviations

AED	Antiepileptic drugs
ATP	Adenosine-5`-triphosphate
BBB	Blood-brain barrier
CA1-3	Cornu Ammonis subfields 1-3
CNS	Central Nervous System
GFAP	Glial fibrillary acidic protein
GLUT	Glucose transporter
HS	Hippocampal sclerosis
ISF	Interstitial fluid
KD	Ketogenic diet
LDH	Lactate dehydrogenase
MCT	Monocarboxylate transporters
MRI	Magnetic resonance imaging
MSO	Methionine sulfoximine
MTLE	Mesial temporal lobe epilepsy, i.e., TLE with hippocampal sclerosis
NAD^+	Nicotinamide adenine dinucleotide
NMDA	N-methyl-D-aspartate
Non-MTLE	TLE without hippocampal sclerosis
TLE	Temporal lobe epilepsy

List of papers

The present thesis is based on the following three original papers:

I. **Lauritzen, F.**, de Lanerolle, N.C., Lee, T-S.W., Spencer, D.D., Kim, J.H., Bergersen, L.H., Eid, T. (2011). Monocarboxylate transporter 1 is deficient on microvessels in the human epileptogenic hippocampus. *Neurobiol Dis* 41, 577-584.

II. ***Lauritzen, F.,** *Perez, E.L., Melillo, E.R., Roh, J-M., Zaveri, H.P., Lee, T-S.W., Wang, Y., Bergersen, L.H., Eid, T. (2012). Altered expression of brain monocarboxylate transporter 1 in models of temporal lobe epilepsy. *Neurobiol Dis* 45, 165-176.

III. **Lauritzen, F.**, Heuser, K., de Lanerolle, N.C., Lee, T-S.W., Spencer, D.D., Kim, J.H., Eid, T., Bergersen, L.H. Redistribution of monocarboxylate transporter 2 on the surface of astrocytes in the human epileptogenic hippocampus. *Submitted*.

Other related publications:

i *Perez, E.L., ***Lauritzen, F.,** Wang, Y., Kang, D., Zaveri, H.P., Ottersen, O.P., Lee, T-S.W., Bergersen, L.H., Eid, T. Increased glutamate in astrocytes: A potential source of extracellular brain glutamate in epilepsy? *Submitted*.

ii Heuser, K., Eid, T., **Lauritzen, F.**, Thoren, A.E., Vinedahl, G.F., Taubøll, E., Gjerstad, L., Spencer, D.D., Ottersen, O.P., Nagelhus, E.A., de Lanerolle, N.C. Loss of inwardly rectifying potassium channel Kir4.1 in the hippocampus in patients with mesial temporal lobe epilepsy. *Submitted*.

iii Eid, T., Lee, T-S.W., Wang, Y., Perez, E., Drummond, J., **Lauritzen, F.**, Bergersen, L.H., Woodruff, J.M., Spencer, D.D., de Lanerolle, N.C., McCullumsmith, R.E. Gene expression of glutamate metabolizing enzymes in the hippocampal formation in human temporal lobe epilepsy. *Submitted*.

* These authors contributed equally

1.0 INTRODUCTION

Epilepsy is a brain disorder characterized by recurrent and unprovoked interruptions of normal brain functions, i.e., seizures (Fisher et al., 2005). Epilepsy is not a single disease entity but comprises many different syndromes and seizure types, all of which are symptoms of underlying brain pathology. About 1 % of the global population has epilepsy, making it one of the most common disorders of the central nervous system (CNS). About one-third of all patients with epilepsy are unable to control their seizures with antiepileptic drugs (AED) (Kwan and Brodie, 2000), and these medication-refractory patients often suffer from partial seizures of temporal lobe origin, commonly known as temporal lobe epilepsy (TLE) (de Lanerolle and Lee, 2005). The seizures in TLE involve a network of temporal lobe structures including the hippocampus, dentate gyrus, entorhinal cortex, lateral temporal neocortex, amygdala, medial thalamus and inferior frontal lobes (Spencer, 2002). The clinical workup and classifications of TLE is often complicated and has resulted in various terminologies (Wieser, 2003). To establish a common nomenclature and a set of concepts to promote communication between physicians and researchers about treatment and research, the first international classification of seizures and epilepsies was presented in 1970 (Gastaut, 1970; Merlis, 1970). Since then, terminology and concepts for organization of seizures and epilepsies have been revised or discussed several times, and most recently in 2010 by the International League Against Epilepsy Commission on Classification and Terminology (Berg et al., 2010). According to the latter report, TLE no longer exists as a separate category. Nonetheless, the term *temporal lobe epilepsy* has been, and is still being widely used in the literature, and will for consistency, also be used in this thesis.

The cause of most epilepsy syndromes are unknown, however in some cases of TLE, the etiology of seizures is often suggested by congenital or acquired lesions which are sometimes found in the temporal lobe (Engel, 1996). Thus, etiology has been used to differentiate between different TLE syndromes such as lesional-, neocortical-, cryptogenic- and mesial TLE (Wieser, 2003) of which the latter constitutes the most common epileptic syndrome among adults (Engel, 1996).

1.1 Mesial temporal lobe epilepsy with hippocampal sclerosis

Due to the prevalent pharmacoresistance of TLE, anteromedial temporal lobectomy with hippocampectomy is sometimes used for seizure control in medication-refractory cases (Spencer et al., 1984). Histopathological analysis of the surgical resected temporal lobe tissue reveals that about 40% to 65% of the hippocampal formations are characterized by hippocampal sclerosis (HS) (de Lanerolle et al., 2003). The histopathological hallmarks of HS were first described by Sommer and Bratz at the end of the 19th century (Bratz, 1899; Sommer, 1880). A recent report has proposed the following pathological criteria for HS (Wieser, 2004): (1) Neuronal loss and gliosis at CA1 and end folium (hilus and dentate gyrus) with sparing of pyramidal neurons in the CA2 and subiculum. (2) All hippocampal regions may show neuronal loss and gliosis, although with variable extent. (3) Functional and structural glial changes. (4) Synaptic reorganization is often present. (5) Dispersion of dentate granular cells (~50 % of cases). (6) Extra-hippocampal pathology. (7) Other pathological findings can be demonstrated with different staining-methods. In this thesis, patients with TLE and concomitant HS are classified as mesial temporal lobe epilepsy (MTLE) (fig. 1).

Depth electrode recordings indicate that the hippocampus, particularly sclerotic regions within it, is the seizure focus in MTLE (Babb et al., 1984). Surgical resection of the sclerotic hippocampus results in excellent seizure control in most cases (de Lanerolle et al., 2003), indicating that structural and/or biochemical alterations in the sclerotic hippocampus are involved in epileptogenesis. Poorer surgical outcome is seen in patients with other types of TLE (e.g., lesional-, neocortical-, or cryptogenic TLE), here classified as non-MTLE (fig. 1). Non-MTLE patients have no sign of HS on magnetic resonance imaging (MRI), but a tumor, vascular malformation or dysplastic lesion in the temporal lobe or elsewhere in the brain is common (Eid et al., 2004). The seizure focus in non-MTLE is more variable than in MTLE and may involve other areas than the hippocampal formation (de Lanerolle et al., 2003). Bouchet and Cazauvielh described the association between epilepsy and HS already in 1825. However, although two centuries have passed since then, it is still controversial whether HS is the cause or the consequence of MTLE.

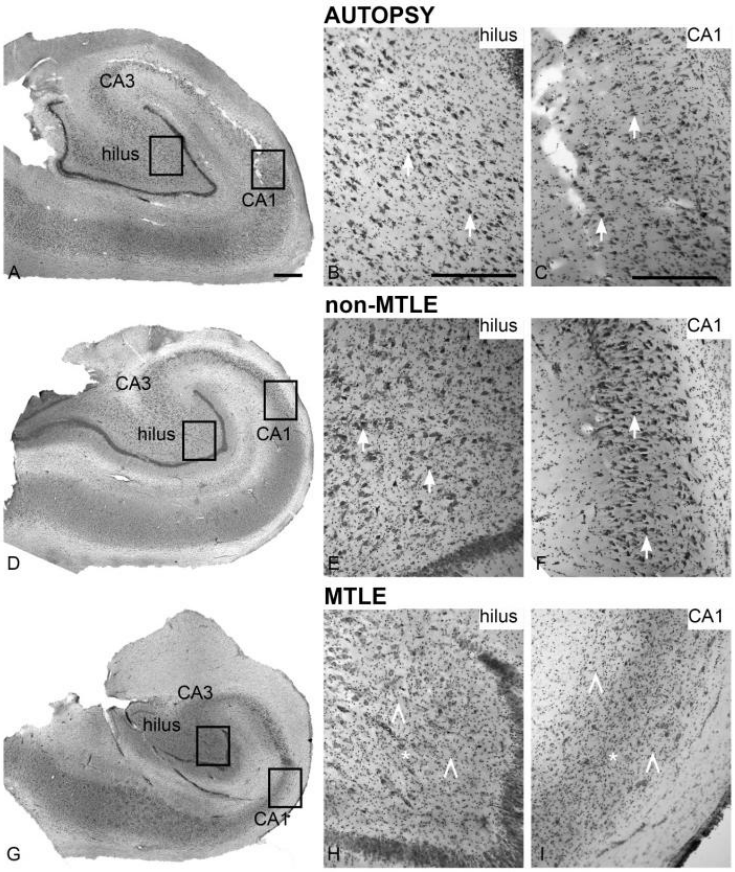

Figure 1. Hippocampal sclerosis. Nissl-stained sections of hippocampal formations from autopsy (A-C), non-MTLE (D-F) and MTLE subjects (G-I). Hippocampal formations from autopsy and non-MTLE subjects are characterized by dense populations of neurons (arrows) in the hilus (B, E) and in the CA1 pyramidal cell layer (C, F). Hippocampal formations from MTLE subjects exhibit hippocampal sclerosis, including hippocampal atrophy (G), neuronal loss (asterisk) in the hilus (H) and CA1 (I), and reactive gliosis (open arrowheads) (G-I). Bars: A, 1mm (same magnification in D, G), B and C, 500 µm (same magnification in E, F, H, I). Modified from publication iii, Eid et al. *Gene expression of glutamine metabolizing enzymes in the hippocampal formation in human temporal lobe epilepsy*. Submitted.

Because so many MTLE patients are refractory to current AEDs and the existing treatment alternatives are often invasive, complicated, and not without complications, there is an urgent need for the development of novel anti-epileptic as well as anti-epileptogenic therapies. The main aim of this thesis is to provide a better understanding of some of the underlying mechanisms of medication-refractory TLE. This new knowledge might in turn facilitate the discovery of novel therapeutic targets for this disease. In this respect, it is important to acknowledge that epilepsy is a multifaceted neurological syndrome with a

vast number of cellular and physiological characteristics. Hence, it is unlikely that the seizures are caused by a single factor. Any new knowledge about the cellular alterations in MTLE would therefore represent an important piece in the puzzle of unraveling the mysteries of HS and drug-refractoriness.

A thorough review of all aspects associated with MTLE is beyond the scope of this thesis. The focus will rather be on a novel concept that recently has emerged as a putative contributor to epileptogenesis, namely impairments in the cerebral energy metabolism in general, and the role played by non-glucose fuels in particular. Before addressing this matter, I will briefly introduce the physiology and pathophysiology of two cell structures that are believed to be particularly involved in cerebral metabolism, the endothelial cells of the blood-brain barrier (BBB) and astrocytes.

1.2 The blood-brain barrier

Precise regulation of the synaptic microenvironment is necessary for reliable signaling between neurons. In the CNS, the endothelial BBB located at the interface between the blood and brain interstitial fluid (ISF) plays a crucial role in this regulation (Abbott et al., 2006; Hawkins and Davis, 2005). In addition to the BBB, other CNS barriers includes the blood-cerebrospinal fluid barrier formed by the epithelial cells of the choroid plexus located between blood and cerebrospinal fluid, and the avascular arachnoid epithelium which separates the extracellular fluid of the CNS from that of the rest of the body (Abbott et al., 2010). Among the CNS barriers, the BBB has the predominant regulatory role. This is due to its vast surface area (150 - 200 cm^2 g^{-1} tissue) that provides a large total area of exchange (12 - 18 m^2 in the adult human brain) (Nag and Begley, 2005), and the short diffusion distance between individual neurons and BBB capillaries (<8-20 µm) (Schlageter et al., 1999).

The protective function of the BBB results from the combination of physical-, transport-, and metabolic barriers (Abbott et al., 2010). The *physical barriers* are made up of continuous tight junctions between adjacent endothelial cells that span the intercellular cleft and seal the diffusional pathway between cells. This forces most of the molecular traffic to take a transcellular route across the BBB, instead of paracellular transport through the junctions (Abbott et al., 2006). Specific transport proteins located on

endothelial luminal and/or abluminal plasma membranes constitutes the *transport barrier*. They facilitate the entry of important nutrients and other solvents into the brain parenchyma, while expelling possibly harmful substances. Finally, several families of enzymes located on the cell membrane provide a *metabolic barrier*. These enzymes metabolize potentially toxic penetrating lipophilic substances so that they cannot travel further across the BBB. In addition to the three barriers, there is a low degree of endocytosis/transcytosis in cerebral endothelium compared to that in peripheral endothelium. Together, the components and properties of the BBB provide strict control of the exchanges between the blood and the brain by limiting passive diffusion of blood-borne solutes while actively transporting nutrients into the brain (Weiss et al., 2009). Notably, the BBB is a dynamic system which can be modulated and regulated under physiological and pathophysiological conditions (Abbott et al., 2006).

1.2.1 The blood-brain barrier and temporal lobe epilepsy

BBB dysfunctions are reported in numerous neurological diseases, including epilepsy. Exactly why a compromised BBB may result in recurrent seizures is not well known. An attractive hypothesis is altered expression of selected BBB transport barrier-components, such as nutrient transporters. This hypothesis is particularly interesting when considering MTLE as a disorder associated with cerebral metabolic dysfunctions. For example, the glucose transporter (GLUT) 1 deficiency syndrome, a rare autosomal dominant disorder attributed to a defect in the *SLC2A1* GLUT1 gene, causes impaired transport of glucose across the BBB, interfering with brain energy metabolism, ultimately leading to seizures (De Vivo et al., 1991). The role of BBB GLUT1 in medication-refractory TLE is unclear. Studies on patients with temporal seizures but with variable MRI-findings, demonstrate that the hypometabolic seizure focus corresponds to decreased BBB glucose transport activity (Cornford et al., 1998a) and downregulation of GLUT1 on endothelial cells (Cornford et al., 1998b). Animal models of MTLE, however, are characterized by an acute upregulation of BBB glucose transporter activity (Cornford et al., 2000) and over-expression of GLUT1 mRNA and protein levels on the BBB (Gronlund et al., 1996; Leroy et al., 2011). The discrepancies between patients and animal models may be due to different cellular adaptations to acute seizures vs. chronic seizures. Additional studies are needed to clarify the role of GLUTs in TLE. The role of non-glucose energy transporters in epilepsy has

not yet received much attention. This is surprising considering the important functions of non-glucose fuels in the brain (for further discussion, see section 1.4). Nonetheless, transport of non-glucose fuels, namely monocarboxylates, is the main focus of this thesis and will accordingly be thoroughly discussed in the following.

BBB dysfunctions in epilepsy comprise a broad specter of factors certainly not limited to dysfunctional transporter proteins. Other known pathophysiological alterations includes but are not limited to: BBB disruption (Marchi et al., 2010; Nitsch and Klatzo, 1983; Rigau et al., 2007), angiogenesis (Morin-Brureau et al., 2011; Rigau et al., 2007) and upregulation of multidrug resistance-associated proteins (Dombrowski et al., 2001). For a thorough review of aspects related to the BBB in epilepsy, please refer to review papers by Oby and Janigro (2006) and Weiss and colleagues (2009).

Anatomically, brain endothelial cells are surrounded by the basal lamina and are in addition closely apposed by several other cell types, including perivascular astrocyte endfoot processes, pericytes and neurons (figs. 2 and 3). Together they constitute a neurovascular unit that is essential for the health and function of the CNS (Hawkins and Davis, 2005). Astrocytes, constituents of the neurovascular unit, play particular important roles in brain homeostasis. These star shaped cells are in close contact with endothelial cells, neurons and other astrocytes via an array of finely branched processes (Peters and Palay, 1965). Such intimate associations make it possible for individual astrocytes to

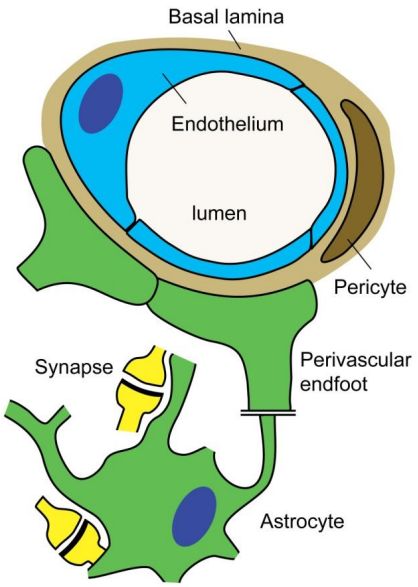

Figure 2. The neurovascular unit. Endothelial cells, basal lamina, astrocytes and perivascular endfeet, pericytes and neurons are all involved in the formation and maintenance of the blood-brain barrier and in the regulation of cerebral homeostasis.

support the function of neurons to communicate with the microvasculature and facilitate the flux of solutes between blood and cells in the brain parenchyma (Nedergaard et al., 2003).

1.3 Astrocytes

Astrocytes, the most numerous of the glial cells, were long considered a passive constituent of the CNS, merely providing structural support to neuronal networks. Research carried out over the past decade has changed this view, and it is now acknowledged that astrocytes are intimately involved in brain function. Astrocytes are not a homogenous cell type, but include a heterogeneous population of cells with different morphological variants and functional differences depending on their localization in the brain and association with other cell types (Matyash and Kettenmann, 2010). Astrocytes have been traditionally divided into two main subgroups: Protoplasmic astrocytes and fibrous astrocytes. Protoplasmic astrocytes are localized within the grey matter of the CNS, while fibrous astrocytes inhabit the white matter. Because synapses are located in the gray matter we will focus on protoplasmic astrocytes here. Protoplasmic astrocytes form highly organized, non-overlapping astrocyte domains which cover all grey matter CNS-regions. Their anatomical and functional features make them ideal to sense their surroundings and respond to changes in the microenvironment (Allaman et al., 2011). In the hippocampus, perivascular endfeet; distal expansions of astrocytic processes adjacent to the blood vessel wall (Glees, 1955), provides a complete covering of the microvessels, thereby controlling the exchange of molecules between blood and brain ISF (Mathiisen et al., 2010) (fig. 3). In the neuropil, i.e., the substance between the neuronal cell bodies, processes from a single astrocyte can contact multiple neurons and envelope more than 100,000 synapses (Bushong et al., 2002). There, astrocyte processes forms so called tripartite synapses together with a presynaptic neuronal terminal and a postsynaptic dendrite (Araque et al., 1999). Moreover, astrocytes are connected with adjacent astrocytes through gap junctions, thus creating a large glial syncytium where small molecules (<1 kDa) can pass freely (Giaume and McCarthy, 1996).

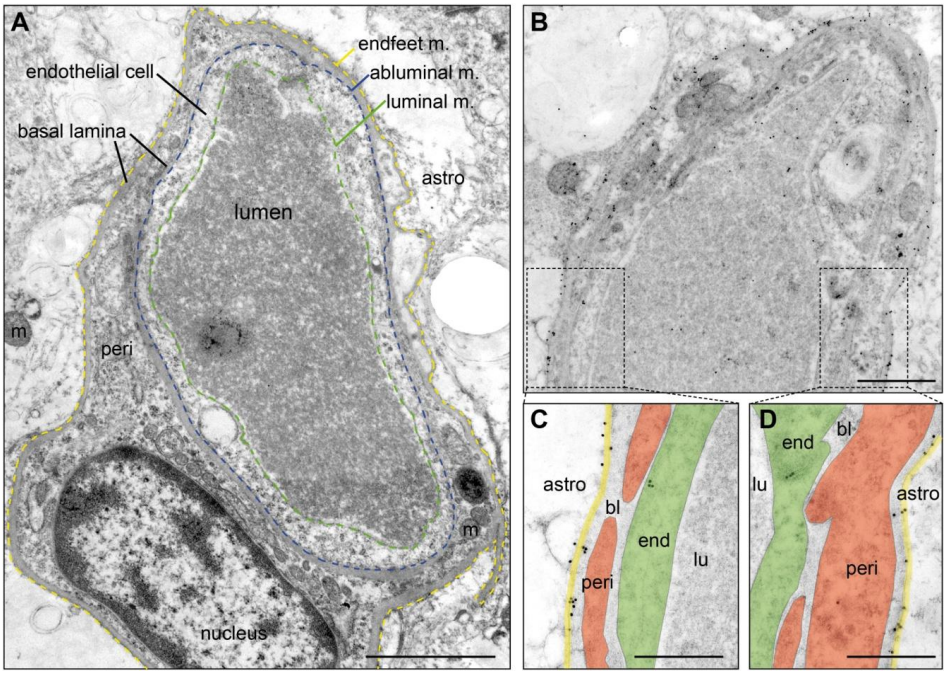

Fig. 3. Electron micrographs of human hippocampal capillaries. (A) The physical barrier of the BBB consists of endothelial cells connected by tight junctions. Transport across the BBB is primarily mediated by proteins localized on luminal (green line) and abluminal (blue line) endothelial plasma membranes (e.g., the transport barrier). The basal lamina separates the endothelial cells and the perivascular endfeet of the astrocytes (yellow line). (B) Part of a cerebral microvessel. The immunogold method is used to detect monocarboxylate transporter 2 on the tissue section, here represented as gold particles/black dots. (C, D) High power images of stippled insets in B. The various components of the BBB are visualized by different colors. The astrocyte endfoot membrane is depicted as a yellow line. Abbreviations: astro, astrocyte; bl, basal lamina; end, endothelial cell; lu, blood vessel lumen; m, mitochondria; peri, pericyte. Bars: A, 2 µm; B, 1 µm; C, D, 500 nm.

Astrocytes are involved in several key “housekeeping” functions in the CNS, including fluid, ion and transmitter homeostasis, neuroinflammation and tissue repair, energy metabolism, modulation of synaptic activity through calcium-induced release of neuroactive agents and synapse formation and remodeling (Sofroniew and Vinters, 2010). Considering the many important roles played by astrocytes, dysfunctions in these cells are likely to affect the brain homeostasis and possibly lead to neurodegenerative processes through the loss of normal function or gain of detrimental effects.

1.3.1 Astrocytes and temporal lobe epilepsy

A bulk of evidence show that following lesions to the CNS, the gene expression and functional properties of astrocytes are altered in ways that are suggested to cause or

contribute to CNS disease (Allaman et al., 2011). This pathological process is often referred to as reactive astrogliosis. In general, reactive gliosis is a non-stereotypic response to CNS injury characterized by finely graded, progressive changes in the gene expression and cellular properties of astrocytes in relation to the severity of the injury (Norton et al., 1992). Characteristics of reactive gliosis are upregulation in the expression of glial fibrillary acidic protein (GFAP) and other genes, hypertrophy of cell body and processes, astrocyte proliferation and tissue reorganization (Sofroniew and Vinters, 2010). In addition to histopathological changes, reactive astrocytes release various mediators, such as cytokines, chemokines, complement factors and reactive oxygen species, all of which potentially mediate neuroprotective and/or neurotoxic effects (Sofroniew and Vinters, 2010). Notably, reactive gliosis and glial scar formation are prominent features of chronic, focal/partial epilepsies, such as TLE (de Lanerolle et al., 2010; Harris, 1975; Pollen and Trachtenberg, 1970). Reactive astrogliosis, visualized by GFAP-positive cells is particularly evident in neuron-depleted areas, such as in the CA1, and positively correlates with seizure frequency in sclerotic hippocamal formations from MTLE patients (Cohen-Gadol et al., 2004). These astrocytes exhibit several unusual features, including altered expression of membrane ion channels, decrements of astrocyte specific enzymes, upregulation of genes involved in cell morphology and immune and inflammatory responses, and changes in the expression of perivascular endfoot proteins (reviewed by de Lanerolle et al., 2010; Seifert et al., 2010). The observations of dysfunctional astrocytes in hippocampal formations of patients of animal models with MTLE have led many to suggest a pivotal role for reactive astrocytes as active contributors to the epileptogenic process (Harris, 1975; Pollen and Trachtenberg, 1970). For example, in hippocampal formations of MTLE patients and in animal models of this disease, the loss of glutamine synthetase (Eid et al., 2004; Wang et al., 2009), an astrocytic glutamate metabolizing enzyme, may impair the conversion of glutamate to glutamine, thus contributing to an accumulation of glutamate in the astrocyte cytosol (Perez et al., submitted, publication i) and consequently a buildup of glutamate in the extracellular space (During and Spencer, 1993; Eid et al., 2008b). The hippocampal formations of MTLE patients are also recognized by loss of inwardly rectifying potassium channels on astrocyte plasma membrane facing the neuropil which is likely to impair spatial buffering of K^+ and contribute to hyperexcitability and epileptogenesis (Heuser et al., submitted, publication ii).

1.4 Impaired energy metabolism and temporal lobe epilepsy

Brain tissue is known to be very dependent on adequate fuel substrate. This is emphasized in the fact that the brain consumes 20 % of the total body resting metabolism although it only constitutes 2 % of the total body weight (Rolfe and Brown, 1997). It is therefore not surprising that impairments in the cerebral energy metabolism may have detrimental consequences on normal brain function. Accordingly, hypometabolism is a common hallmark of several serious neurological disorders, including Alzheimer's disease (Ferreira et al., 2010), Parkinson's disease (Borghammer et al., 2010), Huntington's disease (Browne and Beal, 2004) and medication-refractory TLE , in which several studies have confirmed an association between impaired mitochondrial function and reduced synthesis of adenosine-5'-triphosphate (ATP) in mesial temporal lobe structures (Connelly et al., 1994; Hetherington et al., 1995; Kuhl et al., 1980; Pan et al., 2005; Theodore et al., 1983). Interestingly, there is increasing evidence that perturbations in the brain energy metabolism are not only an accompanying characteristic of epilepsy but that it directly contributes to epileptogenesis.

Glucose either directly supplied from blood or formed from astrocyte-derived glycogen is the predominant substrate for ATP production under normal conditions. However, many *in vitro* and *in vivo* studies have demonstrated that neurons can, and may even prefer to use monocarboxylates to fuel aerobic brain metabolism, particularly during times of neuronal activation (Barros and Deitmer, 2010; Boumezbeur et al., 2010; Dalsgaard et al., 2004; Ivanov et al., 2011; Larrabee, 1995; Pan et al., 2002; Pellerin and Magistretti, 1994; Schurr et al., 1999; Schurr et al., 1988; Smith et al., 2003; Wyss et al., 2011). Monocarboxylates are organic acids such as lactate, pyruvate and ketone bodies. Lactate in the form of lactic acid (lactic acid dissociates into a lactate ion and a proton at physiological pH) was first detected in fermented milk by the Swedish chemist Carl Wilhelm Scheele in the 18th century. Some years later, Jöns Jacob Berzelius, a fellow-countryman of Scheele, observed that lactic acid was produced in muscle during exertion. We now know that lactate, via the enzyme lactate dehydrogenase (LDH), is constantly formed in the cytosol from pyruvate in the last step of glycolysis (fig. 4). The conversion of pyruvate to lactate is essential for cellular ATP production during periods of insuffient oxygen supply because the LDH reaction also forms nicotinamide adenine dinucleotide (NAD^+) from NADH, a step that is

necessary to maintain a high rate of glycolysis. The LDH reaction is bidirectional, that is, if the cell has the capacity for oxidative metabolism and the oxygen supply is sufficient, lactate is converted back to pyruvate together with the conversion of NAD^+ to NADH. In vertebrates, five LDH isoenzymes exist, each with unique subunit compositions and tissue expressions. In the human hippocampus, LDH-1, which is predominantly found in aerobic tissues and generally accepted to be better at oxidizing lactate to pyruvate is expressed in neurons and astrocytes (Bittar et al., 1996; Cahn et al., 1962; Markert et al., 1975), while LDH-5 preferentially converts pyruvate to lactate in lactate-producing tissues and is present in astrocytes only (Bittar et al., 1996; Cahn et al., 1962). The presence of cell-specific LDH isoenzymes indicate that astrocytes both imports and exports lactate, while neurons mainly import lactate. Other have claimed that the LDH isoenzyme pattern is without effect on the intracellular lactate concentration since LDH is regarded a near-equilibrium reaction and the equilibrium constant of all LDH enzymes are the same (Quistorff and Grunnet, 2011). During rest, the human brain simultaneously release and take up lactate, albeit there is a small net release (van Hall et al., 2009). When arterial lactate levels increase, for example during systemic lactate infusion or physical exercise, blood content-, and thus brain uptake increases several fold (Ide et al., 2000; van Hall et al., 2009).

Ketone bodies, that is acetoacetate, β-hydroxybutyrate and acetate, are formed in the liver from fatty acids and can be interconverted by 3-hydroxybutyrate dehydrogenase and oxidized in many tissues, including in the brain (fig. 4) (Morris, 2005). Like lactate and pyruvate, ketone bodies can be metabolized in both the developing and adult brain (Cremer and Heath, 1974; Nehlig, 2004; Nehlig and Pereira de Vasconcelos, 1993; Pan et al., 2002). The rate of cerebral ketone body metabolism is dependent on two main factors: 1) The blood concentration, and 2) the transport of ketone bodies across the BBB (Morris, 2005). The presence of these compounds in the blood at rest is low (Morris, 2005) but can be significantly increased during fasting (Haymond et al., 1982; Owen et al., 1967) or following a high-fat, low-carbohydrate – ketogenic diet (KD) (Morris, 2005; Musa-Veloso et al., 2002). High blood levels is also seen in neonates (Nehlig and Pereira de Vasconcelos, 1993). High blood ketone levels are accompanied by increased transport of these

compounds into the brain (Daniel et al., 1977; Gjedde and Crone, 1975; Pollay and Stevens, 1980).

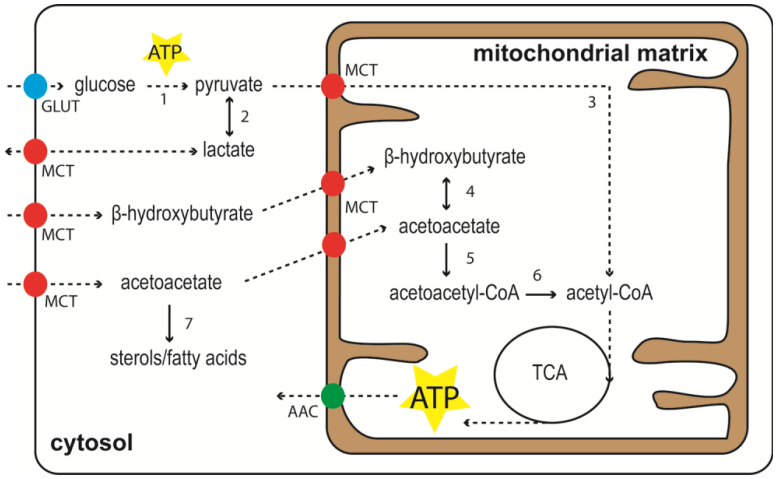

Figure 4. Simplified diagram showing pathways for cerebral metabolism of glucose and monocarboxylates. Enzymes: 1. The glycolytic pathway, 2. Lactate dehydrogenase, 3. Pyruvate dehydrogenase complex, 4. 3-hydroxybutyrate dehydrogenase, 5. Succinyl-CoA-oxoacid CoA transferase, 6. Mitochondrial acetoacetyl-CoA thiolase, 7. Several enzymes depending on direction of the pathway, including acetoacetyl-CoA synthetase, cytoplasmic acetoacetyl CoA thiolase, cytoplasmic hydroxymethylglutaryl-CoA synthase. Abbreviations: *AAC*, ADP/ATP carrier; *GLUT*, glucose transporter; *MCT*, monocarboxylate transporter.

A particular interesting finding that links monocarboxylates to medication-refractory TLE is the remarkable clinical efficacy of the KD on these patients. Ever since Wilder in the beginning of the 20th century observed that a high-fat diet could be used to control intractable seizures (Wilder, 1921), the KD has been prescribed to patients of all ages with significant success (Kossoff et al., 2008; Neal et al., 2008; Sirven et al., 1999). The exact anticonvulsive mechanisms of the KD remain elusive, but efficient transport of circulating ketone bodies across the BBB seems to be a prerequisite. For the ketone bodies and the other monocarboxylates to be used by cells in the brain parenchyma, they must cross the BBB and plasma membranes of individual cells. At very high concentrations, monocarboxylates freely diffuses across phospholipid plasma membranes (Bakker and Van Dam, 1974). However, although not yet discovered at the time, the low diffusion rates of monocarboxylates across plasma membranes at physiological concentrations indicated that there had to be a carrier-mediated mechanism to provide efficient flux of these

molecules between cells and organelles (Henderson et al., 1969; Papa et al., 1971). This was confirmed some years later, when Halestrap and colleagues provided evidence for the presence of solute carriers for monocarboxylates (1974).

1.5 Monocarboxylate transporters

The monocarboxylate transporter (MCT) family comprises 14 members (Halestrap and Meredith, 2004). Only the first four isoforms have been experimentally demonstrated to be functionally expressed, of which MCT1, MCT2 and MCT4 are present in the brain (Bergersen, 2007), and MCT3 in retinal epithelial cells and the choroid plexus (Philp et al., 2001). The transport of monocarboxylate anions is coupled by 1:1 stoichiometry to the symport of a proton (Broer et al., 1998; Halestrap and Price, 1999). The MCT isoforms differ in their substrate affinity (Table 1) and cellular distribution. The transport of monocarboxylates into or out of the cytoplasm is notably determined by the concentration gradient of these molecules as well as protons across the membrane; however, the K_m values of the different isoforms might explain their functional role in various tissues. MCT1 is ubiquitously expressed but preferentially found on cells presumed to import monocarboxylates. In the brain, MCT1 is mainly detected on endothelial cell of the BBB, both in mice (Koehler-Stec et al., 1998), rats (Bergersen et al., 2001; Gerhart et al., 1997; Leino et al., 1999) and humans (Froberg et al., 2001). By being expressed on cerebral microvessels, MCT1 is part of the BBB transport barrier and controls the passage of monocarboxylates between the blood and brain.

The high affinity MCT2 is less widely distributed than MCT1. It also demonstrates substantial species differences in both its amino acid sequence and tissue distribution (Jackson et al., 1997). In rat brain, MCT2 immunoreactivity has primarily been found on perivascular endfeet (Cornford and Hyman, 1999; Gerhart et al., 1998; Hanu et al., 2000), neuronal post-synaptic densities (Bergersen et al., 2001; Bergersen et al., 2005) or both (Baud et al., 2003; Rafiki et al., 2003). Little is known about the MCT2 expression in the human brain. According to the human EST database and Northern blotting analysis done by Price and colleagues (1998), MCT2 mRNA levels in human tissues are minimal if at all present. However, a strong and widespread expression on neurons and astrocytes has been shown on brain autopsy tissue using immunohistochemical methods (Chiry et al.,

2008). The discrepancies regarding the MCT2 distribution emphasize the importance of more studies to clarify the exact localization of MCT2 in the human brain.

Table 1: Comparison of K_m values for various substrates of MCT1 and MCT2 expressed in xenopus oocytes (Broer et al., 1999; Broer et al., 1998).

	K_m (mM)	
Substrate	MCT1	MCT2
Lactate	3.5	0.5
Pyruvate	1.0	0.08
Acetoacetate	5.5	0.8
β-hydroxybutyrate	12.5	1.2

There is indeed a tight metabolic coupling between MCT expression and monocarboxylate levels. We know that during early postnatal development, when the offspring´s dietary needs are supplied mainly by fat-rich milk from the mother, the brain uptake and utilization of monocarboxylates increases dramatically (Cornford et al., 1982; Cremer et al., 1976). At the same time, levels of MCTs on the BBB and on brain parenchymal cells is high compared to in adult animals (Baud et al., 2003; Leino et al., 1999; Vannucci and Simpson, 2003). This effect is less pronounced in human neonates than in suckling rats, due to the higher fat content in rat- than in human mature milk (Morris, 2005). BBB MCT is also increased in adult rats when blood ketone levels are raised following KD (Leino 2001).

Despite the fact that the MCTs facilitate the transport of molecules crucially important for normal brain function, their expression and potential role in neurological disease is not well known. As a first step in clarifying this matter, the goal of this thesis was to examine the distribution of MCT1 and MCT2 in the epileptogenic brain. In the following section, key methodological aspects related to the results presented in this thesis are discussed. Then I summarize the aims and results of the three papers included in the thesis before ending with a general discussion of the findings.

2.0 METHODOLOGICAL DISCUSSION

2.1 The use of human tissue samples

Human hippocampal sections were obtained from the Yale epilepsy program at Yale University School of Medicine and Yale-New Haven Hospital. At the Yale-New Haven Hospital, medication-refractory TLE patients selected for surgery after phased clinical and electrophysiological assessment, underwent anteromedial temporal lobectomy, including hippocampectomy (Spencer and Spencer, 1991). The resected tissue samples used in this thesis were randomly selected from the brain tissue repository of the Yale epilepsy program and categorized as MTLE or non-MLTE based on standard histological criteria of HS by two experienced investigators (F. Lauritzen and T. Eid). In contrast to MTLE, non-MTLE cases exhibit normal histology by visual examination. For this reason, non-MTLE is often used and referred to as controls in the literature. This tradition raises some important questions because non-MTLE patients constitute a widely heterogeneous group with different pathological features ranging from mass lesions and vascular deformations, to no histological abnormalities. Importantly, all non-MTLE cases have recurrent seizures originating from the temporal lobe and are therefore suitable in combination with MTLE when studying cellular mechanisms of HS.

The lack of controls is a pervasive problem when examining pathological mechanisms in human brain tissue. Due to certain moral aspects, brain tissue is rarely removed from healthy individuals, not even for research purposes. One alternative is to include autopsy tissue, as we have done in our studies. These subjects are neurologically normal, however the use of autopsies introduces other challenges. Tissue resected post mortem is usually inferiorly preserved compared to surgical tissue. Because the tissue is “dead” at the time of resection, degeneration may have already started. Also, the lack of standardized procedures regarding tissue preservation may cause the time from death to fixation of the tissue to vary between cases. As a consequence, the tissue morphology at high magnification, such as with electron microscopy, as well as the degree of antibody penetration and binding, will be affected. This must be taken into account when comparing labeling density between autopsy and TLE-groups. Accordingly, the strong labeling of

autopsy presented in papers # I and III may be artifacts caused by partly denatured proteins, and hence, more available epitopes for penetrating antibodies. Nevertheless, although not optimal, autopsy tissue is valuable as it often is the best control available for the researcher. As long as one recognizes the possible limitations of using autopsies, these samples may provide a useful estimate of the protein distribution in normal tissue, assuming that the staining methods used are accurate.

2.2 The use of laboratory models to study MTLE

A goal when using experimental animal models to understand the nature of human disease is that the model of choice involves the pathology and pathophysiology of the specific human condition. In paper # II, three novel laboratory models were used to induce spontaneous, recurrent seizures: The glutamine-synthetase deficiency-/methionine sulfoximine (MSO) model developed in the laboratory of Prof. Tore Eid at Yale University School of Medicine (Eid et al., 2008a; Wang et al., 2009), and two modified versions of the perforant pathway stimulation model first described by Prof. Robert Sloviter and colleagues at the University of Arizona College of Medicine (Harvey and Sloviter, 2005; Sloviter, 1996). Traditionally employed models of hippocampal-onset TLE, such as systemic kainic acid (Ben-Ari, 1985; Nadler and Cuthbertson, 1980) and pilocarpine injections (Cavalheiro et al., 1991; Turski et al., 1983) are characterized by high lethality, extensive and highly variable brain damage among surviving animals and seizures of unknown origin that only minimally involve the hippocampal formation (Harvey and Sloviter, 2005; Schwob et al., 1980; Sloviter, 2005). Consequently, it is difficult to assess which of the many effects produced in chemoconvulsant-treated animals that may be causally epileptogenic, and which are confounding phenomena (Sloviter, 2009). In contrast, the models employed in paper # II more closely replicate key features of MTLE, in addition to being characterized by a high success- and low mortality rate.

2.3 Imaging techniques and quantification of cellular protein

In this project, light- and electron microscopy were used to investigate the distribution of MCTs on epileptogenic hippocampal formations. Histological methods are associated with

both strengths and limitations. In the following discussion, aspects of the postembedding immunogold electron microscopic method will be emphasized.

The use of electron microscopy in histological studies has several advantages over other imaging techniques. Firstly, the high power resolution makes it possible to distinguish subcellular compartments within cells. By using contrasting agents, cellular structures can be visualized directly without the need of markers. In addition, by assuming that there is a linear relationship between the gold particle density and the number of antigens in a tissue (Ottersen, 1989), the immunogold method make it possible to quantify reliably and compare the contents of molecules between different membrane domains or organelles of the same or different cell types (Bergersen et al., 2008). On the critical side, the high resolution makes analysis time-consuming and limits the tissue area and number of cases that can be studied. Inclusion of larger tissue areas from a higher number of specimens can be achieved by supplementing electron microscopic analysis with light microscopic studies. In papers # I-III, light microscopic immunoperoxidase was first done to get an overview of the MCT distribution on hippocampal formations of multiple specimens. Qualitative visual analysis of protein distribution was followed by semi-quantitative stereological analysis. Because autopsy tissue could not be analyzed by electron microscopy, stereological analysis of light microscopic sections made it possible to compare densities of MCT as well as other markers in autopsy vs. non-MTLE and MTLE. In papers # I and III, randomly selected CA1-sections from non-MTLE and MTLE were further analyzed by immunogold quantification to assess the subcellular distribution of MCT1 and MCT2. The postembedding This method is based on a step-wise procedure in which ultrathin tissue sections mounted on nickel grids are immersed in solutions of primary and secondary antibodies, as well as mixtures intended to enhance the specific labeling and inhibit the unspecific labeling (Bergersen et al., 2008). In brief, a primary-antibody binds to a specific antigen on the surface of the section, e.g., MCT1, which are identified in the electron microscope via a colloidal gold-coupled secondary-antibody bound to the primary-antibody (fig. 5). The high electron density of the gold particles makes them highly visible and facilitates detection of the molecule on specific structures. For the immunogold data to be useful and reliable, the labeling should as closely as possible determine the actual quantity and position of a given protein in a tissue. Several factors may alone or in concert

potentially affect the immunosignal, such as antigen epitope availability, labeling efficiency and specificity, and the lateral resolution of the method.

Presence of antigen: Depending on the method, antibody solutions are applied onto the tissue either before (preembedding) or after (postembedding) resin embedding. The main advantage of the latter technique is that each antigen molecule present on the tissue surface has an equal chance of being immunodetected, regardless of its cellular or subcellular localization (Mathiisen et al., 2006). This is in contrast with the preembedding method were diffusional barriers imposed by membranes and other tissue constituents may restrict the degree of antibody binding, and hence distort the relationship between tissue protein content and gold particle density (Mathiisen et al., 2006). For this reason, the postembedding method is preferred if the protein density is of interest.

Labeling efficiency and specificity: The term labeling efficiency refers to the ratio between the number of gold particles and the number of antigen molecules available for immunolabeling (Mathiisen et al., 2006; van Lookeren Campagne et al., 1991). The labeling efficiency is affected by the quality of the antibody, fixation procedure, embedding medium and incubation parameters (Griffiths, 1993; Matsubara et al., 1996). High labeling efficiency is of particular importance when examining proteins expressed at low densities. Low labeling efficiency was not a major issue in this thesis.

A major pitfall associated with the EM immunogold method is binding of the primary antibody to unspecific proteins rather than to the target antigen. It is therefore eminent that careful measures are taken to confirm antibody specificity. A particular powerful negative control can be performed by applying the antibody to tissue derived from a knock-out mouse in which part of the gene encoding the target protein has been deleted (Bergersen et al., 2008). If the antibody is specific, deletion of the respective gene should abolish the labeling. However, one might risk that although selected exons in the target protein's DNA sequence is deleted the protein may still be synthesized, albeit in an inactivated, silent form which may promote antibody binding. Knock-outs are not presently available for any of the MCTs. Further should all antibodies be tested on Western blots of brain homogenates. If the blot provides a clean band of appropriate molecular weight, it indicates that the antibody binds the protein in question. Unfortunately, brain

homogenates of human brain tissue are not easily available. Western blots could therefore not be performed. In our studies, we used standard absorption experiments, i.e., the primary antibody is neutralized by applying excess of the immunization peptide, to test primary antibody specificity. It should be emphasized that this is not an optimal specificity test as antibodies cross-reacting with another protein will also be removed by the pre-absorption (Bergersen et al., 2008). The specificity of the secondary antibody should also be tested, although binding of secondary antibodies to other molecules than the primary antibody is infrequent. The latter was tested by omitting the primary antibody from the staining procedure. When the primary antibody is not present on the tissue, the secondary antibody should not bind anything. Gold particle clumping may pose a problem but is normally easily solved by centrifuging the secondary antibody stock and/or by including polyethylene glycol to the secondary antibody solution (Bergersen et al., 2008).

Lateral resolution: The theoretical lateral resolution of the immunogold method, i.e., the distance from the epitope to the respective gold particle, is maximally about 30 nm (Bergersen et al., 2008) (fig. 5). Therefore, gold particles situated at some distance from the epitope may still represent specific labeling. This may pose a challenge when quantifying gold particle densities in tiny tissue elements, such as along membranes which are in close proximity to structures of surrounding cells. When calculating the protein density on membranes, a line was drawn along the membrane and count gold particles around it. To assure that the included gold particles are actually associated with the membrane, we only include

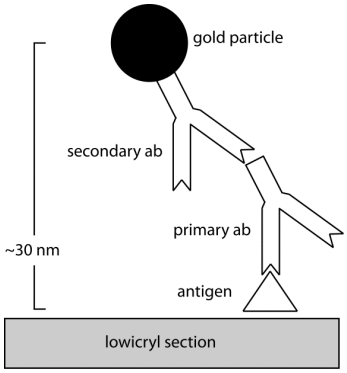

Fig. 5. The immunogold method. The primary ab (antibody) binds an antigen (e.g., MCT1) present on the tissue surface. The protein is visualized in the electron microscope by a colloidal gold particle attached to the secondary ab. Thirty nm is the theoretical maximal lateral resolution of the method when using a 10 nm gold particle.

those located on the membrane itself in addition to those within 25 nm from the membrane on the intracellular side. For detailed description on how gold particles densities on the various cell components were measured, please refer to the original papers (Lauritzen et al., 2011; Lauritzen et al., 2012; Lauritzen et al. Submitted).

All the above-mentioned factors may largely be eliminated by carefully planned and executed experiments. However, the main inherent limitation of imaging is its descriptive nature. Accordingly, the data can only provide information about the status quo and consequently not explain the mechanism behind the present cellular characteristics. Possible implications of our findings are discussed in section 4.

3.0 AIMES AND MAIN FINDINGS

3.1 Paper I

Introduction: Increasing evidence suggests that an impairment of the brain energy metabolism is involved in the pathophysiology of TLE. Monocarboxylates may play an important role in this disorder as these molecules improve brain energy stores, reduce the seizure frequency in patients with medication-refractory TLE, and protect against neuronal loss in animal models of TLE. Monocarboxylates are transported across membranes by proton-linked MCTs. MCT1 is expressed on cells presumed to import monocarboxylates, such as on endothelial cells in the CNS. We hypothesize that the distribution of MCT1 is disturbed in these patients with important consequences for monocarboxylate transport and brain energy metabolism.

Aim: To better understand the metabolic pathways for non-glucose fuels in TLE, we examined the distribution of MCT1 in hippocampal formations from non-MTLE and MTLE patients and from neurologically normal autopsy subjects.

Main findings: Hippocampal formations from autopsy and non-MTLE subjects exhibited unremarkable gross anatomy and histology. In contrast, hippocampal formations from MTLE subjects were characterized by gross atrophy and reactive gliosis and neuronal loss in the dentate hilus, CA3 and CA1. Using immunohistochemistry, a widespread network of microvessels positive for MCT1 was found in autopsy and to a lesser degree in non-MTLE, whereas labeling for MCT1 was nearly absent in neuron depleted areas of MTLE. The loss of MCT1 on microvessels was replaced by granular labeling, particularly in CA1. Immunogold electron microscopy revealed that labeling for MCT1 on luminal and abluminal endothelial cell membranes were reduced by 37 % and 48 % respectively in MTLE vs. non-MTLE hippocampal formations ($P<0.05$).

Conclusion: We hypothesize that the downregulation of MCT1 on endothelial cells may be implicated in the pathophysiological process leading to epilepsy through impaired uptake of blood-derived monocarboxylates by the brain and that a re-expression of MCT1 on endothelial cells may represent a novel therapeutic approach in medication-refractory TLE.

3.2 Paper II

Introduction: Based on the discoveries made in paper # I about the altered distribution of MCT1 in sclerotic hippocampal formations we proposed that carefully controlled studies were needed to further explore the role of MCTs in medication-refractory TLE. Because such studies cannot be easily performed in human subjects, we wanted to establish whether the results from paper # I could be replicated in newly developed and highly relevant laboratory models of TLE. To study this, recurrent seizures were induced in rats by either (i) continuous unilateral hippocampal infusion of MSO, an antagonist of glutamine synthetase, or by (ii) electrical stimulation of the perforant pathway. These laboratory models are associated with a low mortality rate, consistent appearance of recurrent seizures, pathological changes similar to human HS, and minimal neuronal loss outside the hippocampal formation.

Aim: To study whether the alterations in MCT1 distribution previously observed in hippocampal formations of medication-refractory TLE patients could be replicated in relevant laboratory models of this disease.

Main findings: MSO and perforant pathway stimulation-models induced recurrent limbic seizures and pathology similar to that found in human MTLE. Immunohistochemistry and stereological analysis revealed that MCT1 labeling was reduced by 70 to 80 % on microvessels in epileptic animals compared to in control hippocampal formations ($P<0.05$). RECA-1, an endothelial cell marker, was not altered between control and epilepsy groups, indicating that the loss of MCT1 was not due to a general loss of proteins on endothelial cells. The deficiency of MCT1 on microvessels in models of TLE was replaced by a strong upregulation on cells recognized as astrocytes by using immunofluorescence and markers for GFAP and aquaporin 4.

Conclusion: By determining that the MCT1 labeling follows the same pattern in laboratory models of TLE as in patients with this disorder, these models can now be used to further examine the role of MCT1 in the pathophysiology of medication-refractory TLE.

3.3 Paper III

Introduction: The MCT family comprises 14 members, of which MCT1, MCT2 and MCT4 are expressed in the brain. The results from studies # I and # II indicated that the loss of MCT1 on microvessels is a universal characteristic of epileptogenic hippocampal formations, possibly mechanistically involved in the pathophysiology of MTLE. Our hypothesis presumes that the loss of MCT1 is not compensated for by increased cellular protein synthesis and expression of other brain MCT isoforms.

Aim: Examine the distribution of MCT2 on hippocampal formations of non-MTLE and MTLE patients and in neurologically normal autopsy controls

Main findings: Immunohistochemistry and stereological analysis revealed a significant loss of MCT2 labeling on microvessels in hippocampal formations of non-MTLE (-52 %, $P=0.003$) and more notably MTLE (-87 %, $P<0.001$) compared to autopsy. β-dystroglycan, a protein associated with the dystrophin complex localized on perivascular endfeet, was not altered between groups ($P=0.159$), indicating that MCT2 was not lost in epileptogenic hippocampal formations due to a general loss of proteins on perivascular endfeet. By immunogold electron microscopy, MCT2 was primarily localized to perivascular endfeet in non-MTLE. However, in MTLE, MCT2 densities on perivascular endfeet was reduced by 42 % compared to non-MTLE ($P<0.001$). The granular up regulation of MCT2 labeling in areas with neuronal loss and reactive gliosis in MTLE as observed with light microscopy, was identified as a 170 % increase in labeling density on membranes of astrocyte processes in the neuropil in MTLE vs. non-MTLE ($P<0.001$).

Conclusion: The loss of MCT1 on endothelial cells previously observed in MTLE, is not compensated for by an upregulation of MCT2, but is rather accompanied by a loss of MCT2 on perivascular endfeet, and an overexpression on membranes of astrocyte processes facing the neuropil. These findings support the notion that the transport of monocarboxylates is disturbed in the epileptogenic brain. Presumably, the flux of monocarboxylates between the blood and the brain ISF is impaired, while there is an increased shuttling of these molecules between astrocytes, and between astrocytes and the ISF.

4.0 GENERAL DISCUSSION

The results presented in this thesis raise several critical questions. 1) Is the redistribution of MCT critically implicated in the pathophysiological process leading to epilepsy, i.e., epileptogenesis, or is it an adaptive mechanism that protects the brain during seizures? 2) Is the redistribution of MCT1 and MCT2 clinically relevant? I.e., can the results from the studies be used to develop novel diagnostics of or therapeutics for epilepsy?

It is important to note that the results from the studies presented here are correlative and that additional studies are necessary to assess causality and clinical relevance. Even if there are striking similarities in the expression patterns of MCT1 and MCT2 among patients with TLE and animal models of the disease, we do not know if the alterations in MCTs cause seizures or are a result of seizures. Nor do we know whether MCTs can be targeted diagnostically or therapeutically in epilepsy. Thus, the purpose of this section is to discuss these issues in more detail through a critical review of the literature.

Although we did not measure the monocarboxylate flux, a loss of MCT1 on endothelial cells and of MCT2 on perivascular astrocyte endfeet processes implies a reduced transport of these molecules between blood and brain. Based on the properties of the transporters, the direction of the flux depends on the concentration gradient of monocarboxylates and protons across the cell membrane at a given time (Bergersen, 2007). Possible consequences on cerebral homeostasis resulting from either a reduced transport of monocarboxylates into the brain or out of the brain will be discussed below.

4.1. Reduced brain influx of monocarboxylates impairs cerebral energy metabolism and contributes to seizures

Blood monocarboxylates are critical for normal brain function. We hypothesize that a reduced availability of these molecules in the brain following a loss of their transporters on the BBB and associated structures may contribute to the disturbed brain homeostasis observed in medication-refractory TLE.

Increasing evidence supports the notion that mitochondrial dysfunction and oxidative stress contributes to epileptogenesis in TLE (Kudin et al., 2009; Waldbaum and Patel,

2010). Monocarboxylates, particularly ketone bodies, may be necessary to maintain normal mitochondrial function (Bough et al., 2006). A microarray study on rats reported that among several hundred upregulated transcripts following a calorie-restrictive diet, genes attributed to energy metabolism were most prominent, of which many was involved oxidative phosphorylation. Rats on the diet also displayed about 50 % increases in mitochondrial density compared to controls (Bough et al., 2006). The findings are concurrent with reports showing improvements in the energy metabolism following KD-induced increase in brain ketone levels (Pan et al., 1999).

Ketone bodies have been claimed to exert multiple antiepileptic effects in the brain, (for a review, see Morris, 2005). β-hydroxybutyrate may indirectly regulate excitatory neurotransmission through increasing the synthesis of kynurenic acid, an endogenous antagonist of glutamatergic and α7-nicotinic receptors, which via interaction with presynaptic n-methyl-D-aspartate (NMDA) receptors may reduce the release of glutamate (Chmiel-Perzynska et al., 2011). Acetoacetate, another ketone, modulates vesicular glutamate release and suppresses seizures evoked with 4-aminopyridine (Juge et al., 2010). Ketone bodies have also been reported to increase the levels and activity of mitochondrial uncoupling proteins, thus acting neuroprotective by decreasing the production of reactive oxygen species (Sullivan et al., 2004). Some have claimed that ketone bodies are themselves anti-convulsive (Likhodii et al., 2003), but this has been refuted by others (Morris, 2005). Both monosynaptic and polysynaptic inhibition is decreased in dentate granule cells in MTLE relative to non-MTLE patients (Williamson et al., 1999). Metabolic studies have suggested that synaptic inhibition is particularly energetically demanding due to the high fire frequency of these neurons (Attwell and Laughlin, 2001). Accordingly, the strength of polysynaptic granule cell inhibition positively correlated with brain PCr/ATP levels (Williamson et al., 2005). Furthermore, ketone bodies have been reported to enhance GABAergic inhibition (Cantello et al., 2007), increase the brain levels of GABA through suppressing degradation (Suzuki et al., 2009) and increase GABA synthesis (Lund et al., 2011; Yudkoff et al., 2004), ultimately lowering the neural excitation within the cortex.

Liver hepatocytes have traditionally been considered the only cells capable of ketogenesis (Garber et al., 1974; Hawkins et al., 1971; Reichard et al., 1974). Although this view has

recently been challenged (Auestad et al., 1991; Bixel and Hamprecht, 1995), the dominant opinion is that ketone bodies are exogenous to the brain. Furthermore, the high rate of ketone transport into the brain during periods of high blood levels of these molecules (reviewed in Nehlig, 2004) supports the notion that even if endogenous brain ketogenesis is present, it is quantitatively inferior to liver ketogenesis, at least during prolonged ketonemia. Thus, the brain needs blood-derived ketone bodies to meet its needs. Since ketones do not readily diffuse across the BBB they need to be transported from the blood across the BBB and into astrocytes through MCT1 and MCT2 respectively, before they can be utilized by the brain. We propose that the perturbations in cerebral energy metabolism which are suggested implicated in the pathophysiology of TLE (Cendes et al., 1997; Connelly et al., 1994; Hetherington et al., 1995; Pan et al., 2005; Theodore et al., 1983) is at least partly due to a reduced availability of energetic monocarboxylate fuels, particularly ketone bodies, in the epileptogenic brain following the loss of MCT1 and MCT2. This may impair the ATP production and thereby disturb key energy-demanding processes possible resulting in accumulation of extracellular glutamate, distorted ionic gradients, impaired synaptic inhibition and promotion of excitatory neurotransmission, thus increasing the likelihood for seizure occurrence.

4.2 Redistribution of MCTs on astrocytes contributes to epileptogenesis

Since the transport of monocarboxylates across the BBB via MCTs is bidirectional, the described loss of MCT1 on endothelial cells and of MCT2 on perivascular endfeet may impair the efflux of monocarboxylates from the brain to the blood. This hypothesis may seem contradicting to the previous section where low brain levels of ketone bodies were suggested as a contributor to epileptogenesis. However, since ketone bodies are exogenous to the CNS, and blood ketones are rapidly consumed and does not accumulate within the brain once they have crossed the BBB (Pan et al., 2002), the loss of MCTs will likely impair the influx and not efflux of ketone bodies as suggested in the previous section. Lactate on the other hand is produced throughout the brain and particularly in regions with few mitochondria, such as in astrocytes. Although astrocytes have the capacity for oxidative metabolism, their narrow surface extensions which are in close contact with synapses and account for 80 % of the astrocyte surface area, are to narrow for

mitochondria and thus depend on glycolysis and glycogenolysis when increases in energy demand is triggered, for example following fluctuations in the extracellular contents of ions and excitatory transmitters (Hertz et al., 2007). In normal tissue, the presence of MCT2 on perivascular endfeet suggests a function of monocarboxylates removal from the brain by providing a microenvironment around the abluminal endothelial membrane high in lactate in turn resulting in preferential efflux of lactate to the blood via MCT1 on the BBB (Cornford and Hyman, 1999). The loss of MCT2 on endfeet processes and of MCT1 on endothelial cells may impair the clearance of lactate from astrocytes to blood. Accumulation of lactate and protons in astrocytes would negatively affect cytosolic ATP production through inhibition of phosphofructokinase-1, the key regulatory enzyme in the glycolytic pathway. However, sustained accumulation of lactate in astrocytes in MTLE seems unlikely due to the high capacity of these cells for lactate dispersal through the glial syncytium (Gandhi et al., 2009) and through release of lactate into the ISF through MCTs on membranes of astrocytes processes in the neuropil. The ability for the latter may in fact be augmented in hippocampal formations in MTLE because the MCT levels on plasma membranes of astrocyte processes in the neuropil are significantly increased in these subjects (Lauritzen et al., 2011; Lauritzen et al., 2012; Lauritzen et al. submitted). An increased expression of MCT1 and MCT2 on membranes of astrocyte processes in the neuropil may be caused by a higher rate of anaerobic metabolism in astrocytes, and hence, an increased demand to remove lactate and protons from the cell cytoplasm to the brain ISF. Due to an increased release of glutamate during seizures (During and Spencer, 1993), the need for glutamate buffering by astrocytes is likely to be enhanced. Because cellular uptake of extracellular glutamate is energy-dependent (Danbolt, 2001), more ATP is needed during conditions of excessive glutamate clearance, and thus more lactate and protons needs to be cleared from the cell. In peripheral tissues, neural activation of a cell regulates the MCT expression. For example, in skeletal and heart muscle cells of rats, activation through electrical stimulation or exercise increases the maximal rate of lactate transport (McCullagh et al., 1996) and significantly increase MCT1 expression (Baker et al., 1998). Similar results have been obtained in humans following a period of physical training (Bonen et al., 1998; Pilegaard et al., 1999). Overexpression of MCTs seems appropriate considering the increased buffering needs for lactate and protons in activated muscle cells. Although changes in MCT expression in astrocytes following brain stimulation has not been

studied, the presence of a similar mechanism in these cells as is present in muscle seems plausible. The released lactate may be used as fuel for oxidative metabolism in neurons and neighboring astrocyte during high-energy yielding convulsions (Hertz et al., 2007; Pellerin and Magistretti, 1994). According to the astrocyte-neuron lactate shuttle model, astrocytes respond to glutamatergic activation by increasing the rate of glucose utilization from blood or from glycogen stored in astrocytes (Pellerin and Magistretti, 1994; Pellerin et al., 1998; Tsacopoulos and Magistretti, 1996). Lactate formed during astrocytic glycolysis is transported out of the cytosol through MCTs. The resultant increase in extracellular lactate favor uptake of these molecules by neurons through MCTs present on post-synaptic dendrites (Bergersen et al., 2001; Bergersen et al., 2005) to meet the activity-induced energy demands of these cells. In this regard, over-expression of MCT on membranes of perisynaptic astrocytes may increase the flux of lactate to help sustain epileptogenic activity by fueling hyperexcitable neurons. This hypothesis is supported by the fact that interictal extracellular concentrations of lactate is 48 % higher in the epileptogenic hippocampal formation of TLE subjects than in control, and 90 % higher during, and in the first hour after spontaneous complex partial seizures with secondary generalization (During et al., 1994).

4.3 Redistribution of MCT on astrocytes is neuroprotective

Reactive astrogliosis is a common hallmark of MTLE (de Lanerolle et al., 2010; Harris, 1975; Pollen and Trachtenberg, 1970), however, it has been questioned whether this process should be regarded as detrimental or beneficial. Several recent studies using genetically modified animals indicate that the former belief of reactive astrogliosis as a uniformly negative phenomenon is no longer tenable (Bush et al., 1999; Okada et al., 2006; Sofroniew, 2005). Instead, reactive astrogliosis is likely involved in the healing process after CNS injury by regulating the immune response, facilitating BBB repair and protecting neurons. Could the redistribution of MCTs on astrocytes in the epileptogenic hippocampal formation be part of the neuroprotective effects of reactive astrocytes during seizures? Because MCT2 is lost on astrocyte endfeet in epileptogenic hippocampal formations (Lauritzen et al., submitted), lactate must take alternative routes out of the astrocyte cytosol, e.g., through MCTs on the perisynaptic membrane, so that high rates of glycolysis

can be maintained. The concomitant elevated lactate levels in the brain ISF will lead to decreased pH in the epileptic focus (Jasper and Erikson, 1941; Meyer et al., 1966; Tschirgi et al., 1957). Low pH in turn, may exert an antiepileptic effect by reducing currents through the NMDA receptor, causing arrest of seizure activity and postictal refractoriness (Tang et al., 1990). However, others have reported that even high concentration of lactate cannot block the occurrence of epileptic depolarization (Stittsworth and Lanthorn, 1993).

4.4 Regulation of monocarboxylate transporters

The molecular process that governs the regulation of the MCTs in epileptogenic hippocampal formations is not well understood. Ancillary proteins may be involved. These molecular chaperones, CD147[1] for MCT1 and gp70 (also known as embigin) for MCT2 (Kirk et al., 2000; Poole and Halestrap, 1997; Wilson et al., 2005) are suggested to modulate the transport of monocarboxylates across cell membranes through regulating the catalytic activity of the transporters, or through regulating the transporters translocation to the membrane (Juel and Halestrap, 1999). Disruption of the transporter/chaperone interaction inhibits monocarboxylate transport, suggesting that ancillary proteins are required to maintain the catalytic activity of MCTs (Ovens et al., 2010; Wilson et al., 2005). In peripheral tissue, it has been proposed that MCT1 respond to increased metabolic activity by upregulation of CD147 (Nehme et al., 1995). Whether the same mechanism is present in the brain is unknown.

It is tempting to speculate that the loss of MCT1 on the endothelial BBB and of MCT2 on perivascular endfeet is caused by a loss of neurons in the MTLE hippocampal formation, and hence, a lesser demand for energy under interictal conditions. However, studies have been unable to demonstrate a correlation between neuron loss and concentration of high-energy compounds (Kuzniecky et al., 2001; Petroff et al., 2003). Instead, MCT may be downregulated due to local tissue inflammation. Studies on patients with inflammatory bowel disease reported that MCT1 expression was downregulated on intestinal epithelial cells in these subjects (Thibault et al., 2007). The downregulation of MCT1 was closely associated with the concentration of proinflammatory cytokines in a dose-dependent

[1] CD147 has been characterized under a variety of names in different species, including MMP-1, TCSF, hBasigin, M6, Hab18G, EMMPRIN (human), OX-47, CE9 (rat), GP42, basigin (mouse), HT7 neurothelin, 5A11 (chicken).

manner, and was mediated by inhibition of MCT1 transcription. A similar mechanism may be present in medication-refractory TLE and animal models of this disease as inflammatory changes such as increased levels of cytokines are present in the epileptogenic hippocampal formation of these cases (for a review, see Vezzani and Granata, 2005).

Recent findings in human intestinal epithelial cells suggest that somatostatin, a peptide hormone; exert a modulatory effect on monocarboxylate transport through blood vessels. Studies on human intestinal epithelial Caco-2 cells showed that application of somatostatin increased membrane levels of MCT1 while the intracellular MCT1 pool decreased (Saksena et al., 2009). Somatostatin also enhanced membrane levels of CD147 and its association with MCT1. This finding is interesting because patients and laboratory models of TLE display a loss of somatostatin-immunoreactive interneurons in the hilus of the dentate hilus (Mathern et al., 1995; Robbins et al., 1991; Sloviter, 1991; Sundstrom et al., 2001). The loss of hilar somatostatin-positive interneurons reduce functional inhibition of granule cells of the dentate gyrus and lower the seizure threshold (de Lanerolle et al., 1989). In the normal brain, the somatostatin system is believed to exert an inhibitory modulation of hippocampal circuitries (Binaschi et al., 2003), thus suppressing the spread of epileptiform events through the hippocampal formation (Tallent and Siggins, 1999). Hence, a loss of somatostatin-releasing neurons in the epileptogenic hippocampal formation may result in downregulation of MCT1. Loss of MCT may further augment the damage caused by less inhibition through reducing available energy substrate and anti-convulsive ketone bodies.

4.5 Clinical applications and future projects

When AEDs fail to control seizures as they frequently do in MTLE patients, few alternative, non-invasive therapies are presently available. One exception is KD, which is remarkably effective in both children (Neal et al., 2008) and adults (Kossoff et al., 2008; Sirven et al., 1999). Despite its proven efficacy, KD is not used by a large group of medication-refractory patients. This may partly be due to the demanding lifestyle changes associated with the diet combined with its unpalatebility which makes adherence to KD is difficult, especially for adults (Mosek et al., 2009). In addition, compliance to the diet is associated with various early- and late-onset complications, some of which may force the patient to cease the treatment (Ballaban-Gil et al., 1998; Bergqvist et al., 2003; Best et al., 2000; Kang et al.,

2004). The mechanisms behind the efficacy of the KD remain largely unknown, although the current opinion is that the diet leads to "secondary" adaptations following ketonemia that prevent epileptic seizures (Bough and Rho, 2007). One documented cellular adaptation to KD in rats is increases in the transporter process that carries ketone bodies into the brain (Daniel et al., 1977), notably of MCT1 expression on the BBB endothelium (Leino et al., 2001). Based on these findings, one could argue that the KD likely restores and perhaps even increases the flux of monocarboxylates into the brain. This effect may be particularly important in medication-refractory TLE, where unknown processes cause a loss of BBB MCT1 as well as other alterations in the MCT distribution (Lauritzen et al., 2011; Lauritzen et al., 2012; Lauritzen et al., submitted). If the regulation of MCTs could be influenced in a similar fashion through pharmacological intervention as following a KD, this would potentially be an attractive alternative therapy against drug-refractoriness. We are currently conducting studies which seek to clarify the relationship between MTLE, monocarboxylate transporters and the ketogenic diet. Future studies should aim to examine the exact mechanisms by which ketone bodies regulate MCT expression and MCT activity.

The results presented in this thesis are not without limitations. However, these papers are the first to document the MCT distribution in epileptogenic hippocampal formations. The pattern of MCT labeling observed between MTLE and epileptogenic rats were strikingly similar which indicates that these transporters are involved in the pathogenesis of medication-refractory TLE. Based on our findings, highly relevant animal models can now be used to rigorously test the exact role of the MCTs in medication-refractory TLE and hopefully establish a causal link between altered MCT distribution and increased seizure propensity. The main questions that have risen in the aftermath of this work are whether the altered MCT labeling contributes to epileptogenesis, or if it is simply a secondary effect of the recurrent seizures. For now, this question remains to be answered.

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Errata

- P. 9 (12), para 1, line 1: ”In this regard” **changed to** “Hence”.
- P. 9 (12), para 3, line 8: “The predominant role” **changed to** “This”.
- P. 11 (14), para 2, line 10: “Among the constituents of the neurovascular unit, a particular important regulator of brain homeostasis, is astrocytes” **changed to** “Astrocytes, constituents of the neurovascular unit, play particular important roles in brain homeostasis”.
- P. 12 (15), para 2, line 9: “among the most numerous neuroglial cells” **changed to** “localized”.
- P. 18 (21), para 1, line 1: “Later, Halestrap and colleagues…” **changed to** “This was confirmed some years later, when Halestrap and colleagues…”.
- P. 18 (21), para 2, line 10: **removed** “for lactate”.
- P. 19 (22), para 2, line 9: “…rised…” changed to “…raised…”.
- P. 26 (29), para 1, line 8: “MCT 1” **changed to** “MCT1”
- P. 28 (31), para 3, line 1: “revealing” **changed to** “revealed”
- P. 29 (33), para 4, line 1: “A reduced availability…” **changed to** “We hypothesize that a reduced availability”.
- P. 30 (34), para 1, line 6: “findings is concurrent” **changed to** “findings are concurrent”.
- P. 32 (35), para 1, line 1 (2): “Nevertheless” **changed to** “Furthermore”.
- P. 32 (36), para 1, line 14 (15): “…the MCT levels on membrane of astrocyte…” **changed to** “…the MCT levels on plasma membranes of astrocyte…”.
- P. 33, para 1, line 2: “...neighboring astrocyte during…” **changed to** “…neighboring astrocytes during…”.
- P. 33 (37), para 2, line 8: “…MCT on astrocytes…” **changed to** “…MCTs on astrocytes…”.
- P. 34 (38), para 3, line 1: “…the loss of BBB MCT1 and MCT2 on…” changed to “…the loss of MCT1 on the endothelial BBB and of MCT2 on…”.
- P. 36 (40), para 1, line 10: “…in a similar through pharmacological…” **changed to** “…in a similar fashion through pharmacological…”.
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Page, para and line numbers refer to the original manuscript. Numbers in parenthesis refer to the revised manuscript.

I

II

III

Redistribution of monocarboxylate transporter 2 on the surface of astrocytes in the human epileptogenic hippocampus

Fredrik Lauritzen,[a,b] Kjell Heuser,[f] Nihal C. de Lanerolle,[c] Tih-Shih W. Lee,[d] Dennis D. Spencer,[c] Jung H. Kim,[e] Tore Eid,[b] and Linda H. Bergersen[a]

[a]The Brain and Muscle Energy Group, Department of Anatomy and Centre for Molecular Biology and Neuroscience, University of Oslo, P.O. Box 1105 Blindern, NO-0317 Oslo, Norway
[b]Department of Laboratory Medicine, Yale University School of Medicine, P.O. Box 208035, New Haven, CT 06520, USA
[c]Department of Neurosurgery, Yale University School of Medicine, P.O. Box 208035, New Haven, CT 06520, USA
[d]Department of Psychiatry Yale University School of Medicine, P.O. Box 208035, New Haven, CT 06520, USA
[e]Department of Pathology, Yale University School of Medicine, P.O. Box 208035, New Haven, CT 06520, USA
[f]Department of Neurology, Oslo University Hospital, Rikshospitalet, Oslo, Norway

Corresponding Authors:
Linda H. Bergersen, PhD
The Brain and Muscle Energy Group
Centre for Molecular Biology and Neuroscience
Department of Anatomy and Institute of Basic Medical Sciences
University of Oslo
P.O. Box 1105 Blindern
NO-0317 Oslo, Norway
Tel: +47 22851496
Fax: +47 22851278
l.h.bergersen@medisin.uio.no

Tore Eid, MD PhD
Department of Laboratory Medicine
Yale University School of Medicine
330 Cedar Street, P.O. Box 208035
New Haven, CT 06520-8035, USA
Tel: 203-785-4928
Fax: 203-785-2236
tore.eid@yale.edu

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ABSTRACT

Emerging evidence points to monocarboxylates as key players in the pathophysiology of temporal lobe epilepsy (TLE) with hippocampal sclerosis (mesial temporal lobe epilepsy, MTLE). Recently, we reported that monocarboxylate transporter (MCT) 1, a protein that facilitates the co-transport of monocarboxylates and protons across cell membranes, was reduced on endothelial cells of microvessels and upregulated on astrocytes in the neuropil in the hippocampal formation in MTLE patients and in animal models of the disease. Because the perivascular endfeet of astrocytes comprise an important part of the blood-brain barrier, we sought to assess the distribution of MCT2 – an astrocyte and neuron specific MCT in rodents – in the hippocampal formation in TLE patients with (MTLE) and without hippocampal sclerosis (non-MTLE). Light microscopic immunohistochemistry revealed significant decreases in perivascular MCT2 immunoreactivity in the hippocampal formation in MTLE vs. non-MTLE, and to a lesser degree in non-MTLE vs. non-epilepsy controls. Immunogold electron microscopy showed that the decrease was confined to perivascular endfeet. β-dystroglycan, a dystrophin complex protein localized to astrocyte endfeet, was not altered between groups, asserting that the deficiency in MCT2 was not caused by a general loss of astrocyte endfeet proteins. Interestingly, the loss of MCT2 on astrocyte endfeet in MTLE was accompanied by an upregulation of the protein on astrocyte membranes facing synapses in the neuropil. We propose that the redistribution of MCT1 and MCT2, in concert, may reduce the flux of monocarboxylates across the blood-brain barrier and enhance the flux within the brain neuropil in TLE, especially in MTLE.

INTRODUCTION

The epilepsies represent one of the largest groups of serious neurological brain disorders, affecting nearly 1 % of the global population. Despite recent advantages in the development of new antiepileptic drugs and invasive treatments, it still remains a challenge to control seizures in about one-third of all epilepsy patients (Kwan and Brodie 2000; Sander 2003). These medication-refractory cases are often patients with temporal lobe epilepsy (TLE), generally defined by seizure involvement of the temporal lobe, particularly the hippocampus (de Lanerolle and Lee 2005). Surgical resection of the seizure focus is the treatment of choice for many drug-refractory patients, and such treatment results in excellent outcome in the majority of cases (McIntosh et al. 2001; Wiebe et al. 2001). However, detection of the seizure focus is complicated and often more than one brain structure is involved (Spencer 2002). Also, temporal lobe resection is invasive and also contraindicated in some patients (Helmstaedter et al. 2003). Hence, there is an urgent need for more less invasive and more efficacious therapies for TLE. A better understanding of the molecular mechanism underlying TLE is expected to facilitate the discovery of such therapies.

Emerging evidence points to monocarboxylates – i.e., lactate, pyruvate and the ketone bodies β-hydroxybutyrate and acetoacetate – as key players in the pathophysiology of medication-refractory TLE. Monocarboxylates require a specific transport mechanism to cross cell membranes (Poole and Halestrap 1993). This mechanism is provided by proton-linked monocarboxylate transporters (MCTs) (Halestrap and Meredith 2004). Recently, we found that MCT1 was deficient on the endothelium of microvessels in surgically resected hippocampal formations from patients with medication-refractory TLE (Lauritzen et al. 2011). Similar findings were present in three newly developed animal models of TLE (Lauritzen et al. 2012). We proposed that the loss of MCT1 on endothelial cells is implicated in the increased seizure susceptibility in medication-refractory epilepsy by impairing the transport of ketone bodies across the blood-brain barrier. This hypothesis presumes that other MCT isoforms do not compensate for the altered expression of MCT1. At least two additional isoforms are present in the brain: MCT2 and MCT4 (Halestrap and Meredith 2004). The kinetic properties of MCT2 are similar to those of MCT1; however, the cellular expression of the two MCTs is different. There are some discrepancies in the literature on the distribution of MCT2; however, most studies in rodents report that the protein is expressed along the post synaptic density of excitatory synapses and on perivascular astrocyte endfeet (Baud et al. 2003; Bergersen et al. 2001; Bergersen et al. 2005; Gerhart et al. 1998; Hanu et al. 2000; Rafiki et al. 2003) or both . Whether MCT2 is expressed in the human brain in significant quantities remains controversial (Chiry et al. 2008; Price et al. 1998).

The aim of this study was twofold: First, to assess whether MCT2 is expressed in significant quantities in the human hippocampal formation. Second, to evaluate possible changes in the distribution of MCT2 in the hippocampal formation in patients with TLE compared to non-epilepsy control subjects.

MATERIALS AND METHODS

Human subjects and tissue preparation

Patients with medication-refractory TLE underwent phased presurgical evaluation at the Yale–New Haven Hospital, and those elected for surgery had their hippocampus resected according to standard procedures (Spencer and Spencer 1991). Informed consent from each patient and institutional approval were obtained for the surgery and for the use of tissue for this study. Randomly selected hippocampal formations from 23 TLE patients were included in the study (Table 1). Six hippocampal formations obtained at autopsy from non-epilepsy subjects were included as additional comparison controls.

The TLE patients were classified into two categories. 1) Patients with mesial temporal lobe epilepsy (MTLE, n=12) were characterized by: (a) complex partial seizures that appeared to originate from the hippocampal formation, (b) atrophy and increased T2 signal intensity of the hippocampal formation by magnetic resonance imaging (MRI), (c) decreased interictal phosphocreatine/ATP ratio and n-acetyl aspartate (NAA) concentration in the atrophic hippocampal formation by in vivo magnetic resonance spectroscopy (MRS) (Chu et al. 1998; Hugg et al. 1993), and (d) hippocampal sclerosis (i.e. glial proliferation with preferential loss of neurons in CA1, CA3 and the dentate hilus) of the resected hippocampal formation (de Lanerolle et al. 2003; Sommer 1880). 2) Patients with other types of TLE, here referred to as non-MTLE (n=11), were characterized by: (a) complex partial seizures that often (but not always) appeared to originate from the hippocampal formation, (b) no signs of atrophy or increased T2 signal intensity by MRI; however, other findings such as a tumor, malformation or dysplastic lesion in the mesial temporal lobe or elsewhere in the brain might be present, (c) an unremarkable interictal phosphocreatine/ATP ratio and NAA concentration in the hippocampus, and (d) minimal glial proliferation and modest, if any (<25%), loss of neurons in the resected hippocampus (de Lanerolle et al. 2003; Sommer 1880). Tissue preparation was done as previously described (Lauritzen et al. 2011). The category to which each sample belonged was concealed from the investigators during stereological and quantitative immunogold analysis.

Light microscopic immunohistochemistry and stereological point count analysis

Fifty µm thick Vibratome sections were incubated free floating in solutions of β-dystroglycan (B-DG-CE, Novocastra; 0.1 µg/ml) or MCT2 (AB1287, Millipore, Billerica, MA; diluted 1:100) antibodies for 72 h at 4 °C and processed according to the avidin biotin peroxidase method (Hsu et al. 1981) using the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA). The immunostained sections were mounted on gelatin coated glass slides and examined in a light microscope.

To quantify the density of β-dystroglycan and MCT2-positive structures on hippocampal formations, stereological point count analysis was performed as described by Gundersen and colleagues

(Gundersen et al. 1988). A test system with a set of regularly spaced points was created on a transparent A4-sized foil and superimposed on printed A4-sized pictures of β-dystroglycan or MCT2 labeled hippocampal sections. The areal density of positive profiles was estimated by counting points hitting β-dystroglycan- or MCT-labeled structures, divided by points hitting the hippocampal formation. Densities were expressed as positive counts per 100 counts.

Electron microscopic immunocytochemistry

Freeze substitution of the brain tissue and the postembedding immunogold method were done according to procedures described by Bergersen and colleagues (Bergersen et al. 2008). Small tissue blocks (0.3 x 0.5 x 1 mm^3) of area CA1 were dissected from 500-µm thick Vibratome sections and subjected to freeze substitution. Single immunogold labeling for MCT2 was performed on ultrathin sections from 3 non-MTLE and 3 MTLE patients using a rabbit anti-MCT2 primary antibody (kindly provided by A. Halestrap, University of Bristol, UK, (Jackson et al. 1997), 1:100) and a colloidal gold-conjugated secondary antibody (goat F (ab) anti-rabbit 10 nm, BB International, Cardiff; 1:20). Images were taken with a FEI Technai 12 transmission electron microscope (Hillsboro, OR). The specificity of the primary and secondary antibodies was tested by preabsorbtion with the peptide used for immunization and by omitting the primary antibody from the immunogold protocol, respectively, both of which prevented labeling of tissue components (Bergersen et al. 2008).

Immunogold quantification

The MCT2-gold particle (gp) densities were determined on astrocyte membranes facing microvessels, i.e. endfeet specializations, and on astrocyte processes in the neuropil. Morphological criteria according to Peters and colleagues were used to identify the profile types (Pan et al. 2002). Astrocyte endfeet were identified based on their localization in juxtaposition to the basal lamina, which separates the endothelial cells, and pericytes, from the brain parenchyma. Astrocyte processes in the neuropil were identified by their relatively electron lucent cytoplasm and the presence of intermediate filaments in bundles.

The MCT2 labeling densities were quantified using the following procedures: i) *Astrocyte endfeet*. Ten random microvessels were examined from each patient (non-MTLE, n=30; MTLE, n=30). Since astrocyte endfeet form an essentially continuous cover around the microvessels in the brain (Mathiisen et al. 2010), all membranes facing the basal lamina were included in the analysis. The length of each membrane profile was measured using imaging software (Image J, National Institutes of Health, Bethesda, MD), and associated gp recorded, including gp with their centers located on the membrane itself or within 30 nm on the intracellular side. As about 90 % of the gp that represent a membrane protein are found within 40 nm of the midline of the membrane profile (Chaudhry et al. 1995), the 30 nm distance includes most of the relevant gp, at the same time minimizing the contribution of any immunoreactivity elsewhere than in the membrane; gp within 30 nm on the

extracellular side were excluded to minimize the contribution of gp representing antigenic sites in neighboring cells. The areal densities of membrane-associated gp were calculated by dividing the number of gp by the "membrane area" (membrane length in nm x 30 nm). Densities were expressed as gp/μm^2. ii) *Astrocyte processes in the neuropil.* Fifteen random astrocyte processes localized in the neuropil were examined for each patient (non-MTLE, n=45; MTLE, n=45). Astrocyte gp densities were divided into three categories: 1) *astrocyte*, includes all gp on the membrane or within the cytoplasm, 2) *astrocyte cytoplasm*, includes intracellular gp, excluding gp localized $\leq$ 30 nm from the plasma membrane, and 3) *astrocyte membrane*, includes gp on the astrocyte plasma membrane or within 30 nm on the intracellular side. Gold particle areal densities were calculated and expressed as described for i).

Statistical analysis

A one-way ANOVA and a Tukey HSD post-hoc test were used for multiple comparisons (point count analysis of β-dystroglycan and MCT2 in non-epilepsy autopsy, non-MTLE and MTLE), while a Mann-Whitney U test was used for comparisons between two groups (immunogold quantification of MCT2 in non-MTLE and MTLE). A p value of < 0.05 was considered a statistically significant difference. Unless otherwise stated, the data are presented as mean $\pm$ SD.

RESULTS

The general histopathology was examined using Nissl-stained sections of hippocampal formations from the following subject categories: non-epileptic autopsy controls (n=6), non-MTLE (n=11) and MTLE (n=12). Consistent with earlier reports (de Lanerolle et al. 2003; Eid et al. 2004; Lauritzen et al. 2011), the hippocampal formations from autopsy (Figs. 1B, C) and non-MTLE patients (Figs. 1D, E) were impossible to distinguish visually, and did not exhibit significant neuronal loss or reactive gliosis. However, neuronal cell loss and reactive gliosis were prominent in sections from MTLE patients, particularly in the dentate hilus and CA1 (Fig. 1F, G respectively).

We next used immunohistochemistry to compare the expression of MCT2 to that of β-dystroglycan in hippocampal formations from autopsy (n=4 and 4 respectively), non-MTLE (n=6 and 6) and MTLE patients (n=6 and 6). β-dystroglycan, a member of the dystrophin associated protein complex has been hypothesized to play a key role in the integrity of the blood brain barrier (Zaccaria et al. 2001) and was used as a marker for perivascular endfeet (Tian et al. 1996). As noted also by Heuser and colleagues (Heuser K et al. *Loss of inwardly rectifying potassium channel Kir4.1 in the hippocampus in patients with mesial temporal lobe epilepsy*. Unpublished manuscript), the β-dystroglycan labeling was present along microvessels and did not reveal differences among specimens from control autopsy (Fig. 2A, D, E), non-MTLE (Fig. 2B, F, G) or MTLE patients (Fig. 2C, H, I). In contrast, such homogeny among groups was not seen on sections labeled for MCT2 (Fig. 2J-R). The labeling pattern

for MCT2 in non-epilepsy autopsy tissue (Fig. 2J, M, N) was similar to that for β-dystroglycan (Fig. 2A-I), indicating that MCT2 and β-dystroglycan are expressed on the same microvessels. However, in both non-MTLE (Fig. 2K, O, P) and MTLE patients (Fig. 2L, Q, R), the MCT2 labeling along microvessels was reduced compared with labeling for β-dystroglycan. The greatest loss of labeling was seen in MTLE, particularly in areas exhibiting neuronal loss and reactive gliosis, such as the dentate hilus (Fig. 2Q) and CA1 (Fig. 2R). In these areas the vascular labeling found in control autopsy specimens was replaced by diffuse granular labeling in the neuropil (Fig. 2Q-R).

MCT2 was also detected on structures other than the microvessels, albeit in lower quantity. In all groups, MCT2 was present in the alveus of the hippocampal formation. However, no obvious differences in labeling were seen among the patient categories. The densities of microvessels labeled for β-dystroglycan and MCT2 were then analyzed in the hippocampal formation using quantitative stereological analysis (Fig. 2S). As expected from the visual examination, there were no differences in the density of β-dystroglycan-positive microvessels among groups ($p = 0.159$). However, densities of MCT2 labeled structures were reduced by 52 % in non-MTLE (11 ± 5 positive counts per 100 counts, $p=0.003$), and by 87 % in MTLE (3 ± 2, $p<0.001$) compared to non-epilepsy autopsy specimens (23 ± 5). A 73 % reduction in MCT2-density was found in MTLE vs. non-MTLE ($p =0.018$). The ratios between MCT2 and β-dystroglycan labeling densities were 1.0 ± 0.2, 0.5 ± 0.2 and 0.1 ± 0.1 for autopsy, non-MTLE and MTLE respectively, showing that essentially all microvessels expressed MCT2 protein in non-epilepsy hippocampal formations (ratio = 1), whereas many microvessels expressed β-dystroglycan but not MCT2 in non-MTLE vs. autopsy ($P=0.001$) and in MTLE vs. autopsy ($p<0.001$) (Fig. 2T). A smaller but significant reduction in the MCT2/β-dystroglycan ratio was found in MTLE vs. non-MTLE ($p=0.011$), supporting our hypothesis that there is a significant decrease in MCT2-positive microvessels in the MTLE vs. the non-MTLE hippocampal formation.

Immunogold electron microscopy revealed that MCT2 was primarily present on the surface membranes of perivascular astrocyte endfeet and astrocyte processes in the neuropil, in non-MTLE as well as MTLE hippocampal formations (Fig. 3). In contrast to MCT1 (Lauritzen et al. 2011), MCT2 was not significantly present on the endothelium of microvessels. Due to the poor ultrastructural quality of the autopsy tissue, the electron microscopic analysis was limited to non-MTLE and MTLE brains, which were chemically fixed immediately after surgical resection. In non-MTLE hippocampal formations, dense MCT2-labeling was found along membranes of astrocyte endfeet (Fig. 3A), whereas scant labeling was found on astrocytes in the neuropil (Fig. 3C). A reverse pattern was found in MTLE, in which there was a substantial decrease in labeling on astrocyte endfeet compared to non-MTLE (Fig. 3B). In addition, MCT2 was strongly upregulated on astrocytes in the neuropil in MTLE, especially on the plasma membrane (Fig. 3D). Labeling on perivascular astrocyte endfeet membranes was reduced by 42 % in MTLE vs. non-MTLE (73 ± 26 vs. 126 ± 44 gp/um^2, $p<0.001$) (Fig. 4). However, for astrocytes in the neuropil, labeling was increased by 43 % in MTLE vs. non-MTLE (11 ± 8 vs. 8 ± 9

gp/um^2, p=0.003) (Fig. 4). The total astrocyte labeling was then divided into a membrane fraction and a cytoplasm fraction (see method). Although no significant difference was found on the cytoplasm between MTLE and non-MTLE (4±4 vs. 6±7 gp/um^2, p=0.4), MCT2 was upregulated by 170 % on the plasma membrane (50±31 vs. 18±17 gp/um^2, p<0.001) in MTLE compared to non-MTLE (Fig. 4).

DISCUSSION

This is the first study of the cellular and ultrastructural distribution of MCT2 protein in the hippocampal formation in patients with medically intractable TLE. The hippocampal formations in patients with TLE vs. non-epilepsy controls were characterized by: Loss of MCT2 on perivascular astrocyte endfeet, and upregulation of MCT2 on astrocyte plasma membranes in the neuropil. The redistribution of MCT2 was most prominent in patients with TLE and concomitant hippocampal sclerosis (i.e., MTLE). The distribution of β-dystroglycan, an astrocyte endfeet marker, showed no differences among groups confirming that the deficiency of MCT2 in MTLE and to a lesser degree in non-MTLE, is not caused by a general loss of proteins on the perivascular astrocyte endfeet.

Increasing evidence suggests that astrocytes, previously considered passive constituents of the central nervous system, may contribute to the development of neurological disorders including epilepsy through a process known as reactive gliosis (de Lanerolle et al. 2010; Harris 1975; Pollen and Trachtenberg 1970; Sofroniew and Vinters 2010). Notably, the sclerotic hippocampal formation in MTLE contains reactive astrocytes with unique molecular features that may predispose to epileptic seizures (de Lanerolle et al. 2010; Eid et al. 2008; Tian et al. 2005). Our findings provide evidence for yet another molecular alteration of reactive astrocytes in TLE. The potential significance of the aberrant expression of MCT2 on these cells will be discussed below.

MCT2 deficiency on perivascular endfeet

In rodents, the monocarboxylate fuels, lactate, pyruvate and ketone bodies, are transported between the blood and the brain extracellular space via the vascular endothelial compartment through MCT1 (Gerhart et al. 1997; Leino et al. 1999; Terasaki et al. 1991). MCT2, which we here show to be present on perivascular astrocyte endfeet in human hippocampus, shuttles the monocarboxylates between the extracellular space and the astrocyte compartments (Bergersen 2007; Gerhart et al. 1998; Hanu et al. 2000). As the endfeet cover most of the circumference of the blood vessels (Mathiisen et al. 2010), monocarboxylate flux via the astrocyte compartment may greatly exceed flux through the extracellular space. The loss of MCT1 (Lauritzen et al. 2011) and MCT2 (present study) in human epileptogenic hippocampal suggests that the uptake of blood-derived monocarboxylate, as well as export of brain-derived ones, are perturbed in the epileptogenic hippocampal formation. Such perturbation may contribute to the brain hyperexcitability in TLE.

An adequate supply of monocarboxylates is critical for normal brain function and a deficiency in these fuels, especially in ketone bodies, may promote excitability. High fat, low carbohydrate, adequate protein – i.e., ketogenic diets (KD), have for almost a century been prescribed to patients with intractable seizures with significant reductions in the frequency of seizures (Kossoff et al. 2008; Neal et al. 2008; Sirven et al. 1999; Wilder 1921). Although the underlying mechanism of the KD is unknown, sustained ketonemia (i.e. high blood ketone concentrations), appears to be a prerequisite for its effect (Bough and Rho 2007). The current opinion is that the ketogenic diet leads to a series of secondary ("adaptive") changes that prevent epileptic seizures via mechanisms secondary to ketonemia itself (Bough and Rho 2007). One such change is the upregulation of MCT1 in blood vessels (Leino RL et al. 2001 Neurochem Int), which might counteract the downregulation observed here, thereby augmenting the entry of ketone bodies into the brain. Ketone bodies are metabolized in the normal human brain, even during euglycemic, non-ketonemic conditions (Pan et al. 2002). It is plausible that ketone bodies, even in small quantities, are necessary for normal brain activity, such as excitatory and inhibitory neurotransmission. Notably, Juge and colleagues recently discovered that acetoacetate modulates vesicular glutamate release and suppresses seizures evoked with 4-aminopyridine (Juge et al. 2010). Furthermore, ketone bodies increase the brain energy stores (Pan et al. 1999), stabilize the neuronal membrane potential (Bough 2008) and enhance GABA-mediated inhibition (Williamson et al. 2005), thus resulting in more sustained GABA-mediated inhibition (Cantello et al. 2007).

The transport of monocarboxylates between cells is bidirectional, and dependent on the concentration gradient of these molecules as well as of protons across the plasma membrane. For example, when lactate increases above the blood concentration in skeletal muscle during exercise, excess lactate is exported to the blood via MCTs (Brooks 2009). The same phenomenon also occurs in the brain: The brain exports small amounts of lactate at rest, but imports large amounts of lactate when blood levels rise, such as during sensory stimulation or exercise (van Hall et al. 2009). The loss of MCT1 and MCT2 on and around microvessels in TLE suggests that the clearance of brain lactate to the blood, as well as lactate import from the blood, may be impaired in this disease. Microdialysis studies have indeed demonstrated that lactate increases in the extracellular space during seizures (Cavus et al. 2005; During et al. 1994) and sustains elevated interictally in the epileptogenic hippocampal formation in patients with TLE.

The density of mitochondria in astrocytes is lower than in neurons, and astrocytes need to export lactate whenever the rate of glycolysis exceeds that of oxidative phosphorylation. Impaired buffering of lactate and protons due to a loss of MCT2 on perivascular endfeet may acidify the astrocyte cytoplasm, slow the rate of glycolysis through inhibition of phosphofructokinase, and inhibit the synthesis of ATP. Because astrocytes are critically dependent on ATP for extracellular glutamate uptake via energy dependent high-affinity glutamate transporter molecules (Danbolt 2001), a deficiency in astrocyte ATP is likely to impair extracellular glutamate uptake. Persistently high

extracellular glutamate concentrations are indeed present in the epileptogenic hippocampal formation in human TLE, and the extracellular glutamate levels are particularly high when hippocampal sclerosis is present (Cavus et al. 2005; During and Spencer 1993). The excess of glutamate in TLE is postulated to cause hyperexcitability and epileptic seizures in these patients.

Another possibility is that the observed downregulation of MCT1 in the endothelium (Lauritzen et al. 2011; Lauritzen et al. 2012) and endfeet (present study) are adaptive changes to prevent loss of locally produced lactate to the blood in order to provide an anticonvulsive effect of high lactate levels. While lactate itself does not inhibit VGLUT, its product, pyruvate, inhibits vesicular glutamate uptake with affinity comparable to that of the ketone body, acetoacetate (Juge et al. 2010, supplemental information). Furthermore, lactate interacts with the G-protein coupled receptor, GPR81 also called HCA_1, to lower cAMP (Offermanns et al. 2011 Pharmacol Rev). As β-adrenergic receptors elevate cAMP levels, elevated lactate would therefore be expected to mimic the known anticonvulsive effect of β-adrenoceptor blockers (Luchowska E et al. 2002 Pharmacol Biochem Behav; De Sarro G et al. 2002 Eur J Pharmacol). An analogous mechanism may contribute to the antiepileptic effect of ketone bodies through the HCA_2 receptor, which binds β-hydroxybutyrate (Offermanns et al. 2001). Third, oxidation of lactate may provide energy for preserving the membrane potential glutamate uptake, preventing hyperexcitability (see above).

Redistribution of MCT2 to the plasma membrane of astrocyte processes

It is possible that the increased levels of lactate in the epileptic tissue, presumably ensuing from the observed loss of MCT1 and MCT2 at microvessels, can be counteracted by increased flux of lactate through distant areas through the astroglial network (Dienel and Cruz 2003; Rouach et al. 2008). The lactate excess in astrocytes may then be released into the extracellular space via the increased expression of MCT1 and MCT2 on the astrocyte membranes in the neuropil. Because MCT2 and MCT1 are rapidly saturated, an upregulation of the transporters on astrocyte membranes in the neuropil might be a response to the increased buffering needs.

Astrocytes are suggested to play a central role in regulating the brain energy metabolism. During seizures, when neurons have a high demand for energy, glutamate released from nerve terminals stimulates astrocyte glucose use and lactate production via the activation of Na+/K+-ATPase on astrocyte membranes (Pellerin and Magistretti 1994). Astrocyte glucose may be obtained from blood or formed from glycogen stored in the cells. Brain glycogen is only found in significant quantities in astrocytes and may function as a substantial energy source for energy-deficient neurons during high energy demanding convulsions (Gruetter 2003). According to the astrocyte-neuron lactate shuttle model, lactate metabolized from glucose are released by astrocytes and taken up into neurons to complement their own energy substrate use (Bittar et al. 1996; Pellerin et al. 1998). Increased levels of MCT2 on astrocyte membranes facing synapses in the neuropil will secure the supply of glial-derived lactate to neurons during high-energy demanding convulsions and consequently help sustain

neuronal function. Increased levels of extracellular lactate may also have an inhibitory effect on neuronal excitability due to acidification of the extracellular compartment at the site of seizure activity (During et al. 1994), which can inhibit receptor activity, including NMDA receptors.

MCT2 expression and species differences

Different cellular localizations of MCT2 have been reported in different species. In rodent brain, MCT2 protein has been reported to be present on neurons (Debernardi et al. 2003; Pierre et al. 2002; Pierre et al. 2000) on post-synaptic densities (Bergersen et al. 2001; Bergersen et al. 2005), or on perivascular endfeet processes of astrocytes (Gerhart et al. 1998; Hanu et al. 2000), as well as on both compartments during development (Baud et al. 2003; Rafiki et al. 2003). Little is known about the MCT2 expression in the human brain. Price and colleagues (1998) detected small amounts of MCT2 mRNA levels in human brain tissues. In contrast, Chiry and colleagues (Chiry et al. 2008) reported a strong and widespread expression of MCT2 mainly on neurons but also on astrocytes in the cerebral cortex from non-epilepsy controls. We were unable to detect MCT2 on neurons in CA1 of the hippocampal formation in TLE patients. The discrepancies between studies may be related to species differences and to methodological issues and the use of antibodies from different sources.

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FIGURE LEGENDS

Fig. 1. Nissl-stained coronal sections from representative cases. High-power images from the dentate hilus (B, D, F) and CA1 (C, E, G) of the hippocampal formation (A) are presented. Hippocampal formations from autopsy and non-MTLE subjects are characterized by abundant neurons (arrows) in the hilus (B, D) and CA1 (C, E). In contrast, there is marked neuronal loss in the hilus (F) and CA (G) in the hippocampal formation in MTLE subjects. The loss of neurons in these areas is accompanied by

reactive gliosis (open arrowheads in F, G). Little or no reactive gliosis is present in autopsy (B, C) or non-MTLE (D, E). Abbreviations: *al*, alveus; *EC*, entorhinal cortex; *CA1-3*, cornu ammonis 1-3 of the hippocampus; *DG*, dentate gyrus; *fi*, fimbria; *gcl*, granular cell layer; *ml*, molecular layer of the DG; s*l-m*, stratum lacunosum-moleculare; s*o*, stratum oriens; *pcl*, stratum pyramidale; s*r*, stratum radiatum; *SUB*, subiculum. Bars: A, 1 mm: B-G, 200 um.

Fig. 2. Immunohistochemistry quantitation of microvessels labeled with β-dystroglycan and monocarboxylate transporter 2 (MCT2). (A-I) β-dystroglycan is abundantly expressed on microvessels (arrows) throughout the hippocampal formation in autopsy (A, D, E), non-MTLE (B, F, G) and MTLE (C, H, I) subjects. There is no difference in the density of β-dystroglycan positive microvessels in the hippocampal formation among autopsy, non-MTLE or MTLE subjects (p = 0.159) (S). The diagrams depict mean±SD. (J-R) Microvessels are densely labeled with MCT2 in autopsy hippocampal formations (J, M, N). The labeling pattern and density are similar to that of β-dystroglycan (S). However, there is a significant loss of MCT2-positive microvessels in non-MTLE (K, O, P) vs. autopsy (J, M, N, S) and in MTLE (L, Q, R) vs. non-MTLE and autopsy (S). The reduction of MCT2-positive microvessels is particularly evident in areas with considerable neuronal loss and reactive gliosis, such as the dentate hilus (Q) and CA1 (R). Here, the transporter is completely lost (asterisk) or strongly reduced (open arrowhead), compared to similar regions in autopsy (M, N) and non-MTLE (O, P). The ratios of the labeling densities in MCT2 vs. β-dystroglycan confirm that the loss of MCT2 on astrocyte endfeet is not due to a loss of β-dystroglycan associated microvessels. Bars: Large images (A-C, J-L), 1 mm; insets (D-I, M-R), 200 μm.

Fig. 3. Immunogold electron microscopy of monocarboxylate transporter 2 (MCT2) on perivascular astrocyte endfeet membranes (green) and on astrocyte plasma membranes in the neuropil (yellow). Dense labeling of MCT2 (arrowheads) is present along perivascular astrocyte endfoot membranes facing the basal lamina (stippled line) in non-MTLE (A), whereas labeling of the same compartment is significantly reduced in MTLE (B). MCT2 labeling is also found on astrocytes plasma membranes in the neuropil, and to a lesser extent on organelles in the cytoplasm of these cells (C, D). Compared to non-MTLE (C), the density of MCT2 on astrocytes in the neuropil is significantly upregulated in MTLE (D). The over-expression in MTLE is particularly evident on the plasma membrane (stippled line in D). Abbreviations: *astro*, astrocytes; *AT*, axon terminal; *bl*, basal lamina; *end*, endothelial cell; *lu*, lumen; *m*, mitochondria. *Arrow*, bundle of intermediate filaments. Bars: 500 nm.

Fig. 4. Quantitation of immunogold labeling for monocarboxylate transporter 2 (MCT2) on astrocytes in CA1 of the hippocampal formation. MCT2 was significantly reduced by 42 % on perivascular astrocyte endfoot membranes in MTLE compared to in non-MTLE (p < 0.001). The density of MCT2 on astrocytes in the neuropil was divided into three categories: 1) labeling of the plasma

membrane and cytoplasm combined and expressed per total profile (astrocyte), 2) labeling of the cytoplasm only, excluding the plasma membrane (astrocyte cytopl), and 3) labeling of the plasma membrane only (astrocyte membrane). A significant upregulation of MCT2 is seen on astrocytes in MTLE compared to in non-MTLE (p = 0.003). The upregulation of MCT2 in MTLE is confined to the plasma membrane (p < 0.001) and not to the cytoplasm (p = 0.4). The particle densities for plasma membranes represent particles located in a 30 nm wide zone on the inner side of the membrane corresponding to the resolution of the immunogold method, see text. The bar diagrams depict mean±SD.

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Table 1. Characteristics of patients selected for the study

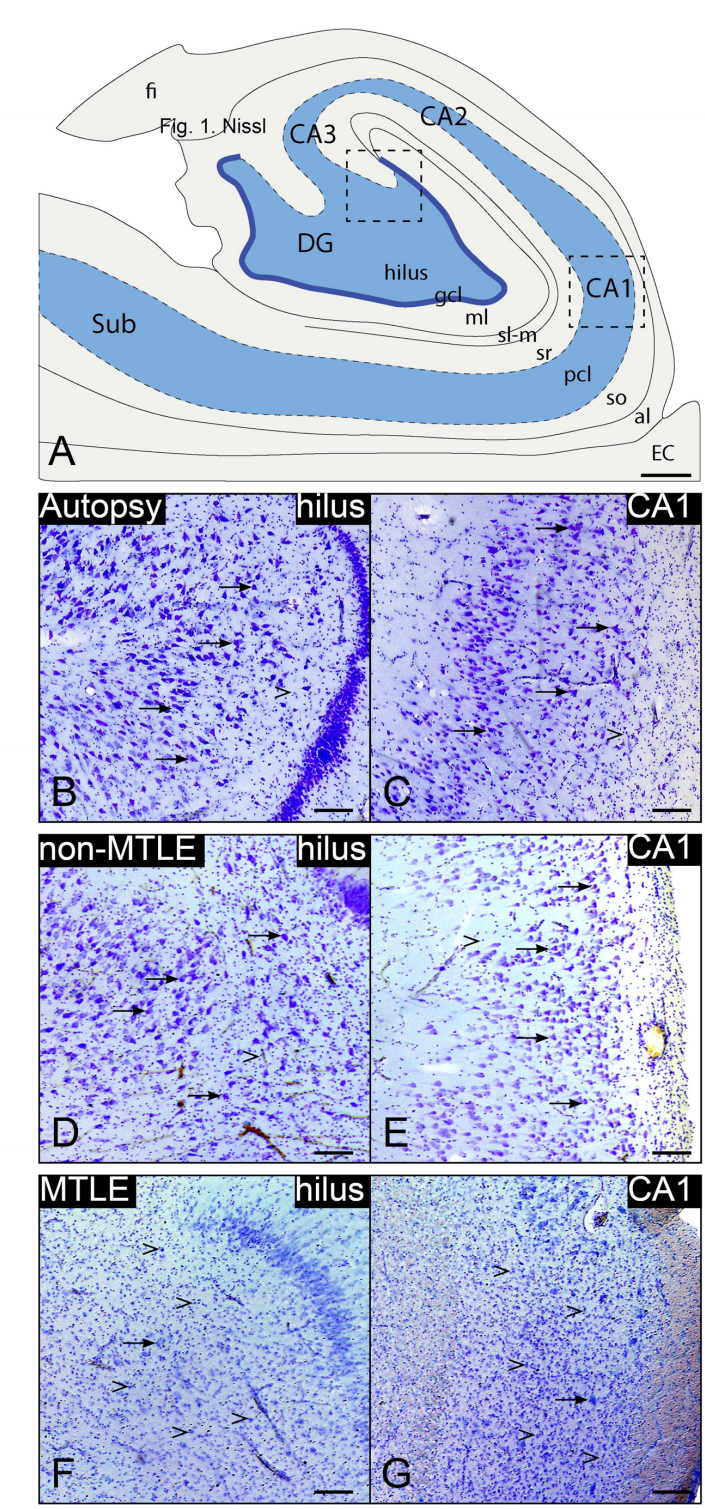
Patient and classification	Sex	Age (years)	Time since first unprovoked seizure (years)	Antiepileptic drugs at surgery	MRI findings	Pathology	Immunohistochemical labeling
Non-MTLE 1	F	18	3	Valproate, lamotrigine	Open lip schizencephaly, R hemisphere	Marked gliosis in cortex and white matter	LM: β-dystroglycan
2	F	28	21	Lamotrigine, zonisamide, phenytoin	Nonspecific, focal T2 signal hyperintensities in subcortical white matter, R frontal lobe	Mild to moderate neuronal loss with severe reactive gliosis. Abnormal lamination and clustering of neurons.	LM: MCT2, β-dystroglycan
3	M	4	2	Oxcarbazepine	Abnormal signal of R amygdala, hippocampal head and parahippocampal gyrus with cyst near R hippocampal head	Oligodendroglioma	LM: β-dystroglycan
4	F	49	13	Carbamazepine XR, phenytoin, lamotrigine	Large mass in R frontal and R temporal lobes	Oligodendroglioma and ganglioneurocytoma	LM: MCT2, β-dystroglycan
5	F	38	<1	Levetiracetam	Nonenhancing R hippocampal lesion	Glioma	LM: MCT2, β-dystroglycan
6	M	13	9	Phenytoin, levetiracetam, carbamazepine	Large porencephalic cavity in the R MCA distribution with surrounding gliosis. Compatible with remote R MCA infarct.	Frequent heterotopic neurons in molecular layer of cerebral cortex	LM: MCT2
7	M	6		Levetiracetam, lamotrigine	Abnormal gray matter with local mass-effect involving L frontal cortex. Possible localized hemimegalencephaly and cortical dysplasia.	Cortical dysplasia	LM: MCT2, β-dystroglycan
8	M	69	7	Phenytoin	Neoplastic lesion, R temporal lobe	Oligodendroglioma	LM: MCT2
9	F	10	5	Lamotrigine	R temporal tumor	Low-grade astrocytoma	EM: MCT2
10	M	28	26	Carbamazepine, acetazolamide	Ectopic gray matter adjacent to L anterior horn. High-signal by FLAIR in R posterior temporal lobe with corresponding low T1 signal. Possible bilateral hippocampal atrophy.	Heterotopic neurons in the molecular layer of the dentate gyrus. Subpial glial cell proliferation.	EM: MCT2
11	M	44	26	Carbamazepine, clonazepam	Possible R mesial temporal sclerosis	Hippocampus w/mild, diffuse neuronal loss and mild to moderate increase in number of white matter glial cells.	EM: MCT2

Patient and classification	Sex	Age (years)	Time since first unprovoked seizure (years)	Antiepileptic drugs at surgery	MRI findings	Pathology	Immunohistochemical labeling
MTLE 12	F	17	5	Oxcarbazepine, lamotrigine, carbamazepine	Heterotopic gray matter in posterior R temporal lobe white matter with extension to the atrium and body of the right lateral ventricle.	Hippocampal sclerosis	LM: MCT2
13	M	57	39	Phenytoin, carbamazepine, topiramate, levetiracetam	Mesial temporal sclerosis	Hippocampal sclerosis	LM: β-dystroglycan
14	F	51	48	Levetiracetam, pregabalin	R mesial temporal sclerosis	Hippocampal sclerosis	LM: MCT2, β-dystroglycan
15	F	40	28	Felbamate, topiramate, gabapentin, carbamazepine	L hippocampal sclerosis	Hippocampal sclerosis	LM: MCT2
16	F	42	7	Levetiracetam, oxcarbazepine	R hippocampal sclerosis	Hippocampal sclerosis	LM: MCT2, β-dystroglycan
17	F	40	37	Carbamazepine, levetiracetam, zonisamide	L hippocampal sclerosis	Hippocampal sclerosis	LM: β-dystroglycan
18	M	62	56	Valproate, levetiracetam, phenytoin	L mesial temporal sclerosis	Hippocampal sclerosis	LM: MCT2
19	F	40	27	Carbamazepine, levetiracetam	Increased T2 signal, L hippocampus	Hippocampal sclerosis	LM: MCT2, β-dystroglycan
20	F	30	22	Zonisamide, carbamazepine XR	R mesial temporal sclerosis	Hippocampal sclerosis	LM: β-dystroglycan
21	F	51	11	Carbamazepine	L mesial temporal sclerosis	Hippocampal sclerosis	EM: MCT2
22	F	37	11	Primidone, lamotrigine	R mesial temporal sclerosis	Hippocampal sclerosis	EM: MCT2
23	F	15	5	Carbamazepine XR, lamotrigine	L mesial temporal sclerosis	Hippocampal sclerosis	EM: MCT2

Patient and classification	Sex	Age (years)	Cause of death	Immunohistochemical labeling
Autopsy 1	M	33	Adenocarcinoma of lung with widespread metastases	LM: MCT2, β-dystroglycan
2	M	52	Myocardial infarct, coronary atherosclerosis	LM: MCT2, β-dystroglycan
3	F	49	Ruptured berry aneurysm, R posterior inferior cerebellar artery	LM: MCT2
4	F	76	Peritonitis	LM: MCT2
5	M	61	Myocardial infarct, cardiovascular atherosclerosis	LM: β-dystroglycan
6	F	62	Dementia, mixed type	LM: β-dystroglycan

Abbreviations: L, left; MCA, middle cerebral artery; R, right; XR, extended release

Figur 1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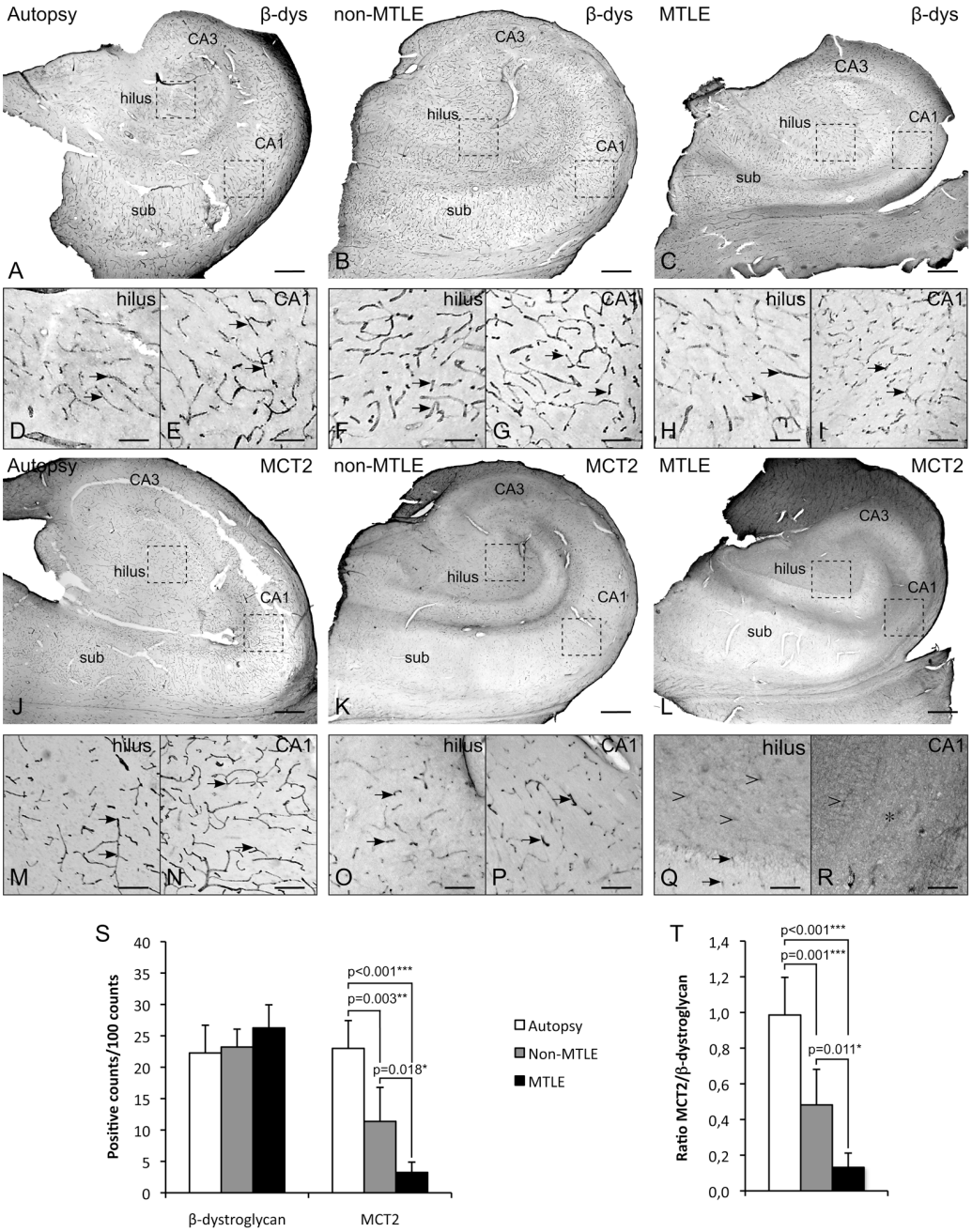
Figur 2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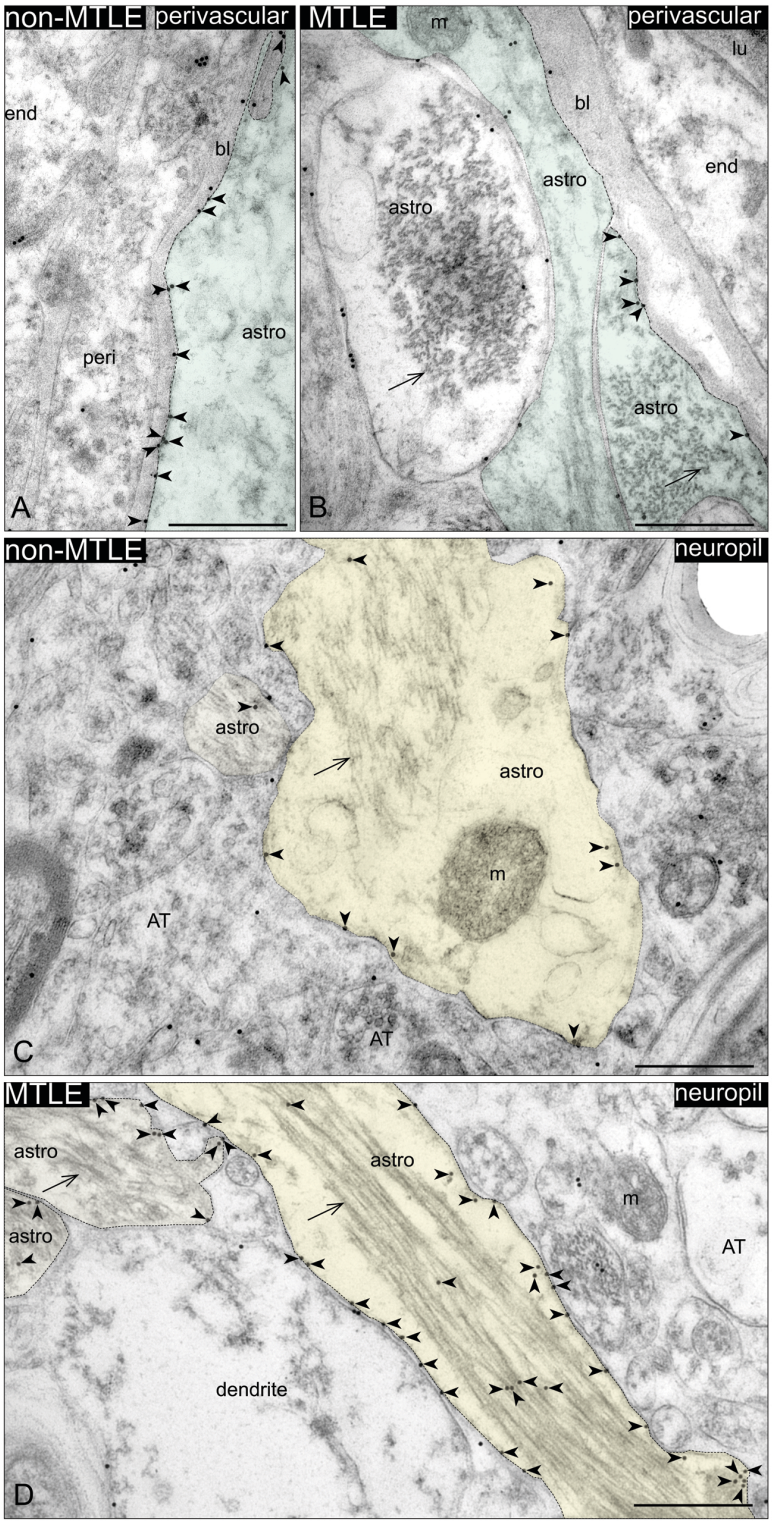
Figur 3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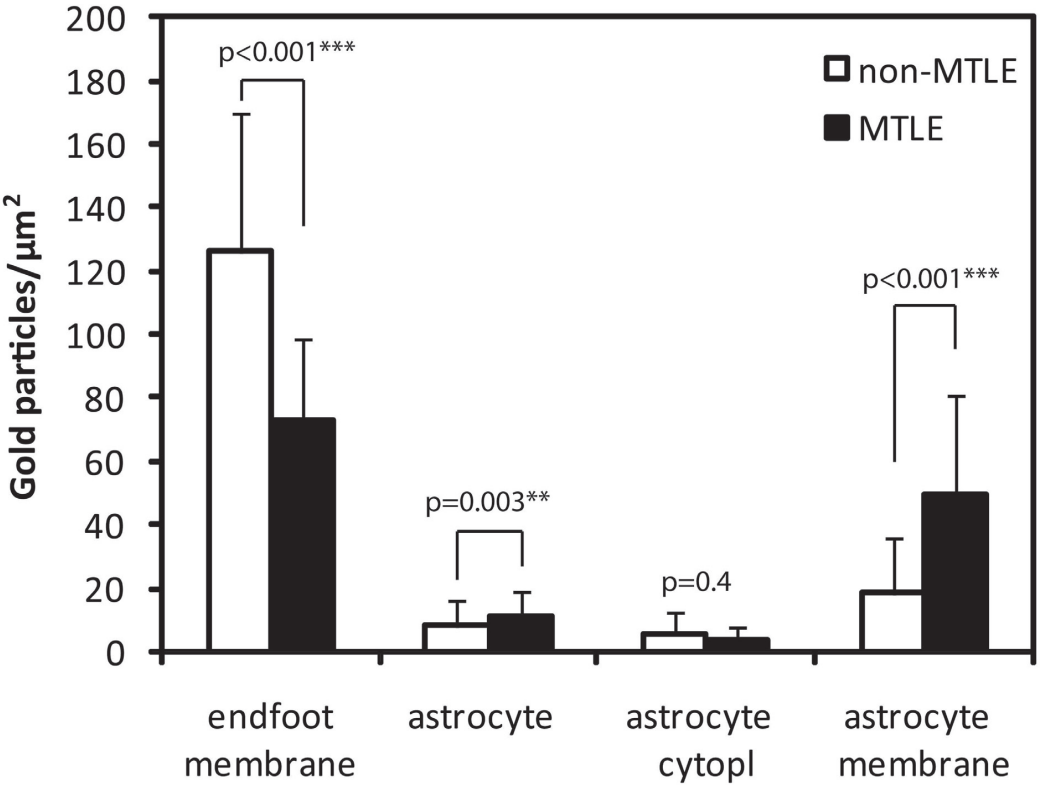
Figur 4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