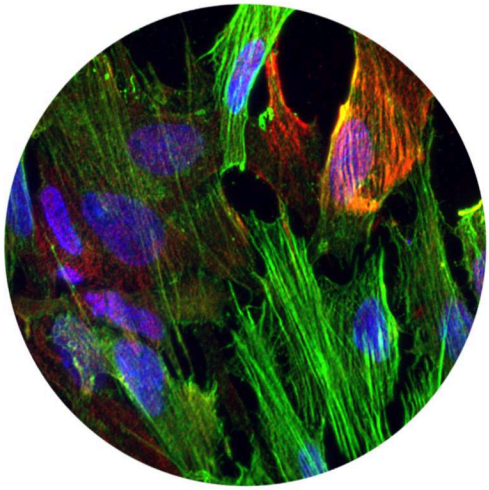
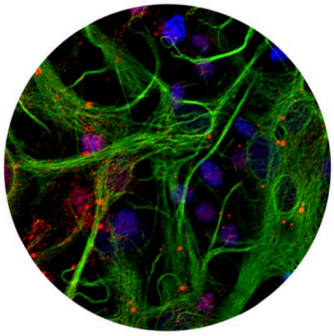
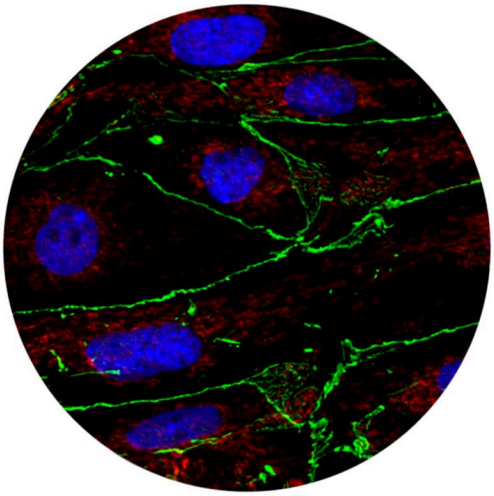
Epigentic regulation of *Cpt1a* and *Cpt1c* and link to multiple sclerosis

Master thesis

Katrine Jønsson







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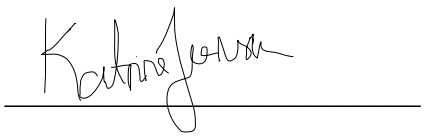

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Abstract

Introduction: Multiple sclerosis (MS) is a disabling disease that affects more than 2 million people worldwide. Carnitine palmitoyl transferase 1A (CPT1A) is an enzyme involved in fatty acid oxidation and mutations in the gene coding for this protein decreases the activity of the enzyme, which has shown to decrease the incidence of multiple sclerosis. Epigenetics of multiple sclerosis is a relatively new field and only a few studies has to date investigated the role of epigenetics of *Cpt1a* and *Cpt1c,* whereas there are no studies describing the expression and regulation of both genes in blood brain barrier (BBB)-associated cells and an experimental autoimmune encephalomyelitis (EAE) model. **Aim:** The aim of this study was to investigate the expression of *Cpt1a* and *Cpt1c in vitro* in rat primary BBB cells and *in vivo* in EAE mouse model of MS and investigate the epigenetic landscape of the two genes. **Methods:** This study investigates the relative mRNA expression of *Cpt1a* and *Cpt1c in vitro* in primary rat endothelial cells, astrocytes, and pericytes before and after treatment with histone deacetylase (HDAC) inhibitors and *in vivo* in brain tissue of a murine EAE model by reverse transcriptase-quantitative polymerase chain reaction. Immunocytochemistry was applied to investigate the amount of protein in primary cells before and after treatment with HDAC inhibitors. DNA methylation analysis was performed to investigate the fraction of DNA methylation *in vitro* before and after treatment and *in vivo* in controls and EAE animals. This study further investigated histone modifications *in vivo* by chromatin immunoprecipitation. **Results:** The results showed that both genes were expressed in BBB-associated cells, which was never shown before. The endothelial cells presented with the highest expression of *Cpt1a*, whereas pericytes presented with the lowest. Furthermore, astrocytes presented with the highest expression of *Cpt1c*. After treatment with HDAC inhibitors an increased expression of *Cpt1a* and *Cpt1c* is seen in endothelial cells and pericytes, whereas astrocytes presented with a decreased expression of both genes after treatment. Immunocytochemistry supported these results. DNA methylation analysis showed increased fraction of DNA methylation in endothelial cells and astrocytes after treatment compared to controls and decreased DNA methylation after treatment in pericytes compared to controls. *In vivo*, EAE animals presented with a decreased expression of *Cpt1c* compared to controls. DNA methylation analysis demonstrated a tendency of increased DNA methylation in EAE animals compared to controls. Chromatin immunoprecipitation demonstrated a decreased acetylation of histone 4 of *Cpt1c* in EAE mice compared to controls. **Conclusion:** *Cpt1a* and *Cpt1c* are epigenetically regulated *in vitro*. Furthermore, the expression of *Cpt1c* decreases *in vivo* in EAE animals and is epigenetically regulated.

Resumé

Multipel sklerose er en invaliderende sygdom, der påvirker mere end 2 millioner mennesker på verdensplan. Carnitin palmitoyl transferase 1A (CPT1A) er et enzym, der er involveret i fedtsyre metabolismen. Dens funktion er at omdanne acyl-CoA til acylcarnitin, så denne kan passere den impermeable mitokondriale membran for dernæst at kunne fortsætte i β-oxidation. I to populationer har det vist sig, at en mutation i genet, der koder for CPT1A, medfører en nedsat incidens af multipel sklerose. Epigenetik af multipel skerose er et relativt nyt felt, hvor kun få studier har undersøgt epigenetikken bag *Cpt1a* og *Cpt1c*. Der er på nuværende tidspunkt ingen der har undersøgt udtrykkelsen og reguleringen af disse to gener i blod hjerne barriere-associerede celler og i en eksperimentiel autoimmun encephalomyelitis (EAE) model. Dette studie undersøger den relative mRNA udtrykkelse af *Cpt1a* og *Cpt1c in vitro* i rotte primærceller, endothelceller, astrocytter og pericytter, både før og efter behandling med histon deacetylase (HDAC) inhibitorer og *in vivo* i hjernevæv fra en EAE model. Dette undersøges ved RT-qPCR. Desuden bliver immuncytokemi brugt for at undersøge udtrykkelsen af CPT1A proteinet i primærceller før og efter behandling med HDAC inhibitorer. Både *in vitro* før og efter behandling og *in vivo* i EAE modellen bliver DNA methylering undersøgt. Derudover bliver histon modifikationer undersøgt *in vivo* ved hjælp af kromatin immunprecipitation. Dette studie finder, at begge gener er udtrykt i blod hjerne barriere-associerede celler, hvilket ikke før er blevet vist. Endothelcellerne havde den højeste udtrykkelse af *Cpt1a*, mens pericytterne havde den laveste udtrykkelse af genet. Derudover havde astrocytterne den højeste udtrykkelse af *Cpt1c* sammenlignet med endothelceller og pericytter. Efter behandling med HDAC inhibitorer ses en øget udtrykkelse af *Cpt1a* og *Cpt1c* i endothelceller og pericytter, hvorimod der ses en nedsat udtrykkelse af *Cpt1a* og *Cpt1c* i astrocytter. Immuncytokemi bakkede op om disse resultater. DNA methyleringsanalysen viste en øget DNA methylering i endothelceller og astrocytter efter behandling sammenlignet med kontroller og nedsat DNA methylering efter behandling i pericytter sammenlignet med kontrol. EAE mus udviste nedsat udtrykkelse af *Cpt1c* sammenlignet med kontroller. Kromatin immunprecipitation viste en nedsat acetylering af histon 4 af *Cpt1c* i EAE mus sammenlignet med kontroller. *Cpt1a* og *Cpt1c* er dermed epigenetisk reguleret *in vitro*, hvorimod *Cpt1c* er epigenetisk reguleret *in vivo*.

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Abbreviations

α-SMA – α smooth muscle actin
BBB – Blood brain barrier
BCEC – Brain capillary endothelial cells
CACT – Carnitine acylcarnitine translocase
cDNA – Complementary DNA
CFA – Freud's adjuvant
ChIP – Chromatin immunoprecipitation
CNS – Central nervous system
CPT1 – Carnitine palmitoyl transferase 1
CPT2 – Carnitine palmitoyl transferase 2
DAPI – 4',6-diamidino-2-phenylindole
DNMT – DNA methyltransferase
EDTA – Ethylenediaminetetraacetic acid
EAE – Experimental autoimmune encephalomyelitis
GFAP – Glial fibrillary acidic protein
H3K4me3 – Histone 3 lysine 4 trimethylation
H3K9me3 – Histone 3 lysine 9 trimethylation
H3K27me3 – Histone 3 lysine 27 trimethylation
H4Ac – Histone 4 acetyla
HAT – Histone acetyltransferases
HDAC – Histone deacetylases
HPRT1 – Hypoxanthine Phosphoribosyltransferase 1
ICC – Immunocytochemistry
IL – Interleukin
MBP – Myelin basic protein
MMP – Matrix metalloproteinases
MOG – Myelin oligodendrocyte glycoprotein
MTP – Mitochondrial trifunctional protein
MS – Multiple sclerosis
M/SCHAD – Medium and short chain hydroxyacyl-CoA dehydrogenase
NADH – Nicotinamide adenine dinucleotide
PBS – Phosphate buffered saline
PPAR – Peroxisome proliferator-activated receptors
PPMS – Primary progressive multiple sclerosis
PRMS – Progressing relapsing multiple sclerosis
PTM – Posttranslational modifications
Ptx – Pertussis toxin
qPCR – Quantitative polymerase chain reaction
RRMS – Relapsing remitting multiple sclerosis
RT-qPCR – Reverse transcriptase-quantitative polymerase chain reaction
SB – Sodium butyrate
SPMS – Secondary progressive multiple sclerosis
VLCAD –Very long chain acyl-CoA dehydrogenase
VPA – Valproate/Valproic acid
WT – Wildtype
ZO-1 – Zona occludens-1

1 Introduction

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a disabling and multifactorial disease of the central nervous system (CNS), characterized by degradation of the myelin sheath surrounding the axons, followed by neurological symptoms, such as vertigo, loss of vision, tremor, weakness, and spasms. (1–3) The age of onset of MS is usually between 20-40 years, although the first symptoms start five years before onset of the disease. (4) Furthermore, 20% of MS patients may require a wheelchair. (1) MS affects 2.3 million people worldwide (2013) and is dominated by females. The prevalence of MS varies between countries, where the highest point prevalence is found in North America (140 per 100.000) and Europe (108 per 100.000). (5) MS can be classified as relapsing-remitting (RRMS), primary progressive (PPMS), progressive relapsing (PRMS), and secondary progressive (SPMS) with relapsing-remitting being the most common type (85%). (5) The majority of patients diagnosed with relapsing-remitting MS evolve to secondary progressive MS. (6,7) RRMS is characterized by episodes with lack of neurological function followed by recovery. (8) As mentioned, SPMS often follows RRMS and is characterized by an uninterrupted phase of disease progression. (9) Furthermore, PPMS is characterized by a progressive disease phase from the onset of disease without any acute relapses. (8) There is currently no cure for MS, however, medication that modify the disease course are applied. These encompass interferon-β and monoclonal antibodies, although these medications exert side effects. (10)

1.1.1 Pathology

In MS both environmental and genetic factors are thought to play a role. Environmental factors include smoking, Epstein-Barr Virus, vitamin D deficiency, and geography with the northern latitudes having the highest prevalence of MS (5,8,11). Additionally, HLA-DRB1 is found as a genetic predisposition. Furthermore, a slightly higher risk of MS is found in parents, siblings or children to patients with MS. (1,5,12)

MS is characterized as an autoimmune disease that is affected by both molecular mimicry and bystander activation. In molecular mimicry, the self-antigens share antigen structure with infectious agents and an immune attack can be initiated against both self- and non-self antigens. However, if an antigen-presenting cell is activated within the CNS, T-cells initiate an autoimmune response against epitopes in CNS, known as bystander activation. (8)

In MS, the blood brain barrier (BBB) is disrupted (2). The BBB consists of blood vessels with unique properties. The barrier regulates the movement of ions, cells, and molecules from the blood to the brain and protects the brain from pathogens and toxins. As seen on figure 1.1, the BBB

consists of endothelial cells, supported by pericytes and astrocytes. Tight junctions connect the endothelial cells in CNS, which limits the flux of solutes. Pericytes are located in the basement membrane and contain contractile proteins, which enable contraction of the vessel. Astrocytes are one of the main types of glial cells and surround blood vessels by their end-feet. Astrocytes regulate blood flow depending on neuronal activity, hence, more oxygen is delivered to the active brain regions. (13,14)

1.1.2 Demyelination of neurons

Myelin is a supporting and insulating substrate for neurons, which is synthesized by oligodendrocytes. Damage to myelin or myelin-producing cells compromise both the support and the insulation of the neurons, hence, leading to deficits in the neuronal function. The process of disintegrating myelin is called demyelination. (8) RRMS is the best characterized type of MS, in which the immune response is initiated by activated T-cells, which becomes autoreactive. These T-cells have the ability to cross the BBB by an upregulation of α4-integrins, especially α4β1, which binds to vascular cell adhesion molecules expressed on endothelial cells. (15) The T-cells cross BBB through several pathways: by diapedesis, by α6β1 integrin activation, and by secretion of matrix metalloproteinases (MMP). MMP produced by microglia in the area of migration disintegrates BBB. Antigen-presenting cells, such as microglia in CNS, become activated by pro-inflammatory cytokines secreted from T-cells, which in turn secretes chemokines and cytokines contributing to recruitment of T-cells, macrophages, and dendritic cells. Furthermore, cytokines induce an upregulation of endothelial adhesion molecules and activated microglia secrete interleukin (IL)-17 resulting in disruption of BBB. Hence, more T-cells invade the CNS. (2,8) Activated microglia can establish contact between the microglia itself and the oligodendrocyte-unit, leading to release of e.g. tumor necrosis factor α and other cytokines, where demyelination occurs. (1,15) Furthermore, autoreactive T-cells have an overexpression of β-arrestin 1, a promoter of activated T-cell survival, which unable the cells to undergo apoptosis upon stimulation. (1)

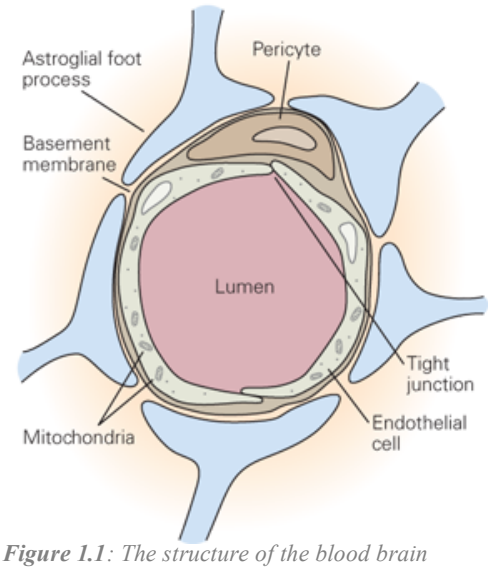

***Figure 1.1**: The structure of the blood brain barrier. From Principles of neural science*

1.1.3 Remyelination of neurons

One of the major factors contributing to MS symptom development is demyelination of neurons. Demyelination is followed by remyelination in RRMS. The process of remyelination consists of proliferation, migration, and differentiation of neuronal progenitor cells and oligodendrocyte progenitor cells into mature oligodendrocytes and lastly the interaction with axons. (16–18) MS patients suffer from attacks, which result in demyelination and glial scar tissue, also called MS plaque. Especially the progressive types of MS have a limited remyelination. Some of the

limitations to remyelination in MS patients are the formation of scar tissue creating a barrier between the oligodendrocytes and the axons and thinner and shorter myelin sheaths than in healthy individuals. (17,18)

1.2 Carnitine palmitoyl transferase 1 (CPT1)

Demyelination of neurons might be caused by an autoimmune reaction as described above, although it is tempting to speculate in a link between fatty acid metabolism and demyelination or remyelination of neurons. One of the major players in fatty acid metabolism is the rate limiting enzyme carnitine palmitoyl transferase 1 (CPT1). It is demonstrated, that blockage of CPT1 reduces both disease severity and demyelination in a mice experimental autoimmune encephalomyelitis (EAE) model. (19) Hence, CPT1 and fatty acid metabolism might play a role in the development of the disease. This means that even though some, but limited, remyelination occurs in MS patients, the myelin sheaths might be destructed as fast as it is repaired. The mechanism behind the possible increased expression of CPT1 is yet unknown. Fatty acid oxidation occurs in the mitochondria but the mitochondrial membranes are impermeable to acyl-CoAs. CPT1 is an enzyme located in the outer mitochondrial membrane and enables transport of acyl-CoA through the membrane. As seen on figure 1.2, CPT1 functions as an enzyme that converts acyl-CoA to acylcarnitine, which is able to cross the membrane in presence of carnitine acylcarnitine translocase (CACT) in exchange for a carnitine molecule. In the inner mitochondrial membrane carnitine palmitoyl transferase 2 (CPT2) is present, which converts acylcarnitine into acyl-CoA and carnitine. Afterwards, acyl-CoA is degraded via β-oxidation. (20–22) Three subtypes of CPT1 exist. CPT1A is mainly found in the mitochondria of the liver, but is widely distributed throughout the body e.g. in pancreas, brain, and blood, CPT1B is located in the mitochondria of skeletal and heart muscle, and CPT1C is an isoform only expressed in the CNS that is uniquely localized in the endoplasmic reticulum in neurons contrary to CPT1A and CPT1B localized to the mitochondria. (11,12) The function of CPT1C is not yet fully understood, although CPT1C has CPT1 activity. (23,24) CPT1A and CPT1C are encoded by *CPT1A* and *CPT1C*, respectively.

In humans, the *CPT1A* consists of 89,791 bp comprising 19 exons and is located on chromosome 11q13.3. Furthermore, the gene has three CpG islands. (25,26) This gene plays an essential role in the lipid metabolism by coding for the CPT1A enzyme. Two populations found in Canada, the Hutterites and Inuit populations have a *CPT1A* mutation that decreases the activity of the protein to 0% or 22%, respectively. (22,27) Interestingly, these populations have a significantly lower incidence of MS. (28) CPT1A mutation is inherited in a autosomal recessive manner. Although, the incidence for MS in cpt1a -/- and cpt1a +/- is lower, the mutation causes other complications such as hypoglycemia in these patients. (29) *CPT1C* consists of 21,353 bp and 24 exons. The gene is located on chromosome 19 locus 19q13.33 and includes three CpG islands. The gene codes for CPT1C, which is a brain specific enzyme. (26)

1.3 Fatty acid oxidation and CPT1

Once the fatty acids are transported to the mitochondria by CPT1, β-oxidation occurs. β-oxidation is a process generating acetyl-CoA by shortening of acyl-CoA. As seen on figure 1.2, after import of the fatty acids to the mitochondria, acyl-CoA is converted to enoyl-CoA by very long chain acyl-CoA dehydrogenase (VLCAD). Mitochondrial trifunctional protein (MTP) catalyzes the reaction from enoyl-CoA to 3-hydroxyacyl-CoA. MTP further catalyzes the steps from 3-hydroxyacyl-CoA to 3-ketoacyl-CoA and at last acyl-CoA + acetyl-CoA. (20)

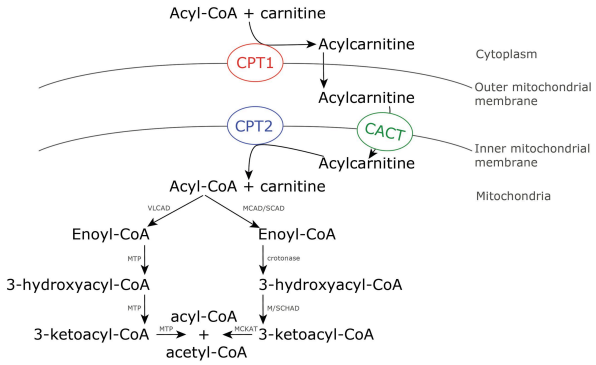

Figure 1.2: *Transport of acyl-CoA through the mitochondrial membrane and β-oxidation, (Abbreviations; CPT1, Carnitine palmitoyl transferase 1; CPT2, Carnitine palmitoyl transferase 2; CACT, Carnitine acylcarnitine translocase; VLCAD, Very long chain acyl-CoA dehydrogenase; MTP, Mitochondrial trifunctional protein; MCAD/SCAD;Medium/short chain acyl-CoA dehydrogenase; M/SCHAD, medium and short chain hydroxyacyl-CoA dehydrogenase; MCKAT, medium chain 3-ketoacyl-CoA thiolase).*

The acyl-CoAs enter another cycle of β-oxidation, although when reduced to medium chain fatty acids, these are metabolized in the mitochondrial matrix. In the mitochondrial matrix, the oxidation products are the same, but enzymes differ. The enzymes catalyzing these products are MCAD, cronotase, medium and short chain hydroxyacyl-CoA dehydrogenase (M/SCHAD), and medium chain 3-ketoacyl-CoA thiolase (MCKAT). After each cycle the acyl-CoA is shortened by two carbon atoms, one acetyl-CoA, one nicotinamide adenine dinucleotide (NADH), and one flavin adenine dinucleotide (FADH2) are released. NADH and FADH2 enter the electron transport chain to produce ATP, while acetyl-CoA enters the citric acid cycle. Acetyl-CoA exerts negative feedback on pyruvate, which inhibits energy production from glycolysis. Fatty acid oxidation is regulated by malonyl-CoA and peroxisome proliferator-activated receptors (PPARs). Malonyl-CoA is derived from fatty acid synthesis and acts as an inhibitor of CPT1A. (20,30) In addition, PPARs function as transcription factors and regulate expression of the enzymes involved in fatty acid oxidation. (20) Fatty acid oxidation can be inhibited pharmacologically by a drug called etomoxir, which blocks CPT1 activity, hence, blocking the use of fatty acids in energy production. (31)

1.4 Epigenetics

All cells in the body have the same genome, although the genes are expressed according to function and cell type. Epigenetics makes it possible to regulate the expression of genes in different contexts

without altering the sequence of DNA. (32,33) Epigenetic regulation can also be referred to as molecular switches and plays an important role in the regulation of genes. (33,34) The epigenetic regulation is inheritable through cell divisions for several generations despite lack of changes in DNA sequence. (35)

One of the most studied epigenetic modifications is DNA methylation by which a methyl group is transferred from S-adenosylmethionine by DNA methyltransferase (DNMT) to the 5'-position of the cytosine ring, where it is covalently bound. (9,32,36) This epigenetic modification often occurs at CpG sites, especially in the CpG rich area called CpG islands. Additionally, CpG islands are usually found near or in promotor regions. (37,38) Hypermethylation is associated with gene silencing and hypomethylation is associated with gene activation caused by a closed or open conformation of chromatin, respectively. (39) Two processes can be responsible for gene silencing: 1) Hypermethylation prevents binding of transcription factors or 2) Methyl binding domains blocks the access to regulatory elements, hence, blockage of transcription factors. (33,40)

Additionally, histone modifications are also a well-known epigenetic factor and play an important role in the chromatin dynamics. The conformation of chromatin determines whether the gene is silenced or activated. The nucleosome consists of DNA wrapped around an octamer of histones that comprises two of each core histone, H2A, H2B, H3, and H4. (37,39) The chromatin structure can be altered by histone posttranslational modifications (PTM). PTMs can alter the chromatin structure to an ‘open’ or ‘closed’ structure leading to gene activation or gene silencing, respectively. (32,34) A variety of PTMs exist including methylation of the amino acids lysine and arginine, phosphorylation of tyrosine, and acetylation of lysine. Acetylation is one of the most studied PTMs and is associated with gene activation, hence, an ‘open’ chromatin structure. This process is carried out by histone acetyltransferases (HATs) and the acetylation can be reverted by histone deacetylases (HDACs). Additionally, methylation of histones is carried out by histone methyltransferases and reverted by histone demethylases. (39)

1.5 Association between multiple sclerosis and epigenetic regulation of *CPT1*

The idea of epigenetic modifications in MS has been available for a decade. Vitamin D has the ability to alter gene expression through histone modifications, which indicates epigenetic regulation. (41,42) Furthermore, the injection of HDAC inhibitors in EAE model of MS decreases phenotype severity. (43) One can speculate in a link between MS and fatty acid metabolism due to the demyelination of neurons, hence, a link between CPT1A and MS. It has been previously demonstrated, that blocking of CPT1A decreases disease severity in mice. (19) Additionally, it is well known that *CPT1A* mutations are inheritable and decreases the incidence of MS as seen in two populations in Canada. A study on monozygotic twins demonstrated a MS concordance rate of 20%, which might indicate that epigenetics might play a role in the development of disease due to identical DNA level. (22,41,44) Therefore, it is interesting to look at the epigenetics of *CPT1* and the possible link to MS. However, the empirical knowledge regarding epigenetic regulation of

CPT1 is scarce. It is demonstrated that fructose consumption induces hypermethylation of *CPT1A* promoter regions in mice, which indicates that *CPT1A* might be epigenetically regulated, in this case, causing a switch from lipid metabolism towards glucose metabolism. (45)

1.6 Experimental autoimmune encephalomyelitis (EAE)

EAE is a commonly used model to investigate demyelinating diseases such as MS. (46,47) One method to induce EAE is to inject myelin oligodendrocyte glycoprotein (MOG)-peptide or myelin basic protein (MBP), which leads to demyelination, inflammation, and clinical symptoms. (31,46) MOG or MBP is often injected subcutaneously with the presence of an adjuvant inducing phagocytosis. In EAE, lesions are found in cerebral cortex, cerebellum, optic nerve, and spinal cord. (46)

1.7 Aim and hypotheses

CPT1A might play a role in the development of multiple sclerosis due to a switch from glucose metabolism to fatty acid metabolism. Furthermore, it is known that *CPT1A* mutation decreases the risk of developing MS, although the concordance rate is only demonstrated to be 20% in monozygotic twins. Hence, *CPT1A* and *CPT1C* might be regulated by other mechanisms. The pathophysiology of MS also indicates disruption of the BBB. *CPT1C* is brain specific, whereas it is interesting to investigate the expression of the gene in BBB. However, to the author's knowledge, no study has investigated the presence of CPT1A and CPT1C in the BBB and the epigenetic regulation of *Cpt1a* and *Cpt1c.* Therefore, the purpose of this study is to characterize the epigenetic landscape of *Cpt1a* and *Cpt1c* both *in vitro* and *in vivo* and investigate the possibility of pharmaceutical intervention in order to influence *Cpt1* expression and obtain a therapeutic effect. The hypothesis is that *Cpt1* genes are epigenetically regulated by e.g. DNA methylation and (or) histone modifications. Pharmacological intervention altering the epigenetic modification will lead to a decrease in *Cpt1* expression and as a consequence a shift in metabolism towards glucose consumption, thereby protecting neurons from demyelination.

The aim of the in vitro study was to:

- Culture primary cells associated with the blood-brain barrier, i.e. rat brain capillary endothelial cells, astrocytes, and pericytes
- Investigate the expression of *Cpt1a* and *Cpt1c* in these cells
- Investigate the expression of *Cpt1a* and *Cpt1c* after treatment with HDAC inhibitors
- Investigate the DNA methylation pattern

The aim of the in vivo was to:

- Investigate the expression of *Cpt1a* and *Cpt1c* in cerebrum from WT mice and EAE mice
- Investigate the DNA methylation pattern and histone modifications

2 Methods

The present study investigated the expression of *Cpt1a* and *Cpt1c* in primary rat endothelial cells, pericytes, and astrocytes to clarify the gene expression in the blood brain barrier. Furthermore, the study also investigated the expression of *Cpt1a* and *Cpt1c* in wild type (WT) mice, mutation mice (Cpt1a +/-), and EAE mice. The epigenetic landscape of *Cpt1a* and *Cpt1c* was investigated by DNA methylation analysis and chromatin immunoprecipitation (ChIP).

2.1 Study design and sample population

The study consisted of an *in vitro* and *in vivo* sub-study.

2.1.1 *In vitro* model: Primary cell model and cell culturing

This sub-study consisted of the rat primary cells associated with the BBB; endothelial cells, pericytes, and astrocytes.
Brain capillary endothelial cells (BCEC) were isolated from 2-3 weeks old Sprague-Dawley rats. Primary BCECs were cultured in a T75 bottle coated with fibronectin/collagen IV and further cultured in Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F12, Glutamax, Invitrogen, cat no 31331-028) with 10% plasma derived bovine serum (First Link, Wolverhampton, United Kingdom, UK), heparin (Sigma-Aldrich), insulin, transferrin, and sodium selenite (Roche), gentamicin sulphate (10 µg/ml), and basic fibroblast growth factor (1 ng/ml, Roche). To ensure a pure BCEC culture, puromycin (1 µl/ml, Sigma-Aldrich) was added to the medium once and left for three days. The cells were cultured in an incubator with 5% CO_2 and 95% O_2 at 37°C until confluent. After three days or when the cells reached the desired confluency, the cells were washed three times with phosphate buffered saline (PBS), trypsinized (Gibco, cat. no 15090), and cultured in 12-well plates (Appendix 1).

Primary pericytes were also obtained from 2-3 weeks old Sprague-Dawley rats. The capillaries containing both BCEC and pericytes were cultured in an uncoated T75 bottle in DMEM (low glucose, GlutaMAX(TM), Invitrogen, cat no. 10106-169), fetal calf serum (Gibco, cat. no 10106-169), and gentamicin sulphate (10 µg/ml, cat. No 17-518Z) cultured in an incubator with 5% CO_2 and 95% O_2 at 37°C in approximately 10 days or until confluent. Afterwards, the cells were trypsinized (Gibco, cat. no 15090) and cultured in 12-well plates. (Appendix 2)

Primary astrocytes were isolated from neonatal Sprague-Dawley rats and cultured in a poly-L-lysine-coated T75 bottle in DMEM (low glucose, GlutaMAX(TM), Invitrogen, cat no. 10106-169), fetal calf serum (Gibco, cat. no 10106-169), and gentamicin sulphate (10 µg/ml, cat. No 17-518Z). The cells were further cultured in an incubator with 5% CO_2 and 95% O_2 until confluent. Afterwards, the cells were trypsinized (Gibco, cat. no 15090) and cultured in 12-well plates (Appendix 3).

After cell culturing the cells were either used as control cells, by which the media was removed and the cells was frozen at -80°C, or as treated cells. The cells were treated with 2 mM and 4 mM sodium butyrate (SB) (Sigma-Aldrich) or sodium valproate (VPA) (Sigma) for 6- and 24 hours in the appropriate media. Each group consisted of three samples.

2.1.2 *In vivo* model: EAE mice experiment

This sub-study consisted of two animal experiments conducted by PhD student Anne Mørkholt. The study is approved by the Danish Experimental Animal Inspectorate under the Ministry of Food and Agriculture (2017-15-0201-01240). All mice used were C57/BL6 mice. As seen in table 2.1, the first study consisted of WT mice (Control/Cpt1a) and heterozygotic CPT1A (Cpt1a +/-) mice. The WT mice are in house bred mice originating from a Cpt1a +/- mother (Control/Cpt1a).
All mice were subcutaneously immunized with 100 µg MOG and Freud's adjuvant (CFA) in the concentration 1:1 and intraperitoneally with 200 ng of pertussis toxin (Ptx). Control/Cpt1a mice were treated with either placebo or etomoxir (Control/Cpt1a EAE placebo and Control/Cpt1a EAE etomoxir).

Table 2.1: *Overview of the three groups in the first study. All groups were immunized with MOG. Control/Cpt1a mice were either treated with either placebo or etomoxir (*Control/Cpt1a EAE placebo and Control/Cpt1a EAE etomoxir*) and Cpt1a +/- did not receive any treatment. (Abbeviations; Cpt1a, Carnitine palmitoyl transferase 1a; MOG, Myelin oligodendrocyte glycoprotein).*

Group	Immunization/treatment	No.
Control/Cpt1a EAE placebo	MOG, placebo	4
Control/Cpt1a EAE etomoxir	MOG, etomoxir	5
Cpt1a +/- EAE	MOG	5

The mice were scored from 0-4 on a disease score, due to ethical reasons the animals were put down when reaching a disease score of 4. None of the mice reached a score above 1, which indicates that the animals did not get appropriately immunized with EAE. Therefore, another study was conducted. The second study consisted of in house bred WT mice (Control/Cpt1a) and WT mice (Control). The mice were subcutaneously immunized with either 200 µg MBP or MOG and CFA in the concentration 1:1 and injected intraperitoneally with 400 ng Ptx as seen in table 2.2.

Table 2.2: *Overview of the four groups in the second study. Control/Cpt1a and Control were induced with either MOG or MBP. (Abbreviations; Cpt1a, Carnitine palmitoyl transferase 1a; MOG, Myelin oligodendrocyte glycoprotein; MBP, Myelin basic protein).*

Group	Immunization	No.
Control/Cpt1a	MOG	4
Control/Cpt1a EAE	MBP	4
Control	MOG	4
Control EAE	MBP	4

The mice were scored from 0-4 on a disease score. Only two of the animals from Control/Cpt1a immunized with MBP (Control/Cpt1a EAE) reached a score of 2. Therefore, the mice immunized with MBP were used for further analysis.

To investigate the epigenetic background and the difference in expression of *Cpt1a* and *Cpt1c* in the EAE model, 4 WT mice from a non-CPT1A background (Control) and 3 WT mice from a CPT1A background (Control/Cpt1a) were not immunized with EAE and used as controls, as seen in table 2.3.

Table 2.3: *Overview of controls. None of the animals are immunized. (Abbreviations; Cpt1a, Carnitine palmitoyl transferase 1a).*

Group	Immunization	No.
Control/Cpt1a	Not immunized	3
Control	Not immunized	4

2.2 Expressional analyses

Expressional analyses were performed to determine the expression of *Cpt1a* and *Cpt1c in vitro* and *in vivo* in all groups.

2.2.1 Purification of RNA and DNA

RNA and DNA from all samples were purified using AllPrep DNA/RNA Mini kit (Qiagen, cat no 80204) according to the manufacture's protocol (Appendix 4). As seen on figure 2.1, 30 mg of animal tissue was used and disrupted/homogenized with a rotor stator homogenizer in the appropriate amount of Buffer RLT Plus and β-mercaptoethanol. The tissue suspension was centrifuged for 3 min at maximum speed and the supernatant was used further on. Primary cells were lyzed in the appropriate amount of Buffer RLT Plus and β-mercaptoethanol and homogenized by vortexing. The homogenized lysate was placed on an AllPrep DNA spin column in a 2 ml collection tube and centrifuged 30 s at 10,000g. The flow-through was used for RNA purification.

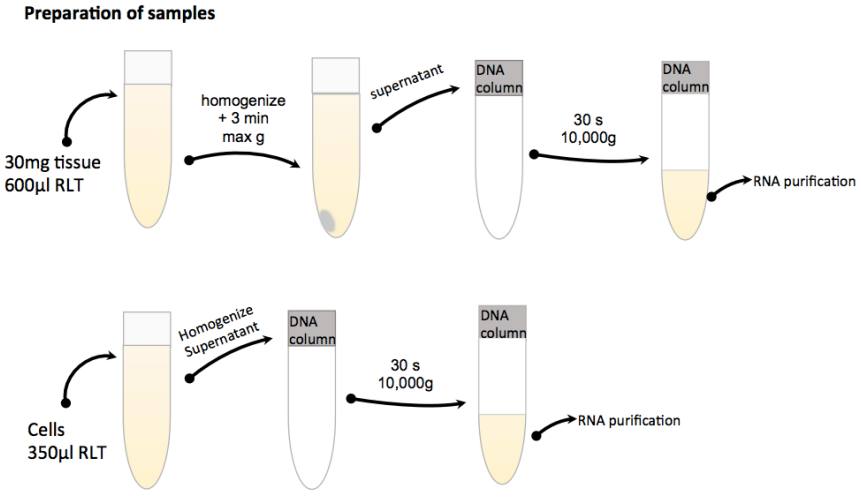

Figure 2.1: *Preparation of RNA and DNA purification from tissue or primary cells.*

As seen on figure 2.2, 1 volume of 70% ethanol was added to the flow-through and mixed. The RNeasy spin column was placed in a vacuum and the sample was transferred to the column. The column was washed with buffer RW1 and RPE and placed in a 1.5 ml collection tube. 30 µl RNase-free water was added directly on the membrane and the sample was centrifuged for 1 min at 10,000g to elute RNA. The DNA spin column was placed in the vacuum and washed with buffers AW1 and AW2. The column was placed in a 1.5 ml collection tube and 100 µl buffer EB was added, following centrifugation for 1 min at 10,000g to elute DNA. The concentration of the eluted RNA and DNA was measured using a spectrophotometer (DeNovix, DS-11 FX).

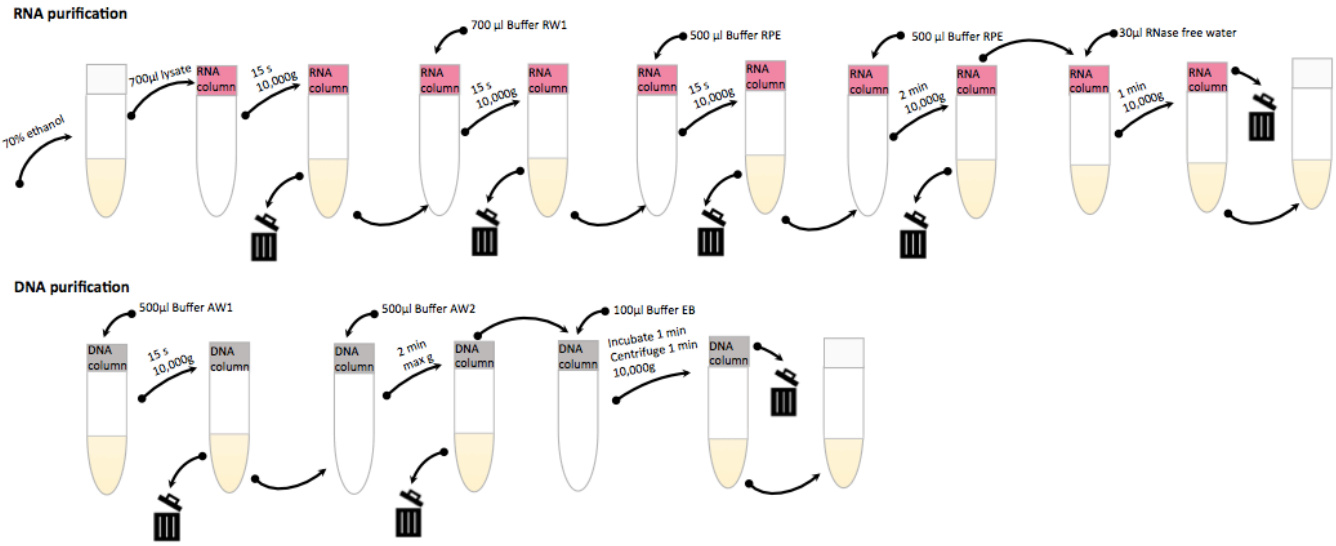

Figure 2.2: *Overview of RNA and DNA purification.*

2.2.2 DNase treatment and complementary DNA synthesis

Genomic DNA was removed from RNA preparations using a DNase kit (Thermo Scientific, cat. no EN0525) following the manufacturer's protocol (Appendix 5). In an RNase-free tube, 500 ng RNA, 1 µl 10X reaction buffer with $MgCl_2$, 1 µl DNase I, and DEPC-treated water to 10 µl was added. The sample was incubated for 30 min at 37°C. 1 µl 50 mM ethylenediaminetetraacetic acid (EDTA) was added to each sample and incubated for 10 min at 65°C. Complementary DNA (cDNA) was synthesized using Maxima H Minus First Strand cDNA synthesis kit (Thermo Scientific, cat no K1651) according to the manufacturer's protocol (Appendix 6). A mastermix containing oligo $(dT)_{18}$ primer and random hexamer primer, 10 mM dNTP mix, and 5X RT buffer was mixed. In each tube the appropriate amount of RNA (to 100 ng cDNA), 5.5 µl master mix, 1 µl Maxima H minus enzyme mix and ddH_2O to 20 µl was added and briefly centrifuged. The samples were incubated for 10 min at 25°C followed by 15 min at 50°C and 5 min at 85°C.

2.2.3 Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) was performed to determine the expression of the genes of interest, *Cpt1a* and *Cpt1c*, using an AriaMX qPCR

machine. Normalization of the samples was performed using hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) and/or *actin-β*. Mouse *Cpt1a* and *Cpt1c* and rat *Cpt1c* primers were designed using the pick-primers-tool on NCBI's webpage, rat *Cpt1a* was ordered from Qiagen. All primers were tested by standard curves and the most appropriate primers were used for RT-qPCR as seen in table 2.4.

Table 2.4: *Primers used for reverse-transcriptase quantitative polymerase chain reaction (Abbreviations; CPT1A/C, Carnitine palmitoyl transferase 1A/C)*

Primer name	Forward primer	Reverse primer
CPT1A mouse	TGCAGACTCGGTCACCACTCA	GGCTTCATGGCTCAGGCGGA
CPT1C mouse	AAGACCTGGCTGGCATTGGT	AGCACAGGGCGTACAGACTC
CPT1A rat (Qiagen)	N/a	N/a
CPT1C rat	AAGACCTGGCTGGCATTGGT	CACAGGGCGCATGGACTCTA

All samples were measured in triplicates. In each well 0.5 µl cDNA, 9.5 µl ddH_2O and 10 µl master mix containing 0.1 µl of forward and reverse primer and 10 µl SYBR green per well were added. The plate was shaken and centrifuged for 1 min at 1000g. The thermo profile used was 10 min hot start and amplification of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C for 40 cycles.

The *in vitro* samples were normalized using *Hprt1* and the *in vivo* samples were normalized with *Hprt1* and *actin-β*. The relative expression of the genes is calculated from the following formula.

$$Relative\ expression = \frac{\left(1 + eff_{target}\right)^{-Cq(target)}}{geometricmean((1 + eff_{HK1})^{-Cq(HK1)}; (1 + eff_{HK2})^{-Cq(HK2)})}$$

2.3 Immunocytochemistry

After the expression analyses cells were cultured and stained for CPT1A by immunocytochemistry (ICC) as demonstrated in appendix 7. Cells were cultured as described previously, however, on coverslips in 24-well plates, and astrocytes were cultured in serum-free media to ensure glial fibrillary acidic protein (GFAP)-positive cells. When confluent the cells were treated with either 2 mM SB or 2 mM VPA for 6 h.

The cells were washed 2 times in PBS prior to fixation with 4% paraformaldehyde for 5 min and washed two times in PBS. The cells were stored prior to immunostaining in PBS at 4°C.

Immunostaining were performed for endothelial cells and astrocytes by adding 0.5 ml incubation buffer containing 0.1% Triton X in PBS to each well for 30 min on a rocking shaker. For pericytes the incubation buffer consisted of 3% BSA and 0.3% Triton X in PBS. The buffer was removed and the primary antibody was added in a concentration of 1:200 in incubation buffer and incubated for 1 h at room temperature on a rocking shaker. All cells were immunostained for CPT1A. Moreover, astrocytes were stained for the marker GFAP, pericytes were stained for α-smooth muscle actin (α-

SMA), and endothelial cells were stained for zona occludens-1 (ZO-1). All samples were washed 2 times for 5 min with washing buffer containing 1% BSA diluted 1:3 in PBS. Secondary antibodies were added with incubation buffer in concentration 1:500 and incubated for 1 h at room temperature on a rocking shaker. The respective secondary antibodies can be seen in table 2.5. A secondary antibody-control was performed for each cell type to ensure specific binding of the antibody (Appendix 8). Cells were washed for 5 min in PBS followed by 4',6-diamidino-2-phenylindole (DAPI)-staining.

Table 2.5: *Overview of primary and secondary antibodies used for immunocytochemistry. (Abbreviations; CPT1A, Carnitine palmitoyl transferase 1A; GFAP, Glial fibrillary acidic protein; ZO-1, Zona occludens-1; α –SMA, α-smooth muscle actin).*

Protein	Primary antibody	Secondary antibody
CPT1A (Endothelial cells and astrocytes)	Anti-CPT1A (abcam, ab128568)	Goat anti-mouse (Invitrogen, A11032)
CPT1A (Pericytes)	Anti-CPT1A (Proteintech, 15184-1-AP)	Goat anti-rabbit (Invitrogen, A11037)
GFAP (Astrocytes)	Anti-GFAP (Dako, Z0334)	Goat anti-rabbit (Invitrogen, A11034)
ZO-1 (Endothelial cells)	Anti-ZO1 (Invitrogen, 61-7300)	Goat anti-rabbit (Invitrogen, A11034)
α –SMA (Pericytes)	Anti-α-SMA (Sigma, A5228-2)	Donkey anti-mouse (Invitrogen, A21202)

The DAPI solution was diluted 1:500 in PBS, added to the wells and incubated for 4 min. The cells were washed two times in PBS and left in PBS after the final wash. The coverslips were mounted with the cells facing down on object slides using DAKO fluorescent mounting media and a special needle.

Pictures of the cells were conducted using an inverted Zeiss-microscope and edited in ImageJ. The assessment of the protein level was blinded. The author randomly numbered the groups, whereas two experts assessed the protein level.

2.4 Epigenetic analyses

DNA methylation analysis and ChIP were performed to determine the epigenetic state of *Cpt1a* and *Cpt1c in vitro* and *in vivo.*

2.4.1 Primer design for epigenetic analyses

Primers for epigenetic analyses were produced based on CpG islands for DNA methylation analysis (table 2.6 *in vitro* and table 2.7 *in vivo*) and up- or downstream for CpG islands as well as areas with transcription factor bindings found on Genome Browser for ChIP (Table 2.8). The primers were designed using BiSearch primer design, tested, and the most appropriate primers were used for analysis. An overview of the different regions and CpG islands can be seen on figure 2.3 *in vitro* and figure 2.4 *in vivo*.

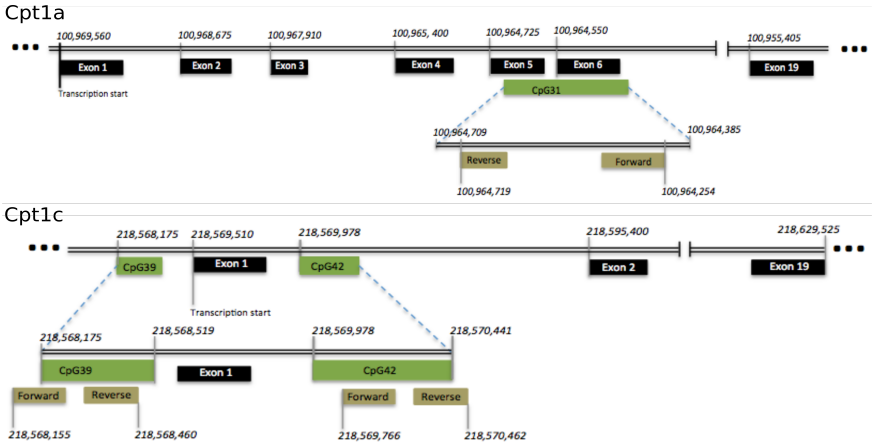

Figure 2.3: Overview of the CpG islands used for DNA methylation analysis in Cpt1a and Cpt1c in vitro. (Abbeviations; Cpt1a/c, Carnitine palmitoyl transferase 1a/c).

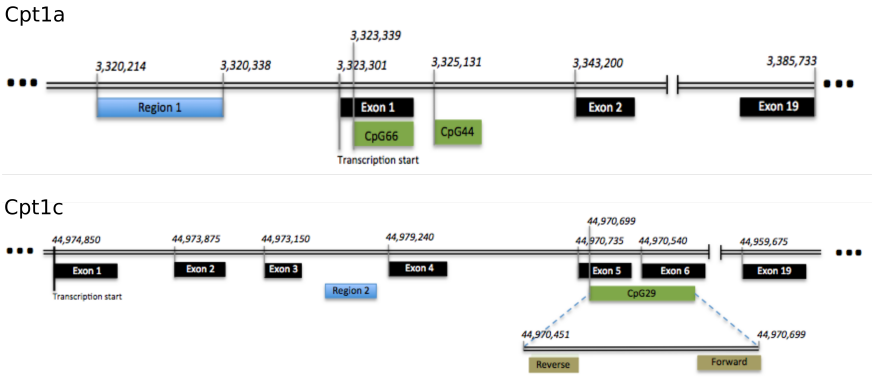

Figure 2.4: Overview of the regions used for ChIP and CpG islands in Cpt1a and Cpt1c in vivo. Only CpG islands for Cpt1c was used for DNA methylation analysis. (Abbreviations; Cpt1a/c, Carnitine palmitoyl transferase 1a/c).

2.4.2 DNA methylation analysis

To investigate whether the CpG islands of *Cpt1a* and *Cpt1c* are methylated, EpiTect Methyl II DNA Restriction Kit (Qiagen) was used (Appendix 9). Samples from the *in vitro* study consisted of DNA from control cells, cells treated with 2 mM SB for 6 h, and cells treated with 2 mM VPA for 6 h, whereas samples from the *in vivo* study consisted of Control and Control EAE. Prior to the procedure, all DNA samples were micro dialyzed. A reaction mix for each DNA samples was prepared from 250 ng DNA, 5x Restriction Digestion Buffer and RNase-/DNase-free water. Four digestion reactions were prepared with each 14 µl of the reaction mix and 1 µl water for M_o, 0.5 µl water and 0.5 µl methylation-sensitive enzyme for M_s, 0.5 µl water and 0.5 µl methylation-dependent enzyme for M_d, and 0.5 µl methylation-sensitive enzyme and 0.5 µl methylation-dependent enzyme for M_{sd}. All samples were incubated at 37°C overnight. A qPCR reaction was set up for each sample containing 1 µl digest and 9 µl water and 0.08 µl reverse and forward primer (see table 2.6 *in vitro* and table 2.7 *in vivo*) in 10 µl SYBR Green.

Table 2.6: Overview of the designed primers used for DNA methylation analysis in vitro. (Abbreviations; Cpt1a/c, Carnitine palmitoyl transferase 1a/c).

Gene	Forward primer	Reverse primer
***Cpt1a*, CpG 39**	TGGCAGGCTGTTTTGTTGCATG	GAGGGAAGGGCACTAGCAACC
***Cpt1a*, CpG 42**	GCTGGGCTCAGCCAATCACC	TCTGGGGACCAAGCATCTTTCC
***Cpt1c*, CpG 31**	GGGCGCATGGACTCTAGG	CCCGAGGCTCTTCAGC

Table 2.7: Overview of the designed primers used for DNA methylation analysis in vivo. (Abbreviations; Cpt1c, Carnitine palmitoyl transferase 1c).

Gene	Forward primer	Reverse primer
***Cpt1c*, CpG 29**	CGTGGTGCGTGCAACC	GGAGGCACCCGAGGCT

The qPCR reaction was performed on an Aria MxPro machine with the amplification thermo profile on 30 s at 95°C, 30 s at 63°C, and 30 s at 72°C for 40 cycles. The methylation status of the samples was assessed from the manufacturer's formula (Appendix 10).

2.4.3 Chromatin immunoprecipitation

ChIP was performed according to appendix 11 to demonstrate the epigenetic state of histones. Three cerebra from Control and three from Control EAE were used.

All buffers were prepared according to the protocol. A piece of mouse cerebrum was transferred to 10 ml cold extraction buffer 1 and homogenized using an Ultra Turrax T25 following centrifugation in 20 min, 3000g, 4°C. The supernatant was discarded and the pellet was resuspended in 1 ml extraction buffer 2 and centrifuged in 10 min, 12000g, 4°C. The pellet was resuspended in dilution buffer, transferred to non-stick tubes and the amount of chromatin is measured using a nanophotometer. 0.5 mg/ml chromatin was used and 50U of micrococcal nuclease was added, following digestion at 37°C, 600rpm, 4 min. The digestion was stopped by adding EDTA and the sample was centrifuged 5 min, 11600g, 4°C. The supernatant was transferred to a new tube (S1) and the pellet was resuspended in resuspension buffer and dialyzed in 4 L resuspension buffer overnight at 4°C on a magnetic stir.

Table 2.8: Antibodies used for Chromatin immunoprecipitation. (Abbreviations; H4AC, Histone 4 acetylation; H3K4me3, Histone 3 lysine 4 trimethylation; H3K27me3, Histone 3 lysine 27 trimethylation; H3K9me3, Histone 3 lysine 9 trimethylation).

Sample	Antibody
Mock	Rabbit serum
H4Ac	Anti-H4Ac, rabbit pAb (Upstate cell signaling solutions, Cat 06-598)
H3K4me3	Anti-H3K4me3, rabbit pAb (abcam, ab8580)
H3K27me3	Anti-H3K27me3, mouse mAb (abcam, ab6002)
H3K9me3	Anti-H3K9me3, rabbit pAb (abcam, ab8898)

The sample was transferred from the dialysis tube to an eppendorf tube, centrifuged for 10 min at 2000g and 4°C, and pooled with S1. Antibodies were added to the samples according to table 2.8 and incubated overnight at 4°C. Dynabeads Protein A (Invitrogen) was prepared by washing 30 µl

of Dynabeads per sample three times with ChIP dilution buffer following resuspension in ChIP equilibration buffer and incubated overnight at 4°C in a rotator.

The beads were washed three times with ChIP dilution buffer and resuspended in the appropriate amount of dilution buffer. 30 µl of beads were added to each sample and incubated 3 h at 4°C in a rotator. 100 µl of the supernatant from mock was used as input DNA, the rest of the supernatants were discarded. The pelleted beads were washed with washing buffers 10 min at 4°C in a rotator (see protocol for further details). 400 µl elution buffer was added to the input DNA. 250 µl Elution Buffer was added two times to the samples, vortexed, and incubate 15 min at 65°C with an agitation at 1000 rpm.

DNA was purified using NucleoSpin Gel and PCR clean-up (Macherey-Nagel) according to the manufacturer's protocol (Appendix 12). DNA binding conditions was adjusted by mixing 1 volume of sample with 5 volumes of buffer NTB. The sample was loaded to a NucleoSpin Gel and PCR clean-up column in a 2 ml collection tube and centrifuged 30 s at 11,000g. The membrane was washed two times with buffer NT3 and centrifuged 30 s at 11,000g. The flow-through was discarded and the membrane dried by centrifugation 1 min at 11,000g. The column was placed in a 1.5 ml eppendorf tube and 15 µl buffer NE was added. The sample was incubated 1 min at room temperature and centrifuged for 1 min at 11,000g to elute DNA. The eluted DNA was used for qPCR. A qPCR reaction is set up for each sample containing 1 µl digest and 9 µl water and 0.08 µl reverse and forward primer (see table 2.9) in 10 µl SYBR Green.

Table 2.9: *Overview of the designed primers used for Chromatin immunoprecipitation. (Abbreviations; Cpt1a/c, Carnitine palmitoyl transferase 1a/c).*

Gene	Position	Forward primer	Reverse primer
Cpt1a (region 1)	Chr19: 3,320,214-3,320,338	GTTTGTTATCCTTGCGACAATG	AGGCCCCACTGAACTTGAA
Cpt1c (region 2)	Chr7: 44,971,556 – 44,971,705	TAGCTCATCTCCCTGCTTCC	CCAAGCTTCTAAAATCACTTTCCT

Results from ChIP was analyzed from the following formula:

$$Fraction\ IP = 2^{Ct(Input)-2.2-Ct(ab)}$$

2.2 correspond to a dilution factor of 5.

2.5 Statistical procedure

All statistical analyses and graphs were performed in Prism GraphPad. One-way analysis of variance (ANOVA) with Tukey's post hoc test was performed for the *in vitro* subjects for comparison of the gene expression between groups for *Cpt1a* and *Cpt1c*, respectively. Two-way ANOVA with a Sidac post hoc test was performed for comparison of expression of *Cpt1a* and *Cpt1c* between groups *in vivo*. Two-way ANOVA with Sidac post hoc test was used for comparison

of ChIP results between the antibodies. $P<0.05$ is marked with an asterisk, $p<0.01$ is marked with two asterisks, $p<0.001$ is marked with three asterisks, and $p<0.0001$ is marked with four asterisks.

3 Results

3.1 *In vitro* study

The present *in vitro* study is the first approach aimed to investigate the expression of *Cpt1a* and *Cpt1c* in the rat primary cells of the BBB: endothelial cells, astrocytes, and pericytes. The expression of *Cpt1* genes was tested before and after treatment with HDACi, which can indicate if an epigenetic regulation through histone acetylation is involved in the transcriptional activation/repression of the genes. The investigations were primarily performed by RT-qPCR. Furthermore, the study also aimed at investigating DNA methylation as the other important factor in the epigenetic landscape.

3.1.1 Expression of *Cpt1a* and *Cpt1c*

In order to investigate the general level of expression of *Cpt1a* and *Cpt1c* in BBB-associated cells, RT-qPCR was performed on rat endothelial cells, astrocytes, and pericytes.

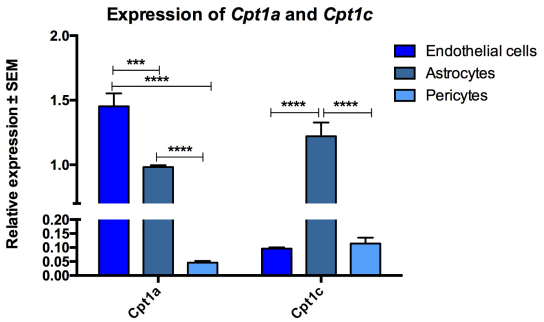

Figure 3.1: *The expression of Cpt1a and Cpt1c in endothelial cells, pericytes, and astrocytes. The results are presented as mean ± SEM. *** p<0.001, **** p<0.0001. (Abbreviations; Cpt1a/c, Carnitine palmitoyl transferase 1a/c)*

As seen on figure 3.1, endothelial cells presented with the highest expression of *Cpt1a* compared to astrocytes and pericytes ($p<0.001$ and $p<0.0001$, respectively). Pericytes presented with the lowest expression of *Cpt1a* compared to endothelial cells and astrocytes ($p<0.0001$ and $p<0.0001$, respectively). Astrocytes presented with the highest expression of *Cpt1c* compared to endothelial cells and pericytes ($p<0.0001$ and $p<0.0001$, respectively). Furthermore, endothelial cells presented with the lowest expression of *Cpt1c*.

In order to investigate the level of expression and the possible effect of treatment with HDACi of primary rat endothelial cells, astrocytes, and pericytes, the expression of *Cpt1a* and *Cpt1c* was examined by RT-qPCR. All samples were normalized with *Hprt1*. The cells were treated with 2- or 4 mM SB and VPA for 6 h and 24 h, after which the expression of *Cpt1a* and *Cpt1c* was investigated.

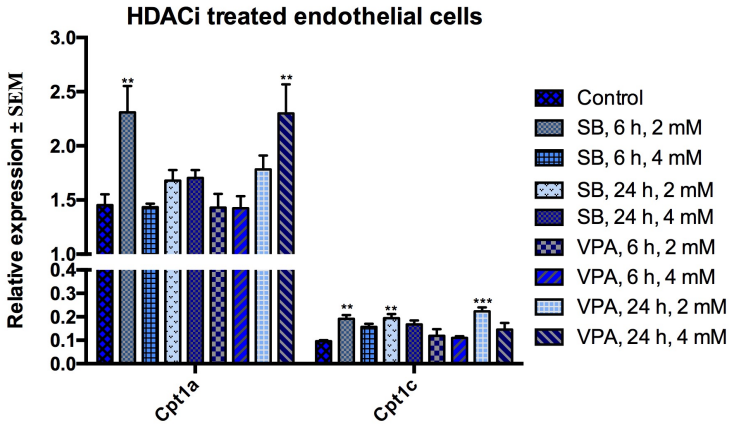

Figure 3.2: *The expression of Cpt1a and Cpt1c in rat endothelial cells after treatment with HDACi (2- and 4 mM SB or VPA for 6- and 24 h). The results are presented as mean ± SEM. ** indicates p<0.01 and *** indicates p<0.001. N=3 in each group. (Abbreviations; Cpt1a/c, Carnitine palmitoyl transferase 1a/c; HDACi, Histone deacetylase inhibitor; SB, Sodium butyrate; VPA, Valproic acid)*

Endothelial cells presented with an increased expression of *Cpt1a* when treated with 2 mM SB after 6 hours and 4 mM VPA after 24 hours compared to controls ($p<0.01$ and $p<0.01$, respectively). Moreover, the expression of *Cpt1c* was also increased after treatment with 2 mM SB after both 6 and 24 hours and 2 mM VPA after 24 hours compared to controls ($p<0.001$, $p<0.001$, and $p<0.0001$, respectively) (Figure 3.2). This indicates that the inhibition of HDAC prevents the deacetylation of histones as expected, hence, an increased expression of the genes. These results indicate that *Cpt1a* and *Cpt1c* are potentially epigenetically regulated by means of histone acetylation.

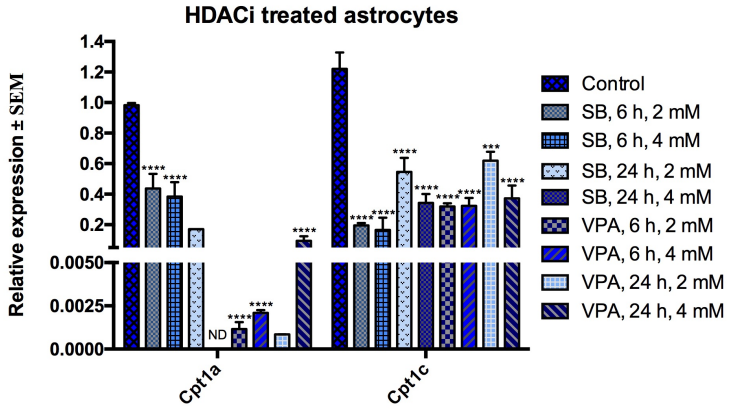

Figure 3.3: *The expression of Cpt1a and Cpt1c in rat astrocytes after treatment with HDACi (2- and 4 mM SB or VPA for 6- and 24 h). The results are presented as mean ± SEM. N=3 in each group. *** indicates p<0.001 and **** indicates p<0.0001. N=3 in each group, except for Cpt1a SB, 24 hours, 2 mM and VPA, 24 hours, 2 mM, whereas two out of three samples were non-detectable. (Abbreviations; Cpt1a/c, Carnitine palmitoyl transferase 1a/c; HDACi, Histone deacetylase inhibitor; SB, Sodium butyrate; VPA, Valproic acid).*

The primary rat astrocytes presented with a significantly decreased expression of *Cpt1a* after all treatments ($p<0.0001$). Moreover, a decreased expression of *Cpt1c* was demonstrated after both treatments compared to the controls ($p<0.0001$ for all samples except VPA 24 h 2mM, which

presented a p<0.001) (Figure 3.3). This unexpected result might indicate an indirect effect, probably through deinhibition of a transcriptional repressor of *Cpt1a* and *Cpt1c* genes.

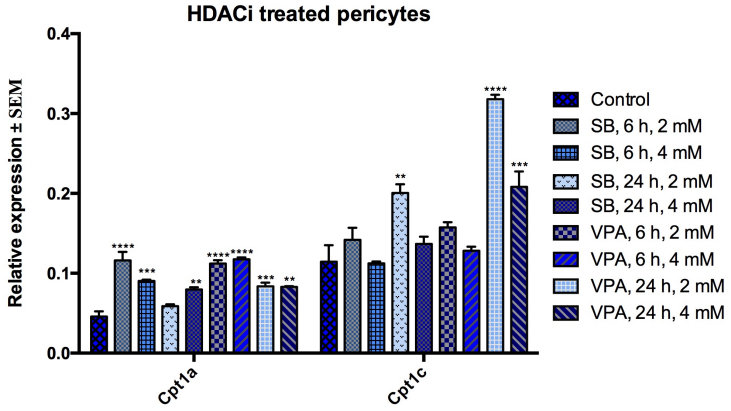

Figure 3.4: *The expression of Cpt1a and Cpt1c in rat pericytes after treatment with HDACi (2- and 4 mM SB or VPA for 6- and 24 h). The results are presented as mean ± SEM. ** indicates p<0.01, *** indicates p<0.001, and **** indicates p<0.0001. N=3 in each group. (Abbreviations; Cpt1a/c, Carnitine palmitoyl transferase 1a/c; HDACi, Histone deacetylase inhibitor; SB, Sodium butyrate; VPA, Valproic acid)*

Pericytes presented with an increased expression of *Cpt1a* after treatment with SB and VPA after both 6 and 24 hours, except treatment with 2mM SB for 24 hours, compared to controls (p<0.0001 for SB (6 h 2 mM), VPA (6 h 2 mM), and VPA (6 h 4 mM), p<0.001 for SB (6 h 4 mM) and VPA (24 h 2mM), and p<0.01 for SB (24 h 4 mM) and VPA (24 h 4 mM)) (Figure 3.4). This indicates a fast and long-lasting effect of the epigenetic regulation. Furthermore, the expression of *Cpt1c* is increased in both treatments after 24 hours, except for SB (24 h, 4 mM), compared to controls (p<0.01, p<0.0001, and p<0.001, respectively) indicating a slower regulation compared with *Cpt1a*.

3.1.2 Protein expression

The expression analysis demonstrated an increased expression of *Cpt1a* and *Cpt1c* in the rat primary endothelial cells and pericytes after treatment with HDACi, whereas astrocytes demonstrated a decreased expression of both genes after treatment with HDACi. Therefore, it is interesting to investigate if the increased or decreased transcript expression is reflected on CPT1A protein level as well. To test this ICC was performed. The treatment of the cells was chosen based on the mRNA expression analysis. For simplicity, all cells were treated with 2 mM SB for 6 h and 2 mM VPA for 6 h.

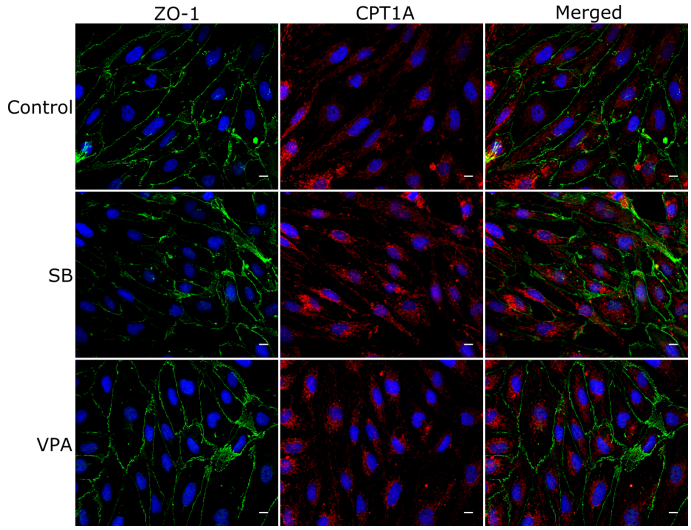

Figure 3.5: *Immunocytochemistry of endothelial cells: Controls, 2 mM SB for 6 h, and 2 mM VPA for 6 h. The endothelial marker ZO-1 is stained with green, CPT1A is stained with red, and the nucleus is stained with DAPI (blue). The scale bar indicates 10µm. (Abbreviations; SB, Sodium butyrate; VPA, Valproic acid; ZO-1, Zona occludens-1; CPT1A, Carnitine palmitoyl transferase 1A).*

The blinded qualitative assessment of the protein level in endothelial cells concluded an increased protein level after treatment with SB compared to controls (Figure 3.5). This supports the finding of an increased mRNA expression after treatment with 2 mM SB for 6 hours. The increased CPT1A protein level should lead to a switch from glucose towards increased lipid metabolism, which would be interesting to test in the future. Furthermore, a decreased level of protein was found after treatment with 2 mM VPA for 6 hours compared to controls (Figure 3.5). This finding does not completely correlate with the finding from mRNA expression, which showed an equal expression after treatment with 2 mM VPA for 6 hours compared to controls. The location of the protein of widely spread over the cells except from the nucleus.

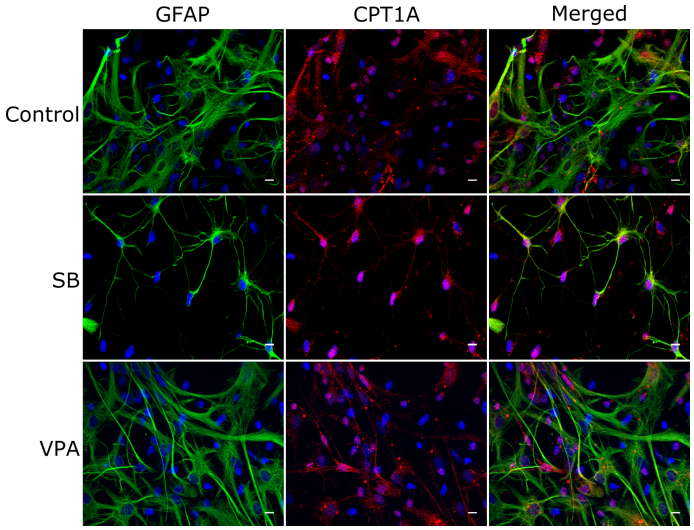

Figure 3.6: *Immunocytochemistry of astrocytes: Controls, 2 mM SB for 6 h, and 2 mM VPA for 6 h. The astrocyte marker, GFAP, is stained with green, CPT1A is red, and the nucleus is stained with DAPI (blue). The scale bar indicates 10µm. (Abbreviations; SB, Sodium butyrate; VPA, Valproic acid; GFAP, Glial fibrillary acidic protein; CPT1A, Carnitine palmitoyl transferase 1A).*

The blinded assessment of the protein level concluded a decreased level of protein after treatment with both 2 mM SB for 6 hours and 2 mM VPA for 6 hours compared to the controls (Figure 3.6). This finding support the mRNA expression results, in which a decreased expression of *Cpt1a* was demonstrated after treatment with SB and VPA compared to controls. This further supports the idea of an indirect effect of the HDACi, as the opposite was expected when treating with HDACi. The location of the protein of widely spread over the cells except from the nucleus.

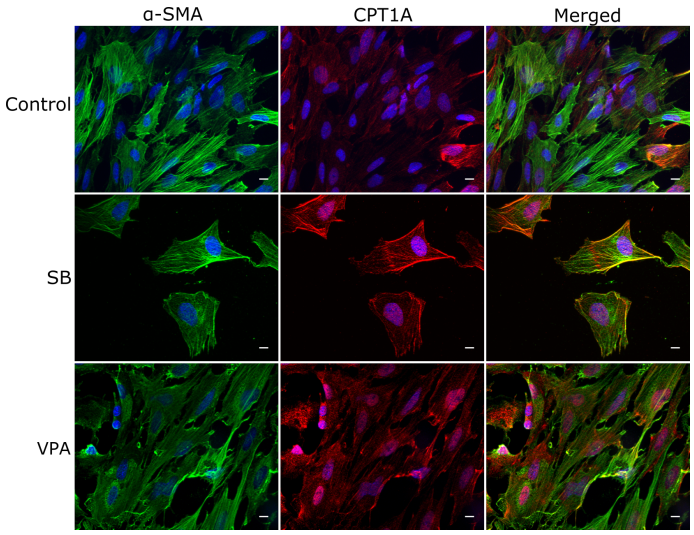

Figure 3.7: Immunocytochemistry of pericytes: Control, 2 mM SB for 6 h, and 2 mM VPA for 6 h. The pericyte marker α-SMA is green, CPT1A is red, and the nucleus is stained with DAPI (blue). The scale bar indicates 10μm. (Abbreviations; SB, Sodium butyrate; VPA, Valproic acid; α-SMA, α-smooth muscle actin; CPT1A, Carnitine palmitoyl transferase 1A).

Immunocytochemistry of pericytes demonstrated an increased protein concentration of CPT1A after treatment with SB and VPA compared to control with SB inducing the highest amount of the protein expression (Figure 3.7). These results support the mRNA expression results, in which *Cpt1a* after SB and VPA treatment has an increased expression compared to controls. This result is expected when treating the cells with HDACi. The location of the protein is widely spread over the cells for controls and VPA. However, for SB the protein is relocated towards the cell membrane.

3.1.3 DNA methylation analysis

Since the *Cpt1* gene expression was affected by the HDACi suggesting epigenetic regulation of both genes, the next study was set up aimed at investigating the DNA methylation state of *Cpt1a* and *Cpt1c* CpG islands. Three CpG islands were investigated: CpG39 (*Cpt1a*), CpG42 (*Cpt1a*), and CpG31 (*Cpt1c*) (Figure 2.3). The samples used were rat BBB-associated primary cells: controls, 2 mM SB for 6 h, and 2 mM VPA for 6 h, as these samples correspond to the samples used for ICC.

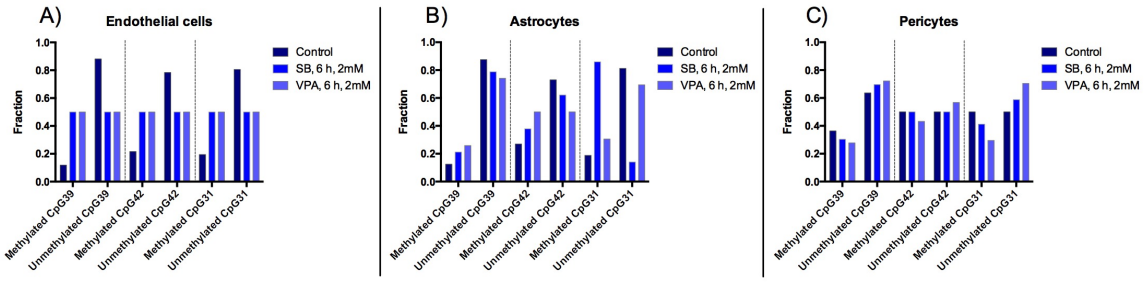

Figure 3.8: *Fraction of methylated and unmethylated DNA from A) endothelial cells, B) astrocytes, and C) pericytes. Samples used are control, SB (6 h, 2 mM), and VPA (6 h, 2 mM) for CpG39 (Cpt1a), CpG42 (Cpt1a), and CpG31 (Cpt1c). N=1 in each group (Abbreviations; SB, Sodium butyrate; VPA, Valproic acid).*

As exemplified on figure 3.8.A, endothelial cells presented with an increased fraction of DNA methylation after treatment with 2 mM SB and VPA for 6 h. This indicates, that the expression of both *Cpt1a* and *Cpt1c* should be decreased. However, this does not correspond with the expression results, whereas the expression is increased after treatment with 2 mM SB for 6 h and equal to controls after treatment with 2 mM VPA for 6 h.

Astrocytes demonstrated an increased fraction of DNA methylation in all CpG islands after both treatments (Figure 3.8.B). This indicates a decreased expression of *Cpt1a* and *Cpt1c*, which corresponds to the expression results.

Pericytes presented with a decreased fraction of DNA methylation status in CpG39 (*Cpt1a)* and CpG31 (*Cpt1c*) after treatment with SB and VPA for 6 hours. However, CpG42 (*Cpt1a*) only decreases after treatment with VPA compared to controls (Figure 3.8.C). Overall, this corresponds to the expression results, which demonstrated an increased expression of *Cpt1a* and *Cpt1c* after treatment. In CpG42 (*Cpt1a*) the equal DNA methylation status between control and SB does not correspond to the expression results.
The above results indicate changes in DNA methylation depending on the expression level of both genes. However, a more detailed study involving sequencing of the gene promoters is needed in order to determine the exact DNA methylation levels and CpG positions affected.

3.2 *In vivo* study

The *in vivo* study aimed at investigating the expression of *Cpt1a* and *Cpt1c* in the EAE mouse model of MS. Two sub-studies were performed due to lack of disease phenotypes in the first sub-study. Furthermore, the sub-study aimed at investigating the epigenetic state of *Cpt1a* and *Cpt1c,* which was demonstrated by DNA methylation analysis and ChIP.

3.2.1 Expression of *Cpt1a* and *Cpt1c*

Expression analysis was performed looking at the difference between Control/Cpt1a EAE placebo, Control/Cpt1a EAE etomoxir, and Cpt1a +/- EAE compared to Control/Cpt1a. For this purpose cerebrum was used.

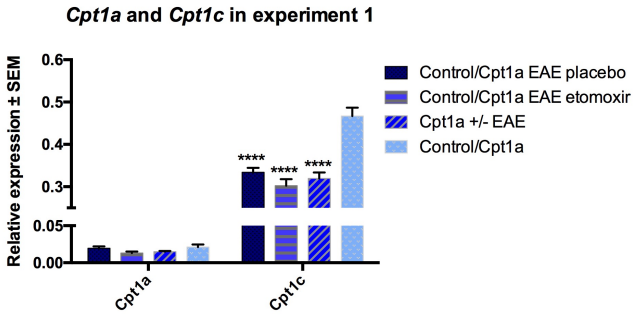

Figure 3.9: *Expression of Cpt1a and Cpt1c in the first study. The results are presented as mean ± SEM. **** indicates p<0.0001. n=4 Control/Cpt1a EAE placebo), n=5 (Control/Cpt1a EAE etomoxir), n=5 (Cpt1a +/- EAE), n=3 (Control/Cpt1a). (Abbreviations; Cpt1a/c, carnitine palmitoyl transferase 1a/c).*

An increased expression of *Cpt1a* in the Control/Cpt1a EAE placebo was expected, due to a hypothesis stating that MS pathogenesis starts with a shift towards lipid metabolism. Furthermore, it is interesting to look at the expression after inhibition of CPT1A by etomoxir. The expression results showed no difference between the groups in *Cpt1a* (Figure 3.9). For *Cpt1c,* a difference between Control/Cpt1a EAE placebo, Control/Cpt1a EAE etomoxir, and Cpt1a +/- EAE compared to Control/Cpt1a were demonstrated (p<0.0001 for all) (Figure 3.9). This indicates that the expression of *Cpt1c* decreases in EAE mice.

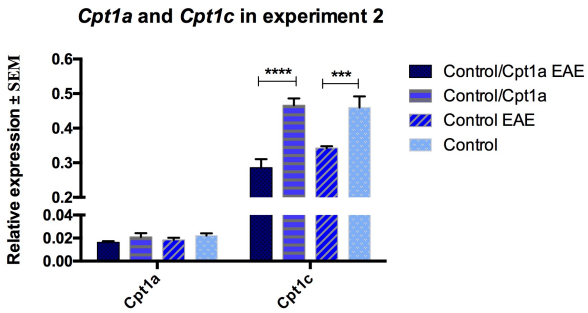

Figure 3.10: *Expression of Cpt1a and Cpt1c in the second study. The results are presented as mean ± SEM. *** indicates p<0.001 and **** indicates p<0.0001. n=4 (Control/Cpt1a EAE), n=3 (Control/Cpt1a), n=4 (Control EAE), n=4 (Control). (Abbreviations; Cpt1a/c; Carnitine palmitoyl transferase 1a/c; EAE, Experimental autoimmune encephalomyelitis).*

Due to variations in phenotypes in the first sub-study, a second study was set up. This sub-study included Control/Cpt1a EAE, Control/Cpt1a, Control EAE, and Control.
The results exhibit no difference between both Control/Cpt1a EAE and Control/Cpt1a and Control EAE and Control in *Cpt1a* expression indicating that the EAE model does not exhibit changes in gene expression of *Cpt1a.* For *Cpt1c* a significant decrease is demonstrated in Control/Cpt1a EAE compared to Control/Cpt1a (p<0.0001) and in Control EAE compared to Control (p<0.0001) (Figure 3.10). This indicates that the expression of *Cpt1c* decreases in the EAE mice.

3.2.2 Epigenetic analyses

This study aimed at investigating the epigenetic status of *Cpt1a* and *Cpt1c*, which was carried out by DNA methylation analysis and ChIP. As no difference was seen in the mRNA expression of *Cpt1a* the DNA methylation analysis was only performed for GpG islands in *Cpt1c*. ChIP was performed for regions near CpG islands in both *Cpt1a* and *Cpt1c,* whereas *Cpt1a* is used as an internal control.

DNA methylation analysis

DNA methylation analysis was performed to investigate the state of DNA methylation in the CpG island, CpG29, for *Cpt1c* (Figure 2.4).

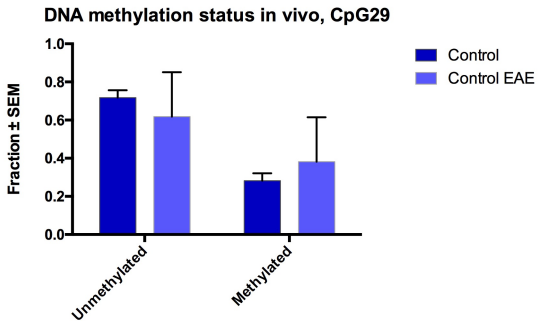

Figure 3.11: *Fraction of unmethylated and methylated DNA from Control and Control EAE in CpG island, CpG29, Cpt1c. The results are presented as mean ± SEM. N=3 (Control), n=2 (Control EAE). (Abbreviations; EAE, Experimental autoimmune encephalomyelitis).*

The DNA methylation analysis of CpG29 in *Cpt1c* demonstrated a tendency towards more methylated DNA in the EAE mice, indicating a more silenced gene as compared to controls (Figure 3.11). This result corresponds with the expression analysis as a decreased expression of *Cpt1c* is demonstrated in the EAE animals.

Chromatin immunoprecipitation

In order to investigate the state of histone modifications regions near CpG islands of *Cpt1a* and *Cpt1c* ChIP was performed on cerebrum from Control and Control EAE. As no difference in the expression of *Cpt1a* is demonstrated it is not expected to see any difference in histone modifications between EAE mice and controls, thus making *Cpt1a* a good internal control for the assay. However, since nothing is known about epigenetic landscape of *Cpt1a* from the literature the obtained results contribute to better understanding of the potential regulatory modifications of the *Cpt1a*.

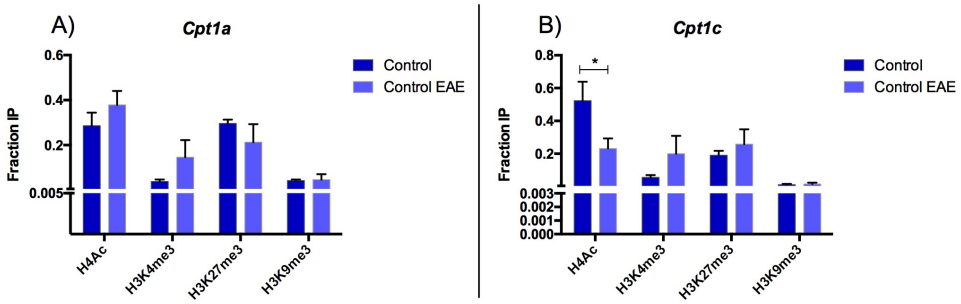

Figure 3.12: *Chromatin immunoprecipitation of Cpt1a and Cpt1c with H4Ac, H3K4me3, H3K27me3, and H3K9me3. The results are presented as mean ± SEM. N=3 in each group. (Abbreviations; Cpt1a/c, Carnitine palmitoyl transferase 1a/c; EAE, Experimental autoimmune encephalomyelitis; H4AC, Histone 4 acetylation; H3K4me3, Histone 3 lysine 4 trimethylation; H3K27me3, Histone 3 lysine 27 trimethylation; H3K9me3, Histone 3 lysine 9 trimethylation; IP, Input).*

No statistical significance was found between Control and Control EAE for *Cpt1a* for any of the investigated histone modifications (Figure 3.12.A). However, a tendency of a higher fraction of input is seen for H4Ac and H3K4me3 in the Control EAE, which might indicate more transcriptional activity. Nonetheless, no significant difference was found in the expression analysis between Control and Control EAE in the second sub-study, which does not correspond to this speculation.

Cpt1c presented with a statistical significant difference between Control and Control EAE for H4Ac (Figure 3.12.B). This indicates transcriptional repression of the gene in the Control EAE, which corresponds to the mRNA expression results, as a decreased expression of *Cpt1c* was demonstrated in the Control EAE (Figure 3.10). However, a higher fraction of input of H3K4me3 in the Control EAE compared to Control seems contradictory, as this would indicate an increased transcriptional activity, which is not the case for *Cpt1c* in Control EAE.

4 Discussion

The purpose of this study was to investigate role of *Cpt1a* and *Cpt1c* in the rat primary BBB-associated cells and in the mouse EAE model of MS. This was primarily performed by expression analysis of rat endothelial cells, astrocytes, and pericytes before and after treatment with HDACi. Furthermore, expression analysis was performed for the two *in vivo* sub-studies. In the first sub-study, the mice were treated with placebo and etomoxir and the analysis were performed before and after treatment. In the second sub-study, expression analysis of EAE mice and the corresponding controls were performed. Furthermore, this study aimed at investigating the epigenetic landscape of *Cpt1a* and *Cpt1c*, which was demonstrated by analysis of DNA methylations and histone modifications.

4.1 The expression of *Cpt1a* and *Cpt1c* in blood brain barrier-associated cells

To the author's knowledge no one has yet investigated the expression of *Cpt1a* and *Cpt1c* in BBB-associated cells. Therefore, it is interesting to investigate the expression of these two genes in endothelial cells, astrocytes, and pericytes. The expression of *Cpt1a* was found to be highest in endothelial cells and lowest in pericytes. The relatively low expression of *Cpt1a* in pericytes might be due to the function of the cell, which is primarily regulation of blood flow through contraction. (48,49) Furthermore, Héliès-Toussaint *et al* demonstrated that metabolism of both glucose and fatty acids are present in human umbilical cord endothelial cells, although, glucose metabolism was more distinct than β-oxidation (50), which corresponds to the relatively high expression of *Cpt1a* compared to astrocytes and pericytes. For *Cpt1c* the expression was found to be highest in astrocytes and lowest in endothelial cells. However, Sierra *et al* demonstrated that *Cpt1c* is mainly expressed in neurons and no co-localization of CPT1C and GFAP was found in astrocytes, which is contradictory to this finding. (23)

The cells were treated with two HDAC inhibitors, SB and VPA, to investigate if an epigenetic regulation through histone acetylation is involved. It is well known that HDAC inhibitors such as SB and VPA inhibit the histone deacetylases regulating acetylation of histones leading to an increased expression of mRNA and protein. (51,52) However, this was only the case for endothelial cells and pericytes. Astrocytes presented with a decreased expression of *Cpt1a* and *Cpt1c* after treatment, which does not correspond with the theory about blocking the enzymes responsible for acetylation of histones. However, Saito *et al*, Chou *et al*, and Mazzarelli *et al* also demonstrated a decreased expression of genes after treatment with HDAC inhibitors. (53–55) This might indicate that a deinhibition of a repressor of *Cpt1a* and *Cpt1c* is present due to HDACi treatment, which affects the expression of *Cpt1a* and *Cpt1c*.

The knowledge regarding CPT1C and the respective gene is scarce. However, Nigel *et al* found *Cpt1c* to be brain specific. (56) In this study, the expression of *Cpt1c* is investigated in primary

endothelial cells, astrocytes, and pericytes and is found to be present in all cell types. However, Sierra *et al* found CPT1C to be expressed in the endoplasmatic reticulum in neurons and not in the mitochondria as CPT1A. (23) Therefore, it can be speculated which function CPT1C exerts and if it is involved in fatty acid metabolism as CPT1A. The present study investigated the amount of CPT1A by immunocytochemistry, which corresponds to the expression of *Cpt1a*. The location of the protein in endothelial cells and astrocytes is widely distributed throughout the cell except from cell nucleus in controls, SB, and VPA. Small foci where the protein is localized are visible. However, CPT1A is relocalized in pericytes after treatment with SB towards the cell membrane. To the author's knowledge no one has demonstrated a relocalization of CPT1A after treatment with HDACi. This result requires more experiments to directly investigate the localization of the protein. An experiment could be co-immunostaining of primary cells with different markers for organelles and the protein, which can show the exact localization of the protein. Furthermore, it would have been interesting to investigate the amount and localization of CPT1C as well, compared to CPT1A.

It could be interesting to investigate the expression of *Cpt1a* and *Cpt1c* in mice treated with HDAC inhibitors due to the decrease of expression seen in astrocytes. It is well known that CPT1A plays a role in fatty acid metabolism (20–22) and it is interesting to speculate in a link between MS and CPT1A due to demyelination of neurons and a decreased incidence of MS in populations with *CPT1A* mutations. Therefore, it would be interesting if HDAC inhibitors have the possibility of decreasing the expression of *Cpt1a* in an EAE model, as seen in astrocytes, thereby shifting the metabolism from lipids towards glucose as desired. To the author's knowledge no one has demonstrated the effect of HDACi on *Cpt1a* and *Cpt1c* in an EAE model. However, a study by Camelo *et al* demonstrated that injection of the HDAC inhibitor trichostatin A in EAE mice reduces inflammation in the spinal cord, axonal loss, and demyelination. Hence, treatment with HDAC inhibitors has the possibility of reverting neuronal dysregulation. (57)

4.2 The expression of *Cpt1a* and *Cpt1c* in an EAE model

An EAE model was employed to investigate the expression of *Cpt1a* and *Cpt1c* in controls, EAE mice and heterozygotic *Cpt1a* mice. Lieury *et al* demonstrated an upregulation of *CPT1A* in MS lesions. (58) However, the first sub-study showed no difference in expression of *Cpt1a* between the groups, indicating that both EAE and treatment with either placebo or etomoxir does not affect *Cpt1a*. In addition, a difference was seen in *Cpt1c* between the groups and controls, which indicates that the decreased expression is associated with EAE. However, the mice in this study did not score above 1, indicating no disease signs. The animals used in this study are mice bred from a *Cpt1a* mutated mother, however, no mutation is present in the pups (CPT1A background). Both the non-significant difference in *Cpt1a* between the treatment groups and control and the breeding gave rise to the idea about transgenerational epigenetic inheritance, where the epigenetic marks can be inherited across generations and affect gene transcription despite lack of genetic mutation. (59) Therefore, the second study was conducted with mice from CPT1A background (Control/Cpt1a) and mice without CPT1A background (Control) to investigate the difference in expression of *Cpt1a*

and *Cpt1c*. In this study no difference was found between the groups in *Cpt1a*, indicating that transgenerational epigenetic inheritance does not take place. For *Cpt1c* a difference was seen between Control/Cpt1a and Control/Cpt1a EAE and between Control and Control EAE. No difference was seen between CPT1A background and non-CPT1A background, indicating no signs of transgenerational epigenetic inheritance. This further supports that the induced EAE decreases the expression of *Cpt1c*. However, the mice in this study did not show signs of disease either.

Several studies use EAE as a model for multiple sclerosis e.g. Delarasse *et al,* Rasmussen *et al,* and Mangiardi *et al.* (60–62) This indicates that EAE is an efficient model that works in rodents. As mentioned, the animals in this study did not develop any disease signs. This might be due to the low number of animals used for this study as to the author's knowledge no study describes how many animals induced with EAE develop disease signs. However, in a new EAE study conducted in the laboratory group the animals developed disease signs. This indicates that the group has a good expertise in this model.

4.3 The role of epigenetics on *Cpt1a* and *Cpt1c*

Epigenetics of multiple sclerosis is a relatively new field. Camelo *et al,* Ge *et al,* and Zhang *et al* have all investigated the effect of HDAC inhibitors on multiple sclerosis. (57,63,64) As described previously, Camelo *et al* demonstrated decreased demyelination, spinal cord inflammation, and axonal loss when treating EAE animals with trichostatin A. (57) Ge *et al* demonstrated that treatment of EAE animals with Vorinostat decreases cytokine production from Th1-cells and inhibited differentiation and maturation of dendrictic cells. (63) Zhang *et al* demonstrated reduced severity of EAE and decreased mRNA levels of inflammatory factors such as TNF-α and interferon-β. (64) Of other studies that investigated the autoimmune aspect of multiple sclerosis Wang *et al* can be mentioned. Wang *et al* demonstrated that trichostatin A promote acetylation of Foxp3, which is essential for the binding to the IL-2-promoter. (65) This indicates that the investigated epigenetics of multiple sclerosis mostly rely on the autoimmune aspect of the disease. Some studies have investigated epigenetics of metabolic diseases, however, not many studies have investigated epigenetics of *Cpt1a* in MS. Lillycrop *et al* demonstrated epigenetic regulation of PPARα after dietary protein restriction. (66) Furthermore, Natarajan *et al* demonstrated inhibition of EAE by a PPAR-ϒ agonist by blocking the immune response. (67) Aslibikyan *et al* found increased plasma adiponectin levels to be associated with increased DNA methylation of *CPT1A*, indicating that *CPT1A* methylation might be a marker for metabolic disease. (68) In addition, Silva-Martínez *et al* demonstrated that arachidonic acid induces global DNA hypermethylation and that inhibition of global DNA hypermethylation can be received by blocking CPT1A by etomoxir. (69) The above mentioned indicates that very little is investigated about epigenetics of the metabolic aspect of multiple sclerosis. Therefore, it is very interesting to investigate the epigenetic landscape of *Cpt1a* and *Cpt1c,* which are key players of fatty acid metabolism.

This study aimed at investigating the epigenetic landscape of *Cpt1a* and *Cpt1c in vitro* and *in vivo.* DNA methylation analysis was performed to investigate whether DNA methylation is present, however, this analysis does not give any information of the exact position of the methylations. Ohashi *et al* demonstrated that DNA methylation of *Cpt1a* increases in rats with metabolic syndrome fed with fructose-rich meals. (45) This indicates that *Cpt1a* is epigenetically regulated, whereas it is interesting to investigate alterations in DNA methylation of CpG islands of *Cpt1a* and *Cpt1c. In vitro,* the DNA methylation results correlate to the expression analysis, which indicates that DNA methylation patterns supports results from HDAC inhibition. However, the result from endothelial cells is less valid due to <1 Ct value difference both between mock and methylation-sensitive enzyme and mock and methylation-dependent enzyme, therefore the fraction of methylated and unmethylated DNA is assigned to 50%. An increased number of samples in each group are necessary to obtain more valid results. However, this experiment was performed to get an idea of an epigenetic regulation. To the author's knowledge no one has investigated DNA methylation of *Cpt1a* and *Cpt1c* in endothelial cells, astrocytes, and pericytes. However, Das *et al* found an association between metabolic syndrome and DNA methylation of *Cpt1a* in CD4+ T-cells, indicating that metabolism and DNA methylation can affect each other. (70) *In vivo,* only the CpG island in *Cpt1c* is investigated due to no difference in relative expression of *Cpt1a* between controls and EAE mice. A tendency of more methylation in CpG31 in the EAE animals is seen. This result corresponds to the decreased relative expression of *Cpt1c* in these mice. However, more samples in each group are necessary to decrease the standard errors. To the author's knowledge no one has investigated DNA methylation of *Cpt1c* in a murine EAE model.

The placement of the CpG islands might have a role in the activation and repression of genes. Only CpG39 for *Cpt1a* is placed in the promoter region, whereas the other CpG islands are placed in the gene. Maunakea *et al* demonstrated that intragenic GpG islands serve as alternative promoters and that histone acetylation and methylation might be more relevant than DNA methylation in the promoter region. (71) In this study, this is demonstrated by CpG31 and CpG42 *in vitro,* which both are intragenic CpG islands that affect the expression of *Cpt1c* and *Cpt1a,* respectively.

Four histone modifications were investigated in areas near CpG islands of *Cpt1a* and *Cpt1c*. Acetylation of histone 4 (H4Ac) and trimethylation of histone 3 lysine 4 (H3K4me3) is associated with gene activation, whereas trimethylation of histone 3 lysine 9 (H3K9me3) and trimethylation of histone 3 lysine 27 (H3K27me3) is associated with gene repression. (39,72) The investigated area of *Cpt1a* is a region placed in the promoter of the gene, as seen on figure 2.4. However, no significant difference was found between controls and EAE mice. In addition, a tendency of increased H4Ac and H3K4me3 in EAE mice compared to controls is present, which indicates transcriptional activity. Transcriptional activity is expected in EAE animals, which indicate an increased expression of *Cpt1a*, hence, assumed more lipids are metabolized. However, this is not demonstrated in the expression results. For *Cpt1c* a significant decrease in H4Ac was found in Control EAE compared to Control. This result corresponds to the decreased expression of *Cpt1c* in Control EAE compared to Control. In addition, a tendency of decreased H3K4me3 is found in

Control compared to Control EAE, which is inconsistent with the increased H4Ac. To the author's knowledge no one has investigated histone modifications of *Cpt1a* and *Cpt1c* in an EAE model.

4.4 *Cpt1c*: Any role in multiple sclerosis?

The knowledge regarding the function of CPT1C is sparse. As mentioned before, Sierra *et al* demonstrated that CPT1C is located in neurons. (23) Furthermore, Price *et al* found CPT1C to bind malonyl-CoA, which is a key substrate in fatty acid synthesis and an inhibitor of CPT1. (56) In addition, it is suggested that CPT1C plays a role in regulation of energy homeostasis. Wolfgang *et al* demonstrated that CPT1C knockout mice had a decreased body weight and food intake. However, feeding these mice a high-fat diet leads to obesity, indicating that CPT1C is protective against over-feeding and obesity. This is consistent with knockout mice having decreased fatty acid oxidation. They further demonstrated that CPT1C could not transfer palmitoyl to carnitine. (73) Dai *et al* demonstrated that CPT1C is highly expressed in hypothalamic neurons and placed in the mitochondria (74), which is contradictory to Sierra *et al* that demonstrated that CPT1C is localized in the endoplasmic reticulum. (23) To the author's knowledge no one has yet investigated the role of CPT1C in an EAE model. The metabolic hypothesis of multiple sclerosis relies on a shift from glucose metabolism towards lipids. If CPT1C is a sensor of malonyl-CoA, hence, fatty acids, then CPT1C could play a role in the development or progression of multiple sclerosis. In this study, it is further demonstrated that the expression of *Cpt1c* decreases after the onset of EAE. It would be interesting to investigate the levels of malonyl-CoA in *Cpt1c* knockout mice in the context of EAE.

4.5 Limitations

In the present study, the EAE murine model should be optimized to obtain animals that exhibit disease signs. Furthermore, more samples in the epigenetic analysis would be preferred to minimize standard error of the mean. Another limitation to the epigenetic analysis is that the used analyses lack information of the precise position of the epigenetic mark. However, to obtain this, next generation sequencing should be performed.

5 Conclusion

In blood brain barrier-associated cells, *in vitro,* the highest expression of *Cpt1a* was found in endothelial cells, whereas the lowest expression of *Cpt1a* was found in pericytes. For *Cpt1c* the lowest and highest expression was found in endothelial cells and astrocytes, respectively. The expression of *Cpt1a* and *Cpt1c* increases in endothelial cells and pericytes after treatment with HDAC inhibitors, whereas the expression decreases after treatment in astrocytes. This was further supported by immunocytochemistry that showed an increased amount of protein in endothelial cells and pericytes after treatment and a decreased amount of protein in astrocytes after treatment. Furthermore, DNA methylation analysis demonstrated an increased fraction of methylated DNA in endothelial cells and astrocytes and a decreased fraction of DNA in pericytes after treatment with HDAC inhibitors. This indicates that *Cpt1a* and *Cpt1c* are epigenetically regulated *in vitro*.

In an EAE murine model, no difference was found in *Cpt1a* between controls and EAE mice. The expression of *Cpt1c* decreased after induction of EAE compared to controls. DNA methylation analysis showed no significant difference between controls and EAE. Chromatin immunoprecipitation showed a tendency of increased H4Ac and H3K4me3 and decreased H3K27me3 was found in EAE compared to controls in *Cpt1a*. For *Cpt1c* a significant decrease of H4Ac was found in EAE compared to control, which supports the finding of a decreased expression of *Cpt1c* in EAE compared to control. This indicates that *Cpt1c* is epigenetically regulated *in vivo.*

6 Acknowledgements

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8 Appendices

Appendix 1 – Cell culturing endothelial cells

Protocol for Culturing of Isolated Capillaries

Opløsninger til RBEC Medie

DMEM-F12 (Glutamax)	Invitrogen 31331-028	opbevares +4°C
Plasma derived bovine serum (PDS)	FIRST LINK Ldt, UK 500 ml.	

Heparin 100µg/ml (15 U final concentrations)
Dissolve 50KU (1 bottle) in 133ml sterile H_20.
Sterilize by filtration (0.2µm)
Put 12 ml/ centrifuge tubes
Langtids opbevaring -20°C (primærlab), ellers 4 °C (primærlab).

ITS 1/100
Dissolve 50mg (1 bottle) in 5ml st. H_2O, swirl to dissolve. Add additionally 45ml st. H_2O.
Sterilize by filtration (0.2µm)
Put 4 ml / centrifuge tube (12 stk.) Vialer står i -20°C fryser primærlab

bFGF 10µg/ml (sterile!)
Dissolve 10 mg bFGF in 1000 µl Sterile PBS
Put 10 µl / small micro-centrifuge tube – Vialer findes i primærlab -20°C fryseren.

bFGF working solution (10,000x stock solution)
Add 90 microL sterile 1mg/ml BSA-PBS or BSA-DME to 10µl bFGF aliquot.
This can be kept for a few weeks at 4 °C. Ekstra vialer finds i primærlab -20°C fryseren.
Final dilution in culture medium 1ng/ml.

Puromycin 4µg/ml
Dissolve 25 mg in 6.25 ml DMEM-F12. Sterilize by filtration (0.2µm.)
Put 0.250 ml /micro-centrifuge tube (25 stk.) store aliquots at -20°C (long term) or at +4°C (short term).
Vialer findes i primærlab -20°C fryseren.

For preparation of 100 ml RBEC Medium

DMEM/F12 (Glutamax)	89ml
10% PDS	10 ml

100 µg/ml Heparin	0.2 ml (kan udelades)
ITS (1/100)	1 ml
Gentamycin	0.020ml (20µl)
1 ng/ml bFGF (freshly added)	0.1 ml (1/1000) -

For passaging:
3cm culture dishes, 25 cm^2 flasks, 75 cm^2 flasks, 12 br. plader, 24 br. plader, 12 br. filter inserts

Trypsin (10X), Gibco Cat.No 15090
Aliqouts diluted 10X in PBS are stored at -20 °C in the freezer.

1XPBS without calcium and magnesium
For Coating

Collagen type VI (CIV) (1mg/mL)
Dissolve 5mg (1bottle) in 5ml 0,25 % acetatic acid in H_2O. Can be difficult to dilute.
Sterilize by filtering (0.2µm).
Put 0,5ml / micro-centrifuge tubes (10stk)

Fibronectin 1mg/mL
Use directly from the vital. Store at +4°C.

Coating:
Coating solutions can be used several times – use old mixtures from the fridge before preparing a new.

Prepare coating solutions dependent on the what type of surface the cells are cultured on:

- **35 mm culture dishes/ culture flasks (T25-T75)/ well plates**
  800 µl water
  50 µl fibronectin
  150 µl Collagen IV

- **Coverslips (***coates to gange – vent mindst 1 time mellem hver coating – gerne flere dage (f.eks. fredag og igen mandag))*
  650 µl water
  100 µl fibronectin
  250 µl collagen IV

- **Filters (***coates to gange – vent mindst 1 time mellem hver coating – gerne flere dage (f.eks. fredag og igen mandag))*
  400 µl water

100 µl fibronectin
500 µl collagen IV

Coat Put the solution into a dish, move it carefully around, then transfer it to the next dish. A thin fluid layer of the matrix will cover the bottom of the dishes. Put the dishes in the CO_2 incubator for at least 1 hour.

Dyrkning af RBECs ud fra frosne kapillære

- 1 vial RBEC kapillærer udsåes i 1xT75 (evt. + 1xT25)

- 1 vial med kapillærer optøes og fortyndes i 9 ml RBEC Medie, 1xPBS eller DMEM-F12.
- Kapillærene spinnes ned (700G i 7 min).
- Mediet hældes fra og pellet opløses i 15 ml (1xT75) eller 20 ml (1xT75 + 1xT25) RBEC medie.
- bFGF og 4µg/ml puromycin tilsættes (begge 1:1000 = 20µl til 20ml). Cellerne udsåes i collagen/fibronectin (”dish coat”) coated flasker og dyrkes i 3 dage (uden medieskift). Udså f.eks. cellerne fredag så er de klar mandag.
- Efter 3 dage bør cellerne være 90% confluente (De må ikke blive 100% konfluente)
- Hæld medie fra og vask cellerne 2 gange i PBS
- Tilsæt trypsin med EDTA – hold øje med cellerne. Hvis trypsin er ny og har stuetemp, bør cellerne løsne sig i løbet af 5 min. Løsen evt. de sidste celler ved at vippe flasken forsigtigt, så trypsinen bliver fordelt på overfladen.
- Stop trypsin ved at tilsætte RBEC medie (med serum). Skyld flaskens bund grundigt med en 10 ml stripette, så alle cellerne løsnes og opsamles. Opsaml cellerne i et 50 ml rør
- Spin evt cellerne ned eller udså dem direkte i det medie, de er opsamlet i. (husk at tilsætte bFGF)
    - For at opnå 100% konfluente celler dagen efter (anbefalet) udsåes cellerne med en celledensitet på 100.000 celler/cm2 eller anvend følgende estimat:
        - Antallet af celler i 1xT75 + 1xT25 svarer til 2-2½ plade med 12 brønds filtre (areal 1.12 cm^2) (til in vitro BBB model) eller 1-1½ 24 brønds plade (areal 1.19 cm^2).

Appendix 2 – Cell culturing pericytes

Protocol for Primary Rat Pericytes

Materials

DMEM (low glucose):

Dulbecco's Modified Eagle Medium
500 ml, Invitrogen Cat. No. 21885025
Store at 4°C in fridge

FCS:

Fetal Calf Serum. Gibco Cat. No 10106-169
Aliqouts stored at -20 °C

Gentamicin Sulfate / or PEN/STEP

Lonza Cat. NO 17-518Z
Store at room temperature

Preparation of 200 ml Medium:

180 ml DMEM
20 ml FCS
40µl gentamicin sulfate
Steril filtered
Store at 4°C

For passaging:

25 cm^2, 75 cm^2 flasks, 60 mm culture dish

Trypsin (10X), Gibco Cat.No 15090
Aliqouts diluted 10X in PBS are stored at -20 °C in the freezer.

1XPBS without calcium and magnesium

Coatning of tissue flasks or dishes:

- No coating needed.

Isolation of Pericytes

- Three days after isolation, the cells are washed thoroughly in PBS (3-4 times)
- Media is changed every 2-3 days.
- The cells will become confluent within 8-10 days, and are ready for passaging or freezing.

For Pericyte passaging and feeding:

- Aspirate medium and rinse the monolayer three times with PBS.

- Treat the monolayer with trypsin for 5-10 min at 37°C until cells detach.
- Add immediately complete medium with FCS to stop trypsin action.
- Spin suspension at 1200 rpm for 5 min, remove the supernatant
- Seed the cells at 10.000-20.000 cells pr cm^2.
- Maintain cultures in humidified 5%CO_2/95% air at 37°C.
- Change medium once or twice a week.
- Do not use the cells after passage #3

For freezing cells:

- Cryoprotectant Medium: Complete growth medium with 30% FCS supplemented with 7.5 % DMSO Sigma Cat. NO D2650

Appendix 3 – Culturing of astrocytes

Protocol for Primary Rat Astrocytes

Materials

DMEM (low glucose):

Dulbecco's Modified Eagle Medium
500 ml, Invitrogen Cat. No. 21885025
Store at 4°C in fridge

FCS:

Fetal Calf Serum. Gibco Cat. No 10106-169
Aliqouts stored at -20 °C

Gentamicin Sulfate

Lonza Cat. NO 17-518Z
Store at room temperature

Preparation of 200 ml Medium:

180 ml DMEM
20 ml FCS
40 µl gentamicin sulfate
Steril filtered
Store at 4°C

Preparation of 10 ml serum-free medium (immunocytochemistry):

10 ml Neurobasal-A medium (10882-022)
100 µl B27
100 µl L-glutamin
100 µl P/S
Steril filtered
Store at 4°C

For passaging:

25 cm^2, 75 cm^2 flasks or different well plates

Trypsin (10X), Gibco Cat.No 15090
Aliqouts diluted 10X in PBS are stored at -20 °C in the freezer.

Poly-L-Lysine (5 mg), SIGMA P6282
5 mg is diluted in 10 ml sterile H_2O. The solution is sterilzed through a 0.2 µm syringe filter.

Aliqouts of 500 µl is stored at -20 °C

1XPBS without calcium and magnesium

Coating of tissue flasks or dishes:

- Add 500 µl poly-L-lysine (500µg/ml) to 50 ml sterile H_2O
- Add sufficient amounts of coating solution to cover the culture surface
- Incubate at 37°C min 30 min. Alternatively overnight.
- Remove the coating solution and let the flasks/plates dry under sterile conditions

For Astrocyte passaging and feeding:

- Aspirate medium and rinse the monolayer three times with PBS.
- Treat the monolayer with 1-3 ml trypsin for 5-10 min at 37°C until cells detach.
- Add immediately 5-10 ml of complete medium with FCS to stop trypsin action.
- Spin suspension at 1100 rpm for 5 min, remove the supernatant and add 10 ml complete medium on pellet.
- Count cells and seed at a final density of $2\text{-}3x10^4$ cells / cm^2 on poly-L-lysine-coated flasks or dishes.
- Maintain cultures in humidified 5%CO_2/95% air at 37°C.
- Change medium once or twice a week.

For freezing cells:

- Cryoprotectant Medium: Complete growth medium with 30% FCS supplemented with 7.5 % DMSO Sigma Cat. NO D2650

Appendix 4 - Purification of RNA and DNA using AllPrep DNA/RNA Mini

Preparation of reagents:

1) Add the appropiate volume of 99% ethanol to Buffer RPE, Buffer AW1, and Buffer AW2 as indicated on the bottle.
2) Add 10 µl β-mercaptoethanol to 1 ml Buffer RLT Plus before use.

Preparation of samples:

1) Tissue samples
    a. Cut 30mg of tissue and put it in an 2ml eppendorf tube
    b. For each sample add 600µl Buffer RLT Plus/β-mercaptoethanol
    c. Disrupt the sample using a rotor-stator homogenizer for approximately 30 s.
    d. Centrifuge the lysate for 3 min at maximum speed
    e. Transfer the supernatant to an AllPrep DNA spin column placed in a collection tube
    f. Centrigufe for 30 s at 10,000 rpm.
2) Cell samples
    a. Add 350µl Buffer RLT Plus/β-mercaptoethanol to each well. Mix well by pipetting the buffer all over the well andadd it to an eppendorf tube.
    b. Homogenize the sample by vortexing.
    c. Transfer the lysate to an AllPrep DNA spin column placed in a collection tube.
    d. Centrigufe for 30 s at 10,000 rpm.

RNA purification

1) Place the DNA spin column in another collection tube and use the flow-through for RNA purification
2) Add 1 volume of 70% ethanol to the flow-through and mix well by pipetting
3) Transfer up to 700µl of the sample into an RNeasy spin column placed in a collection tube. Centrifuge for 15 s at 10,000 rpm or use a vacuum. Discard the flow-through
4) Wash the column by adding 700 µl Buffer RW1 to the RNeasy column. Centrifuge at 15 s at 10,000 rpm or use a vacuum. Discard the flow-through
5) Wash the RNeasy column by adding 500 µl Buffer RPE. Centrifuge for 15 s at 10,000 rpm or use a vacuum. Discard the flow-through.
6) Wash the RNeasy column by adding 500 µl Buffer RPE. Centrifuge for 2 min at 10,000 rpm or use a vacuum. Discard the flow-through.
7) Place the RNeasy spin column in a 1.5mL collection tube. Add 30 µl RNase-free water directly to the membrane and centrifuge for 1 min at 10,000 rpm to elute RNA.
    a. NB! Do not use vacuum.

DNA purification

1) Add 500 µl Buffer AW1 to the AllPrep DNA column and centrifuge for 15 s at 10,000 rpm or use a vacuum. Discard the flow-through

2) Wash the AllPrep DNA column by adding 500 µl Buffer AW2. Centrifuge for 2 min at full speed or use a vacuum.
3) Place the AllPrep DNA column in a new 1.5 ml collection tube. Add 100 µl Buffer EB directly to the membrane. Incubate at room temperature for 1 min and centrifuge for 1 min at 10,000 rpm to elute DNA.
    a. NB! Do not use vacuum.

Appendix 5 – DNase treatment

RNA	500 ng
10X reaction buffer with $MgCl_2$	1 µl
DNase I, RNase-free	1 µl
Nuclease-, and RNase-free water	To 10 µl

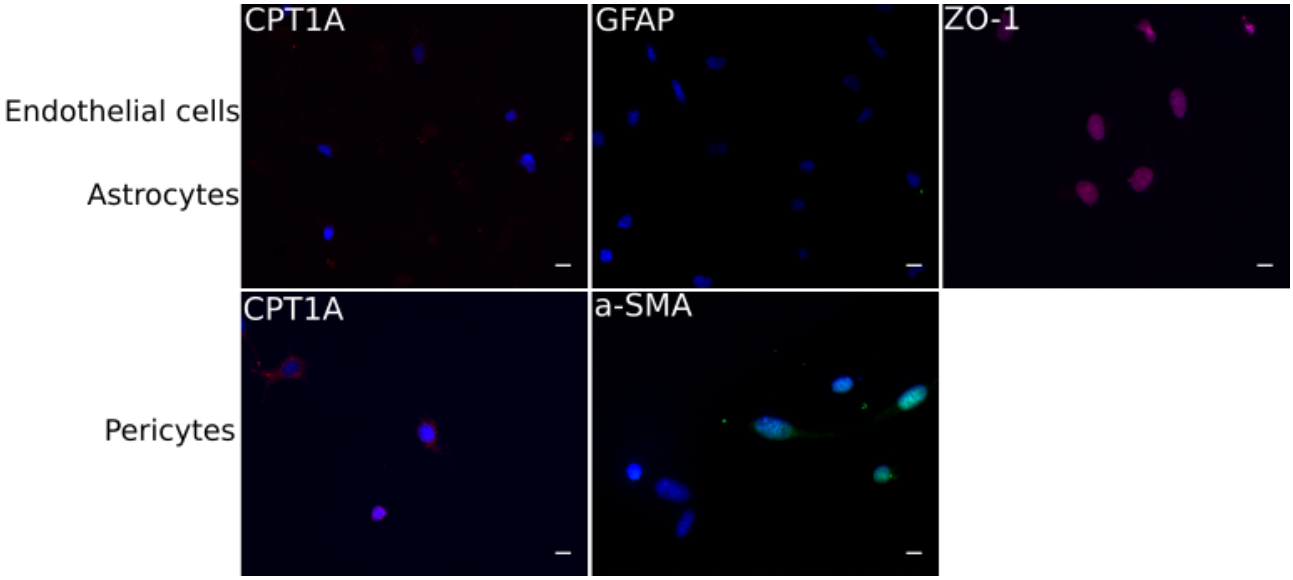
1. Add the abovementioned to a RNase-free tube
2. Incubate the samples at 37°C for 30 min
3. Add 1 µl 50 mM EDTA and incubate at 65°C for 10 min.

Appendix 6 – cDNA synthesis

DNase treated RNA	100 ng
Oligo dT primer	0.25 µl
Random hexamer primer	0.25 µl
10 mM dNTP mix	1 µl
Nuclease free water	To 15 µl
5X RT Buffer	4 µl
Maxima H Minus Enzyme mix	1 µl

1. 100ng DNase treated RNA is added in up to 13.5 µl nuclease free water.
2. Prepare a mastermix containing Oligo dT primer, random hexamer primer, 10mM dNTP mix, and 5X RT buffer.
3. Add 5.5 µl of the mastermix to the RNA/water mix
4. Add 1 µl Maxima H Minus Enzyme mix
5. Incubate for 10 min at 25°C, 15 min at 50°C, and 5 min at 85°C

Appendix 7 – Secondary controls of immunocytochemistry



Appendix 8 – Immunocytochemistry

Immunocytochemistry on fixed cell cultures (24 well plate coverslips)

Fixation: 4 % Paraformaldehyde (PFA)

Blocking buffer: 0.1 % Triton X-100 (only if intracellular antigen) in PBS (for CPT1A endothelial cells and astrocytes)
3% BSA + 0.3 % Triton X-100 in PBS (For CPT1A pericytes)

Washing buffer: 1% BSA in PBS (1:3)

Antibody: Primary antibody (anti-antigen)
Secondary antibody (anti-primary antibody species) (fluorescent)

Fixation:

- Gently wash the cells 2 times in 0.5 ml PBS
- Fix the cells with 300 µl 4 % paraformaldehyde for 5-10 min
- Wash the cells in PBS 2 times
- Store the cells for months at 4°C in 1ml PBS pr. well.

Immunostaining: Use a rocking shaker/belly dancer for wash and incubation

- Add 0.5 ml incubation buffer to each well
- Block for 30 minutes
- Remove the buffer
- Add primary antibody (conc. 1:200) in 150 µl of incubation buffer per well
- Incubate for 1 hour at room temperature or at 4°C overnight
- Wash 2 times for 5 min with the washing buffer
- Add secondary antibody (conc. 1:500) in 150 µl of incubation buffer to each well (light sensitive, cover with tinfoil)
- Incubate for 1 hour at room temperature (light sensitive, cover with tin foil)
- Wash 1 time for 5 min in PBS

DAPI stain (nucleus stain):

- Dilute DAPI stock solution in PBS (1:500)
- Add the solution to the well and incubate for 4 min
- Wash 2 times in PBS; leave the cells with PBS after the final wash.
- Mount the coverslips on object slides using DAKO fluorescent mounting media and a special needle. (Cells should be facing the object slides)

Appendix 9 – DNA methylation by Epitect Methyl II DNA Restiction Kit

- Prepare a mastermix containing 250 ng dNA, 13 µl 5x Restriction Digestion Buffer and RNase/DNase-free water to 60 µl.
    - NB! Make sure to vortex 5x Restriction Digestion Buffer so all precipitate will dissolve
- Vortex thoroughly to mix the components and centrifuge briefly
- Set up 4 digestion reactions according to the table
    - NB! Mix the enzymes gently, do not vortex!

Component	M_o	M_s	M_d	M_{sd}
Reaction mix	14 µl	14 µl	14 µl	14 µl
Methylation-sensitive enzyme A	-	0.5 µl	-	0.5 µl
Methylation-dependent enzyme B	-	-	0.5 µl	0.5 µl
RNase-/DNase-free water	1 µl	0.5 µl	0.5 µl	-
Final volume	15 µl	15 µl	15 µl	15 µl

- Pipet up and down gently but thoroughly.
- Centrifuge briefly
- Incubate alle samples at 37°C overnight in a thermomixer
- After incubation, stop the reaction by heal-inactivation at 65°C for 20 min

Appendix 10 – Calculations of DNA methylation status

$$F_{hm} = \frac{2^{Ct(M_s)}}{2^{Ct(M_o)} - 2^{Ct(M_{sd})}}$$

$$Fum = \frac{2^{Ct(M_d)}}{2^{Ct(M_o)} - 2^{Ct(M_{sd})}}$$

$$Fim = 1 - F_{hm} - F_{um}$$

$$F_m = F_{hm} + F_{im}$$

If no Ct value was present for M_{sd}, then the Ct was assigned to 40.

If ΔCT (M_s – M_o) < 1.0 and ΔCT (M_d – M_o) > 1.0, use following formula to calculate the fraction of hypermethylated DNA:

$$F_{hm} = 1 - F_{um}$$

If ΔCT (M_d – M_o) < 1.0 and ΔCT (M_s – M_o) > 1.0, use following formula to calculate the fraction of unmethylated DNA:

$$F_{um} = 1 - F_{hm}$$

If both ΔCT (M_s – M_o) and ΔCT (M_d – M_o) are less than 1.0, then the fraction of both hypermethylated and unmethylated DNA is assigned as 50 %.

Abbreviations:
F_{hm}: Fraction of hypermethylated DNA
F_{um}: Fraction of unmethylated DNA
F_{im}: Fraction of intermediate methylated DNA
F_m: Fraction of methylated DNA

Appendix 11 – Chromatin Immunoprecipitation

$$x(g) = volumen(mL) \times konc\left(\frac{mol}{1000mL}\right) \times mw\left(\frac{g}{mol}\right)$$

$$volumen_2(mL) = \frac{volumen_1(mL) \times konc_1(M)}{konc_2(M)}$$

Solutions

* Added just before use

PIs: one pill is dissolved in 1400µL milliQ water → vortex until completely dissolved

0.1% SDS: 1µL SDS (20%) pr. 200µL miliQ water (20%/0,1% = factor 200)

TE Buffer	for 100mL
10mM Tris-HCl pH 7.5	1mL (1M)
1mM EDTA	200µL (0.5M)
MilliQ water	98.8mL

Extraction Buffer 1 (ingen mærkning)	for 100mL (20mL pr. tissue)	Make 100mL in 10mL aliquots
0.4M Sucrose		40mL (1M)
10mM Tris-HCl, pH 7.9-8	1mL (1M)	
5mM Na^+ Butyrate	500µL (1M)	
MilliQ water	58.465mL	
*5mM β-mercaptoethanol (BME)	35µL (14.3M) → 3.5µL pr. 10mL!	
*Protease Inhibitors (PIs)	500µL pr. 10mL!	

Extraction Buffer 2 (lokalirrit.)	for 10 mL (1 mL pr. tissue)
Make10mL in 1mL aliquots	
0.25M Sucrose	2500µL (1M)
10mM Tris-HCl, pH 7.9-8	100µL (1M)
1% Triton X-100	1000µL (10%)
10mM $MgCl_2$	100µL (1M)
5mM Na^+ Butyrate	50µL (1M)
MilliQ water	7500µL
*5 mM BME	3.5µL (14.3M) → 0,35µL pr. mL!
*PIs	50µL pr. mL!

Digestion Buffer	for 10 mL (1 mL pr. tissue)
Make 10mL in 1mL aliquots	
0.32M Sucrose	3.2mL (1M)
50mM Tris-HCl, pH 7.5	500µL (1M)
4mM $MgCl_2$	40µL (1M)
1mM $CaCl_2$	100µL (100mM)

5mM Na^+ Butyrate		50µL (1M)
MilliQ water		
*PIs		50µL pr. mL!

Resuspension buffer		for 4L
10mM Tris HCl, pH 7.9-8	40mL (1M)	
1mM EDTA, pH 8		8mL (0.5M)
5mM Na^+ Butyrate		2.2g
MiliQ water		

ChIP dilution buffer (lokalirrit.)		for 100 mL
1.1% Triton X-100		11mL (10 %)
1.2mM EDTA		240µL (0.5 M)
16.7mM Tris-HCl pH 7,9-8		1670µL (2 x 835µL) (1 M)
167mM NaCl		3.34mL (3 X 1000µL + 340µL)
(5 M)		
MilliQ water		

ChIP equilibration Buffer (lokalirrit.)	for 1mL
Sonicated λ DNA	20µL (0,3µg/µL)
BSA	60µL (50mg/mL)
ChIP dilution buffer	930µL

Elution Buffer (freshly made)		for 10mL
1% SDS		0.5mL (20%)
0.1M NaHCO3	86mg	
MilliQ water		

Low Salt Wash Buffer (lokalirrit.)	for 50mL
150mM NaCl	1.5mL (5M)
0.1% SDS	0.25mL (20%)
1% Triton X-100	5mL (10%)
2mM EDTA	200µL (0.5M)
20mM Tris-Cl pH 7.9-8	lmL (1M)
MilliQ water	

High Salt Wash Buffer (lokalirrit.)	for 50mL
500mM NaCl	5mL (5M)
0.1% SDS	0.25mL (20%)
1% Triton X-100	5mL (10%)
2mM EDTA	200µL (0.5M)
20mM Tris-Cl pH 7.9-8	1mL (1M)
MilliQ water	

LiCl Wash Buffer (lokalirrit.)	for 50mL
0.25M LiCl	3.125mL (4M)
1% Igepal (NP-40)	5mL (10%)
1% sodium deoxycholate	0.5g
1mM EDTA	100µL (0.5M)
10mM Tris-Cl pH 7.9-8	0.5mL (1M)
MilliQ water	

Procedure

In general

- Tissue must not defrost unless in buffer!!!
- Max. capacity of magnetic rack is 16 samples.
- Avoid freezing samples more than once during the protocol.
- Avoid eppendorf tubes with "click-lid" (opens spontaneously during shaking in Thermomixer comfort), avoid 2mL eppendorf tubes because too large to fit into Thermomixer comfort.

Make Extraction buffer 1 +2 and Digestion buffer
Precool the centrifuge to 4°C (DNases are inactivated at 4°C)
Work on ice! –except when working with SDS

Day 1 – Isolation of chromatin

1. Add PI and BME to the appropriate solutions (EB1, EB2, DB)
2. Transfer one frozen rat hippocampus to 10mL of cold Extraction Buffer 1:
   a little EB1 into the tube with the frozen hippocampus → loosen the tissue → back into centrifuge tube
3. Homogenize immediately using an Ultra Turrax T25 with 8mm pistil knife:
   10sec level 3, 10sec level 4, 10sec level 5 – make as little foam as possible
4. Centrifuge the homogenate for 20min, 3000g (= RCF), 4°C using centrifuge tube rotor (F-35-6-30).
5. Change rotor to normal eppendorf tube-rotor → pre-cool to 4°C.
6. Discard the supernatant.
7. Resuspend the pellet (gently pipette up and down until lump-free) in 1mL of cold Extraction Buffer 2 and transfer to an eppendorf tube. Spin down 12000g, 10min, 4°C.
8. Discard the supernatant.
9. Resuspend the nuclei in 1mL Digestion buffer.
10. Transfer to non-stick tubes – use these from now on (except when working with SDS).
11. Check the amount of chromatin by nanophotometry:
    a. Transfer 1µL sample to 50µL SDS 0.1% (dilution factor = 50 → multiply concentration with 50).
    b. Blank the nanophotometer with 3µL 0.1% SDS, use Factor 10 lid.

c. Apply 3µL sample – pipette/vortex the eppendorf tube well before!!
(A_{260} = 1 corresponds to ≈50 mg/ml of chromatin DNA).
Example: concentration = 28.0ng/µL * 50 = 1.4µg/µL * 800µL sample = 1120µg = 1.12mg

12. Dilute to a chromatin concentration of 0.5mg/mL (if possible)
Example: 800µL sample + 1.2mL digestion buffer → split into two → proceed with 1mL and store the other in the fridge.
13. Make sure thermomixer is preheated to 37°C
14. Enzymatic digestion:
    a. Add 50U of micrococcal nuclease (mnase)[1] pr. 0.5mg chromatin:
    300U/µL (stock) → 1µL stock-enzyme + 5µL digestion buffer = 50U enzyme/µL → add desired amount
    Example: 900µL sample of 0.5mg/mL requires 0.9µL enzyme
    b. Invert 5times
    c. Digest the sample immediately in a thermomixer at 37°C, 600rpm, 4min
15. Stop the MNase digestion:
    a. Add 0.5M EDTA immediately to a final concentration of 5mM (dilution factor = 100)
    Example: 1mL sample requires 10µL EDTA (0.5M)
    b. Invert 5times
    c. Place on ice
16. Centrifuge the sample for 5min, 11600*g*, 4°C
17. Transfer the supernatant (S1) to a new eppendorf tube (by pipetting!).
18. Resuspend the pellet in 500µL Resuspension buffer
19. Prepare the dialysis tube
20. Dialyze the resuspended pellet in a small piece of dialysis tube closed with two clamps in 4L Resuspension buffer at 4°C overnight on magnetic-stir

[1] is a very sticky solution, so only put the very tip of the pipette into the solution to avoid excess transfer by sticking to outside of pipette-tip

Day 2 – Final isolation and immunoprecipitation

1. Transfer the fluid (= sample) in the dialysis tube to a non-stick tube by pipetting
2. Centrifuge the sample for 10min, 2000g, 4°C
3. Transfer the supernatant (S2) to a non-stick tube by pipetting
4. Pool S1 and S2 (= sample)
5. Use all the sample for immunoprecipitation in non-stick tubes
   Example: Add 100µL sample[2] + 400µL ChIP dilution buffer + 6µL rabbit serum (= mock control)
   Add 100µL sample + 400µL ChIP dilution buffer + 6µL antibody to a non-stick tube
6. Incubate on rotating rack (20rpm), 4°C, overnight
7. Prepare the magnetic beads (30µL/tube[3]) : work on magnetic rack
   a. Add 60µL magnetic beads to an eppendorf tube
   b. Remove the buffer by pipetting
   c. Add 1mL ChIP dilution buffer → resuspend beads →remove the buffer
   d. Repeat step c
   e. Add 1mL ChIP equilibration buffer (blocks unspecific binding)
   f. Incubate overnight (as in step 8.)

[2] In case of high chromatin content, only use 50µL sample to reduce mock and unspecific binding
[3] 1mock + (1sample * 3Ab's) = 4tubes/animal * 6animals/group = 24tubes per group

Day 3 – Final immunoprecipitation

When working with immune complexes, work in 4°C (fridge)

1. Place the tubes with the beads on a magnetic rack and discard the buffer
2. Wash the beads 3times with 1mL ChIP dilution buffer (work on magnetic rack)
    a. Add buffer
    b. Invert quickly to resuspend the beads
    c. Discard buffer
3. By flicking, resuspend the beads in the same amount of ChIP dilution buffer as initially taken from the bead stock
   Example: Add 60µL ChIP dilution buffer
4. Add 30µL of the resuspended beads to each sample (mock + template)
   Remember to cut tip off the pipette
5. Incubate on rotating rack (20rpm), 3h, 4°C
6. While waiting – make buffers: Elution buffer, low + high salt wash buffer, LiCl wash buffer, TE-buffer
7. On magnetic rack:
   Mock: transfer buffer to a new tube (=input chromatin[4])
   Template: discard buffer
8. Wash the beads (both mock and template) 5 x 10min on rotating rack (30rpm), 4°C:
    a. 1mL Low salt wash buffer
    b. 1mL High salt wash buffer
    c. 1mL LiCl wash buffer
    d. 1mL TE-buffer
    e. 1mL TE-buffer
    f. 1mL TE-buffer
9. Input chromatin:
    a. Take 100µL input and add 400µL elution buffer (volume matches the final eluate volume)
    b. Invert
    c. Incubate with templates on thermomixer in step 10

10. Elute the immune complexes:
    a. Add 250µL Elution buffer to the beads
    b. Invert
    c. Incubate in thermomixer, 15min, 65°C, 1000rpm
    d. Pellet the beads on magnetic rack
    e. Transfer the eluate to a new tube (regular)
    f. Repeat step a-e
    g. Combine the two eluates and discard the beads

[4] works as negative control for the beads (shows no unspecifik binding) AND positive control for the pcr because it contains all DNA (if >5Ct difference between mock and template it's good, because mock will always show something)

11. Freeze/fridge until further use

The DNA should be purified before performing qPCR (e.g. by Qiagen's PCR purification kit)

Appendix 12 - NucleoSpin Gel and PCR Clean-Up, Manchery-Nagel

Before starting the preparation:

- Add the appropriate amount of ethanol 96-99% as indicated on the bottle

Adjust DNA binding conditions

- Mix 1 volume of sample with 5 volumes of NTB buffer
    - NB! If SDS starts to precipitate add 1 volume of isopropanol or warm the sample to 20-30°C

Bind DNA

- Place a NucleoSpin Gel and PCR Clean-Up column in a 2 mL collection tube and load up to 700 µl sample
- Centrifuge for 30 s at 11,000*g*. Discard the flow-through and place the column back into the collection tube
- Load the remaining sample if necessary

Wash silica membrane

- Add 700 µl Buffer NT3 to the NucleoSpin Gel and PCR Clean-Up column
- Centrifuge for 30 s at 11,000*g*. Discard the flow-through and place the column back into the collection tube

  NB! Recommended to repeat washing step to minimize chaotrophic salt carry-over and improve A_{260}/A_{230} values

Dry silica membrane

- Centrifuge the samples for 1 min at 11,000*g* to remove buffer NT3 completely
    - NB! Make sure the membrane does not come in contact with the flow-through while removing it from the collection tube

  NB! Residual ethanol from the NT3 buffer can inhibit the enzymatic reactions. Ethanol can be removed by incubating the sample for 2-5 mins at 70°C

Elute DNA

- Place the NucleoSpin Gel and PCR Clean-Up column in a new 1.5 mL eppendorf tube
- Add 15 µl Buffer NE and incubate at room temperature for 1 min
- Centrifuge the sample 1 min at 11,000*g*