The effect of cardiolipin synthase deficiency on the mitochondrial function and barrier properties of human cerebral capillary endothelial cells

by

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ABSTRACT

The blood brain barrier (BBB), formed by endothelial cells lining the lumen of the brain capillaries, is a restrictively permeable interface that only allows transport of specific compounds into the brain. Cardiolipin (CL) is a mitochondrial- specific phospholipid known to be required for the activity and integrity of the respiratory chain. The current study examined the role of cardiolipin in maintaining an optimal mitochondrial function that may be necessary to support the barrier properties of the brain microvessel endothelial cells (BMECs). Endothelial cells have been suggested to obtain most of their energy through an-aerobic glycolysis based on studies of cells that were obtained from the peripheral vasculatures. However, here, we showed that the adult human brain capillary endothelial cell line (hCMEC/D3) appeared to produce ~60% of their basal ATP requirement through mitochondrial oxidative phosphorylation. In addition, RNAi mediated knockdown of the CL biosynthetic enzyme cardiolipin synthase (CLS), although did not grossly affect the mitochondrial coupling efficiency of the hCMEC/D3 cells, did seem to reduce their ability to increase their mitochondrial function under conditions of increased demand. Furthermore, the knockdown appeared to have acted as a metabolic switch causing the hCMEC/D3 cells to become more dependent on glycolysis. These cells also showed increase in [3H]-2-deoxyglucose

uptake under a low glucose availability condition, which might have served as a mechanism to compensate for their reduced energy production efficiency. Interestingly, the increase in glucose uptake appeared correlated to an increase in [3H]-2-deoxyglucose glucose transport across the knockdown confluent hCMEC/D3 monolayers grown on Transwell® plates, which was used in our study as an in vitro model for the human BBB. This suggests that changes in the brain endothelial energy status may play a role in regulating glucose transport across the BBB. These observations, perhaps, also explain why the brain capillary endothelial cells were previously observed to possess higher mitochondrial content than those coming from non-BBB regions (Oldendorf et al. 1977).

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ABBREVIATIONS

BBB	Blood-brain barrier
CNS	Central nervous system
BMEC	Brain microvessel endothelial cells
CL	Cardiolipin
CLS	Cardiolipin synthase
ACM	Astrocytes conditioned-medium
ABC	ATP-binding cassette
BCRP	Breast cancer resistant protein
MRP	Multidrug resistance protein
GLUT1	Glucose transporter-1
2-DG	2-deoxy-D-glucose
SGLT	Sodium dependent glucose transporter
MCT-1	Monocarboxylate transporter-1
CRT1	Creatine transporter-1
AGAT	L-arginine:glycine amidinotransferase

GAMT	guanidinoacetate methyltransferase
Pgp	P-glycoprotein
IMM	Inner mitochondrial membrane
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
G-3-P	Glycerol-3-phosphate
GPAT	glycerol-3-phosphate acyltransferase
CDP-DAG	cytidinediphosphate-1,2-diacyl-*sn*-glycerol
CDS	CDP-DAG synthetase
PPAR-α	proliferator-activated receptor-α
PG	phosphatidylglycerol
MLCL	monolysocardiolipin
BTHS	Barth Syndrome
KRH	Krebs- Ringer HEPES
OCR	Oxygen consumption rate
ECAR	Extracellular acidification rate

PPR	Proton production rate
CLS-KD	CLS knockdown
bFGF	Basic fibroblast growth factor
TGF-β	Transforming growth factor- β
TEER	Trans-endothelial resistance

1. INTRODUCTION

1.1 The blood brain barrier

The human central nervous system (CNS) is separated from the peripheral circulation by three main interfaces, the arachnoid epithelium, the choroid plexus epithelium and the micro-vessel endothelium that forms the so-called blood brain barrier (BBB) (Saunders et al. 2008). As the brain contains billions of capillaries with each neuron being perfused by its own blood vessel, passing through these micro-vessels become the major route for a substance from the blood to enter the brain (Pardridge. 2007). Being restrictively permeable that only allows selective substances to enter the CNS, the BBB thus appears to be the principal protective interface of the brain (Hawkins and Davis. 2005; Khan. 2005). This is one way in which the brain maintains its optimal ionic and chemical milieu necessary for cerebral function. Three types of cells are known to make up the BBB, the endothelial cells, the pericytes and the astrocytes (Hawkins and Davis. 2005). The association of these three cell types with neurons and extracellular matrix (basal lamina) were suggested to form a functional neurovascular unit that separates the blood from the interstitial fluid of the brain and may be important for cerebral health and for the regulation of cerebral blood flow (Hawkins and Davis.

2005; Abbott et al. 2006). Indeed, disruption of this neurovascular unit has been noted in many neurological disorders either as a consequence of the disease or as a precipitating event and these conditions include ischemic strokes, traumatic brain injury, Alzheimer's disease and diabetes (Rosenberg. 2012; Hawkins and Davis. 2005). In these conditions, strategies to restore the integrity of the BBB appear essential for the maintenance of cerebral health. However, there are also conditions such as in the case of brain tumor that the ability to transiently open the BBB to facilitate the delivery of drugs would also be of great interest.

Morphologically, the endothelial cell layer that lines the brain capillaries is believed to be the site of the BBB (Hawkins and Davis. 2005; Cipolla. 2009). It is because these brain microvessel endothelial cells (BMECs) are characteristically unique from those in the peripheral vasculatures that allow them to perform the following barrier functions (Abbott et al. 2006; Hawkins and Davis. 2005; Cipolla. 2009):

1. BMECs can act as a "**physical barrier**" as they are joined together by highly developed tight junctional complexes. These tight junctions, comprised of junctional adhesion proteins such as

claudin, occludin and junctional adhesion molecules, can form a tight seal between adjacent endothelial cells and thus, restrict the paracellular passage of most hydrophilic solutes including small ions such as Na^+ and Cl^- into the brain. This forces most solutes and macromolecules to take a trans-cellular pathway that can be highly regulated. The fact that the cerebral capillary wall is known to be approximately 39% thinner than that of the peripheral capillaries is believed to act as a compensation for this restrictive permeability, allowing the trans-cellular transport of nutrients to be more efficient (Coomber and Stewart. 1985). It is known that the trans-endothelial resistance (TEER) of the cerebral capillary endothelium can be as high as 1500-2000 ohms.cm^2. This is unlike most endothelia, which are characterized by a low trans-endothelial resistance (~2-20 ohms.cm^2) and the presence of fenestraes that allow most substances to passively diffuse through.

2. BMECs can act as a "**transport barrier**" as they are polarized and express specific transporters on the luminal and abluminal membranes that can either actively transport nutrients from the blood into the brain or efflux potential harmful compounds including waste products from the brain back into the blood. With

such a high metabolic requirement, BMECs are also known to possess an increased mitochondrial content compared to those in in the peripheral tissues (Oldendorf et al. 1977).

Even though, BMECs are known to possess a low rate of pinocytosis, large molecules can still get into the brain through the specific receptor-mediated endocytosis system expressed by these cells. This property allows the trans-cellular pathway across the brain endothelium to be further restricted and better controlled (Abbott. 2002).

3. BMECs can also act as a "**metabolic barrier**" as they express both intracellular and extracellular enzymes such as monoamine oxidase and cytochrome P450 that can inactivate potential toxic lipophilic compounds into polar metabolites so that they cannot further cross the BBB to enter the brain**.**

1.2 Induction and maintenance of the BBB

What causes BMECs to possess such barrier properties is not the innate properties of these cells but is rather the interactions with their surrounding environment because it was shown that when the brain

micro-vessels were taken out of their CNS environment and allowed to vascularize non-neural tissues, they became more "leaky" (Rubin et al. 1991). The reverse was also true as abdominal vessels from young quail embryos that were allowed to vascularize neural tissues of the host chicks reportedly developed BBB characteristics (Stewart and Wiley. 1981). Thus, the interactions of BMECs with the other components of the neurovascular units appear important for the induction and maintenance of their barrier properties.

The role of astrocytes

Due to their anatomical proximity and the fact astrocytic end feet cover approximately 99% of the BBB endothelium (Yadollah Omidi and Jaleh Barar. 2012), it is believed that astrocytes play a central role in the induction and maintenance of BMECs' barrier characteristics. Indeed, results from cell culture studies strongly support this role of astrocytes. It was shown that when BMECs were either co-cultured with astrocytes or cultured in astrocytes conditioned-medium (ACM), their barrier properties that include high resistance tight junctions, low paracellular permeability and expression of γ-glutamyl transpeptidase, an enzyme important for amino acid transport across the BBB but is

either reportedly lost or lowered in cultured BMECs, were significantly enhanced (Rubin et al. 1991; Dehouck et al. 1990). Interestingly, Shivers el al. (Shivers et al. 1988) showed that not only BMECs but also when non-neural micro-vessel endothelial cells were cultured in ACM, they developed tight junctions. These findings suggest that the BBB characteristics of BMECs may not only be induced by the direct interaction with astrocytes but also by the soluble factors secreted by these astrocytes. A number of such factors have been documented and they include the glial-derived neurotrophic factor (GDNF) (Igarashi et al. 1999), transforming growth factor- β (TGF-β) (Tran et al. 1999), basic fibroblast growth factor (bFGF) (Sobue et al. 1999) and hydrocortisone (Hoheisel et al. 1998). Besides its role in maintaining the tightness of the BBB, astrocytic influence was also reported to be important for the up-regulation of specific transport systems in cultured BMECs that include the glucose transporter (GLUT-1) (Regina et al. 2001), the L-system amino acid transporter (Chishty et al. 2002), and P-glycoprotein drug efflux transporter (El Hafny et al. 1997). Reciprocally, the Leukemia inhibitory factor (LIF) secreted by the brain endothelial cells was also shown to have the ability to induce astrocyte differentiation (Mi et al. 2001). Thus, it appears that there is a mutual relationship between astrocytes and BMECs with one inducing and

maintaining the characteristics of the other.

The role of pericytes

Pericytes are embedded in the basement membrane that support BMECs and cover about 20-30% of the brain endothelium (Hawkins et al. 2006; Frank et al. 1987). In contrast to the astrocytic involvement, the role of pericytes at the BBB is less well known (Hawkins et al. 2006). The brain capillary pericytes were shown to express the smooth muscle isoform of actin typical of contractile cells and were thus suggested to play a role in the regulation of cerebral blood flow (Bandopadhyay et al. 2001). They are also believed to act as structural support for the assembly of brain capillaries. It was shown in the brain of the PDGF-B deficient mice, a pericyte deficient model, that these animals experienced endothelial hyperplasia, larger capillary diameter, abnormal BMECs structure and increased vascular leakage (Hellstrom et al. 2001). Consistently, when pericytes were co-cultured with astrocytes and endothelial cells, pericytes were reportedly able to induce the reorganization of these cells into a "capillary like structure" (Ramsauer et al. 2002). Pericytes were also shown to have the ability to inhibit endothelial cell proliferation (Orlidge and D'Amore. 1987) but at the same time making endothelial cells more resistant to apoptosis

(Ramsauer et al. 2002). These observations thus support the important role these cells in the structural maintenance of the brain microvessels.

In addition to its structural role, pericyte derived factor were also shown to up-regulate the expression of claudin, the protein intrinsically important for the formation of endothelial tight junction (Hori et al. 2004). Furthermore, in response to hypoxia and/or traumatic brain injury, pericytes were shown to have migrated away from the microvessels and were thus suggested to be partly responsible for the BBB leakiness observed in these conditions (Hawkins and Davis. 2005). Together, these observations strongly support an important role of pericytes in both the induction and maintenance of the barrier properties of the cerebral endothelium.

Neurons and the basal lamina, the remaining components of the neurovascular unit have also been shown to play both a regulatory and structural role in the BBB function (Hawkins and Davis. 2005). Thus, it appears that the individual components of the neurovascular units are intrinsically important for the maintenance of the structural and functional properties of the BBB. Perhaps, such knowledge could set

ground for further understanding of the many different pathways in which diseases or drugs could modulate the permeability across the BBB.

1.3 Transport systems at the BBB

Despite aggressive research and the advances in the understanding of the brain, neurological disorders still remain a major global burden mostly due to the required long-term care and the small number of effective drugs (Misra et al. 2003). The low availability of effective therapeutics is not due to the lack of potent drug candidates but is mainly caused by the presence of the BBB that limit the penetration of these therapeutics into the brain (Pardridge. 2007; Misra et al. 2003). Therefore, in order to enhance drug delivery into the CNS, transport processes across the BBB and the factors that regulate these processes need to be better understood.

In general, only low molecular weight (<400 Da) and lipophilic compounds such as oxygen and carbon dioxide can diffuse through the BBB (Pardridge. 2007). Glucose, amino acids and other hydrophilic essential nutrients can only cross this barrier via specific influx

transporters expressed by BMECs (Pardridge. 2007). However, not all small and lipophilic compounds can pass through this restrictive interface. In fact, almost 98% of small molecule therapeutics studied reportedly failed to cross the BBB in therapeutically relevant amounts (Pardridge. 2012). It is also surprising that while a hydrophilic drug like levodopa can readily pass through the BBB, highly lipophilic anticancer agents such as doxorubicin and vinca alkaloids have a hard time entering the brain (Misra et al. 2003). What can partially explain for such low permeability of these compounds is the presence of the efflux transporters at the brain endothelium. These transporters mostly belong to the ATP-binding cassette (ABC) transporter superfamily that use the energy from ATP hydrolysis to pump waste products including some therapeutic substrates against their concentration gradient from the brain back into the blood (Pardridge. 2012). Three families of ABC transporters have been predominantly characterized to play important role in the transport of important therapeutics across the BBB and they are P-glycoprotein (P-gp), the multi-drug resistance associated proteins (MRPs) and the breast cancer resistance protein (BCRP) (Loscher and Potschka. 2005). Better understanding of the functioning and the regulators of these influx and efflux transporters may be of central importance for the development

of effective strategies to deliver drugs into the CNS.

Major influx transporters at the BBB

The glucose transporter, the monocarboxylate transporter and the creatine transporter are among the major transporter systems expressed by the human BMECs that facilitate the delivery of nutrients from the blood into the brain.

The glucose transporter-1 (GLUT1)

Glucose is the principle and non-replaceable source of energy of the brain. The human brain is known to be the main consumer of glucose despite the fact it represents just ~ 2% of the total body weight (Mergenthaler et al. 2013). The highly glycosylated 55kDa- GLUT1, a member of the SLC2 family, is the principle facilitative glucose transporter at the brain endothelium that mediates the insulin independent transport of glucose from the blood into the brain (Shah et al. 2012). It is known to be selective for D-glucose enantiomers that include 2-deoxy-D-glucose and fluoro-deoxyglucose (Pardridge. 2007). It is expressed at both the luminal and abluminal membranes of human BMECs and was suggested to be asymmetrically distributed

with a 1:4 luminal: abluminal ratio (Farrell and Pardridge. 1991; Simpson et al. 2001). This asymmetrical distribution of GLUT1, however, still remains un-established as a recent study using bovine micro-vessels suggested that the detection of a higher concentration of GLUT1 in the abluminal membrane of BMECs may be an artifact caused by the C-terminal directed antibody previously employed being unable to detect the GLUT1 in the luminal membrane whose C-terminal may either be missing or modified (Simpson et al. 2001). The mechanism of glucose transport from the blood into the brain has not been fully elucidated (McAllister et al. 2001). The asymmetrical distribution with a higher concentration of GLUT-1 transporters and hexokinase in the abluminal membrane together with a high brain glucose metabolism were suggested to form a polarity and concentration gradient that favor the transport of glucose from the blood into the brain (Simpson et al. 2001; McAllister et al. 2001). A large proportion of GLUT1 transporters were also reported to reside in the intracellular pool of BMECs that may provide the potential for transporter recruitment and redistribution between the luminal and abluminal membranes under conditions of increased demand (Simpson et al. 2001). As astrocytic processes cover ~99% of the endothelium and also express the less glycosylated 45kDa-GLUT1, it was suggested

that in order for glucose to enter the brain, it may have to cross through both the astrocytic end feet and the endothelial GLUT1 transporters, which may not be a very efficient process. However, modeling study (Simpson et al. 2007) showed that a sufficient amount of glucose could be delivered into the brain just by diffusing through the gaps in between the astrocytic end feet. The half saturation constant (Km) for Glut1 is known to be 1-2 mM for glucose influx and 20-30 mM for glucose efflux (Siegel et al. 2012). This kinetic property suggests that under a physiological plasma glucose concentration, GLUT1 could be saturated that favors the influx of glucose from the blood into the brain. A number of factors have been shown to regulate the expression of GLUT1 at the BBB and these include chronic hypoglycemia (Simpson et al. 1999), a rich n-3 polyunsaturated diet (Pifferi et al. 2007) and chronic hypoxia or ischemia (Harik et al. 1994; Harik et al. 1995).

The Sodium-dependent glucose transporter

Even though GLUT1 is the principal glucose influx transporter at the BBB, BMECs are also known to express the sodium-dependent glucose transporter (SGLT1) that also contributes to the brain glucose

importation process (Qutub and Hunt. 2005). This transporter co-transports $2Na^+$/glucose into the brain and is facilitated by the Na^+ gradient established by the Na^+/k^+ ATPase pump. Being expressed on the luminal side of BMECs and is regulated by the intracellular glucose concentration (Nishizaki and Matsuoka. 1998), SGLT1 is believed to be important for maintaining a constant endothelial glucose concentration, especially under stressful conditions such as hypoglycemia. Under such condition, increase in glucose uptake into the endothelial cells by the SGLT1 was proposed to stimulate the abluminal GLUT1 to transport more glucose into the brain and thus serve as a protective mechanism to maintain a constant cerebral glucose concentration that is essential for life (Qutub and Hunt. 2005)

The monocarboxylate transporter (MCT) system

Even though glucose is the non-replaceable energy source of the brain, under conditions of increased neural demand such as starvation or increased physical activity, the brain is also known to use the metabolic products such as ketone bodies and lactate as alternative energy sources (Mergenthaler et al. 2013). The monocarboxylate transporter-1 (MCT1) is the main monocarboxylate transporter at the

BBB (Mergenthaler et al. 2013). It is known to be expressed on both the luminal and abluminal membranes of BMECs and is thus proposed to play roles in both the transport of carboxylated molecules from the blood into the brain and from the brain back into the blood (Tsuji. 2005). A number of substrates have been identified for this transporter and they include metabolic compounds such as acetoacetate, puryvate and lactate (Qutub and Hunt. 2005) or carboxylated pharmaceutical agents such as salicylic acid, nicotinic acid, and some β-lactam antibiotics (Tsuji. 2005). Study in rat brain found the co-localization of GLUT1 and MCT1 and suggested a positive correlation between their densities (Maurer et al. 2004). This also suggests a correlation between local brain energy demand and MCT1 expression. Indeed, in children who have impaired cerebral glucose import by GLUT1 mutations, it was shown that a ketogenic diet early in the course of the disease was able to ameliorate the condition (Maurer et al. 2004). Such an improvement may partly be due to the increase in MCT1 expression induced by the ketogenic diet (Leino et al. 2001). MCT1 expression was also shown to be much higher in the developing rat brain as compared to that of adult rats, which further supports the role of MCT1 under a high-energy demand condition.

The Creatine transporter

In organs that need a high burst of energy supply during activation such as the muscle and the brain, ATP is often stored in the form of phosphocreatine that can serve as an immediate mean for ATP regeneration known to be 12 times faster than oxidative phosphorylation (OX-PHOS) (Rae et al. 2003). Indeed, this is supported by the observation that phosphocreatine level rapidly decreased during brain activation while the ATP level remained quite constant (Rae et al. 2003). Whether creatine is mainly synthesized inside the brain or is imported from the peripheral circulation remains a subject of debate. BMECs were shown to express the creatine transporter-1 (CRT-1), encoded by the SLC6A8 gene, that has the ability to uptake creatine into the brain against their concentration in a Na^+, Cl^- dependent manner (Ohtsuki et al. 2002; Beard and Braissant. 2010). However, as expression of this transporter is absent in the astrocytic end feet covering the endothelium, it was suggested that the permeability of creatine into the brain might be limited (Beard and Braissant. 2010). This may indeed be true as oral administration of 20g of creatine per day consecutively for 4 weeks in healthy volunteers only resulted in a moderate 5-10% increase in the brain creatine level (Dechent et al. 1999). As the brain is also known to

express both the L-arginine:glycine amidinotransferase (AGAT) and the guanidinoacetate methyltransferase (GAMT) that are sufficient for endogenous creatine synthesis, it is suggested that the brain can self supply most of its required creatine (Beard and Braissant. 2010). This can be supported by the fact that treatment of AGAT and GAMT deficient patients with a high oral dose of creatine only slowly and partially replenished the cerebral creatine level and still left these patients with CNS developmental problems (Braissant. 2012). These observations appear to challenge the importance of CRT1 in supplying creatine to the brain. However, the fact that patients with defects in the SLC6A8 gene experience worst CNS outcomes that includes progressive brain atrophy, cognitive disability and speech deficit that cannot be treated by creatine oral supplementation (Braissant. 2012) still highly indicate the essential role of CRT1 in maintaining a healthy brain creatine level.

Major efflux transporters at the BBB

The P-glycoprotein (Pgp)

The human BMECs are known to express the efflux transporter P-glycoprotein type I (aka. MRD1) at its abluminal surface, that is

responsible for keeping many lipophilic substances capable of entering the endothelium from getting into the brain (Loscher and Potschka. 2005). A wide range of pharmaceutical agents with molecular weights ranging from 3000-4000 Da has been reported to be substrates of this transporter. These compounds include drugs such as antibiotics, immunosuppressive agents and anti-cancer agents (Loscher and Potschka. 2005; Miller et al. 2008). Not only restricting drug access into the CNS, Pgp expression was also reportedly increased in neurological conditions such as brain tumor and epilepsy that may partly be responsible for the multi-drug resistance observed in these conditions (Loscher and Potschka. 2005; Miller et al. 2008). Thus, strategies to either inhibit Pgp or down-regulate its expression appear fruitful in the treatment of these neurological disorders. Indeed, many Pgp inhibitors have been developed and are in the different stages of clinical trials (Loscher and Potschka. 2005). However, most of these inhibitors have failed to be successfully used in the clinics (Loscher and Potschka. 2005). This may partly be due to the fact sufficient expression of Pgp is needed under physiological conditions to keep a wide range of neurotoxins from accumulating inside the brain (Loscher and Potschka. 2005). Indeed, animal studies showed that drugs that are normally well tolerated could become neurotoxic in the absence of

Pgp due to high accumulation inside the brain (reviewed in (Schinkel. 1999)). Thus, maintenance of an appropriate expression and activity of Pgp appears important for cerebral health.

The multi-drug resistance associated proteins (MRPs)

The MRP (aka. ABCC) family is another member of the ABC superfamily that has also been suggested to play an important role in multidrug resistance. They are known to transport mainly anionic drugs such as methotrexate but their substrates can also include neutral agents such as glutathione and its derivatives (Borst et al. 2000). Having such a wide range of substrates, MRPs are known to have overlapping substrates with the Pgp and thus further restrict the penetration of these compounds into the brain (Borst et al. 2000). This also further enhances drug resistance instances. Twelve homologues of the MRPs have been identified and at least six of these (MRP1-6) have been reported to be expressed by BMECs of different species including human (Loscher and Potschka. 2005).

The Breast Cancer Resistant protein (BCRP)

BCRP was first discovered in a multi-drug resistant breast cancer cell line that showed reduction in accumulation of chemotherapeutic drugs even in the absence of Pgp and MRPs (Doyle et al. 1998). Even though the BCRP gene was isolated from and named after this cell line, there has been no indication that the transporter's expression and activity are specific to chemotherapy resistant breast cancer cells alone (Doyle et al. 1998). Due to its sequence and structural homology, BCRP was suggested to belong to the ABCG family, a subfamily of the ABC superfamily of transporters (Schinkel and Jonker. 2003). Humans are known to express at least 5 ABCG homologues, namely ABCG1, ABCG2, ABCG4, ABCG5, and ABCG8 (Sarkadi et al. 2006). However, ABCG2 is believed to play the primary role in drug transport across the BBB (Sarkadi et al. 2006). Similar to the other ABC efflux transporters, ABCG2 substrates include a broad range of compounds from cytotoxic drugs and their metabolites to neurotoxins and carcinogens found in food (Sarkadi et al. 2006). Both endogenous and exogenous factors have been found to regulate ABCG2 expression and they include growth factors such as TGFβ (Yin et al. 2008), tumor necrosis factor-alpha or interleukin 1 (Evseenko et al. 2007) and hormonal factors such as estradiol and progesterone (Hahnova-Cygalova et al. 2011).

1.4 Cardiolipin

Phospholipids serve important structural and functional roles in the body. They can act as an energy storage source, as signalling molecules and most importantly, as the main structural and functional components of cellular membranes (van Meer et al. 2008). Cardiolipin, also known as bis-(1,2-diacyl-sn-glycero-3-phospho)-1'-3'-sn-glycerol, is the major polyglycerophospholipid in mammalian tissues. CL is unique in that it is synthesized and localized exclusively in the mitochondria of mammalian tissues (Hatch. 2004; Houtkooper and Vaz. 2008). This is unlike most phospholipids, which are synthesized in the ER and later imported into the mitochondria for use (reviewed in (Hatch. 2004). CL is most abundant in cardiac tissues as it makes up 15-20% of the total phospholipid phosphorus mass of the heart (Hostetler. 1982). Given its high abundance in the heart, CL has been proposed to be important for ATP production via the oxidative phosphorylation (OXPHOS) system embedded in the inner mitochondrial membrane (IMM) (reviewed in (Claypool and Koehler. 2012). Indeed, studies have shown that CL is critical for the optimal activity of several enzymes involved in OXPHOS (reviewed in (Hatch.

2004; Houtkooper and Vaz. 2008)). This is particularly evident in a recent report by (Arnarez et al. 2013a) in which a molecular dynamic simulation model of cytochrome c oxidase complex (complex IV) was used to show that there are precise CL binding sites at the entrance to the proton channels on the matrix side of the complex. With the ability of CL to trap protons, these authors suggested that CL might also actively take part in the proton transport across complex IV to the inter-membrane space. CL is also believed to act as a "glue" that holds the mitochondrial complexes together in the supercomplex formation to ensure efficient electron flow and proton transport (Arnarez et al. 2013b; Zhang et al. 2002). Therefore, maintenance of the appropriate levels of CL in the mitochondria is essential for proper cellular function. CL was first isolated from beef heart by (Pangborn. 1942) and its mammalian biosynthesis was first described in rat liver by (Hostetler et al. 1971).

1.5 Cardiolipin biosynthesis and remodeling

The biosynthesis of phospholipids is highly conserved from yeast to mammals (Schlame. 2008)). In mammalian tissues, more specifically in the heart, the *de novo* synthesis of CL occurs in the IMM via the

cytidine-5'-diphosphate-1,2-diacyl-*sn*-glycerol pathway (Hatch. 1994). The pathway begins with the addition of an acyl group from acyl-CoA to the sn-1 position of glycerol-3-phosphate (G3P) by glycerol-3-phosphate acyltransferase (GPAT) to form 1-acyl-*sn*-glycerol-3-phosphate (or lysophosphatidic acid) (Schlame. 2008). The product of GPAT is then acylated by the enzyme 1-acyl-*sn*-glycerol-3-phosphate acyltransferase to form 1, 2-diacyl-*sn*-glycerol-3-phosphate (or Phosphatidic Acid). Then, a condensation reaction catalyzed by cytidinediphosphate-1,2-diacyl-*sn*-glycerol (CDP-DAG) synthetase (CDS), occurs between PA and CTP to form the high energy intermediate phosphatidyl-CMP (or CDP-DAG) (Schlame. 2008). Studies suggest that PA conversion to CDP-DAG is a rate-limiting step in the biosynthesis of phosphatidylglycerol and CL (Cheng and Hatch. 1995; Hatch. 1994). Mammalian CDS is believed to exist in both the mitochondria and the ER. In humans, two isoforms (Cds1 and Cds2) have been cloned and sequenced (Halford et al. 1998). Originally, Cds1 was thought to be the enzyme mainly responsible for CDP-DAG synthesis in the mitochondria (Shen et al. 1996; Kuchler et al. 1986). However, recent study in yeast suggested that Cds1 is localized in the ER exclusively (Tamura et al. 2013) and Tam41, a mitochondrial protein originally described as a maintenance protein for the TIM 23

complex (Tamura et al. 2006), was found to have CDS activity and is now believed to be the mitochondrial CDS (Kutik et al. 2008; Tamura et al. 2013).

The committed step in the CL synthesis pathway is the transfer of the activated phosphatidyl group from CDP-DAG to the sn-1 position of *sn*-glycerol-3-phosphate by phosphatidyl glycerol phosphate (PGP) synthase to form PGP (Hatch. 2004). Data from our laboratory showed that peroxisome proliferator-activated receptor α (PPAR-α) could up-regulate the *de novo* synthesis of CL by regulating the enzymatic activity of PGP-synthase (Jiang et al. 2004). The newly formed PGP is then hydrolyzed by a phosphatase to form phosphatidylglycerol (PG). This phosphatase has recently been identified to be the protein tyrosine phosphatase mitochondrion 1 (PTPMT1) (Zhang et al. 2011).

The final step in the CL biosynthesis is the transfer of an activated phosphatidyl group from another CDP-DAG molecule to PG to form the nascent CL. This reaction is catalyzed by the enzyme CL synthase (CLS) that is localized exclusively in the IMM (Hostetler et al. 1971). CLS has been purified and characterized in rat liver, yeast and *Arabidopsis* (reviewed in (Schlame. 2008)). Its properties include the

requirement of divalent cations (Mg^{2+}, Mn^{2+}, or Co^{2+}) to function, an alkaline pH optimum (pH 8-9), and no preference for specific acyl groups on PG or CDP-DAG (Houtkooper et al. 2006). In humans, CLS is encoded by the *hCLS1* gene, and high levels of this gene have been detected in mitochondria rich tissues such as the liver, kidneys, pancreas and the small intestine (Chen et al. 2006a; Lu et al. 2006a).

As CLS has limited acyl substrate specificity (Houtkooper et al. 2006) and the acyl composition of the mature CL is significantly different from those of its precursor lipids (Schlame. 2008), remodeling has been proposed to take place shortly after the de novo synthesis so that CL can obtain the tissue specific acyl groups necessary for its functions. CL acyl chains can be first de-acylated to monolysocardiolipin (MLCL) (Chicco and Sparagna. 2007; Schlame. 2008). The secretory, cytosolic and calcium-independent phospholipase A_2 have been shown in vitro to have to ability to deacylate CL to MLCL (Hsu et al. 2013). MLCL may then be resynthesized to the mature CL by tafazzin, MLCL AT-1 and ALCAT1.

1.6 The role of cardiolipin synthase in normal cell physiology

In yeast lacking CLS, it was shown that CL was absent and there was accumulation of PG (Chang et al. 1998). Surprisingly, CL deficiency did not grossly affect the yeast's mitochondrial function as well as their viability, especially when the yeasts were grown on a fermentable carbon source (glucose media) (Chang et al. 1998). However, the yeasts' growth was reportedly poorer on a non-fermentable carbon source or at an elevated temperature (Chang et al. 1998; Jiang et al. 1999). These observations are believed to be due to the ability of PG to substitute for the essential functions of CL under normal condition (Chang et al. 1998; Jiang et al. 1999). However, under a more stressful condition such as when the yeast was grown on a non-fermentable carbon source (glycerol/ethanol media) that forced them to strictly depend on mitochondrial respiration for energy, the presence of CL still appeared necessary for their optimal growth. Similarly, in a mammalian cell model where CLS was knock down with only about 25% of CL remaining, it was shown that mitochondrial function and cell viability were not grossly affected (Choi et al. 2007). However, when these cells were challenged with an apoptotic stimulus, the CLS-RNAi treated cells had a more prominent decrease in mitochondrial membrane potential and were also more susceptible to apoptosis (Choi et al. 2007). Taken together, these data suggest that

CL deficiency may not be detrimental for the cells under normal condition but may affect their ability to cope with a more stressful condition.

Besides its role in mitochondrial function, CL externalization to the outer mitochondrial membrane (OMM) in response to mitochondrial damage was also recently suggested to act as a signal for mitophagy. Mitophagy is a physiological process that eliminates damaged mitochondria to prevent its detrimental effect on the cells (Chu et al. 2013). It was shown in primary cortical neurons that rotenone, a complex I inhibitor, could induce CL transport from the IMM to the OMM that resulted in increased mitophagy (Chu et al. 2013). Interestingly, knockdown of CLS or scramblase-3, a protein translocator of CL from the IMM to the OMM, significantly reduced the rotenone-mediated mitophagy (Chu et al. 2013). This can also be supported by the fact that abnormal mitochondria with larger size and reduced cristae formation are often observed in the cells of Barth Syndrome' patients (Finsterer and Frank. 2013), a condition caused by deficiency of the mature polyunsaturated CL. Thus, it appears that the removal process of damaged mitochondria might have been impaired in Barth Syndrome and this also stresses the essential role of CL for

mitophagy. As disruption in mitophagy has also been implicated in many neurodegerative diseases (Chen and Chan. 2009), maintenance of an appropriate level of CL appears essential not only for the optimal mitochondrial function of the cells but also for the clearance of damaged mitochondria critical for maintaining cellular health.

1.7 Cardiolipin biosynthesis alterations in diseases

Given the importance of CL for mitochondrial oxidative function, it appears unsurprising that alterations in CL biosynthesis have been associated with a variety of pathological conditions that include Barth Syndrome and diabetes.

Barth Syndrome

Barth Syndrome (BTHS) is a rare, x-linked recessive disorder of lipid metabolism in young boys that is characterized by wide spectrum of symptoms that include cardio-skeletal myopathy, lethal delayed growth and weakened immunity that are associated with abnormal mitochondrial respiratory function (Hauff and Hatch. 2006; Houtkooper et al. 2006; Aprikyan and Khuchua. 2013; Schlame. 2008; Clarke et al. 2013). It is the only human disease in which the primary defects are

both a reduction in CL content and changes in its molecular acyl composition (Chicco and Sparagna. 2007; Schlame and Ren. 2006). It has been suggested that BTHS is caused by mutations in the TAZ gene, which codes for tafazzin, a phospholipid acyltransferase. This acyltransferase is believed to remodel newly synthesized CL with tissue specific acyl side chains to form the mature CL necessary for adequate mitochondrial activity (reviewed in (Schlame and Ren. 2006)). At the molecular level, BTHS is characterized by a dramatic decrease in unsaturated CL species, accumulation of MLCL and abnormal cristae morphology in the mitochondria (Schlame and Ren. 2006; Acehan et al. 2011; Soustek et al. 2011). However, it is not known how these individual aspects affect the biochemistry of the different cell types in our body. BTHS patients are often presented with variable clinical symptoms from very mild to severe that cannot be predicted by the type of mutations in the TAZ gene (Schlame and Ren. 2006). Therefore, the effects of CL alterations must be better understood in different tissues of our body to allow the development of treatments for BTHS that are currently unavailable.

Diabetes

Reduction in mitochondrial activity has been reported both in human patients (Kelley et al. 2002; Morino et al. 2005) and a mouse model of diabetes (Choo et al. 2006). Having an important role for mitochondrial function, changes in CL biosynthesis and remodeling have also been investigated to have a potential contributing role in the pathogenesis of this disease. Indeed, decrease in myocardial CL content has been reported in streptozotocin treated mice (a model of type I diabetes) as a direct result of decreased CL biosynthesis (Han et al. 2007). A similar CL reduction was also reported in brain mitochondria of streptozotocin treated rats but no substantial change in mitochondrial function was noted (Moreira et al. 2004). This is contradictory to the results reported by (Raza et al. 2011), which showed mitochondrial dysfunction in different tissues of these diabetic rats including the brain. In mononuclear cells of type 2 diabetic patients, CL content was reportedly reduced that were associated with smaller and more spherical mitochondria, higher mitochondrial trans-membrane potential as well as higher superoxide production in a patient subgroup (Widlansky et al. 2010). These are the characteristic triggers of the inflammatory activation process of monocytes and T-cells in type 2 diabetes that have previously been observed (reviewed

in(Widlansky et al. 2010). As monocyte activation and inflammation are believed to be an important cause of insulin resistance (King. 2008), this study provided correlative evidence suggesting that impaired mitochondrial function by alterations in CL content could play a central role in the pathogenesis of type 2 diabetes.

2. OBJECTIVES OF THE STUDY

1. To lower CL content in the human brain capillary endothelial cells by RNAi-mediated knockdown of the CL biosynthetic enzyme cardiolipin synthase (CLS).
2. To examine the effect of CLS knockdown on cellular mitochondrial function.
3. To study the short-term effect of reduced CL content on the barrier and trans-cellular transport properties of the brain capillary endothelial cell monolayer, an in vitro model for the human BBB.

3. MATERIALS AND METHODS

3.1 Materials

Primary human brain microvessel endothelial cells (HBMECs) derived from normal human brain cortical tissues and the cell culture medium (CSC complete medium supplemented with CultureBoost and Bac-Off®) and passaging reagents were obtained from Cell Systems Corporation (Kirkland, WA, USA). The human adult brain endothelial cell line hCMEC/D3 was generated by (Weksler et al. 2005) and was kindly provided to us by Dr. P-O Couraud from Institut Cochin, France. Endothelial Basal Medium -2 (EBM-2) was purchased from Lonza, fetal bovine serum (FBS) and other medium supplements and cell culturing reagents were obtained from Life Technologies Inc. (Burlington, ON, Canada) and Sigma-Aldrich (St. Louis, MO, USA). Opti-MEM reduced serum medium, Lipofectamine® RNAiMAX Transfection Reagent and the Silencer® Select Pre-designed siRNA for hCLS1 were obtained from Life Technologies Inc. RNeasy® Plus Mini Kit used for RNA extraction was obtained from Qiagen (Cambridge, MA, USA). Primers and reagents used for qPCR were obtained Invitrogen™. [14C]oleic acid and [3H]-2-deoxy-glucose were obtained from PerkinElmer (Boston, Massachusetts, USA.) and [14C]-Glycerol-3-phosphate was

purchased from American Radiolabelled Chemicals, Inc. (St. Louis, Missouri, USA). Phosphatidyl-[14C]-glycerol was synthesized from [14C]glycerol-3-phosphate according to previously described protocol (Hatch and McClarty. 1996). Ecolite scintillant was obtained from ICN Biochemicals (Montreal, QC, Canada). Transwell® polycarbonate/polyester membrane inserts (0.4 μm; 24 mm diameter) were obtained from Corning Life Sciences (Lowell, MA, USA). Fluorescein dextran was purchased from Life Technologies Inc. Reagents used for mitochondrial stress test and glycolysis stress test experiments were obtained from either Seahorse Bioscience (North Billerica, MA, USA) or from Sigma-Aldrich. All other biochemical agents and drugs were ASC grade and were obtained from either Sigma-Aldrich or Fisher Scientific.

3.2 Culturing of HBMEC and hCMEC/D3 cells

HBMEC were received in a cryopreserved vial at passage 3 and were cultured according to the supplier's instructions (Cell Systems). In brief, cells were quickly seeded in a T-75 flask coated with Attachment Factor™, an extracellular matrix layer that promotes the cells to attach to the culture surface and also encourages them to establish the

correct polarity. The cells were maintained in CSC-complete medium, a medium that is supplemented with 10% CultureBoost (10% bovine serum albumin supplemented with 50 μg/ml of bovine growth factor and porcine heparin) and ciprofloxacin, until 80-90% confluence. Then, they were further passaged into additional T-75 flasks with a 1:4 splitting ratio. Spent medium was replaced every 2 days until the cells reach 60-70% confluence and was replaced everyday thereafter. Cells were maintained at 37°C in a 5% CO2 incubator. HBMEC were used for experiments at passages 5 and lower to ensure no loss in BBB properties.

The HCMEC/D3 cell line was established by (Weksler et al. 2005) via lentiviral transfection of the catalytic unit of telomerase (hTERT) and SV 40 T-antigen into human brain microvascular endothelial cells isolated from the temporal lobe of a female donor. Cells were received in a cryopreserved vial at passage 27 and were cultured according to the author's instructions (Weksler et al. 2005). In brief, cells were plated into one T-75 flask having a thin layer of collagen type I coating. They were cultured at 37°C in a 5% CO2 incubator and were maintained in EBM-2 media supplemented with 5% fetal bovine serum,

1% chemically defined lipid concentrate, 1ng/ml of human Basic Fibroblast Growth Factor (bFGF),1.4uM hydrocortisone, 10mM HEPES, 5ug/ml Acid ascorbic and 1% antibiotic-antimycotic (penicillin, streptomycin and amphotericin-B). Upon reaching confluence, cells were further passaged into additional collagen I coated flasks or petri dishes at a density of 18000-25 000 cells per cm sq depending on the experiment. Cells typically reach confluence after 2-3 days of passaging and were used for experiment at passages 35 and lower to ensure no loss of BBB properties (Vu et al. 2009).

3.3 siRNA transfection studies

Transfection of HBMECs and HCMEC/D3 cells were performed using siRNA against the hCLS1 gene and lipofectamine RNAiMAX transfection reagent, partly, according to the protocol described for human umbilical vein endothelial cells (HUVEC). The siRNA target sequence for hCLS1 was 5'-GGACAAUCCCGAAUAUGUUtt-3'. Silencer® Select Negative Control No.2 siRNA (Life Technologies), which has been tested using microarray analysis to have minimal effect on gene expression profile, was used in the mock transfection as a control. In brief, HBMECs were seeded at a density of 50 000 cells/cm2 onto 6-

wells Transwell® inserts or at 30 000 cells/cm2 onto 100mmm cell culture dishes. HCMEC/D3 cells were seeded at a density of 45000 cells/cm2 onto 6 wells Transwell® inserts and 15000 cells/cm2 onto 100mm dishes and 6 wells culture plate. The cells were left to attach either overnight (HBMECs) or for 4-6hrs (HCMEC/D3), after which the culture medium was replaced by OptiMEM I reduced serum media. 20nM and 30nM of siRNA-lipofectamine complexes were added drop-wise, respectively to the HBMEC and hCMEC/D3 cells. The siRNA lipofectamine complexes were formed by mixing siRNA and RNAiMAX transfection reagents with OptiMEM in separate tubes and the two mixtures were combined and incubated at room temperature for 5min (as recommended by the new optimized protocol for RNAiMAX transfection reagent). Cells were incubated with these complexes overnight and the medium was changed with fresh culture medium the next day.

3.4 Real time PCR analysis

HBMECs and hCMEC/D3 cells were grown and transfected in 100mm cell culture dishes. After 48- 72hrs of transfection, cells were harvested and total RNA was obtained using the RNeasy® Plus Mini kit

and Qiashredder homogenizer columns (Qiagen). The integrity of total RNA was confirmed by running the RNA sample on a denaturing agarose gel. Gene expression analysis was measured using the Mastercycler ep *realplex* system (Eppendorf). Primers were designed using NCBI/Primer-Blast and were synthesized by Invitrogen (Ontario, Canada). Quantitative PCR analysis of human CLS, Creatine transporter (SLC6A8), P-glycoprotein (ABCB1), Breast cancer resistant protein (ABCG2), multidrug resistant proteins (ABCC1, ABCC3) and glucose transporter (GLUT1) were carried out with either on step qPCR using the Quantitect Probe RT PCR SYBR Green kit (Qiagen) or two steps qPCR using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). The cDNA used for the two steps qPCR was synthesized from 2ug of RNA with SuperScript® II Reverse Transcriptase (Invitrogen) according to the manufacturer's instruction. PCR amplification of the reference gene 18S RNA was performed for each sample to control for equal sample loading and also to allow normalization among samples. For one step PCR, the samples were heated for 15mins at 50°C for reverse transcription, which was followed by activation of HotStarTaq DNA Polymerase at 95°C for 15 min and then 40 cycles of 15 sec denaturation at 95C, 30sec annealing at 60-62C (depending on the primer's optimal annealing temperature)

and extension for 30 sec at 72°C. All primers were tested for optimal annealing temperature and $MgCl_2$ concentration. To ensure that the correct DNA segment was amplified in each reaction, the PCR product was either run on an agarose gel to make sure only one single band is seen at the predicted MW or melting curve was analyzed to have one peak melting temperature. For the PCR of CLS and Creatine transporter, standard curves were run to ensure that the PCR amplification efficiency of the target genes and the reference gene were similar. The PCR results were analyzed using the comparative Ct method. The relative gene expression, ΔΔCt values, was determined by taking the difference between ΔCt sample and ΔCt Control. The 18S-RNA reference gene was normalized to 1 and each target gene was normalized and represented as the amount of gene expression remaining. All primer sequences and optimal PCR conditions can be found in Table 1.

Table 1: Primers used for real-time PCR analysis

Primer	Forward 5′-3′	Reverse 5′-3′	Annealing Temp
18S	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA	60-65°C
hCLS1	AATGACGAGAATTGGCTTGG	TCTTTGATTGGCCCAGTTTC	60°C
ABCC1 (MRP1)	ACGCCCTTTCTGGTGGCCTT	TTGACAGGCCGTCGCTCGAT	62°
ABCC3 (MRP1)	CGCGCCTTCCAGGTAAAGCAAA	TGTGCCAAGCCTCACCAGGA	60°C
P-gp	TGCTCAGACAGGATGTGAGTTG	AATTACAGCAAGCCTGGAACC	62-63°C
ABCG2 (BCRP)	GGCCTTGGGATACTTTGAATC	GAATCTCCATTAATGATGTCCA	60°C
GLUT1	GGGGCATGATTGGCTCCTTCTCTGTG	AGGCCGCAGTACACACCGATGATGA	62°C
FATP1	ACTCGGCAGGAAACATCATC	TCTCCCCGATGTACTGAACC	60°C
CRT1	AGTCCTTTACCACCACGCTG	GACAAAGGGTCACCTCCCAG	62°C
MRP4v1	CCATCTGTGCCATGTTTGTC	ACTGAAACATCCCCATGAGC	60°C
MRP4v2	CCATCTGTGCCATGTTTGTC	ACTGAAACATCCCCATGAGC	60°C

3.5 Isolation of mitochondrial fraction and CLS enzyme activity assay

HBMECs and HCEMC/D3 cells were transfected with hCLS1 siRNA for 48h. Cells were washed with PBS and were scraped and transferred into test tubes. Mitochondrial fractions were obtained using the Mitochondrial Isolation Kit For Profiling Cultured Cells (Sigma-Aldrich) according to the manufacturer's protocol. All isolation procedures were performed at 4°C. Briefly, cells were washed by re-suspension in ice cold PBS, centrifuged at 600xg for 5 minutes and the PBS was removed. Cells were then incubated in 1 ml of 1x Extraction Buffer A for 15 minutes. This was followed by homogenization by 50-60 strokes of a tight fitting Dounce A homogenizer to damage at least 50% of the cells. Cellular damage was checked by staining an aliquot with trypan blue and observed under a phase contrast microscope. The homogenate was then centrifuged at 600xg for 10 min to remove cellular debris. The supernatant was then transferred to a new tube and centrifuged at 11000x g for 10 minutes. The resulting pellet was the mitochondrial fraction and was re-suspended in either 1x storage buffer for CLS enzyme activity assay or in CelLytic M Cell lysis reagent for downstream western blot. CLS activity assay was performed as previously described (Hatch and McClarty. 1996).

3.6 Western blot analysis

Mitochondrial protein concentration was determined by the detergent compatible Bradford protein assay (Biorad). Samples were then solubilized in a 5:1 ratio with 6X SDS sample buffer and boiled at 100°C for 5 minutes.

Equal amounts of mitochondrial proteins (40ug/lane) were loaded and separated by electrophoresis on a 12% SDS-PAGE gel. The proteins were then transferred onto a PVDF transfer membrane (Immobilon, Millipore, Bedford, MA). The presence of transferred proteins on the membrane was confirmed by staining with Ponceau S (Sigma). Membranes were blocked for 2 hours at room temperature with 5% non-fat milk in 0.1% tween-20/TBS (TBS-T). Then, membranes were incubated overnight at 4°C in blocking buffers with rabbit primary antibodies against CLS (1:150dilution). Expression of Hsp60, a mitochondrial matrix protein, was used as the loading control. After several washes with TBS-T, membrane was incubated with HRP-linked monkey anti-rabbit antibody (1:5000, GE Healthcare Life Sciences) at room temperature for 1 hour. Protein bands in the membranes were then visualized by enhanced chemiluminescence. The relative intensities of the bands were analyzed by Image J software.

3.7 Glucose uptake assay

Glucose uptake assay using 2-deoxy-D-(3H)-glucose was performed according to the protocol published by (Yamamoto et al. 2011). 2-deoxy-D-glucose (2DG) is a non-metabolizable analogue of D-glucose. When taken inside the cells, 2-DG can be phosphorylated by hexokinase to be trapped inside the cells and cannot be further metabolized. Thus, the glucose uptake can be measured by lysing the cells open and measure the radioactivity that was incorporated. Briefly, cells were plated and transfected for 48h in a 6-well culture plate. They were then maintained in FBS-free medium for 4h prior to assay to eliminate the potential influence of growth factors in FBS on cellular glucose uptake. Each cell well was subsequently washed with Krebs-Ringer HEPES (KRH) buffer (50mM HEPES, 136mM NaCl, 4.7mM KCl, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, 0.1% BSA) and then incubated in 1ml of hot KRH containing 0.5, 1, 2, or 5 mM of cold 2-DG and 1uci/ml of 2-deoxy-D-(3H)-glucose for 10min at 37°C. To determine non-GLUT1 specific glucose uptake, some wells were pre-treated for 30min with 20uM of Cytochalasin-B, a glucose transporter inhibitor, prior to addition of the test solution. The assay was terminated by removal of

the hot solution and the cells were washed for four times with ice-cold KRH buffer. Cells were then lysed in 800ul of 0.05M NaOH at 37°C for 2hr. Then, 700ul was transferred to a scintillation vial containing 5ml of scinctillation fluid to determine the radioactivity incorporated into the cells. The remaining lysate was used for protein quantification. A mixture of 800ul of 0.05M NaOH in 5ml scintillation fluid was used as the blank. The non-specific uptake and blank were subtracted from all the glucose uptake measurements. Specific radioactivity was determined by dividing the total radioactivity incorporated into the cells by the number of moles of 2-deoxy-D- glucose applied to each sample.

3.8 Permeability and Transport studies

The monolayer used for permeability and transport studies was established by plating cells (50 000-70 000 cells/cm^2) onto either Attchment factor™ coated (HBMECs) or collagen type I coated (HCMEC/D3) Transwell® polycarbonate membrane permeable supports with a 0.4 uM pore size. After being attached, cells were subsequently transfected and permeability/transport studies were performed after the monolayer become confluent (typically 3 days after transfection).

To control for confluence after transfection, cells were grown and transfected in parallel on Polyester Transwell-Clear inserts, which allowed the visibility of the cell monolayer under a phase contrast microscope.

Permeability studies were performed by addition of assay buffer #2 (122 mM sodium chloride, 2.9 mM potassium chloride, 1.9 mM calcium chloride, 2.5 mM magnesium sulfate, 25 mM sodium bicarbonate, 10 mM HEPES, 10 mM glucose and 0.4 mM dipotassium phosphate, pH 7.4) that contained 0.1mM BSA and 1 uM fluorescein isothiocyanate-labeled dextran (FDX), a paracellular tracer, to the apical side of the confluent monolayer. The basolateral chamber consisted of 0.1mM BSA in assay buffer #2 to maintain equal oncotic pressure on both sides. At 0, 2, 5, 10, 20 and 30 min, a 20 ul aliquot was taken from the basolateral chamber, diluted in 200ul of assay buffer and measured for fluorescence intensity. Flourescence was detected at Ex (λ) 485 nm and Em (λ) 528 nm using a Biotek Synergy HT plate reader.

Glucose and Oleate transport studies were carried out by incubating the monolayer apically with 3 mM 2-deoxy-D-glucose with a tracing dose of 2uci/dish of 2-deoxy-D-(3H)-glucose in KRH buffer and 0.1mM Oleate bound to 0.1mM BSA with 2uci/dish of [1-14C]oleate in assay

buffer #2, respectively. The basolateral medium consisted of either KRH buffer (glucose transport studies) or 0.1 mM BSA in assay buffer #2 (Oleate transport studies). 50 ul of the basolateral medium was collected and measured temporally for up to 60min to determine the radioactivity incorporated into the basolateral chamber.

3.9 Measurement of mitochondrial and glycolysis function using Seahorse XF24 Extracellular Flux analyzer

After 2 days of transfection in 100mm culture dish, hCMEC/D3 cells were passed onto an XF24 cell culture microplate pre-coated with type I collagen. Cells were left to attach for 4h at 37°C in a 5% CO_2 incubator, after which the medium was replaced with XF assay medium supplemented with 1mM pyruvate, 25mM glucose for mitochondrial stress test or with glycolysis stress test assay medium (143mM NaCl, 2mM L-glutamine in Bicarbonate-free, low phosphate DMEM) for glycolysis stress test. Cells were then maintained at 37°C in a CO2 free incubator for 1h prior to be assayed.

A mitochondrial stress test was performed to assess cellular mitochondrial function. The test was done by first measuring the

baseline oxygen consumption rate (OCR). This was then followed by sequential OCR measurements after injection of oligomycin (1uM), FCCP (0.75uM) and Rotenone + Antimycin A (1uM) to obtain the key parameters of mitochondrial function that includes basal respiration, coupling efficiency, spare respiratory capacity and proton leak.

Glycolytic function was assessed by performing a glycolysis stress test. The XF24 analyzer measures glycolysis based on the fact when glucose undergoes glycolysis, it results in the extrusion of metabolic products such as lactate, CO_2 and protons that can acidify the medium surrounding the cells. This extracellular acidification can be measured by the XF24 analyzer and is reported as extracellular acidification rate (ECAR). ECAR measurement is now the gold standard in measuring glycolytic function in cells. The test was performed by first measuring ECAR in the absence of glucose to assess the non-glycolytic acidification value. This was then followed by sequential injection of glucose (10 mM final conc.), oligomycin (2.5 uM final conc.) and 2-deoxy-D-glucose (100 mM final conc.) to allow ECAR measurements associated with key glycolysis parameters such as glycolysis, glycolytic capacity and glycolytic reserve.

3.10 Statistics

All data were expressed as mean + S.D. The differences between the experimental and the control groups were evaluated by Student's t-test. All values with $p < 0.05$ were considered statistically significant. All statistical tests were performed using GraphPad Prism version 6.00 for Mac OS

4. RESULTS

Previously, our laboratory demonstrated that long chain fatty acid transport across the human brain microvessel endothelial cell (HBMEC) monolayer, an in vitro model for the human blood brain barrier (BBB), is a trans-cellular process that is mediated, at least in part, by fatty acid transport protein-1, and fatty acid translocase/CD36 (Mitchell et al. 2009; 2011). However, it is not known what factor may regulate such transport processes across the BBB. As BMECs appear to have a higher metabolic activity than endothelial cells in other vascular beds, we hypothesize that optimal mitochondrial function may be required to power their barrier properties and that an appropriate content of CL, a mitochondrial phospholipid known to be important for both the activity and integrity of the respiratory chain, may be important in regulating these processes. To test this role of CL, we manipulated cardiolipin synthase (CLS) activity, the enzyme that catalyzes the last CL synthesis step, by transfecting both HBMECs and hCMEC/D3 cells with CLS siRNA for 48h. Mock transfected cells or cells that were transfected with a scrambled siRNA sequence (siCTL) were used as the controls. Expression of CLS was then determined by real-time PCR and the 18S rRNA was used as the reference gene for the relative

quantification. It was observed that CLS mRNA expression was significantly reduced in the HBMEC by 75% ($P<0.05$) compared to mock transfected cells **(Fig1.1 A)** and in hCMEC/D3 by more than 90% ($p<0.05$) compared to both mock and siCTL transfected cells **(Fig1.1B).** Enzyme activity of CLS was determined after the transfection by measuring the rate of 14C-PG incorporation into CL. It was observed that consistent with the mRNA expression profile, CLS enzyme activity was reduced by ~40% in the HBMEC **(Fig1.2A)** and by ~50% ($p<0.05$) in the hCMEC/D3 cells **(Fig 1.2B)**. Western blot analysis also confirmed this knockdown both at 48h and 70h post-transfection in the hCMEC/D3 cells (**Fig 1.3A-C).** CLS protein expression was reduced by approximately 43% at 48h and this reduction was relatively maintained (~35% reduction) up to 70h post-transfection.

As HBMECs quickly lose their BBB characteristics, especially their transporters expression despite limited passaging; the major part of the remaining experiments was done using the hCMEC/D3 cell line. To examine the bioenergetics changes associated with CLS knockdown, we measured oxygen consumption rate (OCR) by performing a

mitochondrial stress test using the seahorse XF-24 analyzer. As shown in **Figure 2.1 (A**-**B),** the CLS knockdown (CLS-KD) hCMEC/D3 cells appeared to have a moderate decrease in basal respiration compared to mock-transfected cells, although this change was not statistically significant due to the large variations among the different cell preparations and the low number of replicates. As the major part of basal respiration is determined by ATP turnover, its apparent reduction suggests that the CLS-KD cells may have a lower basal ATP demand. This is indeed supported by the fact the coupling rate (determined by the difference between basal respiration and the oligomycin sensitive rate, where oligomycin is an inhibitor of ATP synthase), even though also not statistically different, appeared to be lower in the knockdown cells, which may indicate a lower ATP output from mitochondrial respiration. This ATP output reduction, however, was not the result of reduced mitochondrial efficiency as the coupling efficiency (determined by the ratio between oligomycin sensitive rate and basal rate) did not change with the transfection (**Fig 2.1B**). FCCP is a lipid soluble molecule that is capable of transporting H+ from the inter-membrane space back into the matrix thereby uncoupling electron transport from ATP synthesis. Thus, injection of an optimal concentration of FCCP allowed us to measure the maximal respiratory capacity of the cells.

Interestingly, the spare respiratory capacity, which is the difference between maximal respiration and basal respiration and is a measure of cellular ability to increase their energy production under conditions of increased demand, was significantly reduced in the CLS-KD cells. Together, the presented OCR data suggest that the CLS knockdown might have affected the cell's bioenergetics by altering their basal ATP demand as well as significantly reduced their ability to increase mitochondrial function under conditions of increased demand such as stress.

As the siRNA treatment may have a non-specific effect on the cell's bioenergetics, the mitochondrial stress test experiment was repeated using cells that have been transfected with a scrambled siRNA sequence (siCTL) as the control to ensure the bioenergetics changes observed above was indeed due to CLS knockdown. As shown in **Figure 2.1 (C-D)**, even though not statistically significant due to the inherent noise of the experiment and the low number of replicates, the important mitochondrial parameters measured in the CLS siRNA transfected compared to siCTL transfected cells appeared to have similar trend to the data presented in **Figure 2.1 (A-B)**. Although requiring more experimental repeats, this set of data does essentially support the mitochondrial changes caused by CLS knockdown.

As most cells possess the ability to shift dynamically between glycolysis and oxidative phosphorylation, we next measured the extracellular acidification rate (ECAR) in the hCMEC/D3 cells first in the absence of glucose and then after subsequent injection of glycolytic substrates/inhibitors to determine whether the reduction in mitochondrial function observed above will cause the cell to switch to depend more on glycolysis. As shown in **Figure 2.2 (A-B)**, the non-glycolytic acidification rate measured in the absence of glucose did not differ between the CLS-KD and siCTL cells. However, there was a moderate increase in the glycolytic flux in the CLS-KD cells suggesting an increase in preference of these cells for glycolysis under basal condition. Interestingly, when stimulated with an optimal concentration of oligomycin, an inhibitor of ATP synthesis that can shift most of the energy production of the cells to glycolysis, the CLS-KD cells showed increase in glycolytic capacity that was accompanied by a moderate elevation in their glycolytic reserve capacity, a parameter that represents the ability of the cells to increase their glycolytic capacity under conditions of increased demand. Essentially, these data suggest the preference of the CLS-KD cells for glycolysis under both basal and stressful conditions. Perhaps, the increase in the glycolytic flux of these cells at baseline could partially explain for the moderate

decrease in basal oxygen consumption observed in the mitochondrial stress test and suggest that the CLS-KD hCMEC/D3 cells might have been generating more ATP from glycolysis to support their basal ATP requirement. Furthermore, the increase in glycolytic capacity and glycolytic reserve in the knockdown cells might have also served as a compensation for their inability to increase their mitochondrial respiration to cope with an increased ATP demand condition as observed above. In essence, despite the fact these glycolytic results could suitably explain the changes observed in the mitochondrial stress test experiments discussed earlier, this glycolytic stress test has only been repeated twice. Thus, more repeats should be done to further confirm the presented findings.

To assess the relative contribution of glycolysis and oxidative phosphorylation (OX-PHOS) to the basal ATP production in both CLS-KD and siCTL cells, the oligomycin sensitive OCR and the glycolytic proton production rate (PPR) (both measured under a saturating extracellular glucose concentration) were converted to ATP production rate. The proton production rate (PPR) expressed as pmol H^+/ min was automatically converted by the XF-24 analyzer from ECAR (mpH/min)

using the buffer capacity of the media and the chamber volume. The oligomycin sensitive OCR was converted to ATP production using a P/O ratio of 2.3 (Brand. 2005); while the glycolytic PPR was converted to ATP production using a 1:1 ratio based on the fact in glycolysis, one ATP is made per lactate/proton produced. Remarkably, as shown in **Figure 3,** the control hCMEC/D3 cells appear to make ~60% of their ATP via OX-PHOS under basal condition. This, indeed, supports our hypothesis that unlike endothelial cells in the non-BBB regions, the brain capillary endothelial cells may be more dependent on OX-PHOS for their energy need under basal condition. Furthermore, as the glycolytic contribution in the CLS-KD cells appears more prominent than that in the siCTL cells, this also suggests that CLS-KD may have acted as a metabolic switch making these cells more dependent on glycolysis. However, more repeats of OCR/ECAR measurements should be performed to confirm this set of results. In addition, to further confirm the relative contribution of OXPHOS and glycolysis to cellular ATP demand, a more direct method of measuring ATP production such as colorimetric/fluorometric ATP assays should also be done.

As the CLS-KD hCMEC/D3 cells appear to have a lower basal ATP demand from mitochondrial respiration, we next examined whether this reduction in basal energy requirement may be due to changes in the expression of transporters that either are directly involved in nutrient supply to these cells or would require ATP to function. Thus, hCMEC/D3 cells were transfected with either CLS siRNA or siCTL for 48h. mRNA expression of GLUT1, creatine transporter (CRT1), P-glycoprotein (Pgp), breast cancer resistant protein (BCRP) and the ABCC transporter 1 and 3 (aka. MRP1 and 3) were examined by real-time PCR. 18S rRNA was used as the reference gene for the relative quantification. Of the genes analyzed, we found a significant reduction in the expression of CRT1 (~40%), Pgp (~25%) and BCRP (~10%), although the reduction of the two latter genes appeared negligible. As an extension of the experiment, the expression of GLUT1, FATP1 (the fatty acid transporter that was previously shown to play a role in long chain fatty acid transport into the brain (Mitchell et al. 2009)) and the two variants of the multi-drug resistant protein 4 (aka. MRP4 or ABCC4) were also examined in the CLS siRNA transfected and mock transfected HBMEC. Consistent with the trend observed in the transfected hCMEC/D3 cells, the expression of these transporters in HBMEC also did not change with CLS knockdown.

To study the effect of CLS knockdown on the integrity of the endothelial cell monolayer, HBMEC and hCMEC/D3 cells were grown as a monolayer on Transwell® polycarbonate membrane inserts and were either mock transfected or transfected with CLS siRNA or siCTL for 3 days. mRNA expression of CLS was then quantified using real-time PCR to ensure sufficient knockdown when the cells were transfected on the microporous Transwell® plates. Effectively, HBMEC (**Figure 5.1A**) and hCMEC/D3 (**Figure 5.1B**) cells, respectively, showed ~55% and ~60% reduction in the expression of CLS after 48h of transfection. The knockdown was more effective in hCMEC/D3 cells after 3 days of transfection which was ~85% reduction. The monolayers were then incubated apically with 1uM of the paracellular tracer fluorescein isothiacynate dextran (FDX) in assay buffer #2 that contained 0.1mM bovine serum albumin (BSA). The basolateral chamber also contained 0.1mM BSA in assay buffer #2 to maintain equal oncotic pressure on both sides. The FDX flux was monitored for up to 30min and it was observed that the transfection did not affect the FDX flux across both the HBMEC **(Fig 5.2A)** and hCMEC/D3 **(Fig 5.2B)** monolayers, which indicated no effect of the knockdown on the monolayer integrity.

Even though no change in GLUT1 mRNA expression was observed in the CLS-KD hCMEC/D3 cells, with the apparent reduced mitochondrial function and the preference for glycolysis, it is hypothesized that these cells may have to take in more glucose to compensate for their lower ATP synthesis efficiency. Thus, we examined the effect of CLS knockdown on GLUT1 activity in the hCMEC/D3 cells. Cells were plated onto 6-wells plates and transfected with either CLS siRNA or siCTL for 48h. Then, uptake of a tracing dose of 2-deoxy-D-[3H]-glucose ([3H]-2DG) in varying concentrations (0.5, 1, 2 and 5 mM) of cold 2-DG in KRH buffer were monitored over 10min to determine the GLUT1 uptake activity. The short incubation time was chosen to eliminate the contribution of hexokinase/phosphatase system on the glucose uptake so the uptake could better reflect GLUT1 uptake activity. Non-GLUT1 specific glucose uptake was determined by pretreating the cells with 20 uM of Cytochalasin-B, a glucose transporter inhibitor, for 30 min prior to addition of the test solution and these values were subtracted from all final glucose uptake measurements. As shown in **Figure 6**, there was a trend towards an increase in glucose uptake in the CLS-KD compared to siCTL cells observed from 0.5-5 mM of extracellular 2-DG

concentration, with a significant increase of ~25% seen at 1 mM and no difference seen at 5 mM of extracellular 2-DG.

To see whether this increase in GLUT1 uptake activity would correspond to an increase in glucose transport across the cell monolayer, hCMEC/D3 cells were grown and transfected with either CLS siRNA or siCTL for 3 days on Transwell® polycarbonate membrane inserts. The cells were then incubated apically with KRH buffer containing 3 mM of cold 2-DG and a tracing dose of 2 uci/well of [3H]-2DG. A 50ul sample was then taken from the basolateral medium of each well temporally for up to 60 min to determine the radioactivity incorporated from the apical chamber. It was observed that there was a significant increase of ~ 25% in [3H]-2DG transport across the CLS-KD compared to siCTL monolayers as early as 5 min that was maintained up to 20 min and was leveled off after 30 min of incubation **(Figure 7)**.

As patients with metabolic syndrome, a condition that has been closely associated with mitochondrial dysfunction, was shown to have increase in fatty acid uptake into the brain (Karmi et al. 2010), we next

examined whether knocking down CLS, which seemed to have reduced the cell's mitochondrial function will have a similar effect on fatty acid transport across this in vitro model of the BBB. Thus, hCMEC/D3 cells were again grown on Transwell® polycarbonate membrane inserts and transfected with either CLS siRNA or siCTL for 70 hour. As oleic acid was previously shown to cross the endothelial cell monolayer in vitro by a carrier-mediated transport (Mitchell et al. 2009), the effect of CLS knockdown on the transport of oleic acid was evaluated in this experiment. The transfected cells were incubated apically with 0.1 mM oleic acid bound to BSA in a 1:1 molar ratio in assay buffer #2 that contained the usual glucose concentration of 10mM. 14-C oleic acid was then added to this incubating solution in a tracing dose of 2 uci/well. A 50 ul sample was collected from the basolateral medium of each well temporally for up to 30 min to determine the incorporated radioactivity. It was observed that over the 30 min incubation, the transfection did not affect the transport of oleic acid across the hCMEC/D3 monolayer (**Figure 8A**).

Even though glucose is the main energy source of the brain, the brain is also known to use other substrates as an alternative energy source

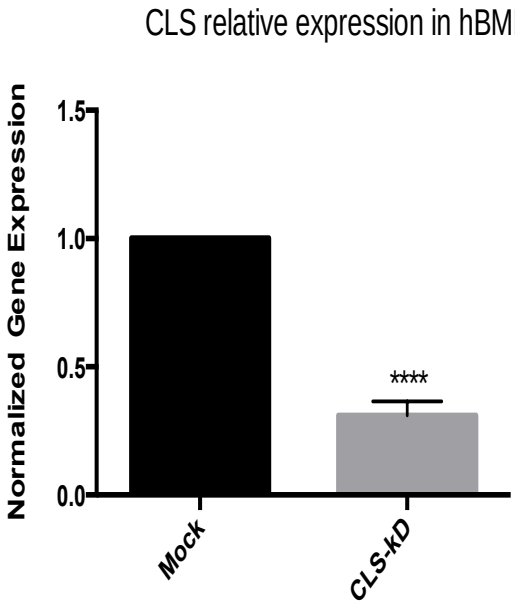
when glucose supply is low. Thus, we decided to lower the glucose concentration in the assay buffer to 1 mM to see whether at such a low glucose concentration the CLS knockdown would enhance the oleic acid transport across the hCMEC/D3 monolayer. However, similar with what observed earlier, the transfection did not affect oleic transport across this in vitro model of the BBB (**Figure 8B**).

Figure 1: RNAi knockdown of CLS reduces its mRNA (1.1), enzyme activity (1.2) and protein expression (1.3)

HBMECs **(A)** and HCMEC/D3 (**B)** cells were transfected with hCLS1 siRNA for 48hr as described in Materials and Methods. Mock-transfected cells with scrambled siRNA sequence (siCTL) or without (mock) were used as the control. (**1.1)** Total RNA was isolated and % of remaining gene expression was determined. Data represent the mean ± SD of three or more independent experiments**. (1.2)** CLS enzyme activity was determined by measuring the rate of incorporation of 14C-PG into CL in both control and knockdown cells. **(A)** Data represent the mean ± SD of two independent experiments. **(B)** Data represent the mean of three independent experiments. **(1.3)** CLS protein expression in hCMEC/D3 cells was analyzed after both 48h and 72h of transfection. Representative blots are shown (**A**) with CLS molecular mass indicated on the left and Hsp-60 loading control shown below. Relative expression of CLS protein after the 48h and 72h of transfection are shown in (B) and (C) respectively. Data represent the mean of two independent experiments. *p<0.05, **p<0.01, ****p<0.0001.

Figure 1.1

A.



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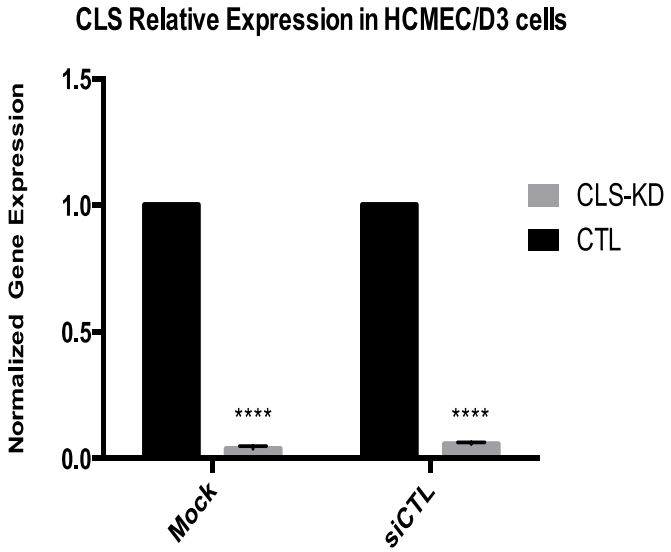
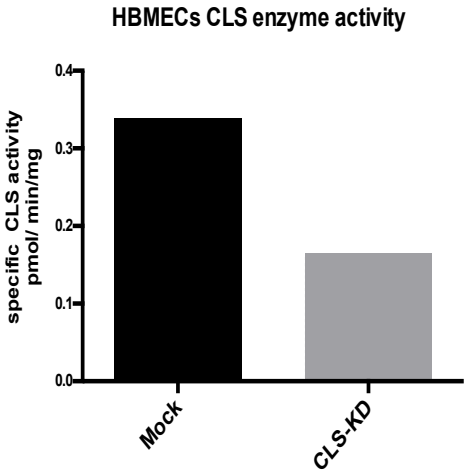


Figure 1.2

A.



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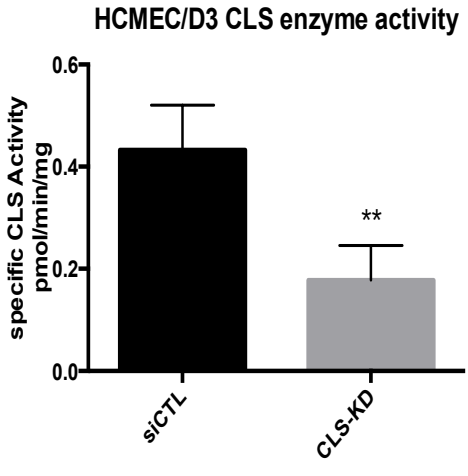


Figure 1.3

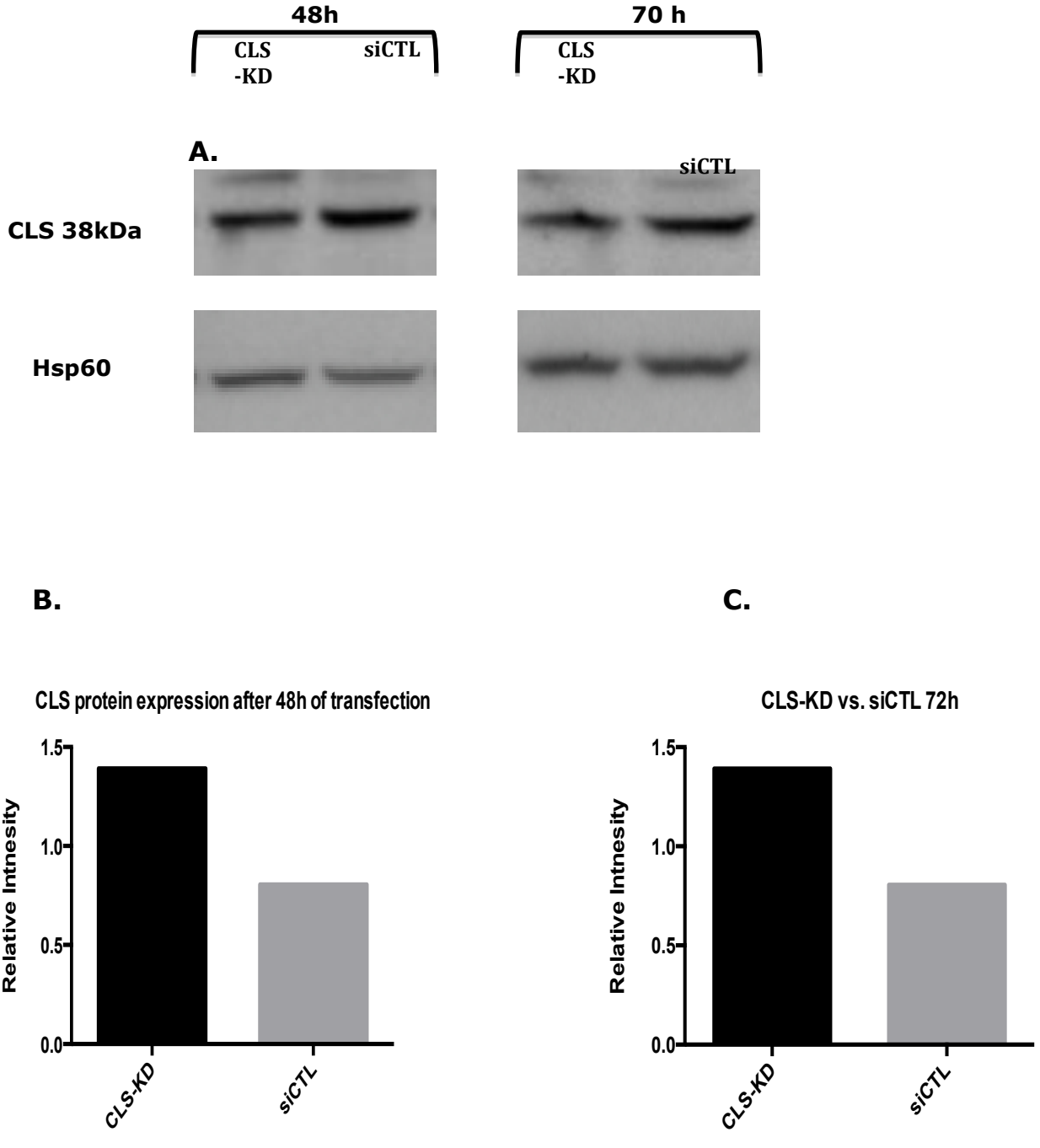

48h
CLS
-KD
siCTL
70 h
CLS
-KD
A.
siCTL
CLS 38kDa
Hsp60
B.
CLS protein expression after 48h of transfection
Relative Intnesity
1.5
1.0
0.5
0.0
CLS-KD
siCTL
C.
CLS-KD vs. siCTL 72h
Relative Intnesity
1.5
1.0
0.5
0.0
CLS-KD
siCTL

Figure 2. Knockdown of CLS reduces the mitochondrial spare respiratory capacity and may act as a metabolic switch making hCMEC/D3 cells more dependent on glycolysis

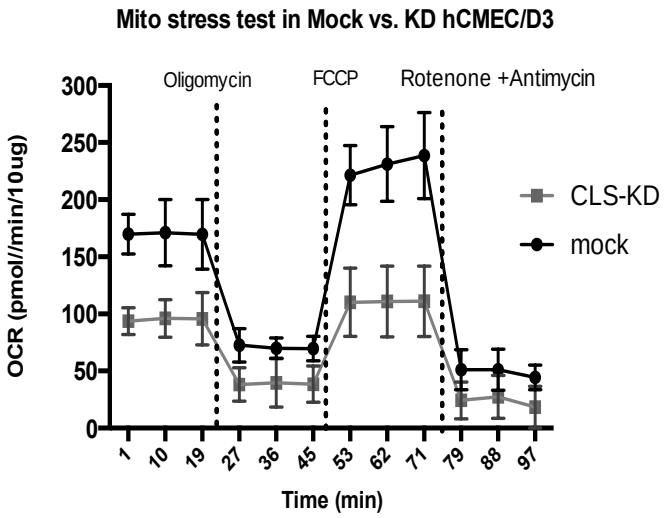
Cells were transfected and the mitochondrial stress test **(2.1)** and glycolysis stress test **(2.2)** were performed as described in Materials and Methods.

(2.1) Oxygen consumption rates (OCR) were measured in real time under baseline conditions and after injection of mitochondrial inhibitors as indicated. **(A)** Mitochondrial stress test in CLS siRNA vs. mock-transfected cells. Graph represents mean ± SD of three independent experiments. **(B)** Relative changes in important mitochondrial parameters in CLS vs. mock transfected cells. **(C)** Mitochondrial stress test in CLS siRNA (CLS-KD) vs. siCTL treated cells. A representative graph of three independent experiments is shown. **(D)** Relative changes in important mitochondrial parameters in CLS-KD vs. siCTL cells. All data represent the mean ± SD of three independent experiments, *$p<0.05$

(2.2) Extracellular acidification rates (ECAR) were measured in the absence of glucose and after subsequent injection of glycolytic substrates and inhibitors as depicted. **(A)** Representative graph of two independent experiments is shown**. (B)** Relative changes in important glycolytic parameters. Data represent the mean of two independent experiments.

Figure 2.1

A.



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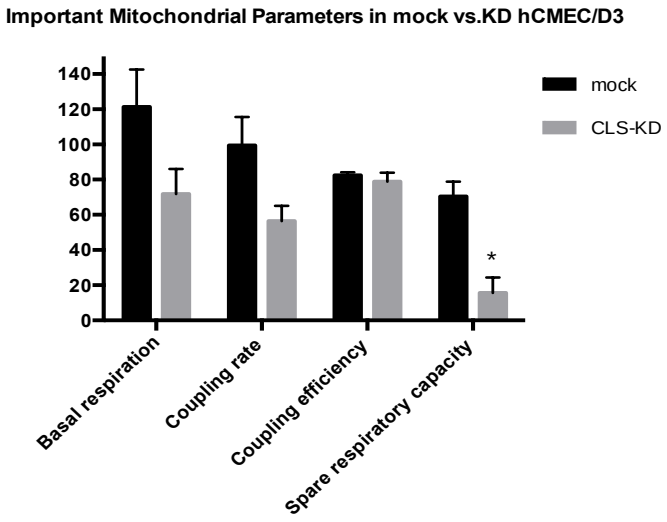
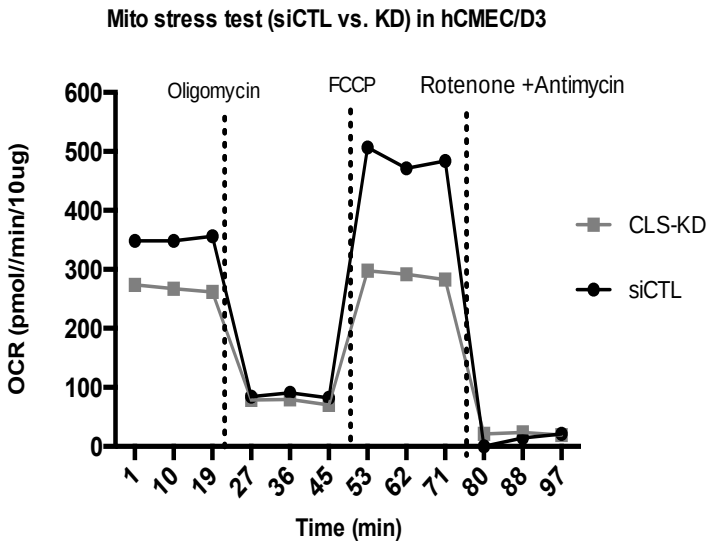


C.



D.

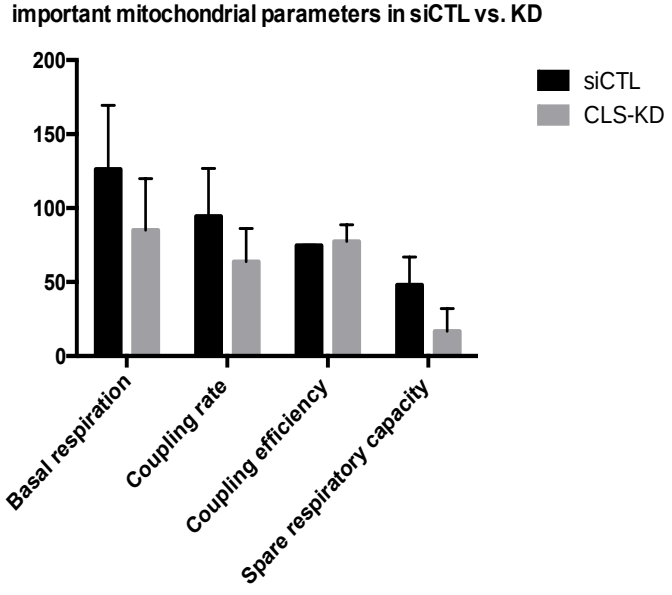
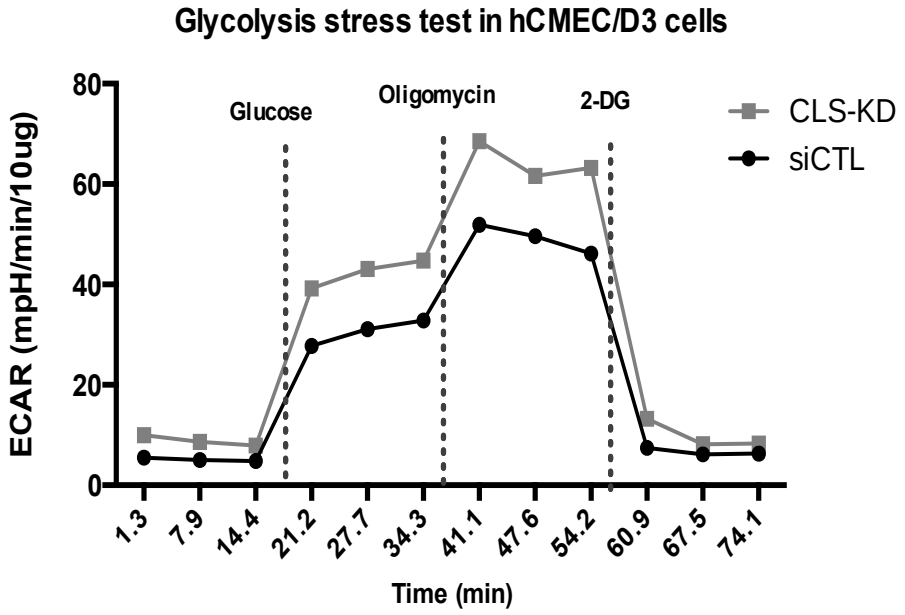


Figure 2.2

A.


Glycolysis stress test in hCMEC/D3 cells
ECAR (mpH/min/10ug)
80
60
40
20
0
Glucose
Oligomycin
2-DG
CLS-KD
siCTL
1.3
7.9
14.4
21.2
27.7
34.3
41.1
47.6
54.2
60.9
67.5
74.1
Time (min)

B.

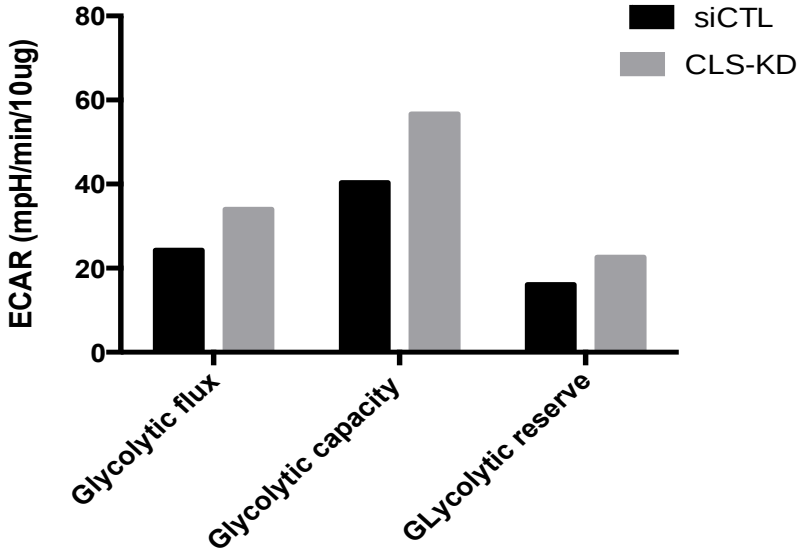

ECAR (mpH/min/10ug)
80
60
40
20
0
siCTL
CLS-KD
Glycolytic flux
Glycolytic capacity
GLycolytic reserve

Figure 3. Relative contribution of glycolysis and OX-PHOS to the basal ATP production rate in hCMEC/D3 cells.

The oligomycin-sensitive OCR was converted to ATP production rate using a P/O ratio of 2.3. The proton production rate (converted from ECAR using the buffer capacity of the assay media) was converted to ATP production rate with a one to one relationship of proton to ATP production. Data represent the mean ±SD of three independent measurements for OX-PHOS and the mean of two independent experiments for glycolysis.

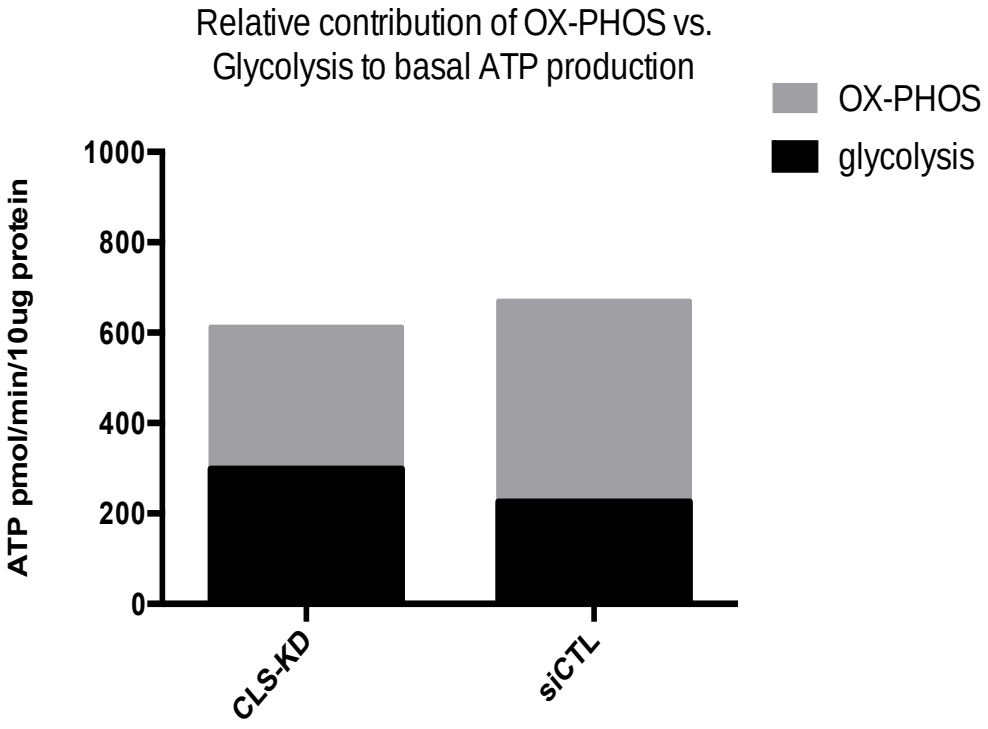
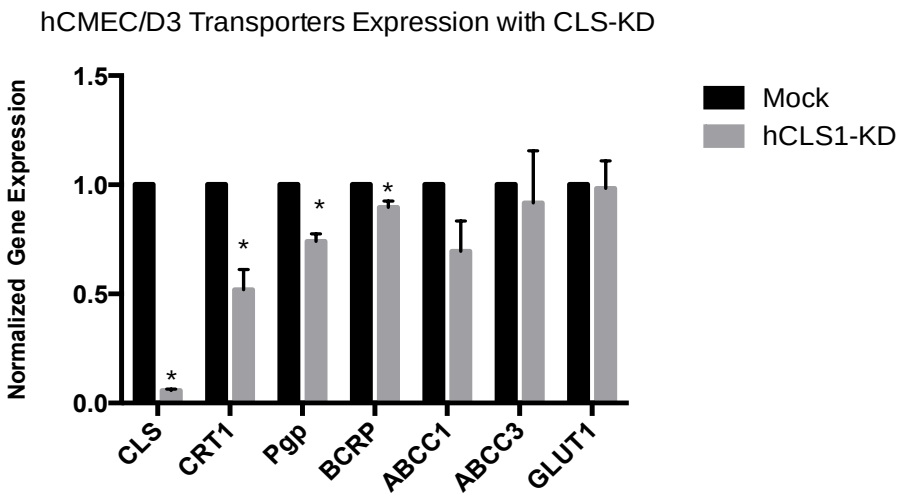


Figure 4. Expression of important BBB transporters with CLS knockdown.

hCMEC/D3 cells **(A)** and hBMEC cells (**B**) were either mock transfected (mock) or transfected with CLS siRNA (CLS-KD) for 48hrs. Total RNA was isolated and mRNA expression of indicated BBB transporters were determined as described in Materials and Methods. Data represent the mean ± SD of three or more independent experiments, *p<0.05.

A.



B.

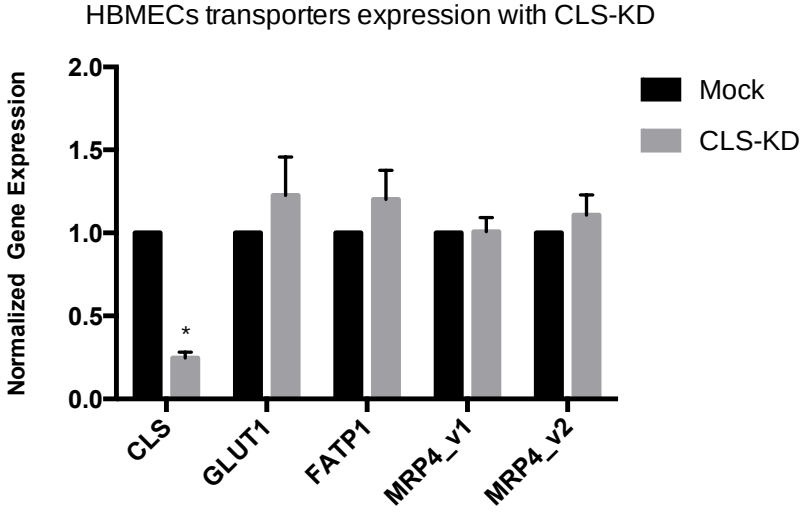


Figure 5. Knockdown of CLS does not alter the permeability across both HBMECs and hCMEC/D3 monolayers.

HBMECs **(A)** and hCMEC/D3 cells **(B)** were plated onto Transwell® polycarbonate membranes insert and were either mock transfected or transfected with CLS siRNA or siCTL. **(4.1)** Total RNA was isolated after 48h and CLS mRNA expression was determined by real-time PCR. **(4.2)** After approaching confluence (typically 70h post-transfection), cells were incubated apically with 1 uM FDX in assay buffer containing 0.1 mM BSA. FDX incorporation into the basolateral medium was determined temporally as described in Materials and Methods. Data represents the mean ±SD of three independent experiments, ****p<0.0001.

Figure 5.1

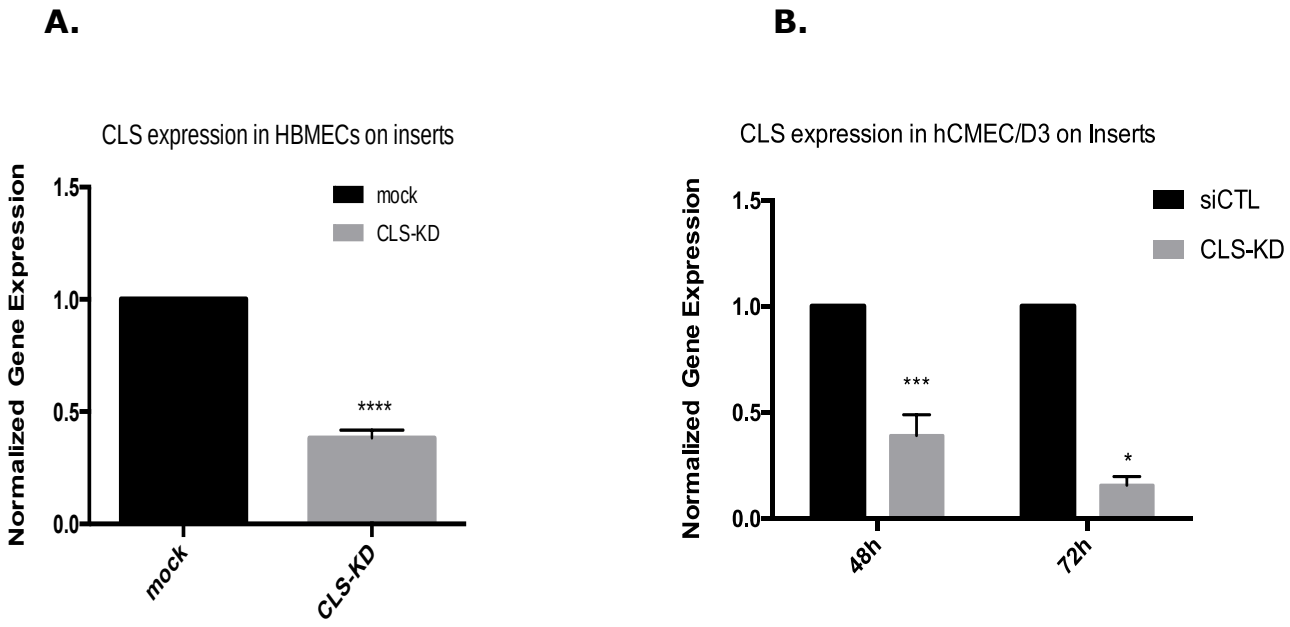
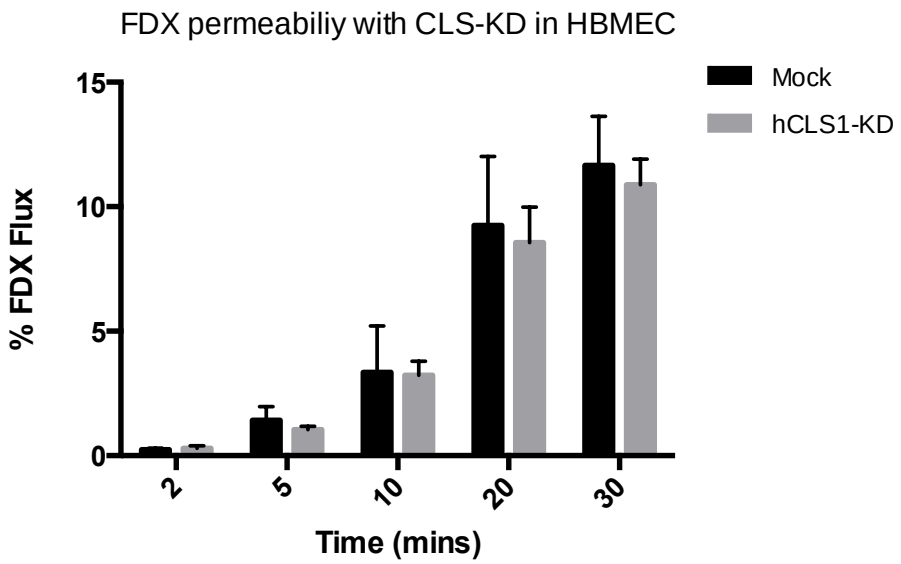


Figure 5.2

A.



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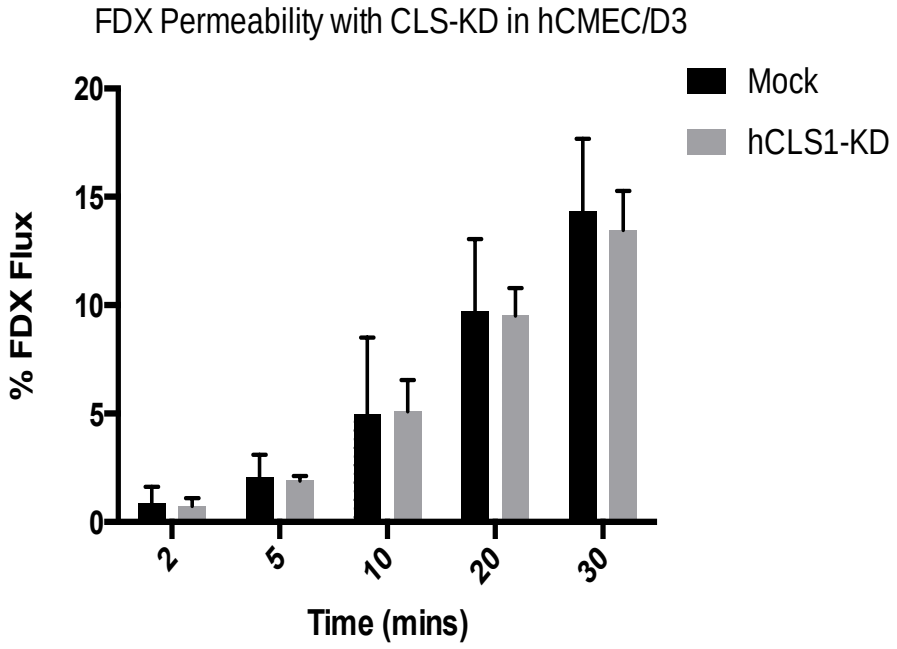


Figure 6.

Knockdown of CLS enhanced GLUT1 uptake activity in hCMEC/D3 cells under hypoglycemic conditions.

hCMEC/D3 cells were plated onto 6 well plates and transfected with CLS siRNA or siCTL for 48hr as described in Materials and Methods. Cells were then incubated in 1ml of KRH buffer containing 0.5, 1, 2, or 5 mM of cold 2-DG and 1uci/ml of 2-deoxy-D-(3H)-glucose for 10min at 37°C. Non-GLUT1 specific glucose uptake was determined by pretreating the cells with 20 uM of Cytochalasin-B for 30 mins prior to addition of the test solution. 2-DG specific uptake was determined as earlier described and the non-GLUT1 specific uptake and the blank were subtracted from all final measurements. Data represents the mean of three independent experiments ±SD, *p<0.05.

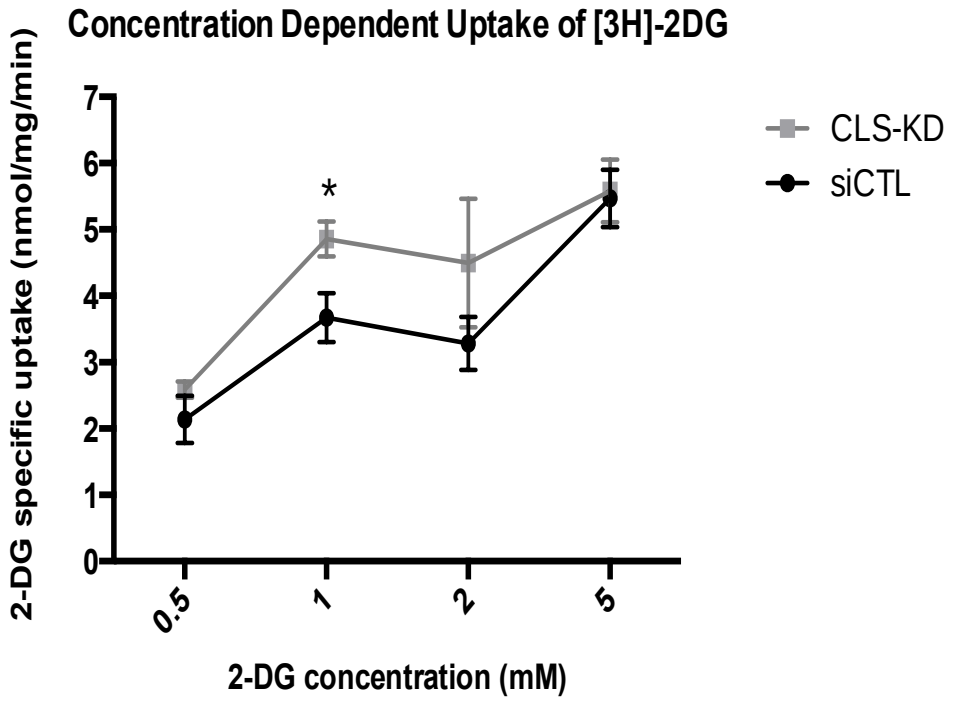


Figure 7. Knockdown of CLS enhanced glucose transport across the hCMEC/D3 monolayer under a hypoglycemic condition

hCMEC/D3 cells were plated onto Transwell® polycarbonate membranes insert and then transfected with hCLS1 siRNA or siCTL for 70h as described in Materials and Methods. Cells were then incubated apically with KRH buffer that contained 3mM of cold 2-DG and a tracing dose of 2uci/well of [3H]-2DG for up to 60 min. Radioactivity incorporated into the basolateral medium was determined as described in Materials and Methods. Data represents the mean ±SD of three independent experiments, *p<0.05.

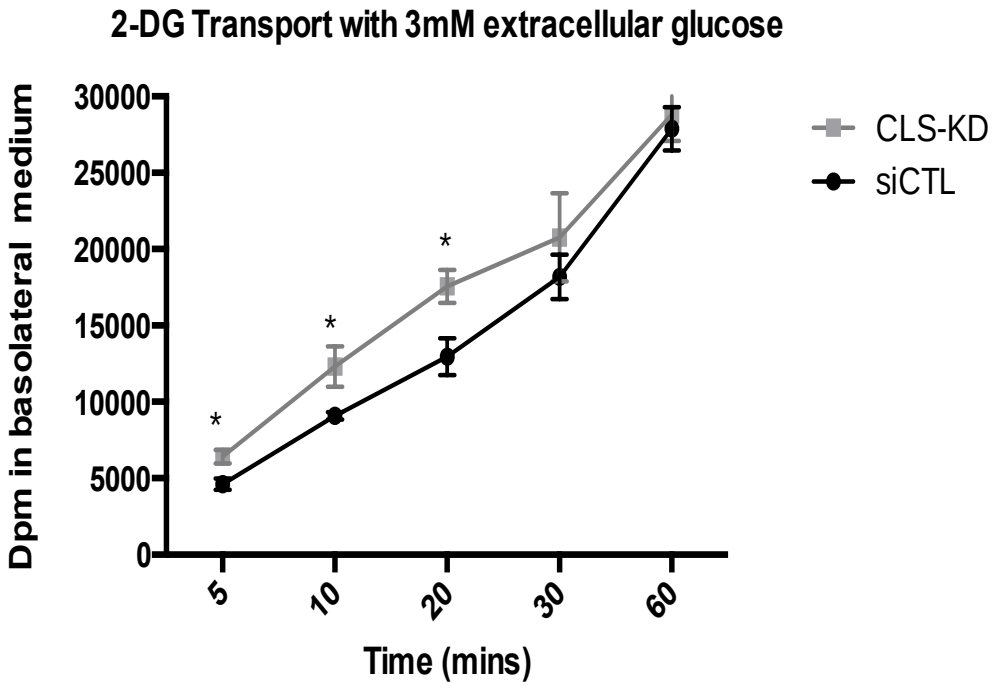
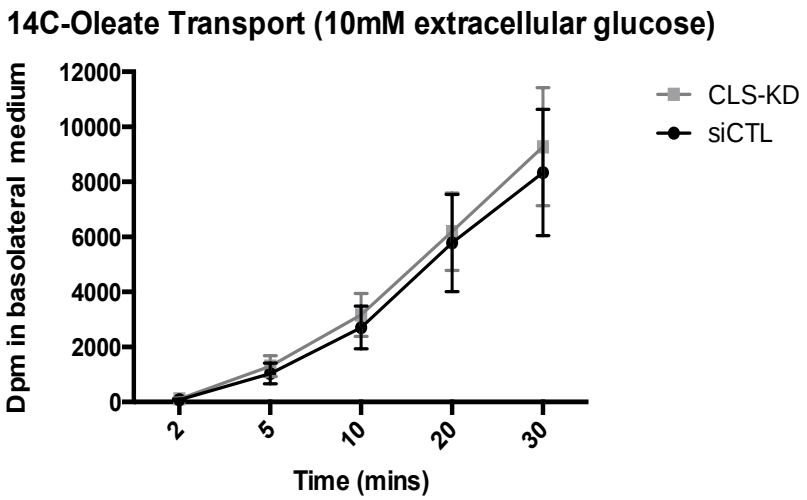


Figure 8. Knockdown of CLS does not affect transport of oleic acid across the hCMEC/D3 monolayer.

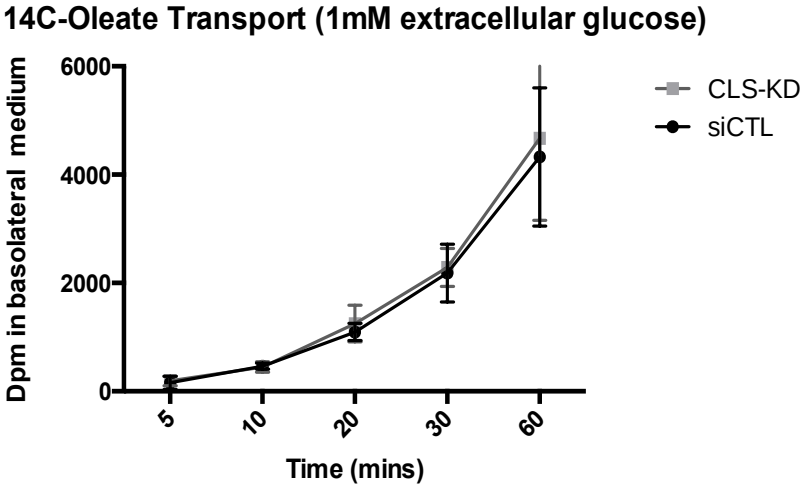
hCMEC/D3 cells were plated onto Transwell® polycarbonate inserts and were transfected with CLS siRNA or siCTL for 70h as described in Materials and Methods.

Cells were then incubated apically with 0.1 mM oleic acid bound to BSA in a 1:1 ratio and [1-14C] oleic acid was added in a tracing dose of 2uci/well. Transport was determined at both 10mM **(A)** and 1mM **(B)** extracellular glucose concentration. Radioactivity incorporated into the basolateral medium was determined as described in Methods. Data represent mean ±SD of three independent experiments.

A.



B.



5.

DISCUSSION

The objective of the current study was to evaluate the effect of lowering CL content by transient knockdown of the CL biosynthetic enzyme cardiolipin synthase (CLS) on the mitochondrial function and the barrier and trans-cellular transport properties of the brain capillary endothelial cells (BMECs). Endothelial cells, in general, are thought to obtain most of their energy through anaerobic glycolysis and this has lead to the lack of investigation on the role of mitochondria on endothelial function. However, this endothelial characteristic has only been characterized in the endothelial cells of the peripheral vascular tissues such as the pig aortic endothelial cells (Culic et al. 1997) and the coronary artery endothelial cells (Spahr et al. 1989; Mertens et al. 1990) but no study has examined this property in the brain capillary endothelial cells specifically. Unlike the endothelial cells in the peripheral vasculatures that are fenestrated, the endothelial cells of the brain capillaries are joined together by highly developed tight junctional complexes that can restrict paracellular passage of most substances from the blood into the brain and thus, selective compounds can only enter or exit the brain through a network of molecule carriers expressed by these cells, many of which require ATP

to function. Being structurally and functionally different as well as having a higher mitochondrial content than endothelial cells in other vascular beds (Oldendorf et al. 1977), we hypothesized that BMECs may be more metabolically active than their counterparts in the peripheral tissues and that an appropriate content of CL, the phospholipid known to be important for the activity and integrity of the respiratory chain, may be required to maintain an optimal mitochondrial function that may be necessary to power their barrier and trans-cellular transport functions.

Two different types of human brain capillary endothelial cells have been employed in our study, the primary human brain microvascular endothelial cells (HBMECs) and the human adult brain endothelial cell line hCMEC/D3. We began our study using the primary HBMECs as this model has been shown to retain some of the characteristics of the human endothelium in vivo (Dorovini-Zis et al. 1991). However, despite limited passaging, these primary cells rapidly lost their BBB characteristics, especially their transporters expression, which made it difficult to study their barrier and transport properties. To overcome such limitation, we decided to perform the major part of our study using the hCMEC/D3 cell line that has been extensively characterized

(Weksler et al. 2013; Poller et al. 2008) to retain many in vivo characteristics of the human brain endothelium that includes expression of tight junctional proteins, restricted paracellular permeability and expression of many functional influx and efflux transporters.

The cDNA enconding the human CLS (termed hCLS-1) had previously been cloned and described by our lab and subsequently confirmed by another lab (Lu et al. 2006b; Chen et al. 2006b). We showed here that RNAi mediated knockdown of the hCLS1 gene effectively lowered the mRNA expression **(Fig 1.1 A-B)** as well as the enzymatic activity of CLS (**Fig 1.2 A-B**) in both HBMEC and hCMEC/D3 cells. Consistently, western blot analysis also confirmed this reduction at the protein level in the hCMEC/D3 cells **(Figure 1.3 A-C).** The protein turnover rate of CLS has been suggested to be slow (Lu et al. 2011), which could possibly account for the only ~ 40% reduction observed in both CLS enzyme activity and protein expression with the RNAi treatment even though the mRNA expression knockdown was significantly higher (~75% in HBMEC and ~90% in HCMEC/D3). In yeast lacking CLS, CL was shown to be deficient (Chang et al. 1998). Furthermore, RNAi mediated knockdown of CLS in both human (Choi et al. 2007; Ji et al.

2012) and animal cells (Ji et al. 2012; Chu et al. 2013) were also shown to reduce CL levels. Thus, we anticipate that with an approximately 40% knockdown in both CLS protein expression and enzymatic activity observed, the CL level must have been decreased, at least in the hCMEC/D3 cells. However, a phosphorus mass assay for CL should also be done to confirm this reduction.

CLS deficiency in yeast, Arabidopsis and Caenorhabditis elegans was previously shown to impair mitochondrial ultrastructure and function (de Paepe et al. 2014)). However, we observed in our experiment that CLS knockdown did not alter the mitochondrial coupling efficiency of the hCMEC/D3 cells under basal condition, although there was a trend towards a decrease in basal respiratory rate **(Figure 2.1).** This is consistent with previous studies in mammalian cells that reported no major change in basal mitochondrial membrane potential as well as ATP production when CL level was reduced to ~44% (Ji et al. 2012; Huang et al. 2008). However, the knockdown seemed to have reduced the ability of the hCMEC/D3 cells to increase their spare respiratory capacity under conditions of stress, when the cells are required to increase their respiratory function to support an increase in

demand **(Figure 2.2)**. This is also consistent with studies in *cls* null yeast (Chang et al. 1998) and CLS-KD Hela cells (Choi et al. 2007), which observed no change in the growth, viability and mitochondrial function of the cells unless they were stimulated by a stress stimulus. These observations, however, are in contrast to the results reported by studies in cell lines generated from Barth Syndrome patients, which also have reduction in CL. In these cells, the reduced CL level reportedly caused abnormalities in both the mitochondrial structure and function of the cells (Gonzalvez et al. 2013; Barth et al. 2004). What could possibly explain for this discrepancy even though both models showed reduction in CL is the fact the CLS deficient cells and Barth Syndrome cells possess a very different phospholipid profile due to the different underlying defects, which make them not directly comparable. In Barth Syndrome, due to the defects in the CL remodeling enzyme, there is a substantial reduction in the level of mature CL as well as accumulation of monolysocardiolipin, while PG level is fairly normal (Choi et al. 2007). In contrast, the CLS deficient cells have an accumulation of PG (Chang et al. 1998). These cells also possess a residual population of mature CL that may be capable of withstanding the baseline requirements for mitochondrial function. Furthermore, the accumulated PG was suggested to have the ability to

substitute for the essential function of CL in yeast (Chang et al. 1998; Jiang et al. 1999) and thus it may also have a similar function in mammalian cells. Essentially, such underlying biochemical differences may partially explain the unchanged basal mitochondrial function observed in our model. Interestingly, it was shown in rat cortical neurons that transient knockdown of CLS significantly reduced the stimulus-induced mitophagy in these cells (Chu et al. 2013). Mitophagy is a physiological process that eliminates damaged mitochondria to prevent their toxic effect on the cells. The fact that the cells of Barth Syndrome's patients who have reduced CL levels are also known to contain abnormal mitochondria with larger size and reduced cristae formation (Finsterer and Frank. 2013) also further support the important role of CL in mediating the clearance of damaged mitochondria. Thus, together with our observed data, we anticipate that reduction of CL level in the brain capillary endothelial cells, especially when this deficiency is chronic, could cause these cells to become less resilient under stressful conditions.

The CLS knockdown did not only cause the reduction in the spare respiratory capacity of the hCMEC/D3 cells, but also resulted in a

moderate increase in the glycolytic rate and glycolytic capacity of the cells **(Figure 2.2)**. This suggests a phenomenon similar to the Warburg effect commonly observed in cancer cells (WARBURG. 1956). Warburg effect is a phenomenon in which cancer cells produce most of their energy need via glycolysis despite the availability of oxygen (aka. aerobic glycolysis) (WARBURG. 1956). This metabolic switch in cancer cells was suggested to be partly caused by their reduced mitochondrial function (Giang et al. 2013). Similarly, the fact glycolytic activity was increased in the CLS knockdown hCMEC/D3 cells could also be another indication that these cells have reduced mitochondrial function.

Studies in both human Umbilical Vein Endothelial cells (HUVECs) (Quintero et al. 2006) and coronary microvascular endothelial cells (Spahr et al. 1989; Mertens et al. 1990) suggest that these cells rely mostly on glycolytic metabolism of glucose for their energy need. However, we observed in our study that under a saturating glucose concentration, the HCMEC/D3 cells appeared to have produced ~60% of their ATP through oxidative phosphorylation (**Figure 3**). Though, these measurements must be further confirmed by a more direct method of measuring ATP production using similar

substrates/inhibitors. Interestingly, our finding appears to be in line with the observation of (Oldendorf et al. 1977) who reported that the brain capillary endothelial cells of rats contain 2-4 times higher mitochondrial volume than those coming from non-BBB regions, which morphologically suggests a higher metabolic activity in these cells. The higher dependence of BMECs on mitochondrial respiration may also be supported by the fact the endothelial cells of the peripheral vasculatures are known to have a high tolerance to hypoxia by their ability to retain both viability and cellular integrity under hypoxic conditions (Tretyakov and Farber. 1995). This has been attributed to their lack of dependence on oxygen for energy production (Tretyakov and Farber. 1995). This is in contrast to BMECs, which have been shown both in vitro and vivo to experience tight junction disruptions (reviewed in(Hawkins and Davis. 2005; del Zoppo and Mabuchi. 2003)) and apoptosis (Zhang et al. 2000; Lee and Lo. 2004) in response to hypoxia-ischemia. Further more, estrogen, the hormone well believed to protect premenopausal women from stroke has been suggested to do so, partly, by enhancing the mitochondrial efficiency and as a result reducing capillary endothelial cell death in response to ischemic insult (Guo et al. 2010). These observations, together with

ours, suggest a higher dependence of brain endothelial cells for aerobic respiration to maintain their integrity and cellular function.

The hCMEC/D3 cells appeared to have a moderate decrease in their basal respiratory rate (**Figure 2.1**), which suggest some changes in basal ATP demand. Thus, we examined the expression of the transporters that may be affected by changes in cellular bioenergetics as indicated in **Figure 4**. Interestingly, we found a significant ~40% reduction in the expression of creatine transporter (CRT1) in the knockdown cells. Whether the majority of creatine in the brain is endogenously synthesized or is imported from the peripheral circulation remains controversial. However, the fact that patients with mutation in the SLC6A8 gene, the gene that encode CRT1, experience serious cognitive and developmental defects (Braissant. 2012) suggest that creatine importation from the periphery must be essential for cerebral health. The process of creatine uptake into the cells, although do not directly require energy, depends on the inward current of Na+ maintained by the Na/K+ ATPase pump that consumes ATP (Beard and Braissant. 2010). Thus, we anticipate that the reduced CRT1 expression observed in our study might be a consequence of the

reduced cellular energy surplus caused by the knockdown that would necessitate less creatine importation for energy storage. Thus, the hCMEC/D3 cells might have reduced CRT1 expression as a mechanism of conserving energy. In support of our claim, study in kidney epithelial cells showed that AMP-activated protein kinase, a "metabolic sensor" that is activated by reduction in cellular energy level, can inhibit the expression of CRT1 (Li et al. 2010). Furthermore, during metabolic stressful states such as fasting, the urinary excretion of creatine was also reportedly increased (Li et al. 2010).

Both HBMEC and hCMEC/D3 cells grown as a monolayer on Transwell Polycarbonate inserts were used in our study as in vitro models for the human BBB. As BMECs have been shown to experience tight junction disruption in response to hypoxia-ischemia (Hawkins and Davis. 2005; del Zoppo and Mabuchi. 2003), a condition that also depresses mitochondrial respiration, we thus examined whether knocking down CLS would have an effect on the integrity of the HBMECs and hCMEC/D3 monolayers. Thus, we incubated the confluent CLS siRNA and siCTL transfected HBMEC and hCMEC/D3 monolayers apically with 1 uM of the paracellular tracer FDX-10 000 and examined its

penetrability into the basolateral compartment. After a 30 min incubation period, we did not observe any change in the permeability with the knockdown in both cell populations. Yet, this result did not surprise us, as the mitochondrial function of these cells was not grossly affected by the knockdown under basal conditions, which may partly explain for the unchanged in permeability. In addition, one limitation of the in vitro, static culture of BMECs is that these in vitro monolayers are known to have a much lower TEER, a measure of monolayer tightness, compared to that of the brain endothelium in vivo (Weksler et al. 2013). Thus, small changes in permeability that might have been caused by the knockdown may not be detected in these models. However, we anticipate that in the presence of a stress stimulus, especially when the CLS knockdown has been maintained over a longer period of time, the knockdown monolayers may be less resilient and may experience a more enhanced leakiness.

As the CLS-KD hCMEC/D3 cells appeared to have increase in glycolytic activity, which is a less efficient energy production process, we postulated that they might need to take in more nutrients to compensate for such inefficiency. Thus, we measured the glucose

uptake activity of GLUT1, the principle glucose transporter at the BBB. Consistent with our postulation, the knockdown cells showed increase in GLUT1 glucose uptake under hypoglycemic conditions, with a statistical significant increase seen at 1 mM extracellular glucose concentration and no difference seen at 5 mM extracellular glucose. What could possibly account for the change observed only at low glucose concentrations may be the fact GLUT1 is known to have a high affinity for glucose with the dissociation constant (Km) in the range of 1-2 mM for glucose influx (Siegel et al. 2012). Thus, under a physiological concentration of glucose (~5 mM), these transporters may be saturated that could provide excess glucose for the cells and thus no change in glucose uptake would be necessary. However, under a low glucose availability condition, the CLS-KD cells, which are more dependent on glycolysis, might have needed to take in more glucose to compensate for their lower energy production efficiency.

Interestingly, the increase in glucose uptake activity appeared to correlate with the increase in glucose transport across the CLS-KD hCMEC/D3 monolayer. This suggests that brain endothelial energy status may play a role in regulating glucose transport across the BBB.

However, how well this in vitro model correlate with the BMECs' behavior in vivo still requires further investigation. Yet, the brain endothelium has been shown in vivo to have the ability to control glucose influx into the brain (Huang et al. 2012). It was shown in mice with endothelial cell-specific deletion of the transcription factor HIF-1α that these animals experienced reduced GLUT1 protein expression that correlated with a reduction in glucose uptake into the brain (Huang et al. 2012). Interestingly, forced expression of GLUT1 cDNA in a parallel in vitro model was able to rescue this defects, which further supported the specific effect of HIF-1α on GLUT1 expression (Huang et al. 2012). In addition, these authors also showed that HIF-1α could induce activation of AMPK, the enzyme that is normally activated under metabolic stress when there is increase in AMP: ATP ratio, thus linking the effect of HIF-1-a on enhancing glucose uptake into the brain to the endothelial energy status. The mechanism through which glucose uptake and transport were increased in our knockdown model is still unclear. The mRNA expression of GLUT1 did not change with the transfection (**Figure 4**). However, the mRNA turnover of GLUT1 in the hCMEC/D3 cells may be quick and we do not exclude the possibility of an increase in GLUT1 expression seen at the protein level. It is also known that ~ 40% of GLUT1 in brain endothelial cells reside in the

intracellular pool that could be recruited to either the luminal or abluminal membranes under conditions of increased demand (Simpson et al. 2001; Shah et al. 2012). Thus, even without a change in GLUT1 mRNA or protein expression, recruitment of GLUT1 from this intracellular pool may also play a role in mediating the increase in glucose uptake and transport observed in our study.

Alterations in glucose transport across the BBB have been reported both in Alzheimer's disease and diabetes but it is unclear what role this has with either the disease etiology or progression (Shah et al. 2012). In human patients and animal models of Alzheimer's disease, glucose transport into the brain were reportedly decreased that correlated with a reduced expression of both GLUT1 and GLUT3 transporters (Shah et al. 2012). However, there has been mixed results on whether the brain glucose influx is decreased or increased in diabetes (Shah et al. 2012). Reduction in CL level has been reported both in the brain of diabetic rats (Moreira et al. 2004; Raza et al. 2011) and in mononuclear cells of type 2 diabetic patients that were correlated with abnormal mitochondrial structure and function (Widlansky et al. 2010). Here, we observed a link between CL reduction and alterations in

glucose transport into the brain under hypoglycemic conditions, which suggest that CL reduction may play a role in the alterations of brain glucose transport observed in diabetic patients who often have alternating hypoglycemic and hyperglycemic episodes. Type 2 diabetes is known to be a major risk factor for Alzheimer's disease(Holscher. 2011) and in both conditions, glucose transport into the brain has been reportedly altered. As the CL profile has not been characterized in AD, whether or not alterations in CL level may serve as causal link between these conditions require further investigation.

Despite the fact metabolically active organs such as the heart and the liver readily oxidize fatty acids, long chain fatty acid still remains a minor energy source of the brain (Schonfeld and Reiser. 2013). This has been partly attributed to the fact fatty acid β-oxidation would require more oxygen, have a slower rate of ATP generation and would generate more reactive oxygen species than the oxidation of glucose (Schonfeld and Reiser. 2013). Thus, selective use of glucose is believed to be a protective mechanism that the brain developed to protect neural cells against hypoxia and oxidative stress. Similarly, despite the increase in glucose uptake and transport observed with

CLS-KD in our study, the knockdown did not seem to affect long chain fatty acid transport across the hCMEC/D3 monolayer at both a saturating and a low extracellular concentration of glucose.

6. STUDY LIMITATION AND FUTURE DIRECTIONS

One major limitation of this study is we have not examined the CL mass in the CLS siRNA and siCTL treated cells to confirm that the knockdown did indeed reduce cellular CL levels. However, mitochondrial fractions have been collected and the CL content of these cells will be examined by performing a phosphophus mass assay in the near future. As the measured mitochondrial and glycolysis parameters appeared highly variable in the knockdown and control cells due to the inherent noise of the experiment and the low number of experimental replicates, we also plan to repeat these experiments to further validate the presented results.

It is interesting that we observed a significant reduction in the mRNA expression of CRT1 with CLS knockdown in the hCMEC/D3 cells. Thus, we also would like to examine its protein expression using Western blot to see whether the same reduction can also be seen at the protein level. CRT1 deficient patients are known to experience serious cognitive defects (Braissant. 2012). Similarly, Barth Syndrome patients, who have reduced CL level, also experience learning difficulties (Mazzocco et al. 2007). Thus, the reduced CRT1 expression at the BBB, especially at the protein level, could potentially explain for

such learning deficit in Barth Syndrome. Therefore, we have also collected the brain capillary fractions of the TAZ-KD mice, an animal model for Barth Syndrome and are planning to examine its BBB CRT1 protein expression in parallel. If similar reduction could also be seen in this model, we anticipate that this would open avenues for development of better treatments for this cognitive phenotype in Barth Syndrome as treatment strategies such as creatine supplementation could also be considered. Last but not least, we would also like to perform a rescue experiment by over expressing CLS in the hCMEC/D3 cells to confirm that the presented changes are indeed due to CLS knockdown.

7. CONCLUSION

1. Transient knockdown of CLS did not significantly alter the basal mitochondrial function of hCMEC/D3 cells.
2. The knockdown did seem to reduce the ability of hCMEC/D3 cells to increase their respiratory capacity under condition of increased demand, which is an indication of reduced mitochondrial function.
3. The CLS knockdown caused hCMEC/D3 cells to switch to being more dependent on glycolysis, which is a less efficient energy production process.
4. Unlike the endothelial cells in the peripheral vascular tissues that are highly glycolytic, the hCMEC/D3 cells appeared to produce ~60% of their ATP via mitochondrial respiration.
5. CLS knockdown significantly reduced the transcriptional expression of creatine transporter (CRT1) in hCMEC/D3 cells, which may indicate a lower ATP reserve in the cells.
6. The CLS knockdown did not alter the permeability across both the HBMECs and the hCMEC/D3 monolayers.
7. The knockdown significantly enhanced GLUT1 glucose uptake activity under hypoglycemic conditions that correlated with an

increase in glucose transport cross the hCMEC/D3 monolayer. Thus, the brain capillary endothelial energy status may play a role in regulating glucose transport across the BBB.

8. CLS knockdown did not significantly alter oleic acid transport across the hCMEC/D3 monolayer possibly due to the fact fatty acid is not a preferred energy source for the brain.

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