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BY
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DENMARK

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PHD DISSERTATION

by

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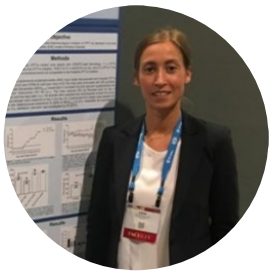

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Publications

Mørkholt AS, Trabjerg MS, Oklinski MKE, Bolther L, Kroese LJ, Pritchard CEJ, Huijbers IJ, Nieland JDV. CPT1A plays a key role in the development and treatment of multiple sclerosis and experimental autoimmune encephalomyelitis. *[Manuscript submitted]*.

Trabjerg MS, Andersen DC, Mørk K, Skjønnemand MLN, Oklinski MK, **Mørkholt AS,** Kroese LJ, Pritchard CEJ, Huijbers IJ, Nielsen S, Nieland JD. Blocking carnitine palmitoyl transferase 1 is highly efficacious in the treatment of Parkinson´s disease: A new way to a cure? *[Manuscript submitted]*.

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Mørkholt AS, Wiborg O, Nieland JGK, Nielsen S, Nieland JD. Blocking of carnitine palmitoyl transferase 1 potently reduces stress-induced depression in rat highlighting a pivotal role of lipid metabolism. *Sci Rep. 2017 May 19; 7(1).*

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Conference presentations

Mørkholt AS. The role of dysregulated lipid metabolism in an EAE model of mice mimicking the Inuit *CPT1A* mutation. Consortium of Multiple Sclerosis Centers (CMSC), 02 June 2018, Nashville, Tennessee, USA.

Mørkholt AS. Looking at multiple sclerosis as a systemic disease; comparing the effect of the lipid metabolism blocker, etomoxir, with the inflammatory blocker, interferon-β. Consortium of Multiple Sclerosis Centers (CMSC), 25 May 2017, New Orleans, Louisiana, USA.

Mørkholt AS. Blocking the lipid metabolism as a new treatment strategy for multiple sclerosis. Consortium of Multiple Sclerosis Centers (CMSC), 02 June 2016, National Harbor, Maryland, USA.

Conference abstracts

Mørkholt AS, Trabjerg MS, Huijbers IJ, Pritchard CEJ, Kroese LJ, Nieland JD. Identifying the role of lipid metabolism in an experimental autoimmune encephalomyelitis mice model. Consortium of Multiple Sclerosis Centers (CMSC), May 30-June 02 2018, Nashville, Tennessee, USA.

Trabjerg MS, **Mørkholt AS**, Nielsen S, Nieland JDN. Identifying the role of lipid metabolism in central nervous systems diseases; is there a common theme for MS, ALS, Parkinson´s disease and depression? Consortium of Multiple Sclerosis Centers (CMSC), May 30-June 02 2018, Nashville, Tennessee, USA.

Mørkholt AS, Kastaniegaard K, Trabjerg MS, Niganze W, Gopalasingam G, Larsen A, Stensballe A, Nielsen S, Nieland JD. Comparison of etomoxir, a lipid metabolism blocker, and interferon-β treatment on antibody recognition of brain proteins in multiple sclerosis. Consortium of Multiple Sclerosis Centers (CMSC), 24-27 May 2017, New Orleans, Louisiana, USA.

Mørkholt AS*, Kastaniegaard K*, Stensballe A, Nielsen S, Nieland JD. Characterization of human autoantibody response to brain proteins in multiple sclerosis patients. Consortium of Multiple Sclerosis Centers (CMSC), 24-27 May 2017, New Orleans, Louisiana, USA.

Mørkholt AS, Larsen A, Wiborg O, Issazadeh-Navikas S, Nieland JGK, Nielsen S, Nieland JD. Highly effective treatment of multiple sclerosis by blocking lipid metabolism. 32nd Congress of the European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS), 14-17 September 2016, London.

Mørkholt AS, Larsen A, Nieland JGK, Issazadeh-Navikas S, Nielsen S, Nieland JD. Blocking lipid metabolism as a new treatment strategy for multiple sclerosis. Consortium of Multiple Sclerosis Centers (CMSC), 01-04 June 2016, National Harbor, Maryland, USA.

Nieland JD, Nieland JGK, **Mørkholt AS**, Larsen A, Corthals A, Issazadeh-Navikas S, Bolther L, Nielsen S. CPT1a mutation leads the way for new medication for the treatment of multiple sclerosis. 31st Congress of the European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS), 07-10 October 2015, Barcelona.

Mørkholt AS*, Routhe LJ*, Moos T. The functional expression of ferroportin in primary rat neuronal culture. European Iron Club, 11-14 September 2014, Verona.

* = Equal contribution

ENGLISH SUMMARY

Multiple sclerosis (MS) is a chronic, inflammatory, autoimmune and neurodegenerative disease of the central nervous system (CNS). Pathological features include loss of oligodendrocytes, demyelination, axonal degeneration, inflammatory plaques and break down of the blood-brain barrier. Several mechanisms for the immunoreactivity affecting the immune system such as molecular mimicry, altered peptide ligand or bystander activation have been proposed. The treatment of MS today includes disease-modifying therapies, ameliorating the inflammatory response which is not able to prevent the progressive neurological decline contributing to disability. This incomplete traditional framework of the understanding of the etiology and pathology of MS and the common comorbid condition, depression, indicates that the pathological pathways of MS and comorbidities remain unexplored and undiscovered.

A new systemic framework of the understanding of MS originates from dysfunction of the lipid metabolism. The brain energy homeostasis is maintained by a competing relationship between oxidation of glucose and fatty acids, depending on the energy demand of the tissue. Glucose is the primary energy substrate in the brain used in glycolysis and oxidation of glucose whereas fatty acids are used in β-oxidation as an alternative energy substrate. In the fatty acid metabolism, a carnitine shuttle with carnitine palmitoyl transferase 1 (CPT1) is required as the outer mitochondrial membrane is impermeable to fatty-acyl CoA. Thereby, CPT1 serves as the regulatory rate-limiting step in β-oxidation of fatty acids.

Several human mutations for *CPT1A* are identified and the mutations *CPT1A P479L* and *CPT1A G710E* are of particular interest since these are associated with low prevalences of MS and depression. Given that the lipid metabolism and thus CPT1 plays a pivotal role in the energy homeostasis, it is tempting to hypothesize that dysregulation of this may underlie diseases of the CNS. The aim of the PhD thesis is therefore to obtain a thorough understanding of the metabolism in health and disease in order to clarify the etiology and pathology of MS and depression, which are presented in *Manuscript I-IV*.

To accomplish the aim of the PhD thesis, state-of-art *in vivo* animal models of MS (experimental autoimmune encephalomyelitis, EAE) and depression (chronic mild stress, CMS) were established. The efficacy of lipid metabolism blockage was evaluated, both pharmacological by using etomoxir targeting CPT1 and genetically by generating *Cpt1a P479L* mice.

In *Manuscript I*, the efficacy of etomoxir was investigated in EAE-induced animals showing decreased clinical score, which indicates amelioration of the disease.

Moreover, the efficacy of etomoxir was compared to interferon-β treatment where the efficacy of lipid metabolism blockage was superior to the standard treatment of MS targeting T cell function. In *Manuscript II*, the efficacy of etomoxir versus interferon-β on the B cell function was evaluated in EAE-induced animals by investigating the autoantibody response. A pallet of autoantigens was modulated after etomoxir treatment, showing that blockage of lipid metabolism causes alterations in the antibody response, thus targeting B cell function. In *Manuscript III*, the efficacy of the *Cpt1a P479L* mutation was evaluated in an EAE model, showing that *Cpt1a P479L* EAE mice were resistant to disease development compared to wild type EAE mice. Moreover, high-fat diet is thought to exacerbate the disease course however the *Cpt1a P479L* EAE mice were unaffected whilst wild type EAE mice were affected. In *Manuscript IV*, the efficacy of etomoxir was investigated in CMS-induced animals, which revealed reduced anhedonic behavior compared to the standard treatment escitalopram. Moreover, etomoxir reversed the depression-like phenotype in 90 % of the animals, compared to 57 % of the animals receiving escitalopram.

In conclusion, the results presented in this PhD thesis provide knowledge supporting the new systemic framework for understanding the etiology and pathology of MS and depression. This indicates a change of paradigm towards development, progression and treatment of CNS diseases, in particular MS and depression, with lipid metabolism playing a central role opening up a pathway to a new cure.

DANSK RESUME

Multipel sklerose (MS) er en kronisk, inflammatorisk, autoimmun og neurodegenerativ sygdom i centralnervesystemet. De patologiske karaktertræk omfatter tab af oligodendrocytter, demyelinisering, nedbrydning af axoner, inflammatoriske plaks samt nedbrydning af blod-hjerne-barrieren. Der er foreslået mange forskellige mekanismer for immunreaktivitet herunder 'molecular mimicry', 'altered peptide ligand' og 'bystander activation'. I dag omfatter behandlingen af MS sygdomsmodificerende terapier, der dæmper det inflammatoriske respons, dog er disse terapier ikke i stand til at forhindre det progressive neurologiske tab, der bidrager til funktionsnedsættelse. Dette ufuldstændige traditionelle perspektiv af forståelsen for ætiologi og patologi af MS og dets mest almindelige komorbiditet depression indikerer, at de patologiske pathways for MS og depression fortsat er uudforskede og uopdagede.

Et nyt systemisk perspektiv af forståelsen for MS omhandler dysfunktionel fedtsyremetabolisme. Hjernens energihomeostase opretholdes ved et konkurrerende forhold mellem oxidation af glukose og fedtsyrer afhængig af energikravet i vævet. Glukose er det primære energisubstrat i hjernen og bruges i glykolysen og oxidation af glukose, hvorimod fedtsyrer anvendes i β-oxidation som en alternativ energikilde. I fedtsyremetabolismen er det nødvendigt med en carnitin transporter kaldet carnitin palmitoyl transferase 1 (CPT1), da den ydre mitokondrielle membran er impermeabel for fedt acyl-CoA. Dermed fungerer CPT1 som det regulerende hastighedsbestemmende trin i β-oxidation af fedtsyrer.

Der er identificeret mange humane mutationer i *CPT1A* og mutationerne *CPT1A P479L* og *CPT1A G710E* er af særlig interesse, da de er associeret med lav prævalens for MS og depression. Givet at fedtsyremetabolismen og dermed CPT1 spiller en afgørende rolle i energihomeostasen, er det oplagt at fremsætte en hypotese, der omfatter at dysregulering af denne ligger til grund for sygdomme i centralnervesystemet. Formålet med denne PhD afhandling er at opnå en grundig forståelse af metabolismen i raske og syge for at kunne afdække ætiologi og patologi af MS og depression, hvilket er præsenteret i *Manuskript I-IV*.

For at opnå formålet med denne PhD afhandling, er *in vivo* dyremodeller for MS (eksperimentel autoimmun encephalomyelitis, EAE) og depression (kronisk mild stress, CMS) blevet etableret. Her blev virkningen af blokering af fedtsyremetabolismen både farmakologisk, ved brug af etomoxir målrettet CPT1, og genetisk, ved generering af *Cpt1a P479L* mus, undersøgt.

I *Manuskript I* blev virkningen af etomoxir undersøgt i EAE-inducerede dyr, der viser nedsat klinisk score, som indikerer afdæmpning af sygdommen. Ydermere

blev virkningen af etomoxir sammenlignet med interferon-β, hvor blokering af fedtsyremetabolismen var bedre i forhold til standardbehandlingen for MS, der er målrettet T celle funktion. I *Manuskript II* blev virkningen af etomoxir versus interferon-β evalueret på B celle funktion i EAE-inducerede dyr ved at undersøge autoantistofresponset. En vifte af autoantigener var ændret efter behandling med etomoxir, hvilket betyder, at blokering af fedtsyremetabolismen forårsager ændringer i antistofresponset og dermed er målrettet B celle funktion. I *Manuskript III* blev virkningen af *Cpt1a P479L* mutationen evalueret i en EAE model, der viste, at *Cpt1a P479L* EAE mus var resistente mod at udvikle sygdom sammenlignet med vildtype EAE mus. Derudover har det vist sig, at fedtrig kost forværrer sygdomsforløbet, men her var *Cpt1a P479L* EAE musene upåvirkede, hvorimod vildtype EAE musene var påvirkede. I *Manuscript IV* blev virkningen af etomoxir undersøgt i CMS-inducerede dyr, hvor denne behandling viste reduceret anhedonisk adfærd sammenlignet med standardbehandlingen escitalopram. Ydermere var etomoxir i stand til at omvende den depressionslignende fænotype i 90 % af dyrene i forhold til 57 % af dyrene der modtog escitalopram.

Det konkluderes, at de præsenterede resultater i denne PhD afhandling bidrager med vigtig viden, der understøtter det nye systemiske perspektiv i forståelsen af ætiologi og patologi af MS og depression. Dette indikerer et paradigmeskifte i udvikling, progression og behandling af sygdomme i centralnervesystemet, særligt MS og depression, hvor fedtsyremetabolismen spiller en central rolle og baner vejen til en ny kur.

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Anne Skøttrup Mørkholt, February 2019, Aalborg

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Manuscript I: **Mørkholt AS**, Oklinski MK, Larsen A, Bockermann R, Issazadeh-Navikas S, Nieland JGK, Kwon TH, Corthals A, Nielsen S, Nieland JD. Pharmacological inhibition of carnitine palmitoyl transferase 1 inhibits and reverses experimental autoimmune encephalomyelitis in rodents. *[Manuscript submitted]*.

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Manuscript III: **Mørkholt AS**, Trabjerg MS, Oklinski MKE, Bolther L, Kroese LJ, Pritchard CEJ, Huijbers IJ, Nieland JDV. CPT1A plays a key role in the development and treatment of multiple sclerosis and experimental autoimmune encephalomyelitis. *[Manuscript submitted]*.

Manuscript IV: **Mørkholt AS**, Wiborg O, Nieland JGK, Nielsen S, Nieland JD. Blocking of carnitine palmitoyl transferase 1 potently reduces stress-induced depression in rat highlighting a pivotal role of lipid metabolism. *Sci Rep. 2017 May 19; 7(1).*

* = Equal contribution

LIST OF ABBREVIATIONS

Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
APOE	Apolipoprotein E
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
CD	Cluster of differentiation
CMS	Chronic mild stress
CNS	Central nervous system
CPT	Carnitine palmitoyl transferase
DRB1	DR beta I
EAE	Experimental autoimmune encephalomyelitis
FATPs	Fatty acid transport proteins
FT	Ferritin
FTH	Ferritin heavy chain
GLUTs	Glucose transporters
HDL	High-density lipoprotein
HFD	High-fat diet
HLA	Human leukocyte antigen
HO-1	Heme oxygenase-1
HPA	Hypothalamic–pituitary–adrenal
IL	Interleukin
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MS	Multiple sclerosis
ND	Normal diet
Nrf2	Nuclear factor erythroid 2–related factor 2
PI3K	Phosphatidylinositol 3´-kinase
PPAR	Peroxisome proliferator-activated receptor
PPMS	Primary-progressive multiple sclerosis
PRMS	Progressive-relapsing multiple sclerosis
PUFAs	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis
SPMS	Secondary-progressive multiple sclerosis
Th	T helper cell
TNF-α	Tumor necrosis factor-α
VLDL	Very low-density lipoprotein
WT	Wild type

CHAPTER 1. INTRODUCTION

This PhD thesis is centered around four manuscripts that collectively focus on metabolic alterations involved in the pathogenesis and treatment of central nervous system (CNS) diseases. Here, a 'zoomed-out' study of well-known mechanisms and extract features that are common to all, and features which stand out are presented. The following paragraphs introduce the diseases with pathology and current treatments. Furthermore, it is explained how genetic, environmental and microbial factors connect to all CNS diseases and problems in the balance of energy and metabolism of both glucose and lipids.

1.1. MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is traditionally understood as an inflammatory, autoimmune and neurodegenerative disease of the CNS. It is a complex disease characterized by pathological features as white matter demyelination, loss of oligodendrocytes, reactive gliosis, axonal degeneration, inflammatory plaques and breakdown of the blood-brain barrier (BBB) (1–4).

MS is estimated to affect 400,000 individuals in the United States and between 2.1 and 2.5 million individuals worldwide with women being predominantly affected (5–7). The symptoms of MS are among others dyscoordination, sensory loss, weakness, vision loss, and bowel and bladder dysfunction. There are four clinical types of MS; relapsing-remitting MS (RRMS), primary-progressive MS (PPMS), secondary-progressive MS (SPMS) and progressive-relapsing MS (PRMS). RRMS is the most common type of MS as it accounts for approximately 85 % of all cases. It is characterized by attacks evolving over days to weeks followed by recovery, and in between attacks there is no worsening of neurological function (1). This subtype may later develop into SPMS in about 65 % of the patients (8). PPMS is characterized by steady decline of function from disease onset with no relapses. SPMS is characterized by initial relapses with following gradual deterioration of neurological function and no acute attacks. The last type of MS is PRMS where function declines steadily from disease onset with presence of acute attacks (1).

The diagnosis of MS is confirmed by magnetic resonance imaging technology, which shows white matter lesions of CNS and spinal cord lesions. Ancillary tests used in problematic cases are cerebrospinal fluid samples detecting inflammation with mononuclear cells and increased levels of immunoglobulins as well as tests of visual, auditory and sensory function (9). Today disease-modifying therapies are effective in RRMS and only few therapies are effective in PPMS and SPMS (4) (Table 1). No current MS therapies, however, reverse the pathological damage or cure the disease. They only slow down the immune-mediated inflammation (1). Other therapeutic approaches have included statins, which decreases blood

cholesterol and peroxisome proliferator-activated receptor (PPAR) agonists treating hypertriglyceridemia (10). In order to develop new drugs for MS, it is essential to understand the mechanisms of actions of the current drugs (Table 1).

The etiology of MS remains unknown, though several mechanisms for immunoreactivity causing autoimmunity have been proposed (7). The first mechanism describes MS triggered in the periphery of the body, while the second mechanism involve inflammation triggered by a viral infection as a primary event and resulting in infiltrative autoreactive T cells in the CNS as a secondary event (11). Normally, most autoreactive T cells are deleted due to central tolerance in the thymus where the production takes place. However, if peripheral tolerance is abrogated as a consequence of defects in regulatory T cell function, autoreactive T cells are activated in the periphery by infections, for example, which can affect the immune system in many ways through processes as molecular mimicry, altered peptide ligand or bystander activation (11,12).

Molecular mimicry, postulated to be the key mechanism in CNS autoimmunity, is a process by which an encounter with a virus, for example, is recognized by a T cell receptor leading to activation of autoreactive T cells that cross-react with self-antigens or have shared epitopes (7,13). Cross-reactivity between the agent and the host is localized at disease-related epitopes, which are peptides of autoantigens like the myelin basic protein (MBP) that are presented by the major histocompatibility complex (MHC) class II molecules on antigen presenting cells to induce autoreactive cluster of differentiation (CD) 4+ T cells. Another proposed autoimmune mechanism is known as altered peptide ligand, in which a peptide with slightly different composition of amino acids can cause disease (7). An example of this mechanism is the post-translational modification termed citrullination, where arginine becomes modified to citrulline, an enzyme which naturally occurs on MBP (14). Bystander activation is yet another proposed mechanism for autoimmunity where a pathogen activates antigen presenting cells, which then stimulate the activation and proliferation of autoreactive T and B cells by presenting a self-antigen (12). These processes all result in activated autoreactive T cells becoming CD8+ T cells, CD4+ T helper (Th) 1 cells, CD4+ Th17 cells, which then migrates into the CNS together with B cells and monocytes (11).

This mechanism of MS etiology is consistent with the pathogenesis investigated in the state-of-art *in vivo* animal model of MS: experimental autoimmune encephalomyelitis (EAE) (10). It mimics some of the clinical and pathological features in MS, where a CNS antigen, such MBP or myelin oligodendrocyte glycoprotein, in an adjuvant is administered together with pertussis toxin producing Th1 and Th17 cells. After entering the blood circulation, these cells cross the BBB and exert effector functions in the CNS (2,11).

Drug	Target	Mechanism of action	Efficacy
Cladribine (15)	Adenosine deaminase	Depletes peripheral T and B lymphocytes	Lower rates of relapses in RRMS
Ocrelizumab (16,17)	CD20+ B cells	Depletes CD20+ B cells	Lower rates of disease activity and progression in RRMS and SPMS
Alemtuzumab (18)	CD52	Depletes CD52-expressing T and B cells via ADCC, CDC and activation of pro-apoptotic pathways	Reduce relapse rates in RRMS
Dimethyl fumarate (19)	Nrf2	Activates Nrf2 and suppresses NF-κB	Reduce relapse rates in RRMS
Teriflunomide (20)	Dihydroorotate dehydrogenase	Inhibits pyrimidine synthesis for DNA replication and reduces T and B cell activation, proliferation and function	Reduce relapse rates and disability progression in RRMS
Fingolimod (21)	Sphingosine-1-phosphate receptor	Inhibits lymphocyte egress from lymph nodes	Reduce relapse rates and disability progression in RRMS
Natalizumab (22)	α4-subunit of α4β1- and α4β7-integrins	Blocks the interaction between α4β1-integrin and VCAM-1 on endothelial cells resulting in reduced leukocyte migration into CNS	Reduce relapse rates in RRMS
Mitoxantrone (23)	Topoisomerase II enzyme	Inhibits T cell activation, inhibits T and B cell proliferation, decreases antibody production and deactivates macrophages	Reduce relapse rates and progression of disability in worsening RRMS and SPMS
Glatiramer acetate (24)	MHC	Binds to MHC II on APCs and displacing peptides from MHC II binding site	Reduce relapse rates, disability and progression in RRMS
Interferon-β (5,25)	Interferon receptor	Inhibits proliferation of T lymphocytes, proinflammatory cytokines, migration of cells across BBB, shifts Th1 response to Th2 response and modulates MHC antigens	Reduce relapse rates in RRMS

Table 1. Overview of food and drug administration (FDA) approved drugs for multiple sclerosis (MS) listed by newest drug. *ADCC: antibody-dependent cellular cytotoxicity, APC: antigen-presenting cell, BBB: blood-brain barrier, CD: cluster of differentiation, CDC: complement-dependent cytotoxicity, CNS: central nervous system, MHC: major histocompatibility complex, NF-κB: nuclear factor-kappa-light-chain-enhancer of activated B cells, Nrf2: nuclear factor erythroid 2-related factor 2, RRMS: relapsing-remitting MS, SPMS: secondary-progressive MS, Th: T helper cell, VCAM-1: vascular cell adhesion molecule-1.*

Besides the T cells, B cells and their production of antibodies are also involved in MS pathology (1). There is limited knowledge about the role of the antibody-antigen response in the induction and progression of MS, however the presence of intrathecal antibodies, known as oligoclonal bands, in the cerebrospinal fluid makes an obvious argument for the involvement of B cells in the pathogenesis (26). The mechanisms by which loss of tolerance to self-proteins result in the production of autoantibodies include altered peptide ligand response and epitope spreading (14).

An incomplete conceptual framework for the understanding of all the factors leading to imbalance in immune homeostasis, demyelination and progressive neurological symptoms (9) indicates that parts of the etiology and pathology remains unexplored and undiscovered.

1.1.1. COMORBIDITIES

Comorbidities, such as depression, diabetes, hyperlipidemia and hypertension, are common in patients with MS and opens up an important and increasing area of interest (27). These comorbid conditions are associated with increased progression of disability (28), delay of diagnosis or disease severity at diagnosis (29), as well as with increased risk of death (30). These factors can potentially describe the heterogeneity of disease outcomes (28) and further highlights the complexity of the treatment regimen of MS patients (27).

Depression is experienced in up to 60 % of the MS patients during disease course and correlates with increased suicide rates (31–34). It is controversial whether abnormalities in the immune system occur prior to onset of depression or as a secondary event to inflammation (35). Immune system abnormality is suggested as a contributor in depression, as increased levels of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 have been found (36). Cytokines are activators of the hypothalamic-pituitary-adrenal (HPA) axis leading to secretion of glucocorticoids and intensification of the stress-response system (37). It is unknown whether depression influences the neurobiological course of MS however stressful life events precede exacerbations (38). Vascular comorbidities such as diabetes, hyperlipidemia and hypertension have adverse effects on MS pathology resulting in increased cognitive decline, brain atrophy, increased peripheral inflammation and oxidative stress (28).

1.2. DEPRESSION

Depression is a common psychiatric disorder, which affects up to 15 % of the population worldwide (39). It is characterized by loss of pleasure (anhedonia), sadness, hopelessness, cognitive impairment and suicidal thoughts (39,40). The diagnosis of depression is confirmed based on the presence of at least five of the following symptoms in the Diagnostic and Statistical Manual present daily or almost

daily for at least two weeks (41). The symptoms include: changes in weight and appetite, insomnia or hypersomnia, loss of energy, loss of interest, psychomotor agitation, worthlessness feelings, diminished ability to concentrate and think, thoughts of death and suicidal ideation. The severity of the disorder is categorized as mild, moderate and severe according to disruption of social and occupational functions (41). As opposed to other conditions, depression is diagnosed based on subjective ranges of symptoms and not on objective diagnostic tests, which highlights the heterogeneity of the disease and difficulties in making a diagnosis.

The areas involved in the pathogenesis of depression are still unknown, however neuropathological changes in the prefrontal cortex, hippocampus, striatum (ventral striatum and nucleus accumbens), amygdala and hypothalamus are associated with the symptoms present in depression (42,43). The prefrontal cortex and hippocampus are related to cognitive impairments as memory loss, feelings as worthlessness and hopelessness, and suicidal ideation (42). The ventral striatum, nucleus accumbens and amygdala are associated with rewards in response to stimuli mediating anhedonia, reduced motivation and anxiety (42,43). The hypothalamus is involved in changes in sleep patterns and appetite (42).

The etiology of depression has not been elucidated yet, however different hypotheses have been proposed: the monoamine, the neurotrophin and the cytokine hypothesis. The monoamine hypothesis involves decreased availability of neurotransmitters such as serotonin, dopamine and noradrenaline in the CNS. These low levels of monoamines have been the ground for developing selective serotonin reuptake inhibitors and serotonin noradrenaline reuptake inhibitors, which are antidepressants increasing the levels of serotonin and noradrenaline, respectively (39). In the neurotrophin hypothesis it is hypothesized that depression is a result of decreased levels of neurotrophic factors, which are growth factors responsible for the regulation of neuronal plasticity in the brain (44). An important, abundantly expressed, neurotrophic factor is brain-derived neurotrophic factor (44,45). Under conditions of stress the expression of brain-derived neurotrophic factor and other neurotrophic factors are decreased leading to alterations in hippocampal structure and function (44,46). The cytokine hypothesis posits that depression is manifested by increased levels of pro-inflammatory cytokines, acute phase proteins, chemokines and adhesion molecules (47). These pro-inflammatory cytokines get access to the brain where they interact with the metabolism of monoamines (serotonin, dopamine and noradrenaline), neuroendocrine function and neural plasticity. The effect of cytokines on the metabolism of neurotransmitters involves the release of cytokines, chemokines, reactive oxygen species (ROS) and reactive nitrogen species (RNS) by microglia in the brain (48). Cytokines such as TNF-α, interferon-γ, IL-1 and IL-6 activates indoleamine 2,3 dioxygenase resulting in breakdown of tryptophan, which is the precursor for serotonin, into kynurenine. This mechanism is suggested to cause the low availability of serotonin (49). In addition, the low levels of dopamine are caused by cytokines influencing tetrahydrobiopterin,

an enzyme involved in dopamine synthesis, via nitric oxide production (48). Furthermore cytokines have an effect on neuroendocrine function, where alterations in the glucocorticoid receptor with following negative feedback regulation of HPA axis leads to glucocorticoid resistance and increased cortisol levels (50). Normally, cytokines as TNF-α, IL-1 and IL-6 provide support to neurogenesis, however prolonged activation of these can cause several abnormalities such as decreased neurogenesis, oxidative stress, apoptosis of astrocytes and oligodendrocytes (51).

Different risk factors contributing to the pathogenesis of depression have been proposed. Environmental factors, for example, such as stress is thought to be a trigger in the development of depression in genetically susceptible individuals (52,53). Internal stress factors include interpersonal, financial, achievement and legal events along with more external stress factors such as hormonal challenges and injuries seem to also be triggers (54,55). Stressful events formed the basis of a rodent animal model of depression, the chronic mild stress (CMS) model. This state-of-art validated model shows that involving realistic stress factors contribute to the induction of depression in humans as well (52,53,56). The theoretical rationale behind the CMS model is a defective reward system which stimulates the cardinal symptom of depression, anhedonia (57,58). This causes a decrease in responsiveness to reward measured as suppressed preference of sucrose solution. Repetitive readout of the hedonic status of the animals and of the depression-like status are then measured (53,55,57).

Drug	Target	Mechanism of action
SSRIs (59,60)	Serotonin transporter	Inhibits the reuptake of serotonin by presynaptic transporters resulting in increased serotonin levels
SNRIs (61)	Serotonin and noradrenaline transporter	Inhibits the reuptake of serotonin and noradrenaline by presynaptic transporters resulting in increased levels of both neurotransmitters
TCAs (53,61)	Plasma membrane transporters of serotonin and noradrenaline	Inhibits the reuptake of noradrenaline (and serotonin) by presynaptic transporters resulting in increased levels of these neurotransmitters
MAOIs (61)	Monoamine oxidase	Inhibits monoamine oxidase resulting in increased concentrations of monoaminergic neurotransmitters (serotonin, noradrenaline and dopamine)

Table 2. Overview of antidepressant classes of drugs for depression. *SSRIs: selective serotonin reuptake inhibitors, SNRIs: serotonin-norepinephrine reuptake inhibitors, TCAs: tricyclic antidepressants, MAOIs: monoamine oxidase inhibitors.*

Currently many different classes of antidepressant drugs exist, all of which have been tested in the CMS model of depression. These classes include selective serotonin reuptake inhibitors, tricyclic antidepressants and monoamine oxidase inhibitors (52) (Table 2). In order to reverse the CMS-induced symptoms in rodents,

it typically requires three to four weeks of antidepressant treatment (62). However, approximately 50 % of the anhedonic animals reject the antidepressant treatment, which is similar to the therapeutic treatment in humans (63). The antidepressant treatments available today have only showed moderate response rates, as up to 40 % of patients do not respond to the treatment (61,64).

1.3. ENERGY METABOLISM OF THE BRAIN

Metabolic pathways have been overlooked in the past, despite the importance of these in both health and disease. Therefore, the metabolic pathways of the brain, though similar to other tissues, will be reviewed in the following paragraphs. The concept behind energy homeostasis denotes processes managing intake, storage and utilization of substrates for the maintenance of stable energy levels (65). In order to maintain the energy homeostasis in the brain there is a competing relationship between oxidation of glucose and fatty acids called the glucose-fatty acid cycle (66,67).

1.3.1. GLUCOSE METABOLISM

Glucose is the primary energy source in the brain, where a constant supply of glucose by tight metabolic regulation is needed to sustain physiological brain function (68–70). Glucose provides functions through neuroenergetics, neurotransmission, energy storage, biosynthesis of amino acids, monosaccharides and carbohydrates, and oxidative defense. The energy-producing pathways using glucose include first glycolysis, in which glucose is converted to pyruvate, and then oxidative glucose metabolism converting pyruvate to carbon dioxide via the Krebs cycle and the electron transport chain (71).

Glucose requires to be transported across the endothelial cells of the BBB achieved by sodium-independent facilitative glucose transporters (GLUTs) localized at the luminal and abluminal side of the cells (68,72). Afterwards it is transported from the extracellular space in the brain through the plasma membranes of neurons, astrocytes, oligodendrocytes and microglia, which is also facilitated by GLUTs (72,73). The expression of the different isoforms of GLUTs is cell type specific (70). GLUT1 mediates glucose uptake into astrocytes, oligodendrocytes and microglia, whereas GLUT3 mediates uptake into neurons (68,72). Once the glucose is taken up, the brain rapidly catabolizes the glucose (70).

There are different pathways for the glucose metabolism however the initial step is common for all pathways (74). The initial step in metabolism involves the enzyme hexokinase 1, involved in glycolysis, which phosphorylates glucose to glucose-6-phosphate, the precursor for glycogen (Figure 1). Then it either can be converted into glycogen for storage creating a glucose pool maintaining a balance between influx, efflux and metabolism. Alternatively, it can be converted to fructose-6-

phosphate and continue in the glycolytic energy production with end products of pyruvate, or lastly it can enter the pentose phosphate pathway generating nicotinamide adenine dinucleotide phosphate, a precursor required for lipid synthesis. When pyruvate enters the mitochondria through the pyruvate dehydrogenase complex it is converted into acetyl-CoA and metabolized in the Krebs cycle (68,70,71,74).

The glucose homeostasis is regulated by neuronal and hormonal components that control production and utilization of glucose. A neuronal component involves glucose-sensing neurons in the hypothalamus and a hormonal component involves insulin, which acts on the melanocortin system, thereby regulating the glucose homeostasis (65,75,76).

The idea of the glucose-fatty acid cycle, which functions as a competing relationship due to a flexible choice of energy substrate in tissues, is that under sparse levels of glucose during conditions of fasting or exercise, the energy source will switch from using glucose to the alternative energy sources as fatty acids and ketone bodies. The purpose of this relationship is saving glucose for the brain when energy demands are scarce (67).

1.3.2. LIPID METABOLISM

Lipid metabolism is of particular importance for the brain due to the high lipid concentration exceeded only by adipose tissue (77). Lipids are divided into eight categories; fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides (78), where fatty acids constitute the major focus.

Fatty acids are essential lipids, which consist of a carbon chain with a carboxylic acid group. These are divided into subtypes based on the carbon chain length. Short-chain fatty acids have 2-4 carbon atoms, medium-chain fatty acids consists of 6-12 carbon atoms, while long-chain fatty acids have 14-18 carbon atoms and lastly very long-chain fatty acids consist of >18 carbon atoms (79). Furthermore, fatty acids can be classified as saturated or unsaturated, where saturated fatty acids are defined by carbons saturated with hydrogen atoms and no double bonds, contrary to unsaturated fatty acids which have double bonds and the number of these determines whether it is mono- or polyunsaturated fatty acids (PUFAs). Saturated fatty acids and monounsaturated fatty acids are synthesized in the brain, whereas PUFAs are supplied by the blood (80).

PUFAs are important for the brain as they serve as vital molecules for membrane structure and cell signaling (79). Predominant PUFAs in the brain are for example omega-6 arachidonic acid and omega-3 docosahexaenoic acid. Fatty acids can be free or esterified to lipids such as phospholipids and cholesterol, where fatty acids in

the brain mostly under undergo esterification to phospholipids for example palmitate and oleate enriching the myelin (80).

There exist three pathways in the metabolism of lipids. Since lipids are insoluble in water, transport proteins, called lipoproteins, are a necessity. The pathways include; the exogenous, the endogenous and the reverse cholesterol pathway (81). The exogenous pathway takes place in the enterocytes of the small intestine, where chylomicrons are synthesized in a process where triacylglycerol, cholesterol esters and apolipoprotein are assembled by microsomal triglyceride transfer protein. After leaving the enterocytes, the chylomicrons enter the lymphatic system and emulsifies. Apolipoprotein from high-density lipoprotein (HDL) activates the endothelial cell walls leading to cleavage of triacylglycerol and release of free fatty acids used for energy utilization. In this process chylomicron remnants are formed and taken up by the liver and further used in the endogenous pathway. The endogenous pathway therefore begins in the liver and involves formation of very low-density lipoprotein (VLDL) from free fatty acids and cholesterol esters. Microsomal triglyceride transfer protein assembles triacylglycerol, cholesterol esters and apolipoprotein producing VLDL, which leaves the liver and enters the serum. Lipoprotein lipase breaks down VLDL into oxidized lipoprotein and intermediate dense lipoprotein resulting in free fatty acid used for energy utilization or storage in adipocytes. The end product is low-density lipoprotein molecules containing cholesterol used for the synthesis of sterol lipids, or uptake into macrophages undergoing apoptosis or emptying the lipids by the reverse cholesterol pathway. The cholesterol reverse pathway involves removal of excess cholesterol esters from the peripheral tissue by the binding of HDL to a receptor on macrophages resulting in cholesterol ester efflux transport to the liver reusing the HDL for VLDL synthesis or catabolism (81).

Peroxisomes, which are specialized organelles, serve as a central regulator of the lipid metabolism through several mechanisms like for example breakdown of long-chain fatty acids necessary for further use in energy production in the mitochondria (81), which are the main site for fatty acid metabolism. The uptake of fatty acids in the mitochondria is mediated by several membrane proteins (67). Transport of fatty acids from the blood to the brain necessitate passage across the BBB and can occur by passive diffusion or by protein-mediated transport pathways dependent on the molecular size of the lipids (82). Short-chain and medium-chain fatty acids have high permeability and cross the plasma membrane easily, whereas long-chain fatty acids are less soluble and therefore need fatty acid transport proteins (FATPs) (83). FATPs are integral transmembrane proteins localized in the plasma membrane that enhance the uptake of long-chain and very long-chain fatty acids (84). FATP1 and FATP4 are predominantly expressed in the brain (83,84). Most of the FATPs possess acyl-CoA synthase activity, which converts fatty acids to fatty acyl-CoA after translocation through the plasma membrane (67) (Figure 1). As the outer mitochondrial membrane is impermeable to fatty acyl-CoA it uses the carnitine shuttle, where the first step includes carnitine palmitoyl transferase (CPT) 1, which

converts fatty acyl-CoA into acyl-carnitine. Carnitine acyl-carnitine translocase transports acyl-carnitine through the mitochondrial membrane. The second step of the shuttle involves CPT2, localized at the inner mitochondrial membrane, which reconverts acyl-carnitine into acyl-CoA and carnitine. Once inside the mitochondria, the degradation of acyl-CoA into shorter units by β-oxidation takes place. The β-oxidation is a cyclic pathway with production of a shortened acyl-CoA, an acetyl-CoA, a nicotinamide adenine dinucleotide and a flavin adenine dinucleotide after each cycle. The turnover of acyl-CoA re-enters β-oxidation (67). After import of the fatty acids, catabolism occurs in the mitochondrial matrix by several steps metabolizing long-chain acyl-CoAs to short-chain acyl-CoAs (67,85–87). CPT1 and CPT2 have a clear cut function allowing β-oxidation of long-chain fatty acids, such as palmitoyl-CoA and oleoyl-CoA, to occur with the end product acetyl-CoA important for the Krebs cycle in the mitochondria (67,85). The energy produced in the Krebs cycle is transferred to the electron transport chain resulting in production of adenosine triphosphate (ATP). Reversible inhibition of CPT1 activity is exerted by malonyl-CoA, thereby possessing a regulatory role of the fatty acid catabolism (66,85).

Other regulators of the fatty acid metabolism involve hormones and transcription factors (88). Insulin is secreted in response to high blood glucose levels resulting in increased transport of glucose by translocation of the GLUTs from intracellular vesicles to the plasma membrane, which also affects the lipolysis by preventing the availability of fatty acids as energy substrate (67,89). PPARs, which are nuclear receptor proteins with transcriptional function widely expressed in the brain, control expression of genes important for both glucose and lipid metabolism along with the function and numbers of peroxisomes (81,89,90). PPARs use free fatty acids as ligands, thus activating PPAR binding to peroxisome proliferator response element resulting in increased expression of target genes (81). PPARs act as metabolic lipid sensors, which make them important transcriptional regulators of the energy balance, the fatty acid metabolism, the glucose homeostasis and insulin sensitivity (89).

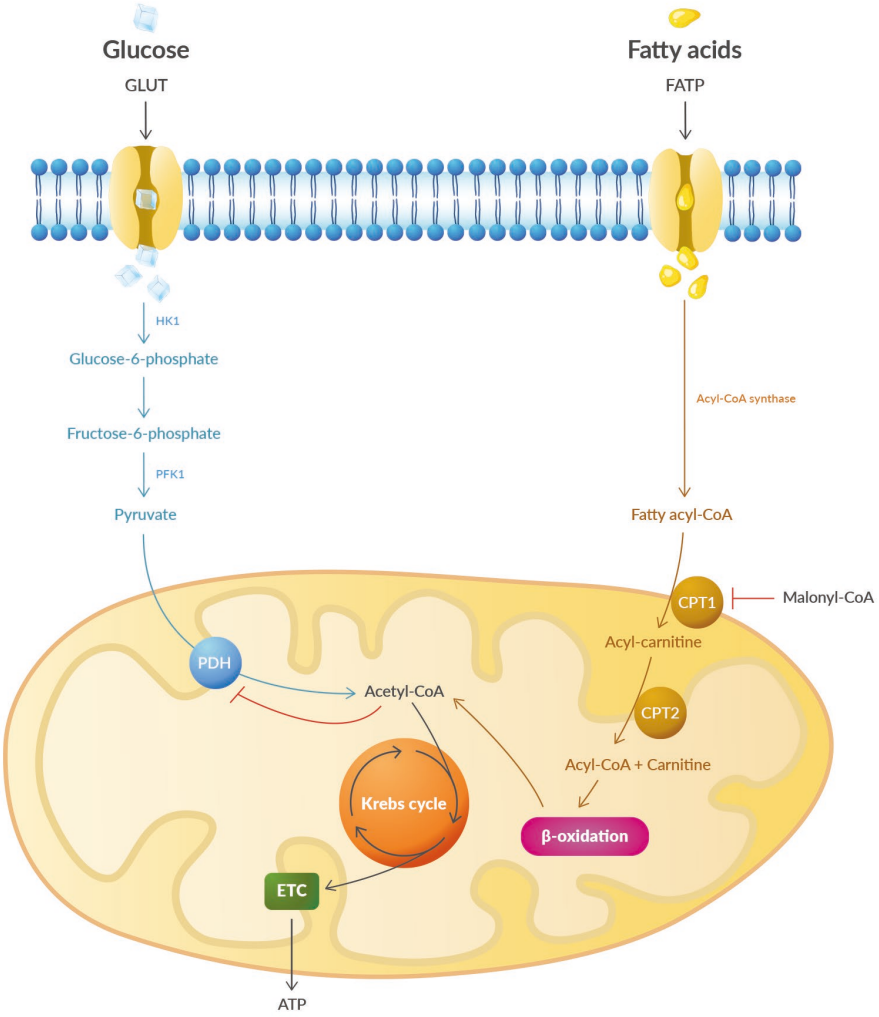

Figure 1. Transport of glucose and fatty acids. *Glucose enters the cell via GLUT and is phosphorylated to glucose-6-phosphate by HK1 followed by conversion to fructose-6-phosphate. Fructose-6-phosphate is converted to pyruvate by PFK1. PDH transforms pyruvate into acetyl-CoA in the mitochondria. Acetyl-CoA is used in the Krebs cycle. Fatty acids enter the cell via FATP, which possess acyl-CoA synthase activity and converts fatty acids into fatty acyl-CoA. CPT1 facilitates the transport across the outer mitochondrial membrane by converting fatty acyl-CoA to acyl-carnitine. CPT2 reconverts acyl-carnitine into acyl-CoA and carnitine. Acyl-CoA is used in β-oxidation producing acetyl-CoA. Acetyl-CoA from glucose pathway and lipid pathway is used in the Krebs cycle. The energy produced in the Krebs cycle passes to the ETC, where it is converted into ATP. Inhibitory mechanisms include inhibition of PDH by acetyl-CoA and inhibition of CPT1 by malonyl-CoA (red inhibiting arrow). ATP: adenosine triphosphate, CPT1: carnitine palmitoyl transferase 1, CPT2: carnitine palmitoyl transferase 2, ETC: electron transport chain, FATP: fatty acid transporter, GLUT: glucose transporter, HK1: hexokinase 1, PDH: pyruvate dehydrogenase complex, PFK1: phosphofructokinase 1.*

1.4. ENERGY METABOLISM OF THE IMMUNE SYSTEM

The different T cell phenotypes have unique metabolic demands thus switching reversibly between quiescent and proliferative states promoting activation of different metabolic pathways (91).

The primary energy source of T cells is glucose, which is required for growth, activation and proliferation (92). The glucose metabolism is activated by signals from T cell receptor and a co-stimulatory signal via CD28. This triggers phosphatidylinositol 3´-kinase (PI3K) leading to activation of protein kinase B (Akt), also known as the PI3K-Akt pathway, which regulates the energy metabolism (91). Akt increases the glucose uptake by upregulation of GLUT1 on T cells. The T lymphocytes Th1, Th2 and Th17 all express GLUT1 thus possessing high glycolytic levels. In contrast, regulatory T cells express low levels of GLUT1 indicating low glycolytic levels and the importance of fatty acid oxidation as energy source (93). Furthermore, Akt stimulates the activity of the rate-limiting enzyme hexokinase important for glycolysis (91). B cell and natural killer cells possess similar metabolic signaling pathways, as these initiated in T cells, though these are activated via CD19 and DNAX-activating protein 10, respectively, instead of CD28.

In addition to the key protein kinase Akt, involved in metabolic control pathways, there is another kinase, adenosine monophosphate (AMP)-activated protein kinase (AMPK), which acts as an energy sensor and is activated by serine-threonine kinase 11. The activation is dependent on the levels of AMP and activated in response to ATP depletion. This AMPK signaling pathway regulate the metabolism and differentiation of CD4+ T cells into effector T cells or regulatory T cells relying on specific metabolic demands, in particular glycolytic and fatty acid metabolism, respectively. The result is suppression of anabolic mechanisms and stimulation of catabolic mechanisms such as fatty acid oxidation by decreasing the levels of acetyl-CoA carboxylase, which then stimulates the activity of CPT1 and thereby ATP production (91,94–96). All these different pathways indicate that metabolism is fundamental in the determination of cell fate, and is important in both health and diseased conditions.

1.5. GENETIC, ENVIRONMENTAL AND MICROBIAL FACTORS

The etiology of MS remains unknown, however it is now accepted as a multifactorial disease influenced by genetic, environmental and microbial factors (4,8). Children or siblings of patients with MS have a risk of 3-5 % for getting MS and monozygotic twins have an even higher risk of 25 % (8). These data show that there is a genetic component in developing MS. Human leukocyte antigen (HLA) variants are associated with immune response genes, whereas non-HLA variants are associated with genes in immune activation and tolerance (4). The *HLA-DR beta I (DRB1)*1501* allele in MHC-II is a dominant risk factor for MS with an odds ratio

of approximately 3 (4,97). Over time more comprehensive investigations were conducted, thereby identifying several alleles related to MS (8,98). More than 50 loci were found susceptible for an association with immune system including genes for IL-2 receptor alpha chain and IL-7 receptor alpha chain, cytokine pathways including a gene for TNF-α, and finally associated with co-stimulatory molecules such as genes for CD80 and CD86 (8). In contrast to these mutations for getting MS, mutations with protective effects against developing MS exist. These mutations include *CPT1A P479L* (99) and *CPT1A G710E* (100,101) and will be elaborated further.

These findings indicate a strong genetic component, however genetic associations in the development of MS are not enough and environmental factors also play a role. Environmental factors include geographic variations where increasing latitude results in increased MS development, as well as vitamin D deficiency increases the risk of MS development due to the correlation between low vitamin D levels and increased latitude (8). Other environmental factors include among others smoking, obesity, Epstein-Barr virus infection and gut microbiota changes. Risk factors as vitamin D, smoking, diet and stress are associated with gut microbiome dysbiosis, since diminished intake of vitamin D causes changes in the immune response by increasing the regulatory T cell amount and decreasing the T cell amount in the gut. Smoking causes changes in the composition of the microbiome (102). Diet and stress enhance the gut permeability mediated by bacteria that drive the immune response and affect the CNS (103,104). These mentioned risk factors increases gut permeability and leads to the transfer of gram-negative bacteria initiating an immune response (103).

Microbiota are able to produce and release neuroregulatory factors such as dopamine, noradrenaline and acetylcholine important neurotransmitters for preventing depression (103), in this context, enhanced gut permeability has been found to be related to depression. The role of genetics in depression is poorly understood and limited data are available (35). Although depression is thought to be a result of both genetic predisposition and environmental factors as stress, head injury, family conflicts, depression is only triggered in genetically susceptible individuals (53,105). Examples of investigated genes implicated in depression are serotonin transporter, serotonin receptor 1A and 2A, involved in the synthesis of serotonin and apolipoprotein E (APOE) involved in the catabolism of lipoproteins (106).

1.6. NEW MODEL OF CENTRAL NERVOUS SYSTEM DISEASES

The traditional way of thinking the pathogenesis of CNS diseases has changed as it rather has become evident that more complex systems underlies the pathology, where MS and depression constitute the major focus in this PhD thesis. Therefore, this opens a question if there is a change of paradigm towards treatments of CNS

diseases with metabolic pathways as the central part? This will be described in the following paragraphs.

1.6.1. LIPID METABOLISM AS A CENTRAL PART

Altered metabolism is proposed to underlie the pathogenesis of several diseases affecting different cellular mechanisms as inflammation, oxidative stress, mitochondrial dysfunction, iron accumulation, insulin resistance and microbiota.

Inflammation is present in several diseases and associated with both MS and depression, which makes the understanding of the impact of lipids on immune cells an important part (107). Lipids serves as regulators of immune cell polarization and proliferation of T lymphocytes is dependent on energy produced by both glycolysis and β-oxidation (107,108). Pathogen entrance and consequently inflammation mediates activation of T cells, which then differentiate into effector T cells or regulatory T cells, having specific metabolic demands, required for the specific environment. When an immune response is initiated and T cells become activated β-oxidation decreases while glycolysis increases. At the end of an inflammatory response the metabolic state switches to decreased glycolysis and increased lipid oxidation, conditions favored by memory T cells (93).

Oxidative stress is a contributor in the pathogenesis of neurodegenerative diseases as MS (109), where lipids serve as the major target. Since the brain contains the highest concentration of lipids next to adipose tissue and with the fact that fatty acids are targets for lipid peroxidation it underlines that the brain, more specific neurons and oligodendrocytes, is particularly vulnerable to oxidative stress and β-oxidation (98,109). Though, it can also cause damage to nucleic acids, proteins and carbohydrates resulting in necrosis and apoptosis (110). ROS and RNS generated by macrophages and microglia cause damage in active MS lesions resulting in structural changes in myelin proteins, which become targets of immune cells as they are recognized as foreign antigens (98). ROS such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals are produced through oxidative phosphorylation by processes as mitochondrial respiratory chain, nicotinamide adenine dinucleotide phosphate oxidases and monoamine oxidases (109). Oxidative stress results from ROS production exceeding the detoxification capacity of these reactive intermediates of the cell (109). The brain consumes high amounts of oxygen for production of energy and has low levels of antioxidants, which makes it even more vulnerable to oxidative stress (110). Moreover, ROS and RNS are able to activate transcription factors such as nuclear factor-kappa-light-chain-enhancer of activated B cells and nuclear factor erythroid 2-related factor 2 (Nrf2) resulting in upregulated expression of TNF-α responsible for activation of neutrophils sensing an infection (111) and induction of antioxidant enzymes as heme oxygenase-1 (HO-1) scavenging free radicals and removing damaged proteins (2).

The mitochondria are the energy factories of the cells and besides this they are also important for cellular functions such as fatty acid oxidation, glycolysis, amino acid biosynthesis and apoptosis. Oxidative metabolic mechanisms generate almost all of the ATP in the cell, which underpin the importance of these (98). However excess amounts of fatty acids can results in mitochondrial dysfunction further contributing to macrophage inflammation and production of superoxide (98,107). Normal mitochondrial respiration produces by-products as ROS and requires detoxification. Though, the lack of protective proteins results in mutated mitochondrial DNA in case of increased ROS levels, thereby compromising oxidative phosphorylation, which results in collapse of the mitochondria (2).

Iron is fundamental to brain metabolism by processes as oxidative phosphorylation and myelination. Normally, iron is present in the brain parenchyma where it is predominantly stored in ferritin (FT) as non-heme iron (ferric iron), whereas a small amount is present as heme iron (ferrous iron) (112,113). There are two subunits of FT: heavy chain (FTH) and light chain, which are both expressed by oligodendrocytes (114). The transport of ferrous iron to other cells in the CNS is facilitated by transferrin, divalent metal transporter and ferroportin. If ferrous iron is not required it will be oxidized into ferric iron and stored in FT (113). In contrast, disrupted brain iron homeostasis in MS, for example, leads to accumulation of ferrous iron generating ROS, which are harmful to lipids. Moreover, the mitochondria are vulnerable to elevated ROS levels and dysfunction of the mitochondria is related to oxidative damage in MS. The oligodendrocytes, which are the main targets of inflammation in MS, are in particular vulnerable to such mitochondrial dysfunction and ROS (112).

Insulin resistance is caused by alterations in metabolism in particular characterized by increased fatty acid oxidation and decreased glycolysis according to the traditional view (88,115–117). Following increased fatty acid oxidation, an increased production of acetyl-CoA from fatty acid catabolism leads to inhibition of pyruvate dehydrogenase. Consequently, levels of glucose-6-phosphate are elevated, which further inhibits hexokinase, which is important for the glucose uptake (88,116,118,119). However other mechanisms are suggested to cause insulin resistance. It is proposed as a reverse mechanism of the traditional glucose-fatty acid cycle describing the glucose metabolism as the controlling part of the rate of fatty acid oxidation in which glucose or insulin limits the entrance of long-chain fatty acids into the mitochondria by inhibiting carnitine acyltransferase. This is different from the traditional mechanism, where it is proposed to be a consequence of a defect in the glucose oxidation rather than increased fatty acid oxidation (115). Additionally, a defect in GLUT4 and hexokinase activity mediates inhibition of glycogen synthesis caused by fatty acid oxidation (120). This failure in the capacity of metabolism stimulates oxidative stress with increased ROS levels, which plays an important role in situations of altered insulin secretion and serves as a contributor to insulin resistance (121). Both insulin resistance and oxidative stress are suggested as

comorbidities in MS particularly SPMS contributing to disease disability and progression (122).

Microbiota is important for tissue development, metabolism, immune system and nutrient synthesis (123). The gut microbiota is able to synthesize and secrete vitamins such as vitamin B12, folate and biotin supporting development of the CNS, endothelial cell growth and regulation of the immune system (102). Moreover it can control signals from the brain via the microbiome-gut-brain axis through the interaction of metabolic, immune system, CNS and endocrine pathways (102). Molecular patterns such as ATP and short-chain fatty acids are proposed to play a role in the interaction between microbiota and the immune system (123). Short-chain fatty acids, metabolized by gut bacteria, serve as a link between the immune system and the microbiota, as they provide beneficial effects to the metabolism through the involvement in cellular processes as gene expression, proliferation, differentiation and apoptosis (123,124). Short-chain fatty acids stimulate expansion of protective regulatory T cells. In contrast, long-chain fatty acids stimulate pathogenic T cell differentiation to Th1 and Th17 cells in the gut and can thereby induce inflammation in the CNS (125). Therefore loss of balance between immune system and microbiota interactions can cause inflammatory diseases and infections (123).

Lipids play a crucial role in health and disease as alterations in lipid metabolism are associated with both neurodegenerative diseases and neurological disorders (77,98,110,126). In neurodegenerative diseases such as MS low levels of PUFAs have been found, which can be related to the function of PPARs to which PUFAs serve as ligands. This results in upregulated lipid metabolism and enhancement of glucose catabolism (81). In neurological disorders such as depression changes in the composition of serum lipids have been found. These changes include low levels of HDL cholesterol, which is a hallmark of depression, low total cholesterol levels and low HDL cholesterol:cholesterol ratio suggesting impairment of efflux transport of cholesterol from tissue to liver, as HDL particles are important for the transport of cholesterol (126). Given that the lipid metabolism plays a pivotal role in energy homeostasis, it is tempting to hypothesize that dysregulation of this may underlie diseases of the CNS.

1.6.2. PHARMACOLOGICAL INHIBITION OF LIPID METABOLISM

CPT1 is located in the outer mitochondrial membrane, where it serves as the regulatory site for fatty acid oxidation. Inhibitors of CPT1 are among others malonyl-CoA (127). Structurally, CPT1 has two transmembrane domains anchoring the enzyme. The active site placed at the C-terminal domain is localized at the cytosolic site of the outer mitochondrial membrane (85). Although the exact role of CPT1 in CNS diseases is not known it can be hypothesized that there is a link between CPT1-mediated lipid transport and these diseases due to its central role in

cellular energy production from lipids. This in combination with the fact that the fatty acid metabolism is dysfunctional in MS and the lipid level in MS patients and depressed patients are reduced. Therefore, inhibition of lipid metabolism through the rate-limiting enzyme in fatty acid oxidation, CPT1, could be a potential target for treating diseases (14,55,81) and several pharmacological approaches of CPT1 inhibitors exist, such as perhexiline (128,129), teglicar (130) and etomoxir (14).

Etomoxir is a potent blocker of CPT1, which is rate-limiting for β-oxidation and thus lipid metabolism. Etomoxir is functioning as a CPT1 antagonist, which specifically binds to CPT1, thereby preventing formation of acyl-carnitine necessary for acyl-CoA transport into the mitochondria (14,55,131). Pharmacological inhibition of etomoxir induces shift in metabolic pathways favoring glucose metabolism as primary energy source rather than lipid metabolism (117,132).

It has been tested as a pharmacological treatment for diabetes mellitus with the result of a reduced hepatic glucose production thereby reducing fasting blood glucose, decreasing triglyceride levels and blocking fatty acid oxidation (117). It has also shown effects in myocardial ischemic injury where etomoxir stimulated the glucose utilization by overcoming fatty acid oxidation (132). However, etomoxir can be provided as a new pharmacological treatment regimen for CNS diseases such as MS and depression aiming at both immune system and metabolism contrary to the current disease-modifying therapies for MS that solely have been effective in RRMS though only revealing symptomatic effects and not reversing the pathological damage and most importantly not curing the disease, and to antidepressant treatments, which are inefficient in up to 40 % of the patients (61,64). This suggests etomoxir as a novel potential target for MS as well as depression.

1.6.3. GENETIC INHIBITION OF LIPID METABOLISM

Regulation of the mitochondrial fatty acid metabolism primarily involves CPT1, which is expressed in various tissues depending on the particular isoform. The protein CPT1A is expressed in the liver, brain, kidney, lung, spleen, intestine, pancreas and ovary (87). CPT1B is expressed in muscle, heart, adipose tissue and testis, whereas CPT1C is expressed in the brain (87,133).

The genes *CPT1A, CPT1B* and *CPT1C* are localized to human chromosomes 11q13.1-q13.5, 22q13.31-q13.32 and 19q13.33, respectively (133,134). The human *CPT1A* gene has an open reading frame of 2319 base pair (135) and *CPT1B* has an open reading frame of 2316 base pair (136).

Several human mutations in *CPT1A* are identified. In this PhD thesis the major focus constitutes two of those mutations. The first is a missense mutation at nucleotide position 1436 C to T predicting a substitution of proline for a leucine at codon 479 (P479L) (99). This variant has 22 % residual activity of the CPT1A protein

(137,138). Carriers of *CPT1A P479L* are identified among Canadian and Greenland Inuits. This frequency of the homozygous mutant allele is high in the Inuits in Canada (93 %), in Nunavut (81 %) and of Greenland (73 %), when taken both homozygous and heterozygous mutant alleles the frequency is up to 98 % (99,139). The second is a mutation at nucleotide position 2129 G to A predicting a substitution of glycine for a glutamic acid at position 710 (G710E) (100,101). This mutant *CPT1A* is totally inactive, thereby dramatically impairing the catalytic function (101). Carriers of *CPT1A G710E* are expressed among the Hutterite community considered as a genetic isolated population similarly to the Amish (100).

CPT1A deficiency is a rare disorder of mitochondrial fatty acid β-oxidation and is inherited as an autosomal recessive disease (100,101). The symptoms comprise hypoketotic hypoglycemia, hepatomegaly, seizures and sudden death and are usually triggered by exogenous stress factors as fasting, fever and illness (101,138), thus making these people more susceptible to infection. Since the catalytic activity of CPT1A is only partially lost in the Inuits, these are expected to have milder clinical consequences compared to the Hutterites, which have lost the enzymatic activity of CPT1A completely. This could be explained by the hindered ability of memory T cells to protect against recurring infections, as these cells are dependent on β-oxidation, which are decreased in these cases (140).

On the other hand, the prevalence for MS (141,142) and depression (143,144) found in the northern indigenous populations, the Inuits and the Hutterites, is remarkably low compared to the non-indigenous Canadian population (145). The prevalence of MS in the Inuit population is 1 per 50,000 (142) and 1 per 1,100 in the Hutterite population (141) compared to the estimated MS prevalence of 240 per 100,000 in Canada (145). The same tendency applies for depression with 3 % in the Inuits (144) and 0.18 % in the Hutterites (143), which can be compared to the prevalence in the Canadian population of 16 % (144). These findings propose a significant role of *CPT1A* mutation in the development of CNS diseases such as MS and depression, although the mechanisms are unknown.

CHAPTER 2. OBJECTIVES

The complex interaction between metabolism and immune system described in CNS diseases are of outermost importance for identifying mechanisms leading to MS thereby finding an effective treatment strategy as there must be aspects of MS development, progression and treatment that we still do not understand. The aim of the PhD thesis has therefore been to obtain a thorough understanding of the metabolism in health and disease using *in vivo* models of MS and the common comorbid condition, depression.

In order to investigate the overall aim of the PhD thesis four studies with the following objectives have been conducted:

Manuscript I: To investigate the role of the lipid metabolism blocker, etomoxir, in rodent EAE models of MS with effects on MBP, CPT1A and FTH expression and cytokine production.

Manuscript II: To investigate the efficacy of the lipid metabolism blocker, etomoxir, and the standard treatment of MS, interferon-β, on the autoantibody response in a rat EAE model of MS in order to identify potential biomarkers for MS.

Manuscript III: To investigate the role of a *Cpt1a P479L* mutation in a mouse EAE model of MS with effects on MBP and CPT1A expression as well as on expression of genes involved in oxidative stress. Furthermore, the impact of high-fat diet (HFD) on the disease course was evaluated.

Manuscript IV: To investigate the efficacy of the lipid metabolism blocker, etomoxir compared to the standard treatment of depression, escitalopram, in a rat CMS model of depression.

CHAPTER 3. RESULTS

3.1. MANUSCRIPT I

Pharmacological inhibition of carnitine palmitoyl transferase 1 inhibits and reverses experimental autoimmune encephalomyelitis in rodents

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Manuscript submitted

Abstract

Multiple sclerosis (MS) is a neurodegenerative disease characterized by demyelination and inflammation. Dysregulated lipid metabolism and mitochondrial dysfunction are hypothesized to play a key role in MS. Carnitine Palmitoyl Transferase 1 (CPT1) is a rate-limiting enzyme for beta-oxidation of fatty acids in mitochondria. The therapeutic effect of pharmacological CPT1 inhibition with etomoxir was investigated in rodent models of myelin oligodendrocyte glycoprotein- and myelin basic protein-induced experimental autoimmune encephalitis (EAE). Mice receiving etomoxir showed lower clinical score compared to placebo, however this was not significant. Rats receiving etomoxir revealed significantly lower clinical score and lower body weight compared to placebo group. When comparing etomoxir with interferon-β (IFN-β), IFN-β had no significant therapeutic effects, whereas etomoxir treatment starting at day 1 and 5 significantly improved the clinical scores compared to the IFN-β and the placebo group. Immunohistochemistry and image assessments of brain sections from rats with EAE showed higher myelination intensity and decreased expression of CPT1A in etomoxir-treated rats compared to placebo group. Moreover, etomoxir mediated increased interleukin-4 production and decreased interleukin-17α production in activated T cells. In conclusion, CPT1 is a key protein in the pathogenesis of EAE and MS and a crucial therapeutic target for the treatment.

3.2. MANUSCRIPT II

Identification of brain antigens recognized by autoantibodies in experimental autoimmune encephalomyelitis-induced animals treated with etomoxir or interferon-β

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Abstract

Multiple sclerosis (MS) is a neurodegenerative autoimmune disease, where chronic inflammation plays an essential role in its pathology. A feature of MS is the production of autoantibodies stimulated by an altered-peptide-ligand response and epitope spreading, resulting in loss of tolerance for self-proteins. The involvement of autoantibodies in MS pathogenesis has been suggested to initiate and drive progression of inflammation; however, the etiology of MS remains unknown. The effect of etomoxir and interferon-β (IFN-β) was examined in an experimental-autoimmune-encephalomyelitis (EAE) model of MS. Moreover, the impact of etomoxir and IFN-β on recognition of brain proteins in serum from EAE rats was examined with the purpose of identifying the autoantibody reactivities involved in MS. Animals treated with etomoxir on day 1 exhibited a statistically significantly lower disease score than animals treated with IFN-β (on day 1 or 5) or placebo. Etomoxir treatment on day 5 resulted in a significantly lower disease score than IFN-β treatment on day 1. After disease induction antibodies was induced to a broad pallet of antigens in the brain. Surprisingly, by blocking CPT1 and therewith lipid metabolism several alterations in the antibody response was observed suggesting that autoantibodies play a role in the EAE animal model.

3.3. MANUSCRIPT III

CPT1A plays a key role in the development and treatment of multiple sclerosis and experimental autoimmune encephalomyelitis

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Manuscript submitted

Abstract

Background: Human mutations in carnitine palmitoyl transferase 1A (*CPT1A*) are correlated with remarkably low prevalence of Multiple Sclerosis (MS) in the Inuits *(P479L)* and Hutterites *(G710E)*. To elucidate the role of CPT1A, we established a *Cpt1a P479L* mouse strain and tested the sensitivity to experimental autoimmune encephalomyelitis (EAE). Moreover, we tested the effect of high-fat diet (HFD) on disease severity.
Methods: EAE was induced in C57BL/6J wild type (WT) and *Cpt1a P479L* mice and clinical score and weight were evaluated daily. The protein expression of myelin basic protein (MBP) and CPT1A were investigated by immunohistochemistry and western blotting, and the gene expression of *Cpt1* and markers of oxidative stress were investigated by RT-qPCR.
Results: The disease symptoms progressed and increased significantly throughout the experiment in WT mice in contrast to *Cpt1 P479L* mice. WT mice receiving HFD, in contrast to mice receiving normal diet (ND) and *Cpt1a P479L* mice receiving ND or HFD, showed significantly exacerbated disease course. After the EAE induction, the expression of MBP was significantly reduced in the WT mice compared to the *Cpt1a P479L* mice. The expression of *Cpt1c* was significantly increased in *Cpt1a P479L* mice compared to WT mice. The WT mice showed significantly increased expression of *Ho-1* compared to CPT1a mice, whereas the expression of *Pgc1α* was increased in *Cpt1a P479L* mice.
Conclusion: In conclusion, these findings support a key role of CPT1A and lipid metabolism in the development of MS and could be a promising pharmacological target in the treatment of MS.

3.4. MANUSCRIPT IV

Blocking of carnitine palmitoyl transferase 1 potently reduces stress-induced depression in rat highlighting a pivotal role of lipid metabolism

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Abstract

Major depressive disorder is a complex and common mental disease, for which the pathology has not been elucidated. The purpose of this study is to provide knowledge about the importance of mitochondrial dysfunction, dysregulated lipid metabolism and inflammation. Mitochondrial carnitine palmitoyl transferase 1A (CPT1A) is a key molecule involved in lipid metabolism and mutations in *CPT1A* causing reduced function is hypothesized to have a protective role in the development of depression. Moreover, *CPT1A* is found to be upregulated in suicide patients with history of depression. Therefore, we hypothesized that inhibition of CPT1A activity can be developed as an innovative treatment strategy for depression. Stress exposure combined with different pharmacological treatment regimens; Etomoxir, CPT1 blocker, and Escitalopram, a favored antidepressant drug, was applied in state-of-the art chronic mild stress model. Etomoxir treatment induced statistical significant reduction of anhedonic behavior compared to vehicle treatment ($p<0.0001$) and reversed depression-like phenotype in 90 % of the rats ($p=0.0007$), whereas Escitalopram only proved 57 % efficacy. Moreover, Etomoxir revealed downregulation of interferon-γ, interleukin-17α and tumor necrosis factor-α. This indicate that alteration in metabolism is pivotal in the pathogenesis of depression, since CPT1 blockage is highly efficient in treating anhedonia and inflammation, thereby opening up for a novel class of antidepressant medication.

CHAPTER 4. DISCUSSION

The objective of the PhD thesis was to obtain an understanding of the role of the metabolism in CNS in health and disease using *in vivo* animal models of MS and depression. The aim with this approach was to come a step closer in clarifying the etiology, development, progression and treatment opportunities of these as well as other CNS diseases. Based on the experimental studies presented in *Manuscript I-IV* and findings of the literature this chapter is a combined discussion of all the projects with metabolism as the central part.

4.1. THE TRADITIONAL AND SYSTEMIC FRAMEWORK OF MULTIPLE SCLEROSIS

The traditional way of understanding the etiology and pathology of MS is as an inflammatory autoimmune disease of the CNS, thus interpreting it as an isolated disease referred to as the traditional framework. However, it has become evident that more complex systems underlie the etiology and pathology of MS, therewith constituting the systemic framework. Both of these frameworks will be discussed in the following paragraphs.

4.1.1. THE TRADITIONAL FRAMEWORK

For a long time MS has been regarded as an inflammatory autoimmune disease influenced by immunological, genetic and environmental pathologic factors, which constitutes the basis of the traditional framework.

This traditional framework covering the immunological part is initiated by exposure to a brain infection or a peripheral infection of an individual, which stimulates the activation of autoreactive T cells in the CNS directly or by mechanisms like for example molecular mimicry, altered peptide ligand or bystander activation (11,12). The T cells recognize CNS antigens and initiate an inflammatory autoimmune response. This response is thought to be the trigger of MS development which is further characterized by the pathological hallmarks such as breakdown of the BBB, demyelination and axonal degeneration (11). According to this framework, the reversible disability seen in RRMS is a result of focal regions of inflammatory demyelination where the oligodendrocytes, the myelin and the axons are destroyed. When monocytes and lymphocytes cross the BBB and enter the CNS, the lesions become edematous and the inflammation can be attributed to this process. After this the onset of neurological decline occurs rapidly and the dysfunction of axons is a result of blocked nerve conduction due to the edema and serum components. The remission phase covers decreased inflammation along with remyelination and therewith restoration of axonal conduction followed by relapses and after years,

patients enter SPMS with no relapses. The transition from RRMS to SPMS is assigned to the progressive loss of axons (3).

Genetic and environmental risk factors of MS influence the function of the immune system. MS has been considered as a disease with an inherited risk for disease susceptibility (3). The risk for MS is increased if you have an affected parent. The *HLA-DRB1* gene is the dominant MS-associated gene accounting for 16-60 % of the genetic susceptibility however this association has not been confirmed for MS patients in general (81,146). Women are also affected more often than men in a 2:1 ratio (9). Furthermore, the MS incidence is increased in Europeans compared to American Indians, Africans and Asians (147). Environmental factors such as microbial infections are also thought as an important trigger of MS by molecular mimicry where T cells recognize an epitope from an infectious agent cross-reacting with a self-antigen. An association between relapses and recent viral or bacterial infections are found, though no specific infectious agent has been identified (148). Moreover, vitamin D has been found to regulate the immune system as well as decreasing the MS incidence (149). These external factors have proved elusive, since exposure of infectious agents in similar populations have different disease incidence and populations obtaining high vitamin D levels have high disease incidence (81).

Until today the immunologic factors have been the logical explanation for the disease development and also the basis for the development of therapies for the treatment of MS. However, other pathologic findings present in MS can neither be explained by focusing solely on the traditional framework. These findings include disease progression from RRMS to PPMS and SPMS, since the inflammation peaks at the time of RRMS, whereas loss of brain volume decreases and axonal loss increases. While transitioning to SPMS, inflammation decreases along with a rapidly increase in loss of brain volume and axonal loss (11). Moreover, the current disease-modifying therapies functioning by slowing down the inflammation have only shown efficacy in RRMS though remaining limited to symptomatic relief and have shown no effect in PPMS and SPMS (1,4). Nevertheless these therapies are not able to stop the progressive neurological decline contributing to disability. Other uncovered findings in the pathology include changes in lipid levels marked by reduced PUFA levels in particular palmitate and oleate lipids (150). Iron accumulation where iron is released from demyelinating lesions contributing to oxidative stress. Glutamate excitotoxicity leading to calcium influx into neurons, which results in neurodegeneration. Microglial production of pro-inflammatory cytokines, ROS and RNS are toxic for neurons and oligodendrocytes thus promoting mitochondrial dysfunction and oxidative stress (4). Mitochondrial dysfunction demonstrated by reduced numbers of mitochondria and energy production contributing to calcium-mediated axonal degeneration. Activation of mitochondrial permeability pores leads to mitochondrial swelling, rupture of membrane and release of apoptotic mediators (98).

Since this traditional framework is incomplete in clarifying the etiology of MS and too simple for explaining the development, progression along with the inefficient treatment therapies, this opens up for a new framework for MS. This is an attempt to clarify the etiology and pathology of MS by covering all aspects with the goal of developing a therapy stopping and preventing the progression of MS.

4.1.2. THE SYSTEMIC FRAMEWORK

It has been evident that more complex systems underlie the pathology of MS, which opens up a new systemic framework challenging the current traditional one. In contrast to the traditional framework, all the well-demonstrated pathological hallmarks characterized in MS are covered by this systemic model, where a shift in metabolism plays a central role. Altered metabolism is therefore suggested to play an essential role in the pathogenesis of MS with lipids as a denominator, since the lipid metabolism affects several cellular mechanisms. Even though the immune system plays a more prominent role in RRMS than in PPMS and SPMS, most of the processes described are out of balance.

The knowledge of disturbed glucose metabolism in the pathology of MS are limited, however much literature hints to a link. The weight of the brain represents 2 % of the total body weight, though 25 % of the total body glucose is utilized by it, which underpins the importance of a continuous glucose supply making it the main energy source in the brain (151). The importance of lipids is proven by the fact that lipids represent the dry weight of 33 % in the grey matter, 55 % in the white matter and 70 % in myelin (152). The lipids are produced by the oligodendrocytes, which are able to produce up to 50 or more segments of myelin (114). This extraordinary high demand for lipids is essential to form myelin sheath for wrapping and insulating axons, thus propagating efficient neuronal impulses. This dependence on lipids is highlighted by pathologies disturbing myelination, such as MS, which affects lipid metabolism (153).

The systemic model is the basis of a new conceptual framework of the pathologic findings in MS (Figure 2). Stimuli such as stress are a broad term which could be induced by physiological, psychological and pathological (viral, bacterial, or fungal infection) factors. Stress affects all the mechanisms proposed, which are connected directly or indirectly to each other and initiates a cascade of events. Therefore it has to be interpreted as complex network with shift in metabolism as the central part.

Stress induces shift in metabolic pathways from glucose-based to lipid-based metabolism. The transcriptional regulators, PPARs, serve as a link between energy balance, lipid homeostasis and immune system by sensing lipid levels. Due to increased lipid metabolism, the expression of PPARs is decreased. This results in dysregulation of PPARs promoting disinhibition of inflammatory response proteins, oxidative stress and myelin degradation (81).

Inflammation induced by the increased lipid metabolism causes increased activity of CPT1 transporting long-chain fatty acids, which stimulates pathogenic T cell (Th1 and Th17 cells) differentiation (125). An acute inflammatory response promotes shift from storage of lipids to sequestration of lipids by immune cells. Favoring lipid metabolism also promotes imbalance in cytotoxic and phagocytic microglia and T cells resulting in chronic activated cytotoxic cells, which lack self-regulatory apoptotic mechanisms inducing neurodegeneration (81).

Activation of the HPA axis affects the CNS and the gut, which causes release of glucocorticoids, mineralocorticoids and catecholamines resulting in altered gut microbiota composition, increased gut permeability and increased immune response (102). Cytokines are able to regulate the HPA axis leading to impairment of the negative feedback mechanism, which is mediated by the hippocampus, thus resulting in overproduction of glucocorticoids (48). Chronic stress causes increased cortisol secretion thus limiting the inhibitory effect of cortisol on the immune response. Due to these high levels of glucocorticoids, macrophages and microglia release pro-inflammatory cytokines. Consequently, this leads to loss of glucocorticoid receptors disinhibiting the HPA axis (40,50). This produces neurotoxic free radicals and decreased glucose transport (40) leading to oxidative stress and insulin resistance, thus entering the vicious cycle.

Oxidative stress could be caused by hypoxia imposing stress to cells, which is a process linked to inflammation. The metabolism is altered in attempt to adapt to the lack of oxygen. This is facilitated by changes in expression of proteins, which stabilizes the transcription factor hypoxia inducible factor. Hypoxia alters oxidative phosphorylation, which is adapted to hypoxia by remodeling the electron transport chain and the Krebs cycle. Changes in the composition of electron transport chain complexes increases the production of ROS, which oxidizes proteins associated with the Krebs cycle as pyruvate dehydrogenase, for example (154). It can also be caused by ischemia, where influx of calcium together with increased levels of ROS and RNS stimulate opening of the mitochondrial permeability transition pores. Consequently, this leads to mitochondrial dysfunction characterized by influx of solutes, loss of the mitochondrial membrane potential and equilibrium of ion gradients. This prevents synthesis of ATP and causes expansion of the mitochondrial matrix, swelling and rupture of the mitochondria membrane with resulting cell death (154,155).

To accomplish the production of the massive quantities of lipids necessary for myelination, enzymes are required. The high levels of iron in the oligodendrocytes could be due to the required enzymes involved in the highly metabolic process when producing myelin (114). However, in neurons and oligodendrocytes the mitochondria are possible areas of iron deposits leading to accumulation of iron (114). Ferrous iron in the presence of hydrogen peroxide generates ROS, which further stimulates oxidative stress (112,156).

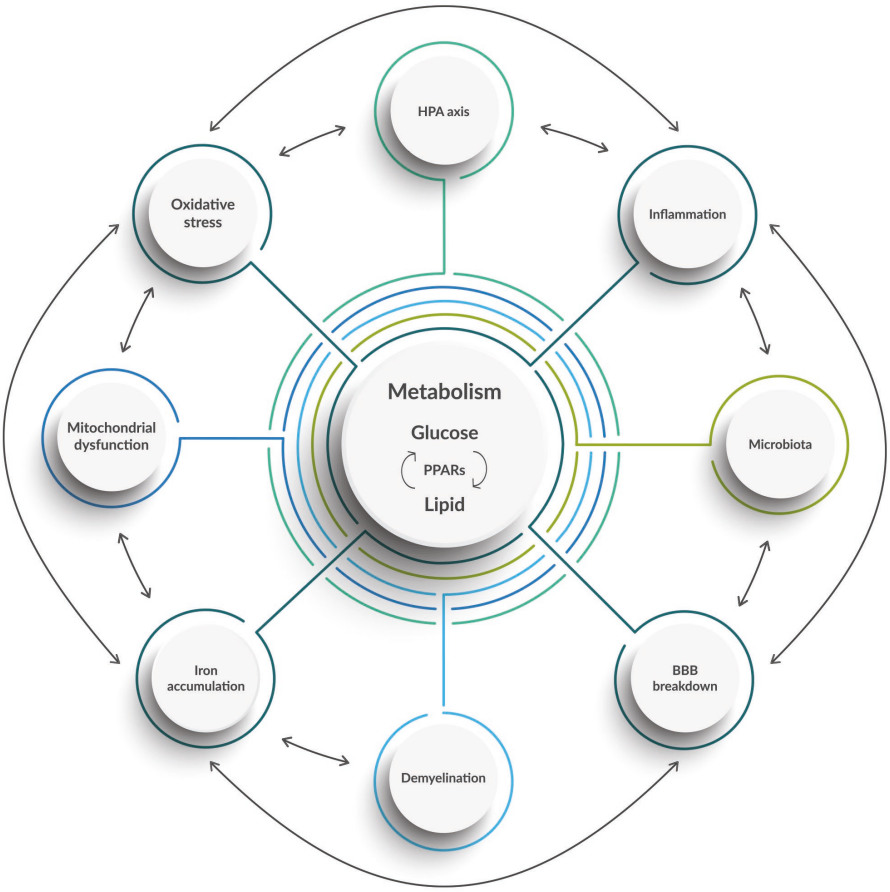

Figure 2. The systemic model. *Description of the systemic model characterized by a complex network with shift in metabolism favoring lipid metabolism instead of glucose metabolism as the central part. Dysregulated metabolism is connected to several pathologic mechanisms inflammation, hyperactivation of HPA axis, oxidative stress, mitochondrial dysfunction, iron accumulation, demyelination, BBB breakdown and dysbiosis of microbiota. BBB: blood-brain barrier, HPA: hypothalamic-pituitary-adrenal axis, PPARs: peroxisome proliferator-activated receptors.*

Myelination requires high demand of lipids. The myelin membrane contains high levels of cholesterol, thus dependent on the cholesterol synthesis. Defects in the metabolism of myelin-enriched lipids, such as cholesterol and long-chain fatty acids, cause demyelination (157). Moreover, lipids become targets for lipid peroxidation, and post-translational modifications such as citrullination modifying arginine to citrulline, which is involved in MBP. This delipidation leads to recognition of

citrulline-modified MBP antigens by the immune cells in the CNS and thus altered peptide ligand causes inflammation as well as autoimmunity (14).

The BBB is compromised due to high levels of circulating fatty acids causing deficient expression PPARs, which regulates the integrity of the BBB. This results in monocytes entering the CNS further stimulating inflammation (81,158). Damage to the BBB causes leakage of transferrin-bound iron, which increases the iron content in the CNS making it more prone to mitochondrial dysfunction and oxidative stress (112).

Insulin resistance is a result of increased lipid metabolism followed by production of increased amounts of acetyl-CoA that inhibits pyruvate dehydrogenase. This elevates the levels of glucose-6-phospaphate and inhibits hexokinase contributing to insulin resistance. Additionally, fatty acids can inhibit glycogen synthesis through GLUT4 causing insulin resistance (120). Thereby, the brain is forced to use lipids as energy substrate due to the reduced influx of glucose.

All these mechanisms underpin a central role of lipid metabolism in MS and during conditions of dysregulated lipid metabolism a vicious cycle where several pathologic processes will be initiated (Figure 2). Despite of which event is the trigger of these processes, the initiating event will affect all the other mechanisms involved. For example intake of diet high in triglycerides promotes inflammation, persistent exposure to stress promotes activation of the HPA axis and smoking causes changes in the microbiota. Common for the all these initiators (diet, stress, smoking) is that they start the cascade of pathological processes leading to disease. This clear cut function of the metabolism of lipids together with the importance of lipids for the brain function underpin that the lipid metabolism and thus CPT1 plays a central role in the etiology and pathology of MS, which are supported by the research presented in *Manuscript I-III.*

4.1.3. RESEARCH CONFIRMING THE SYSTEMIC FRAMEWORK

Manuscript I-III covers research supporting the systemic framework proposed as an explanation of the etiology and pathology of MS. Detailed results are described in the manuscripts (Appendix A-C) (14).

Manuscript I is comparing the traditional and systemic framework by testing the efficacy of the standard immunotherapy of MS, interferon-β, and the lipid metabolism blocker, etomoxir. It covers an attempt to reverse EAE in rodents obtained through the mechanism of action of etomoxir blocking the lipid metabolism by targeting CPT1 resulting in downregulation of β-oxidation. The EAE model showed significantly increased mean clinical score and decreased body weight in placebo-treated animals compared to etomoxir-treated animals, which indicates amelioration of disease. The new therapy, etomoxir, was compared to the

first-line treatment of MS, interferon-β, showing significantly superior effects to interferon-β treatment in the animals. The treatment strategy with both etomoxir and interferon-β was evaluated by investigating the effects of the treatments initiated at different time points, day 1 or day 5. Treatment with etomoxir initiated at day 1 showed increased efficacy compared to day 5. This indicates that etomoxir treatment day 1 revealed a prompt response relevant for the clinical application of etomoxir consistent with findings by Shriver et al. who found beneficial effect of etomoxir treatment (131). In addition, the unsuccessful inhibition of disease by interferon-β underpin that this treatment is not covering all aspects since the animals did not reverse the EAE-induced symptoms.

The effect of etomoxir was evaluated by the protein expression of CPT1A, MBP and FTH in the brain (brainstem and cerebellum). The intensity of CPT1A was significantly lower in etomoxir-treated animals compared to placebo-treated animals confirming the therapeutic efficacy of etomoxir treatment by downregulating the lipid metabolism. This indicates that the metabolism is reversed from increased lipid metabolism in the EAE-diseased animals to mainly relying on glucose metabolism, which is important to sustain physiological brain function. In accordance, the MBP intensity was significantly increased in the cerebellum after etomoxir treatment compared to animals receiving placebo. By the increased MBP levels in the brainstem was also low occurrence of immune infiltrates. This supports the hypothesis of reversed metabolism favoring glucose metabolism by etomoxir, which promotes improved myelination along with low mean clinical scores.

The oligodendrocytes produces lipids incorporated into myelin and this process requires sufficient energy and some of these processes utilize iron (114). However, excessive amounts of redox-active iron are proposed as a generator of ROS in MS and EAE via Fenton reactions facilitating reduction of hydrogen peroxide to highly toxic hydroxyl radicals (156). The FT levels are increased in EAE animals compared to controls (159). Upregulated expression of FT in EAE and MS is associated with protection against oxidative damage in cells and inhibition of inflammation (114,159). This was supported by the findings of reduced FTH expression in the brain after etomoxir treatment. In contrast, the placebo group showed higher FTH expression, which may be due to a protective mechanism limiting the damaging effects of iron. With increased iron load in the cells follows downregulation of transferrin along with upregulation of FT as protection against the toxic iron by restricting the entry of additional iron and binding and storage of released iron, respectively (160). This underpin that etomoxir is functioning by reversing mitochondrial dysfunction demonstrated by data of FTH expression, which illustrates iron deposits. In summary, the experimental data in *Manuscript I* support the hypothesis regarding MS as a systemic disease with lipid metabolism considered as a new target apart from solely inflammation, which has been the focus until today.

Manuscript II covers another new framework for the pathogenesis of MS involving B cells and autoantibodies in MS, thought to play a central role due to the correlation between disease progression and oligoclonal bands. This study was an attempt to investigate the antibody-antigen response in EAE and the efficacy of treatment with etomoxir and interferon-β hereof in order to identify autoantigens and potential biomarkers (14).

The EAE model is associated with inflammation, which was supported by the findings of significantly increased autoantibody reactivity to positive acute phase reactants as α-1-acid glycoprotein, haptoglobin, ceruloplasmin and plasma protease C1 inhibitor, as well as decreased autoantibody reactivity to negative acute phase reactants as transthyretin and α1-inhibitor-3 in the placebo group compared to the control group. These results were consistent with the increased clinical scores presented in the placebo animals, which implies high levels of positive acute phase reactants and low levels of negative acute phase reactants in EAE. Acute phase proteins are synthesized by the liver and released systemically in response to stressful stimuli. The expression of these acute phase proteins is regulated by pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (161).

Several significant differences in the reactivity of autoantibodies to antigens were demonstrated between the treatment groups (etomoxir day 1 or day 5, or interferon-β day 1 or day 5) and the placebo and control group. The relative autoantibody reactivity to APOE, clusterin, plasma protease C1 inhibitor and serum amyloid P component were among others decreased in animals treated with etomoxir, whereas serum albumin was increased compared to the placebo group. The levels of relative autoantibody reactivity to complement component C9 and gelsolin were among others decreased in animals treated with interferon-β compared to animals receiving placebo. The *APOE* gene is associated with MS (162). APOE is important for the transport, uptake and distribution of cholesterol, which is essential for repair of nerve tissue proposing a critical role for lipid metabolism. Moreover, APOE is important for modification of inflammatory responses in brain and regulation of the BBB integrity and function (162,163). All mechanisms important for restoring CNS function during relapses. The increased reactivity to APOE in animals receiving placebo compared to etomoxir-treated animals reveals neuroinflammation in the CNS, which was also demonstrated by Shin et al., who showed infiltrates with dendritic cells expressing high levels of APOE in the CNS of EAE animals (163). In summary, the experimental data in *Manuscript II* demonstrates significant changes in the serum antibodies against several proteins suggesting a significant role of B cells in the pathogenesis of MS. Further supporting an important role of B cells is the efficacy of Ocrelizumab, which is a humanized monoclonal antibody that depletes CD20-expressing B cells. It has shown lower rates of disease progression in RRMS and PPMS compared to placebo (16,17). Continuing with this framework and arranging the autoantigens detected, more than 30 autoantigens were modulated after treatment with etomoxir, where around half of them were involved in

metabolic processes and the other half was involved in stress. This demonstrates that alteration in the metabolic pathways is essential in MS pathogenesis and identification of such autoantibodies against brain antigens opens up for recognition of potential biomarkers for MS.

Until now the experimental data demonstrates that pharmacological inhibition of CPT1 revealed clinical efficacy in the EAE models, effects on the T cell response by downregulating inflammation and effects on the B cell response by downregulating autoantibodies involved in inflammatory processes and metabolic pathways. Overall, this supports the hypothesis of the central role of lipid metabolism and in particular CPT1 suggesting a new systemic treatment strategy in MS affecting metabolism, inflammation, oxidative stress and mitochondrial dysfunction rather than focusing solely on either T cells or B cells.

In order to confirm that lipid metabolism plays a central role in MS, we have generated mice with a *Cpt1a P479L* mutation mimicking the Inuit mutation. It can be speculated whether the findings of low MS prevalence in the Inuit population is a consequence of decreased lipid metabolism due to the *Cpt1a P479L* mutation or because of other risk factors, such as diet, environment or genetics. Studies to test this are covered in *Manuscript III* by the unique use of *Cpt1a P479L* mice, which are hypothesized to be resistant to development of MS, based on the Inuit data and the proposed systemic framework. In the EAE model, significant lower mean clinical score and increased body weight were demonstrated in *Cpt1a P479L* EAE mice compared to wild type (WT) EAE mice supporting the proposed hypothesis of resistance to develop EAE (and MS in humans) obtained by the *Cpt1a P479L* (and *CPT1A P479L*) mutation.

The low prevalence of MS in Canadian and Greenland Inuits is a result of a metabolism where glucose is favored as energy substrate due to the low residual enzymatic activity of CPT1A thereby forcing glucose-based metabolism instead of lipid-based metabolism. Furthermore, the traditional Inuit diet consists of animal-based diet rich in proteins and vitamins. This caloric restricted intake is associated with decreased inflammation, as it is demonstrated that HFD modulates PPARs and induces brain inflammation and oxidative stress, and is associated with increased MS frequency. This underpin that fatty acids are important modulators of inflammation and energy homeostasis (164,165). In order to clarify the role of diet in the Inuits and *Cpt1a P479L* mice, the influence of HFD on disease severity was investigated. It was found that WT EAE mice receiving HFD compared to normal diet (ND) showed exacerbated disease course, which was in accordance to other studies (164). To support the proposed hypothesis of resistance, the effect of HFD was tested. The data showed that diet was not a trigger to exacerbated EAE disease course in *Cpt1a P479L* EAE mice as the mean clinical score was not affected. In addition, *Cpt1a P479L* EAE mice receiving ND and HFD showed significantly lower mean clinical score compared to WT mice receiving HFD.

Excess levels of ROS are mediators of demyelination and axonal damage in EAE and MS, since ROS causes damage to lipids (111). HO-1 is increased in the CNS of EAE mice and in MS lesions (159). The upregulation of HO-1 is induced by ROS activating Nrf2. The increase in *Ho-1* mRNA expression in WT mice compared to *Cpt1a P479L* EAE mice proposes the presence of oxidative stress in WT mice. A regulator of mitochondrial antioxidants in MS, *Pgc1α* (166), was increased in *Cpt1a P479L* EAE mice compared to WT mice indicating a defense mechanism in *Cpt1a P479L* EAE mice by restoring the redox balance. This finding supports the hypothesis that antioxidant pathways protect neurons from inflammatory-mediated oxidative stress in the EAE model and that *Cpt1a P479L* mice possess antioxidant properties, which is another framework for describing the resistance to EAE development. The resistance to EAE development for *Cpt1a P479L* mice is consistent with the low prevalence of MS in the Inuit population carrying the *CPT1A P479L* mutation. Due to this mutation it is assumed that the metabolism has a preference for utilizing glucose compared to individuals with WT *CPT1A* gene with normal prevalence of MS in which the metabolism favors lipids as energy substrate.

The effect of *Cpt1a P479L* mutation on the protein expression of MBP was evaluated in the brain by fluorescent immunohistochemical staining and immunoblotting. *Cpt1a P479L* EAE mice showed markedly increased MBP-labeling with no evident pathological lesions in cerebellum and brainstem compared to WT EAE mice. These observations were confirmed by quantification of the labeling intensity, which demonstrated significant increased values of the integrated density of MBP in both brainstem and cerebellum of *Cpt1a P479L* EAE mice compared to WT EAE mice. This confirms improved myelination in *Cpt1a P479L* EAE mice and corresponds to the lower mean clinical scores in these mice. In summary, the experimental data in *Manuscript III* underpin a pivotal role of CPT1A in the pathogenesis of MS.

By the findings presented in *Manuscript I-III* new treatment avenues of MS through a systemic approach targeting lipid metabolism could be proposed, since both pharmacological and genetic inhibition of CPT1 and *Cpt1a* were demonstrated to attenuate disease. This is in contrast to the current therapeutic approaches, which only treats the collection of symptoms in RRMS and causes relief of these instead of hindering disease disability and progression.

4.2. CENTRAL NERVOUS SYSTEM DISEASES IN THE LIGHT OF THE SYSTEMIC FRAMEWORK

The new conceptual framework sheds new light on the etiology and pathology of MS. By using the metabolism of lipids as the etiology of MS rather than the immune system factors that previously could not be understood within the strict autoimmunity framework can now be explained. It is well-known that depression is

comorbidity to MS. Furthermore other CNS diseases such as amyotrophic lateral sclerosis, Parkinson´s disease and Alzheimer´s disease have shared features to MS. Therefore, it is now interesting to see if the proposed systemic framework can explain connections between as well as the etiology and pathology of depression and CNS diseases with MS.

4.2.1. DEPRESSION

Depression is associated with MS and other CNS diseases, where depression is the most common psychiatric disorder in MS patients. The established hypotheses of depression including the monoamine, the neutrophin and the cytokine hypothesis may still be partly operative, though incomplete in explaining the entire etiology and pathology. Inflammation results from decreased availability of neurotransmitters, decreased levels of neurotrophic factors, and increased levels of pro-inflammatory cytokines. Correspondingly, the traditional framework of MS remains elusive in explaining the comorbidity, depression, both of which are multifactorial diseases and can be associated with psychosocial stress, inflammation and demyelination (98). Thus it is tempting to hypothesize that depression could be explained by the systemic framework.

It is known that in depression the reverse cholesterol transport is impaired due to low levels of HDL, total cholesterol and HDL:cholesterol ratio (126). Moreover, depletion of PUFAs is associated with increased production of pro-inflammatory cytokines such as prostaglandin E2, IL-1, IL-6, TNF-α and interferon-γ, thus promoting inflammation and stress.

Similar to MS, stress induced by internal or external factors is thought to be a trigger in genetically susceptible individuals. Stress induces shift in metabolic pathways from glucose-based to lipid-based metabolism, where dysregulated lipid metabolism plays a central role initiating a cascade of disease-inducing processes (Figure 2). As described in the systemic model, which should now encompass depression, cytokines activate the HPA axis. The loss of negative feedback mechanism of the HPA axis results in increased glucocorticoid and cortisol production (51). In addition, as described in more detail in the systemic model of MS, this will again affect other mechanisms for example altered microbiota, oxidative stress, mitochondrial dysfunction, with dysregulated lipid metabolism playing a central role.

Manuscript IV covers an explanation of depression based on the systemic framework by connecting dysregulated lipid metabolism to the uncovered mechanisms in the pathology. Detailed results are described in the manuscript (Appendix D) (55). This study was an attempt to investigate the role of lipid metabolism and the efficacy of treatment with etomoxir in relative to standard treatment escitalopram in a CMS model of depression.

The CMS rats receiving etomoxir demonstrated significantly increased intake of sucrose solution, illustrating hedonic behavior, in week 4 and 5 compared to rats receiving escitalopram. Further supporting the efficacy of etomoxir is the levels of sucrose intake, which were similar to that of unchallenged rats receiving vehicle. The responding rate to etomoxir and escitalopram was measured and showed a response rate of 90 % and 57 %, respectively. This is in accordance with the response rate of the standard treatments today, which are up to 60 %, thus it underpins the relevance of this comparison (61,64). These results indicate that etomoxir and thereby blockage of lipid metabolism is able to reverse the stress-induced anhedonic behavior in a superior manner compared to escitalopram functioning in a dramatically different way. Normally, a disadvantage of the conventional antidepressants is the slow onset of antidepressant action. However, etomoxir shows a response rate of 70 % after one week of treatment.

Furthermore, results demonstrated significant upregulated expression of *CPT1A* mRNA in the cerebellum of people with history of depression compared to healthy controls. This supports the hypothesis with lipid metabolism and CPT1A playing a key role in the pathogenesis of depression explained by the systemic framework. This means that depression as comorbidity to MS could be explained by the same model, why it is now tempting to hypothesize that other comorbidities to MS or diseases demonstrating similar features could be explained by this as well.

4.2.2. AMYOTROPHIC LATERAL SCLEROSIS, PARKINSON´S AND ALZHEIMER´S DISEASE

Metabolic disturbances are thought to play a possible role in the energy homeostasis of the CNS and found to be implicated in neurodegenerative diseases such as amyotrophic lateral sclerosis, Parkinson´s and Alzheimer´s disease (151). Therefore, it is tempting to hypothesize that dysfunction of lipid metabolism is also playing a central role in these diseases, since these have common features to MS.

In amyotrophic lateral sclerosis, a switch from glucose metabolism to lipid metabolism has been defined as an early pathologic event prior to detectable motor and clinical symptoms (167). Patients with sporadic amyotrophic lateral sclerosis have increased levels of circulating fatty acids (168). This prolonged utilization of lipids as energy substrate increases β-oxidation leading to production of lipid by-products that contribute to lipotoxicity and ROS production. Studies have proposed that the metabolic switch is caused by increased expression of PPARs (167). Restoration of the metabolic balance favoring glucose metabolism is able to protect the mitochondria by preventing oxidative stress (167). Familial cases of amyotrophic lateral sclerosis are often due to mutation in the cytosolic copper-zinc superoxide dismutase 1 gene. Cytosolic copper-zinc superoxide dismutase is an enzyme present in the cytosol and mitochondrial intermembrane space, which catalyzes removal of superoxide radicals (169). In cytosolic copper-zinc superoxide

dismutase 1 mutated mice reduced levels of circulating triglycerides and insulin as well as increased levels of glucagon was demonstrated indicating altered lipid metabolism (170). These can trigger acidosis, which is thought to catalyze onset and progression of amyotrophic lateral sclerosis and other diseases such as MS and Parkinson´s disease by activation of acid-sensing ion channels promoting intracellular calcium influx, inflammation, demyelination, axonal degeneration and neuronal death (170,171).

In Parkinson´s disease, dysregulation of the fatty acid metabolism have been demonstrated (172,173). The reduced level of glucose is deleterious to neurons as it results in oxidative stress important for the formation of Lewy bodies (173). Additionally, it causes increased activation of microglia which is associated with neuronal damage (174).

In Alzheimer´s disease, *APOE* is the strongest genetic risk factor and serves as a regulator of the cholesterol metabolism in the brain by transporting HDL particles. There has been found increased levels of low-density lipoprotein and decreased levels of HDL (175). In Alzheimer´s disease a decline in glucose metabolism of brain has been demonstrated (176). This compromised metabolism is accompanied with oxidative stress, which causes degradation of myelin. Free cholesterol contributes to overproduction of amyloid-β, which will accumulate in the mitochondria further perpetuating oxidation of fatty acids favoring lipid metabolism (176).

The findings in amyotrophic lateral sclerosis, Parkinson´s and Alzheimer´s disease demonstrate dysfunction of lipid metabolism and features which can be described by the systemic framework as proposed for MS. Therefore, it may be assumed that inhibition of lipid metabolism by targeting CPT1A could be efficient as treatment strategy for these diseases as well.

CHAPTER 5. CONCLUSION

The traditional framework of the etiology and pathology of MS as an inflammatory autoimmune disease of the CNS is elusive in both explaining the disease and generation of efficient therapies. Therefore, more complex mechanisms underlie the understanding of the etiology and pathology of MS, which is proposed as the systemic framework. This systemic model constituted the major focus of this PhD thesis hypothesizing that MS and depression originate from a dysfunction of the metabolism of lipids. Without a clear understanding of the etiology and pathology the hope for a cure is limited. Therefore, the open question is: does a change of paradigm in which MS and depression are seen as systemic diseases generate treatments of these diseases with metabolic pathways as the central part?

The results presented throughout the PhD thesis provide convincing evidence of this new framework. Etomoxir treatment promotes the use of glucose as preferred energy substrate instead of lipids, thus preventing disease development. This was demonstrated by efficient blockage of lipid metabolism in mice and rats exposed to EAE resulting in ameliorated clinical scores and remyelination. Etomoxir showed superior efficacy to interferon-β treatment, targeting T cell function, as well modification of the antibody response, targeting B cell function. Moreover, etomoxir was able to reduce the levels of pro-inflammatory cytokines. This proposes multiple functions involved in the pathogenesis, which are affected by etomoxir treatment. The results were further supported by the findings of resistance to disease by the *Cpt1a P479L* or arctic mutation as well as resistance to exacerbated disease severity by HFD. Blockage of lipid metabolism by etomoxir was also efficient in treating rats exposed to CMS by reducing the anhedonic behavior compared to escitalopram.

In conclusion, the research presented in *Manuscript I-IV* in this PhD thesis exemplifies the complexity of diseases such as MS and depression, which has important implications for the development of new therapeutic approaches. The results have provided new insight into MS and depression by proposing a central role of CPT1A in systemic treatment of these diseases, which indicates a change of paradigm towards therapies focusing on CPT1A as the target on a pathway to a new cure.

5.1. FUTURE PERSPECTIVES

This PhD thesis provides a framework for further investigations of the lipid metabolism and in particular the CPT1A molecule in CNS diseases due to the need of efficient treatment opportunities helping individuals for which there is no cure today. The first step is to implicate dysfunction of lipid metabolism in the etiology of MS and understand the systemic framework of the disease, instead of solely

focusing on the immune system. The second step is to evaluate the clinical efficacy of etomoxir in order to aid our understanding of the pathogenesis and progression of MS, which could allow health organizations and health care professionals to better predict the onset of disease in individual patients by monitoring risk factors including clinical history and environmental triggers. Additionally, revisiting the processes of MS in this framework could also improve the current problematic ability to diagnose MS. Overall this systemic framework has opened new and exciting avenues for the development of potential novel therapeutics for CNS diseases.

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Appendix A. Manuscript I

Pharmacological inhibition of carnitine palmitoyl transferase 1 inhibits and reverses experimental autoimmune encephalomyelitis in rodents

Manuscript not yet published

Appendix B. Manuscript II

Identification of brain antigens recognized by autoantibodies in experimental autoimmune encephalomyelitis-induced animals treated with etomoxir or interferon-β

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Identification of brain antigens recognized by autoantibodies in experimental autoimmune encephalomyelitis-induced animals treated with etomoxir or interferon-β

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Multiple sclerosis (MS) is a neurodegenerative autoimmune disease, where chronic inflammation plays an essential role in its pathology. A feature of MS is the production of autoantibodies stimulated by an altered-peptide-ligand response and epitope spreading, resulting in loss of tolerance for self-proteins. The involvement of autoantibodies in MS pathogenesis has been suggested to initiate and drive progression of inflammation; however, the etiology of MS remains unknown. The effect of etomoxir and interferon-β (IFN-β) was examined in an experimental-autoimmune-encephalomyelitis (EAE) model of MS. Moreover, the impact of etomoxir and IFN-β on recognition of brain proteins in serum from EAE rats was examined with the purpose of identifying the autoantibody reactivities involved in MS. Animals treated with etomoxir on day 1 exhibited a statistically significantly lower disease score than animals treated with IFN-β (on day 1 or 5) or placebo. Etomoxir treatment on day 5 resulted in a significantly lower disease score than IFN-β treatment on day 1. After disease induction antibodies was induced to a broad pallet of antigens in the brain. Surprisingly, by blocking CPT1 and therewith lipid metabolism several alterations in the antibody response was observed suggesting that autoantibodies play a role in the EAE animal model.

Multiple sclerosis (MS) is a chronic inflammatory disease of the brain and spinal cord that results in demyelination, neurodegeneration and axonal loss[1]. To date, no cure for MS has been identified, and although treatment for relapse-remitting MS focuses on reducing disease activity and progression, no treatment for the progressive types of MS has been identified[2,3]. One of the first-line therapies for MS is interferon-β (IFN-β), which functions by redirecting immunological responses from pro-inflammatory to anti-inflammatory T cell responses[4]. However, immunomodulatory treatments do not show long-term effects and have no effect on patients entering the progressive phase of MS[2,4,5]. B cells are another potential target for treating MS, and several types of anti-CD20 treatments have shown efficacy in clinical trials[6]. Ocrelizumab is a new recombinant humanized antibody that targets CD20 antigen expressed on B cells and has shown efficacy in phase 3 trials for treatment of both relapse-remitting MS and primary progressive MS[7,8].

Multiple sclerosis as an autoimmune disease

The etiology of MS remains unknown; however, environmental triggers in genetically susceptible individuals have been proposed as a model[1,9,10]. T cells are thought to be activated in the periphery through various mechanisms,

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such as molecular mimicry, by-stander activation and exposure to bacteria present in the colon, and then to traffic to the central nervous system with activated B cells and monocytes. For more than two decades, this hypothesis has been proposed as the framework for understanding and treating MS as an autoimmune disease[9]. However, studies indicate that the T cell biology in MS is complex and that several T cell types play a role in MS pathogenesis[11–13].

In recent years, the involvement of B cells and autoantibodies in MS has been re-examined, and they are both thought to play a central role in MS pathogenesis due to a correlation between disease progression and synthesis of intrathecal antibodies known as oligoclonal bands[9,14–17]. Nevertheless, limited knowledge is available about the antibody-antigen response in the brain and its role in disease induction and progression[14,18,19]. However, recent studies found that treatment with ocrelizumab, which functions by depleting CD20-expressing B cells, had beneficial effects on primary progressive MS and thereby reinforced the contribution of B cells to MS pathogenesis[8]. Specific posttranslational modifications have been shown to result in the loss of tolerance to the modified proteins, thus initiating antibody recognition[20,21]. A candidate for initiation of such an autoimmune response is a posttranslational modification termed citrullination, which naturally occurs on a myelin protein known as myelin basic protein (MBP). Citrullination is highly immunogenic, and antibodies against citrulline proteins are already used as diagnostic biomarkers in rheumatoid arthritis[20,21]. However, treating relapses with immunomodulatory therapies does not stop progression towards neurodegeneration and axonal loss, and as this effect indicates that the causes of lesions and neurodegeneration involve other mechanisms, key questions remain unanswered. Mechanisms underlying disease progression are proposed to involve an interaction between oxidative stress, mitochondrial dysfunction, energy deficit and ion channel dysfunction, which are also suggested to be involved in neurodegeneration in Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS)[22,23].

Multiple sclerosis as a consequence of metabolic dysfunction

The traditional view of MS as a chronic inflammatory autoimmune disease is controversial. Alternatively, MS has been proposed to be a consequence of lipid metabolism dysfunction as well as mitochondrial dysfunction[24]. Studies have supported this hypothesis, as they have shown impaired glucose metabolism in MS[25,26]. Reduced glucose metabolism indicates a shift in metabolism towards lipid metabolism, which is consistent with previous reports of decreased lipid levels in MS lesions[27,28]. In addition, research has shown that T cells exposed to stress also induce a switch from glucose to lipid metabolism as an energy source, which underpins the importance of lipid metabolism in immune cells[29]. Carnitine palmitoyl transferase 1a (CPT1a) is a key enzyme involved in lipid metabolism, as it catalyzes the conversion of acyl-CoA (which is mitochondrial membrane-impermeable) into acyl-carnitine (which is mitochondrial membrane-permeable)[29] [Mørkholt *et al*., submitted for publication]. This indicates that CPT1a serves as a rate-limiting enzyme in beta-oxidation[29,30]. CPT1a expression was found to be upregulated in MS lesions of the spinal cord[31]. Additionally, mutations in CPT1a resulting in either 22% activity or a dysfunctional molecule were found in two human populations in Canada, namely, the Inuits and Hutterites, respectively[32–36]. The incidence of developing MS was found to be 1/50,000 for the Inuits and 1/1100 for the Hutterites, which is rather low compared to the incidence of 1/350 in the Canadian population[37,38]. These findings suggest a role of CPT1a mutations in protecting against the development of MS. Although the particular mechanism involved in the role of CPT1 in MS is unknown, it can be hypothesized that decreased CPT1 activity plays a protective role against mitochondrial dysfunction, as CPT1 plays an essential role in energy production from lipids, which thereby results in diminished production of reactive oxygen species (ROS). Furthermore, ROS and reactive nitrogen species (NOS) are both thought to be mediators involved in demyelination and axonal injury in MS and experimental autoimmune encephalomyelitis (EAE)[39,40].

Chemical blockage of CPT1 by etomoxir enables a shift in metabolism that favors glucose metabolism rather than lipid metabolism as an energy source[41–44] [Mørkholt *et al*., submitted for publication]. This CPT1 antagonist inhibits formation of acyl-carnitine, which is necessary for the transport of acyl-CoA into the mitochondria[41]. CPT1 inhibition presents a new treatment strategy that aims at both metabolism and immune system rather than focusing solely on inflammation as a therapeutic target. Studies by Shriver *et al*. and Mørkholt *et al*. supported this new hypothesis by showing that etomoxir treatment resulted in decreased disease activity and inflammation and increased remyelination in an EAE mouse model characterized by the expression of hallmarks of MS such as demyelination and inflammation[11,29] [Mørkholt *et al*., submitted for publication]. This suggests that etomoxir is a novel potential treatment for MS.

This study aimed to investigate the efficacy of etomoxir and IFN-β treatment on the autoantibody response in EAE-induced and healthy animals using Western blot analysis and to identify and examine the levels of autoantibody reactivity for autoantigens recognized by these autoantibodies by immunoprecipitation and mass spectrometry.

Results

Disease score in an experimental autoimmune encephalomyelitis model. The impacts of the two treatments (etomoxir and IFN-β) and two treatment time points (days 1 and 5) were compared with those of the placebo treatment and control groups (Fig. 1). There were statistically significant differences in the disease score between animals treated with etomoxir on day 1 and animals treated with IFN-β on days 1 and 5 and placebo ($p < 0.05$ and $p < 0.001$). Moreover, animals receiving etomoxir on day 5 exhibited a significantly lower disease score than animals treated with IFN-β on day 1 ($p < 0.01$).

Detection of autoantibodies against brain proteins. Western blot analysis was performed to investigate the immunoglobulin G (IgG) antibody response against brain self-antigens in EAE-induced rats receiving placebo, IFN-β on day 1 or 5, or etomoxir on day 1 or 5 (Fig. 2). Autoantibodies against brain antigens were

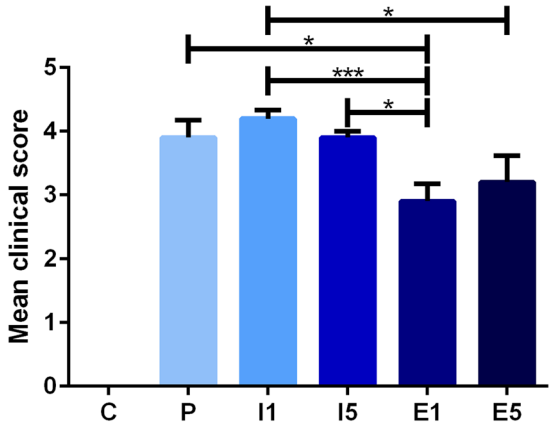

Figure 1. Mean clinical score for animals in the EAE model; control (C, n = 5), placebo (P, n = 10), IFN-β on day 1 (I1, n = 10), IFN-β on day 5 (I5, n = 10), etomoxir on day 1 (E1, n = 10) and etomoxir on day 5 (E5, n = 10). All data are presented as the mean ± SEM. Results from unpaired t-tests showed a statistically significant difference in the disease scores between E1 and I1, I5, and placebo. Treatment on E5 resulted in a significantly lower disease score than that on I1. Number of asterisks indicates level of statistical significance (*$p < 0.05$ and ***$p < 0.001$).

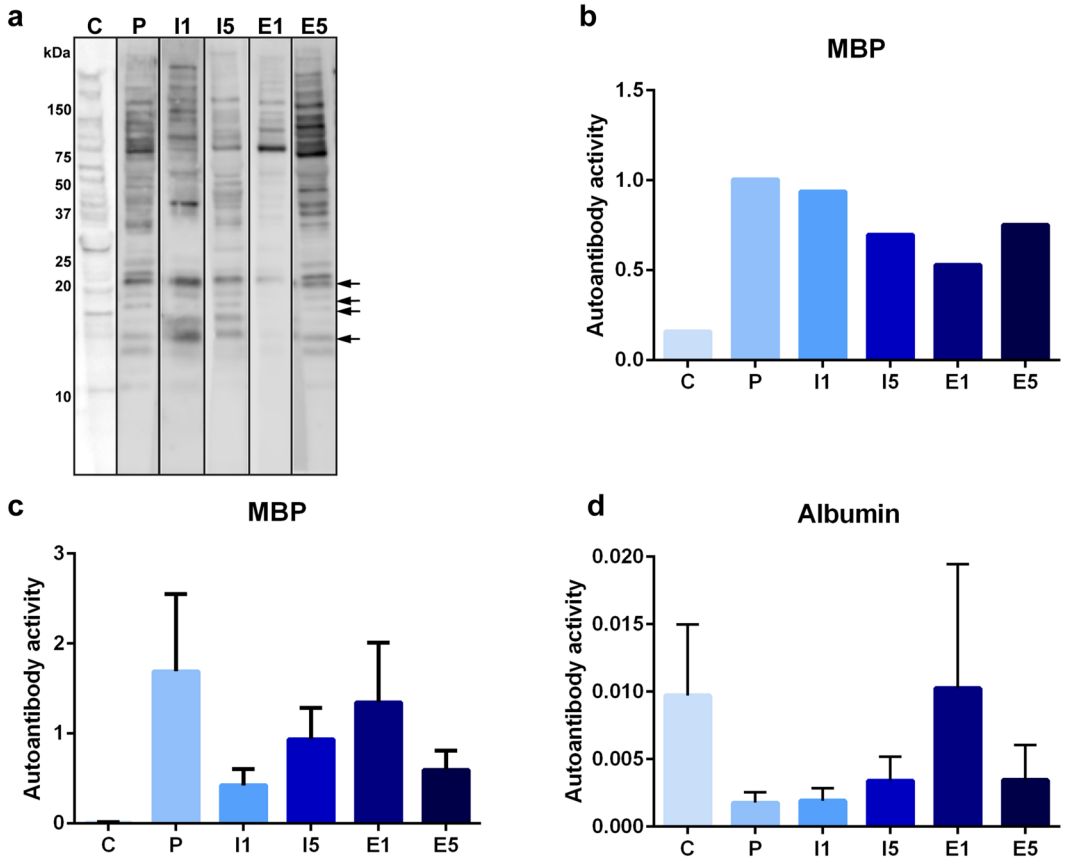

Figure 2. Western blots showing expression of brain antigens recognized by serum-derived antibodies; control (C), placebo (P), IFN-β on day 1 (I1), IFN-β on day 5 (I5), etomoxir on day 1 (E1) and etomoxir on day 5 (E5). Protein marker positions are indicated to the left (kDa). MBP bands are marked with arrows. Serum samples are derived from the same experiment. Each blot represents a sample from the different treatment groups, and blots were cropped and processed in parallel (**a**). Indirect ELISA showing the concentration (μg/ml) of serum-derived autoantibodies towards MBP in the presented blots (**b**). Indirect ELISA showing the concentration (μg/ml) of serum-derived autoantibodies towards MBP (**c**) and albumin (**d**) in all the animals. Data are presented as the mean ± SEM.

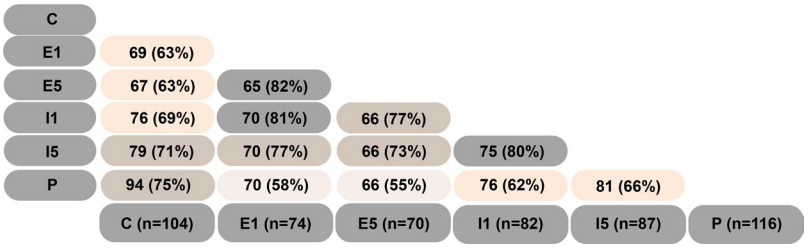

Figure 3. Venn diagram of the immunoprecipitated proteins identified using mass spectrometry. Number of co-identified proteins corresponding to number of autoantibodies and percentages of similarity are shown in the treatment groups; control (C), placebo (P), IFN-β on day 1 (I1), IFN-β on day 5 (I5), etomoxir on day 1 (E1) and etomoxir on day 5 (E5).

detected in all animals, and interestingly, EAE- induced rats treated with etomoxir on day 1 showed weaker antigen binding than the other treatment groups. All six groups revealed expression of MBP (Fig. 2a). The autoantibody response to MBP was validated by ELISA (Fig. 2b and c).

Detection of autoantigens recognized by autoantibodies in the brain. To investigate detection of antibodies to brain proteins further, immunoprecipitation was performed, and precipitated proteins were identified and quantified using label-free mass spectrometry. A Venn diagram of the identified proteins showed a high similarity in the number of precipitated proteins between groups (Fig. 3). The highest number of detected proteins in a sample was 123; of these proteins, seven were directly related to IgG (these proteins were discarded because they could have originated from serum antibodies), and 116 were suggested as self-antigens, including MBP and myelin proteolipid protein (PLP).

Interestingly, certain antigens in the control, placebo and treatment groups were highly similar; the lowest similarity percentage was 55%. The differences in the relative abundance, which potentially represents the antibody response, between the treatment groups and the placebo and control groups were investigated.

There were several significant differences in the quantification of autoantibody reactivity for antigens between the treatment groups and the placebo and control groups (Fig. 4, Supplementary Figure S1).

Some of the significant differences between the placebo and treatment groups were found on both treatment days (Fig. 5). The levels of relative autoantibody reactivity's for apolipoprotein E (ApoE), clusterin, plasma protease C1 inhibitor, and serum amyloid P component (SAP) were all found to be decreased in etomoxir-treated animals, whereas antibody reactivity for serum albumin was found to be increased. The same changes were found in the control group for plasma protease C1 inhibitor and serum albumin levels. In IFN-β-treated animals, the autoantibody reactivity for complement component C9 and gelsolin were decreased, whereas the relative autoantibody reactivity for PLP was upregulated, and only antibody reactivity for complement component C9 was the same as that in control animals.

Discussion

The clinical efficacy of the CPT1 inhibitor etomoxir was compared to that of the current first-line treatment IFN-β in an EAE model of MS. Animals treated with etomoxir on day 1 had a statistically significantly lower disease score at day 11 than animals receiving placebo, IFN-β on day 1 and IFN-β on day 5. Moreover, the group treated with etomoxir on day 5 obtained a significantly lower disease score than the IFN-β on day 1 treatment group. These lower disease scores obtained after etomoxir treatment indicate that blocking beta- oxidation in the mitochondria is a target for treating EAE and potentially MS. This result is consistent with the finding of Shriver and colleagues, who showed a beneficial effect of etomoxir treatment in an EAE model[29]. Furthermore, Bakshi *et al*. found that anti-lipid IgG antibodies were associated with the worsening of brain MRI measurements in MS patients[45]. Gonzalo *et al*. found significantly increased amounts of autoantibodies to lipoxidized proteins, indicating that lipid peroxidation is a pathogenic pathway in MS[46]. Regarding IFN-β-treated groups, Shirani *et al*. found that IFN-β treatment did not slow the progression of disability in MS patients, which is consistent with the findings of the present study[5].

Western blot analysis showed weaker antibody signals following treatment with etomoxir on day 1 compared to the other treatments. This result could indicate a lower abundance of antibodies in the serum, which could be an effect of etomoxir treatment. As the blotting was directed against IgG antibodies, it is important to consider that the half-life of these antibodies is four to eight days in mice and could thus be similar in rats[47]. However, it is unlikely that such a decrease in antibody concentration would be substantial enough to have an obvious effect in late-treated animals. The high similarity between these potential autoantigens indicated that autoantibodies were present even in healthy animals, raising the question of why the autoimmune response was not active in control animals. This result could be due to low concentrations of antibodies, low affinity towards the antigen or an intact blood-brain barrier (BBB).

In the current study, the placebo group had significantly higher serum concentrations of antibodies against positive acute phase reactants (α1-acid glycoprotein, complement component C9, haptoglobin, ceruloplasmin, and plasma protease C1 inhibitor) and significantly lower serum concentrations of antibodies against negative acute phase reactants (transthyretin and α1-inhibitor-3) than the control group. These findings are consistent with the literature[48–54]. Furthermore, complement component C9 has been found to be directly correlated with

	Compared to placebo					Compared to control				
Protein name	**C**	**E1**	**E5**	**I1**	**I5**	**P**	**E1**	**E5**	**I1**	**I5**
T-kininogen 1	**					**	**		**	**
Myelin basic protein	*					*				*
Alpha-1-acid glycoprotein	**					**	**	**	**	**
Transthyretin	*					*	**	*	**	*
T-kininogen 2	**					**	**	*	**	**
Serine protease inhibitor A3N	*					*			**	*
Ceruloplasmin	**					**	*	*	*	*
Alpha-1-inhibitor 3	**					**	*	**	**	**
Hemopexin	**					**			*	**
Neurocan core protein	*									
Serum albumin	**	**	*			**			**	*
Plasma protease C1 inhibitor	*	**	*	*		*				
Keratin, type II cytoskeletal 1	**		*	**		**				
Complement component C9	*			*	*	*				
Haptoglobin	**				*	**	**	**	**	**
Apolipoprotein C-III		**					**			
Beta-2-microglobulin		*						*		
Protein AMBP		**								
Apolipoprotein E		*	*							
Clusterin		*	**							
Serum amyloid P component		*	**							
Hemoglobin subunit alpha-1/2			*					*		
Hemoglobin subunit beta-1			**							
Hemoglobin subunit beta-2			*							
Apolipoprotein C-I			*					**		
Myelin proteolipid protein			*	**	*					
Complement C3				*						
Fibronectin				*						
Carboxylesterase 1C				*			*	*	**	**
Neural cell adhesion molecule 1				*						
Murinoglobulin-1				*			**	*	**	**
Keratin, type I cytoskeletal 10				**						
Amphoterin-induced protein 1				*						
Gelsolin				*	*				*	*
Apolipoprotein A-IV					*		*			*
Heparin cofactor 2					*					
Apolipoprotein A-II							*	*	**	**
Serine protease inhibitor A3L							*	*	**	*
Rab3 GTPase-activating protein non-catalytic subunit							*	*		*
Voltage-dependent anion-selective channel protein 1							*		*	*
V-type proton ATPase 16 kDa proteolipid subunit								*		
Alpha-2-HS-glycoprotein								*	**	*
Ral GTPase-activating protein subunit alpha-1									**	
Neurofilament light polypeptide									**	
Beta-2-glycoprotein 1									*	
Guanine nucleotide-binding protein subunit beta-2									*	
60S ribosomal protein L23a									*	
Leucine-rich repeat protein SHOC-2						*			**	
Serine protease inhibitor A3K									*	*
Corticosteroid-binding globulin									*	*
Retinol-binding protein 4										*
Peptidyl-prolyl cis-trans isomerase A										*

Figure 4. Significant differences in the relative quantification between the treatment groups and the placebo (P) and control (C) groups. Treatments: E1 = etomoxir on day 1; E5 = etomoxir on day 5; I1 = IFN-β on day 1; I5 = IFN-β on day 5. SD under 2.0 on log2 scale. ■ significantly increased or ■ significantly decreased by at least a factor of 2. Number of asterisks indicates level of statistical significance (*$p < 0.05$ and **$p < 0.01$). More information can be found in Supplementary Figure S1.

demyelination of the spinal cord[55]. A previous study showed elevated levels of hemopexin during the progression of disease in the EAE model, which is consistent with our findings[56]. In this study, EAE was induced by MBP suspended in complete Freund's adjuvant; therefore, an antibody response to MBP was expected and was used as a positive control in this model[57,58]. The autoantibody response to MBP was validated by ELISA. The intensities of serum antibodies in the placebo group were consistent with the fact that the placebo group obtained a high

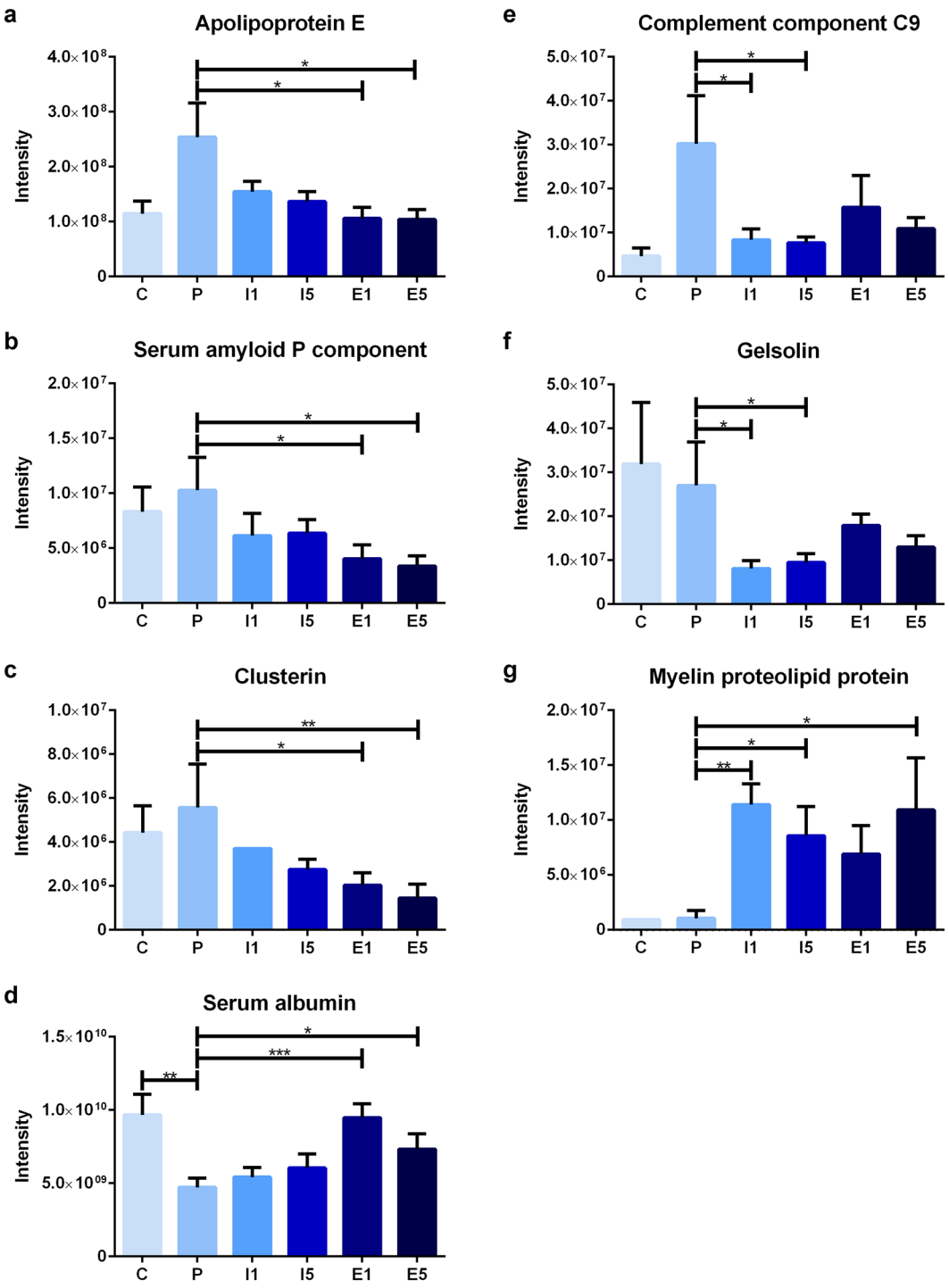

Figure 5. Intensity of immunoprecipitated protein for all groups; apolipoprotein E (**a**), serum amyloid P component (**b**), clusterin (**c**), serum albumin (**d**), complement component C9 (**e**), gelsolin (**f**) and myelin proteolipid protein (**g**); control (C, n = 5), placebo (P, n = 10), IFN-β on day 1 (I1, n = 10), IFN-β on day 5 (I5, n = 10), etomoxir on day 1 (E1, n = 10) and etomoxir on day 5 (E5, n = 10). All data are presented as the mean ± SEM. Results from the unpaired t-tests showed statistically significant differences in the immunoprecipitated protein intensities between P and E1 and E5 (**a**–**d**) and between P and I1 and I5 (**e**–**g**). Number of asterisks indicates level of statistical significance (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

disease score, which implies that high levels of positive acute phase reactants and antigens and low levels of negative acute phase reactants are associated with EAE and possibly MS.

Furthermore, the two etomoxir-treated groups had significantly lower serum concentrations of antibodies against ApoE, clusterin, SAP, and plasma protease C1 inhibitor and a significantly higher serum concentration of anti-albumin antibody than the placebo group. Studies have shown an association between ApoE and MS[59,60].

Of note, Zhao *et al*. examined the effect of ApoE mimetic peptides in a mouse model of diffuse brain injury and found that ApoE mimetic peptides improved memory function and protected against neuronal apoptosis by inhibiting the ERK1/2 pathway and Bax expression. Moreover, malondialdehyde content was significantly decreased, and superoxide dismutase content was significantly increased[61]. Pang *et al*. showed that treatment with ApoE mimetic peptides in an experimental model of subarachnoid hemorrhage resulted in reduced degradation of the BBB, which further resulted in less severe brain edema and neuron apoptosis, increased cerebral glucose uptake, and improved neurological functions. Moreover, the peptides inhibited cyclophilin, nuclear factor-kappaB, interleukin-6, tumor necrosis factor-α, and interleukin-1 and thus inflammation and BBB disruption[62]. This finding could indicate that the significant reduction in serum autoantibodies reactivity for ApoE resulted in decreased inactivation of ApoE and thereby a lower degree of inflammation, apoptosis and breakdown of the BBB, and this effect could explain the significantly lower disease score in etomoxir-treated animals than in placebo-treated animals. Furthermore, based on observations by Pang and colleagues, blocking lipid metabolism by etomoxir could also have an effect on the level of ApoE and result in normalization of glucose metabolism in the brain. Clusterin has been found to be upregulated in acute and chronic plaques in MS[52]. A previous study indicated that clusterin could be a marker of neurodegeneration in AD and PD and that its expression is increased during progression of AD[63]. Moreover, clusterin and complement factors leak into the brain during BBB breakdown[64]. These findings raise the question of whether the significant reduction in the relative autoantibody reactivity for clusterin in etomoxir-treated animals could be the result of reduced degradation of the BBB. Moreover, clusterin facilitates the removal of apoptotic cells and thus diminishes autoimmune responses following cell death[65]. The lower levels of serum anti-clusterin antibodies and the lesser degree of disease in etomoxir-treated animals at day 1 and day 5 could indicate that clusterin facilitates the clearance of apoptotic cells. Ji *et al*. found that SAP might have a protective role in the development of EAE[66]. In support of their findings, Grant and colleagues found that treatment with amyloid β−40 or amyloid β-42 peptides reduced motor paralysis and brain inflammation in four different EAE models[67]. Furthermore, Kurnellas *et al*. showed that amyloidogenic peptides such as tau, amyloid-β A4 and SAP had anti-inflammatory properties, reduced serum levels of interleukin-6 and reduced paralysis in EAE[68,69]. This finding could indicate that the significant decrease in serum anti-SAP antibodies resulted in decreased inactivation of SAP and thereby a lesser degree of inflammation. A review by Levine indicated that serum albumin has two side effects in the brain[70]: anti-pathogenic properties because it is a target for ROS and pro-pathogenic properties because it is pro-inflammatory, and high levels of serum albumin can lead to seizure. Furthermore, serum albumin leaks into the brain during BBB breakdown and is taken up by macrophages and cleaved during the acute phase of MS. In most healthy individuals anti-albumin antibodies have been found[71], and it was therefore expected to detect these antibodies in control animals. Interestingly, in the EAE-diseased animals anti-albumin antibodies were decreased. These results were validated by ELISA.

IFN-β-treated animals at days 1 and 5 had significantly lower serum concentrations of antibodies against complement component C9 and gelsolin than the placebo group. An earlier study found a direct correlation between the level of complement component C9 and demyelination of the spinal cord in an EAE model[55]. The lower serum antibody reactivity for complement component C9 could indicate that it is more active, which would result in demyelination and thereby a high disease score in IFN-β-treated animals[72]. Gelsolin is involved in myelin wrapping and is decreased in cerebrospinal fluid and serum in MS but elevated in brain tissue[73,74]. The low serum concentration of anti-gelsolin antibody could indicate that myelin wrapping in IFN-β-treated animals was affected. Furthermore, IFN-β-treated animals at days 1 and 5 had significantly higher serum concentrations of antibodies against PLP than placebo-treated animals, which is consistent with the higher disease score in IFN-β-treated animals. However, etomoxir-treated animals at day 5 also showed higher serum levels of antibodies against PLP than placebo-treated animals, highlighting a potential topic of interest for further investigation.

In conclusion, this study indicated that etomoxir is a more effective treatment for MBP-induced EAE than IFN-β and placebo, as it resulted in a significantly lower disease scores. Additionally, the results indicate that etomoxir-dependent inhibition of mitochondrial beta-oxidation in the EAE rat model is effective because of the regulation of serum autoantibodies against proteins such as ApoE, clusterin, SAP, and serum albumin, suggesting a role of these antigens in the progression of EAE and their potential use as diagnostic biomarkers for MS.

Materials and Methods

Experimental autoimmune encephalomyelitis rat model. Animal experiments were conducted according to NIH guidelines and were approved by the Danish National Committee for Ethics in Animal Experimentation (2015-15-0201-00647). Two-month-old female Lewis rats were obtained from Charles River Laboratories, Inc., Germany. The rats were housed under standardized conditions with 12 h light/dark cycles and free access to food and water. The rats were anesthetized with isoflurane (Baxter Laboratories) and then intradermally injected in the base of the tail with an emulsion consisting of 100 µg of MBP (Sigma-Aldrich) suspended in saline and complete Freund's adjuvant (Becton Dickinson) and supplemented with 0.2 µg of *Mycobacterium tuberculosis* (Becton Dickinson). EAE was induced in 45 rats, and five rats were used as controls and thus were not EAE-induced. Rats were treated daily with either etomoxir (Meta-IQ ApS, Denmark) or IFN-β (Extavia, Aalborg University Hospital, Denmark) on the first or fifth day of induction. Etomoxir was heated to 37 °C and dissolved in olive oil. Etomoxir was subcutaneously administered every day at a dosage of 1 mg/kg. Interferon-β was subcutaneously administered every other day at a dosage of 200,000 IU. The placebo group received daily injections of olive oil. All rats were weighed and clinically scored on a daily basis according to a scale from zero to five[75]: zero, no clinical signs of EAE; one, limp tail; two, paresis of one or two hind limbs; three, unilateral hind leg paralysis; four, bilateral hind leg paralysis; and five, bilateral hind leg paralysis and incontinence or moribund. Moreover, animals were not permitted to lose more than 20% body weight compared to their weight at the time of EAE induction. Rats were euthanized. Then, blood samples were obtained by cardiac puncture, and collected blood samples were labeled according to the groups. Blood samples were used for Western blot, ELISA and immunoprecipitation.

Western blot. *Preparation of rat brain sample.* Cerebellum from three-week-old Sprague-Dawley rats was isolated, and protein homogenates were prepared by transferring the cerebellum to a dissection buffer containing 10% sucrose (VWR), imidazole (Merck Millipore), EDTA (Sigma-Aldrich), Pefabloc (Sigma-Aldrich) and leupeptin (VWR). The mixture was homogenized (T10 basic ULTRA-TURAX homogenizer IKA®) for 20 sec; then, samples were centrifuged at 1000 g for 15 min. Supernatants were stored at −20 °C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Brain homogenate samples were diluted in dissection buffer. Then, sample buffer (Bio-Rad) and 5% beta- mercaptoethanol (Sigma-Aldrich) were added to the brain homogenate solution at a 1:1 ratio. Samples were incubated for 15 min at 65 °C. Precision Plus Protein Standard Ladder (Bio-Rad) was used as a reference. Western blots of total rat brain tissue were separated on 4–15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) using a Mini-PROTEAN Tetra Cell system (Bio-Rad).

Blotting. Proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad) by using a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were stained with 0.1% Ponceau S (MP Biomedicals; diluted in 1% acetic acid (VWR) and Milli-Q water) for 10 min, and gels were stained with Coomassie (Sigma-Aldrich) for 15 min. After washing three times in Milli-Q water, membranes and gels were respectively destained in 1% acetic acid in Milli-Q water for one hour and 10% ethanol, 7.5% acetic acid in Milli-Q water overnight, respectively. Membranes were cut vertically into lanes and blocked in 5% skimmed milk (VWR) diluted in 0.1% Tween- 20 (Sigma-Aldrich) in PBS (PBS-T) for one hour on a table shaker at room temperature. Then, membranes were washed three times in PBS-T and incubated overnight on a table shaker at 4 °C with sera (1:100; diluted in 1% BSA, 0.1% NaN_3 in PBS-T) from healthy rats or EAE-induced rats that received placebo, etomoxir or IFN-β. After washing three times in PBS-T, membranes were incubated with rabbit anti-rat secondary antibody conjugated with HRP (cat#61-9520, Thermo Scientific) diluted in 5% skimmed milk in PBS-T (1:1000) for one hour on a table shaker at room temperature. Membranes were washed three times in PBS-T and subsequently incubated in Amersham ECL Prime Western blot Detection Reagent (GE Healthcare) for 1 min. Membranes were then visualized using an Odyssey Fc Imaging System (Li-Cor Biosciences) with LI-COR Image Studio™ software.

Indirect Enzyme-Linked ImmunoSorbent Assay. Wells were coated with a protein concentration of 10 µg/ml of rat MBP (cat#2295, Sigma-Aldrich) or rat albumin (cat#A6414, Sigma-Aldrich) overnight at 4 °C. The coated wells were blocked by adding blocking buffer containing 0.1% Tween-20 (Sigma-Aldrich) and 5% skimmed milk (VWR) in PBS, and incubated for one hour at room temperature. The wells were washed three times in PBS. Afterwards, serum samples diluted in blocking buffer (1:100) were added and incubated for one hour at room temperature. At the same time, standards were prepared in a range of 1:10 to 1:100000 using rat anti-MBP (cat#ab7349, Abcam) and rabbit anti-albumin (cat#ab207327, Abcam) antibodies diluted in blocking buffer and incubated for one hour at room temperature. All samples and standards for MBP were run in triplicates and in duplicates for albumin. After incubation, all wells were washed three times in PBS. Secondary antibodies, rabbit anti-rat conjugated with HRP (cat#61-9520, Thermo Scientific) and goat anti-rabbit conjugated with HRP (cat#P0448, Dako) diluted in blocking buffer (1:2000), were added and incubated for one hour at room temperature. All wells were washed four times in PBS and then 3,3′5,5′-tetramethylbenzidine (Sigma-Aldrich) were added and incubated for 15 min at room temperature. Following incubation, stop solution containing MilliQ-water and 2 M H_2SO_4 (1:1) were added. Subsequently, the optical density was read at 450 nm using EnSpire Multimode Plate Reader. The concentration of autoantibodies was calculated using the standard curves in GraphPad Prism 7.

Immunoprecipitation. *Preclearing.* A mixture of antibody buffer containing 1% BSA, 0.1% 2 M NaN_3 in PBS-T and A/G magnetic beads (Pierce Protein Biology, Thermo Scientific) was prepared and transferred to a tube in a magnetic stand. Then, the supernatant was discarded, and the magnetic beads were collected. Antibody buffer was added to the magnetic beads mixture and vortexed. The mixture was then added to brain homogenate obtained from cerebellum of a three-week-old Sprague-Dawley rat and incubated on ice for one hour. Next, this solution was transferred to the magnetic stand, and the beads were collected, washed three times in antibody buffer and subsequently stored at 4 °C. The supernatant was collected, and additional magnetic beads were added and incubated on ice for 30 min. This step was repeated three times, and antibody buffer was added to the supernatant.

Immunoprecipitation. A total of 1 µl of serum from healthy rats and EAE-induced rats receiving placebo, etomoxir or IFN-β, was used for immunoprecipitation experiments. Precleared brain homogenate, which was the same for all groups, was mixed with serum from EAE animals and incubated on ice for one hour. Magnetic beads were added to each sample and incubated on ice for one hour. After incubation, samples were placed in a magnet stand to isolate the beads, which were washed three times in antibody buffer and then eluted by incubating in 120 mM sodium deoxycholate (SDC) in 50 mM triethylammonium bicarbonate (TEAB) (pH 8.5) for 10 min at 95 °C.

Proteomics sample preparation. Proteomic sample preparation was performed according to a FASP digestion protocol by Bennike *et al.*, in which samples are prepared with ethyl acetate phase inversion to facilitate surfactant removal[76]. The eluate was transferred to individual YM-10 kDa spin filters for digestion (Millipore, Billerica, MA, USA) and centrifuged at 14,000 g for 15 min at room temperature, which were the settings for all future performed centrifugation steps. Protein disulfide bonds were reduced with 12 mM tris(2-carboxyethyl) phosphine (Thermo Scientific) and alkylated with 50 mM chloroacetamide (Sigma-Aldrich) for 30 min at 37 °C, followed by centrifugation. Reducing and alkylating agents were dissolved in 120 mM SDC/50 mM TEAB (pH 8.5). In preparation for digestion, 10 mM CaCl and 50 mM TEAB buffer was added to the spin filter and

centrifuged. A 1:25 (w/w) chymotrypsin:protein ratio dissolved in digestion buffer was added to the spin filter, and the samples were digested overnight at 37 °C. Flow-through, containing the peptides, was retrieved by addition of 50 mM TEAB buffer and centrifugation. To facilitate SDC removal, a phase separation was performed with 3:1 (v/v) ethyl acetate:sample and acidified by addition of trifluoroacetic acid (TFA) to a final concentration of 0.5%. Total phase separation was achieved by 2 min agitation followed by centrifugation. The aqueous phase was collected and vacuum centrifuged overnight, and the dry peptide product was stored at −80 °C until analysis.

Mass spectrometry analysis. Peptides were resuspended in a solution containing 2% acetonitrile (ACN), 0.1% formic acid (FA) and 0.1% TFA, and then, peptides were briefly sonicated. Five micrograms of total peptide material were analyzed per liquid chromatography–mass spectrometry analysis. Samples were analyzed using a UPLC-nanoESI MS/MS setup with a NanoRSLC system (Dionex). The system was coupled online with an emitter for nanospray ionization (New Objective PicoTip 360-20-10) to a Q Exactive HF mass spectrometer (Thermo Scientific). The peptide material was loaded onto a 2-cm trapping reversed-phase Acclaim PepMap RSLC C18 column (Dionex) and separated using an analytical 75-cm reversed-phase Acclaim PepMap RSLC C18 column (Dionex). Both columns were kept at 60 °C. The sample was eluted with a gradient of 90% solvent A (0.1% FA, 0.1% TFA) and 10% solvent B (0.1% FA, 0.1% TFA in ACN), which was increased to 7% solvent B on a 1-min ramp gradient at a constant flow rate of 300 nL/min. Subsequently, the gradient was raised to 30% solvent B on a 45-min ramp gradient. The mass spectrometer was operated in positive mode, selecting up to 20 precursor ions with a mass window of m/z 1.6 based on highest intensity for higher-energy collisional dissociation (HCD) fragmenting at a normalized collision energy of 27. Selected precursors were dynamically excluded for fragmentation for 30 sec.

Protein identification and quantitation analysis. A label-free relative quantitation analysis was performed using MaxQuant 1.5.7.4 software. Raw files were searched against the *Rattus norvegicus* UniProt database (proteome ID UP000002494)[77,78]. All standard settings were employed with carbamidomethylation (C) as a static peptide modification and deamidation (NQ), oxidation (M), formylation (N-terminal and K), and protein acetylation (N-terminal) as variable modifications. The output contained a list of proteins identified below 1% false discovery rate, and their abundances were further filtered and processed using Perseus v1.5.6.0 platform. All reverse hits that identified proteins were removed from further analysis, and the data were log2-transformed in order to approximate normal distribution. Two or more unique peptides were required for protein quantitation. Additionally, a non-zero quantitation value in at least 70% of the samples in one of the groups was required for the quantifiable proteins. A Venn diagram was generated using FunRich software to compare the number of proteins that were present in 70% of the samples in each group[79]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD008835[80,81].

Applied statistics. Disease score data were analyzed by unpaired t-tests. ELISA data were quantified by interpolating samples according to a standard curve and groups were analyzed by one-way ANOVA. Quantifiable proteins identified in the different groups were compared in a Venn diagram, and to investigate differences in the expression of proteins between the groups, unpaired two-tailed t-tests were performed. All data are presented as the mean ± SEM. P values of 0.05 were used as a cut-off.

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Author Contributions

A.S.M., K.K. and M.S.T. contributed to experimentation, data analysis and wrote the manuscript. G.G. and W.N. contributed to experimentation and data analysis. A.L. contributed to animal experimentation. A.S. reviewed the manuscript. S.N. and J.D.N. contributed to study design and reviewed the manuscript.

Additional Information

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Appendix C. Manuscript III

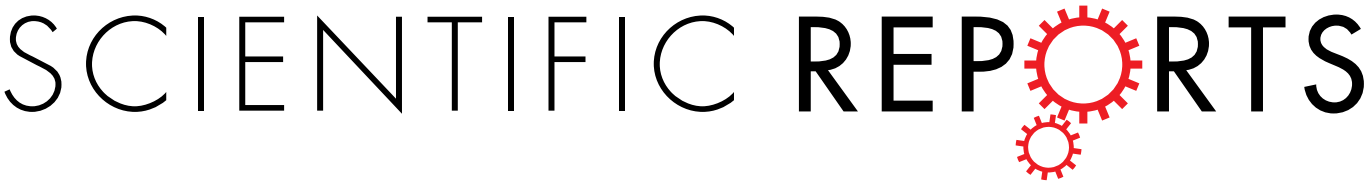
CPT1A plays a key role in the development and treatment of multiple sclerosis and experimental autoimmune encephalomyelitis

Manuscript not yet published

Appendix D. Manuscript IV

Blocking of carnitine palmitoyl transferase 1 potently reduces stress-induced depression in rat highlighting a pivotal role of lipid metabolism

Manuscript published in Scientific Reports, 19 May 2017



OPEN

Blocking of carnitine palmitoyl transferase 1 potently reduces stress-induced depression in rat highlighting a pivotal role of lipid metabolism

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Major depressive disorder is a complex and common mental disease, for which the pathology has not been elucidated. The purpose of this study is to provide knowledge about the importance of mitochondrial dysfunction, dysregulated lipid metabolism and inflammation. Mitochondrial carnitine palmitoyl transferase 1a (CPT1a) is a key molecule involved in lipid metabolism and mutations in CPT1a causing reduced function is hypothesized to have a protective role in the development of depression. Moreover, CPT1a is found to be upregulated in suicide patients with history of depression. Therefore, we hypothesized that inhibition of CPT1a activity can be developed as an innovative treatment strategy for depression. Stress exposure combined with different pharmacological treatment regimens; Etomoxir, CPT1 blocker, and Escitalopram, a favoured antidepressant drug, was applied in state-of-the-art chronic mild stress model. Etomoxir treatment induced statistical significant reduction of anhedonic behavior compared to vehicle treatment ($p < 0.0001$) and reversed depression-like phenotype in 90% of the rats ($p = 0.0007$), whereas Escitalopram only proved 57% efficacy. Moreover, Etomoxir revealed downregulation of interferon-γ, interleukin-17α and tumor necrosis factor-α. This indicate that alteration in metabolism is pivotal in the pathogenesis of depression, since CPT1 blockage is highly efficient in treating anhedonia and inflammation, thereby opening up for a novel class of antidepressant medication.

Major depressive disorder is a common and complex disease characterized by prolonged periods of suppressed mood and anhedonia, which is defined as loss of interest or pleasure in all or almost all activities[1–3]. Depression affects 350 million people worldwide and in 2030 predictions from WHO indicate that depression will be one of the largest causes of the disease burden globally[4]. The most commonly prescribed antidepressant treatment is selective serotonin reuptake inhibitors (SSRIs)[5,6]. The antidepressant treatment used today has only shown moderate response rates of up to 50–60%[5]. This indicates that there is a large number of patients that respond inadequately to treatment, thereby underscoring the need for more effective treatment of depression. The pathology of depression has not been elucidated yet, but different theories have been proposed. This study focuses on a novel concept concerning upregulated lipid metabolism, based on several studies have shown an association between serum lipid concentrations and depression in patients[7–9]. A study by Chen *et al.* showed a significant reduction in serum triglycerides and high-density lipoprotein cholesterol in depressed patients[7]. This indicates that alteration in lipid metabolism is involved in the pathology of depression[7,8,10].

Glucose and fatty acids are both used for cellular energy production during glycolysis and beta oxidation, respectively[11]. In beta oxidation, fatty acyl groups are transported from the cytosol and through the outer and inner mitochondrial membrane[12]. However, the impermeability of the mitochondrial membrane to acyl-CoA molecules necessitates the shuttling of acyl-CoA through the outer mitochondrial membrane by carnitine,

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Tissue	CPT1a expression (Control)	n	CPT1a expression (Depression)	n	Fold change	P-value
Cerebellum	5.96	10	16.55	10	2.78	0.0021
Inferior temporal gyrus	8.60	8	15.01	14	1.75	0.0614
Nucleus accumbens	6.12	7	10.93	13	1.78	0.0682
Hippocampus	10.25	9	11.91	13	1.16	0.4831

Table 1. mRNA expression of CPT1a.

which is catalyzed by carnitine palmitoyl transferase 1 (CPT1)[13]. CPT1 facilitates transfer of the acyl group from CoA to carnitine, thereby enabling transport of the acyl-carnitine through the outer mitochondrial membrane towards the mitochondrial matrix. Via the translocase the acyl-carnitine is transported to the matrix and there converted back to acyl-CoA catalyzed by carnitine palmitoyl transferase 2 (CPT2)[13]. Conversion of acyl-CoA to acyl-carnitine by CPT1 is the rate limiting step and CPT1 thus becomes a key regulator of the metabolism of the cell[12]. In diseases like cardiomyopathy, and psoriasis it has been shown that increased CPT1 expression is directly correlated with disease state[14,15]. Although the exact role of CPT1 in depression is not known it is tempting to hypothesize that there is a link between CPT1 mediated lipid transport and depression due to its central role in cellular energy production from lipids and the fact that there is reduced lipid levels in depressed patients.

A potent blocker of CPT1, rate limiting for fatty acid metabolism and hence lipid metabolism, called Etomoxir is able to reverse the shift in metabolism, thereby favouring glucose metabolism as an energy source rather than lipids[16–18]. Etomoxir, a CPT1 antagonist, binds specifically to CPT1 and thus prevents the formation of acyl-carnitine, which is a necessary step for the transport of fatty acyl-CoA into the mitochondria[16]. This treatment regimen focusing on the metabolism is fundamentally very different from the most common treatment rationales of depression, which are generally based on the use of antidepressants stimulating monoaminergic transmission[5]. Under conditions with stress exposure metabolism shifts from using glucose, the primary energy source of the central nervous system, to fatty acids as energy source[12,19–21]. Stress can be caused by internal or external events. Internal events include traumatic head injury and hormonal challenges, whereas external stressors encompass major adverse life events like bereavement or accumulation of minor stressors for example poverty, unemployment and family disharmony[6]. These different types of stressors formed the basis of establishing the chronic mild stress (CMS) model in rodents[22]. The CMS model is a state-of-art validated model mimicking depression by relying on the application of realistic stressors and thus incorporating the cardinal symptom, anhedonia, and thereby inducing a decrease in responsiveness to reward[1,22]. The CMS protocol consists of exposure to a variety of stressors, e.g. food or water deprivation, tilting of cages, isolation or crowded housing, changing dark-light cycle etc., which results in behavioural deficits and decreased reward sensitivity[1]. Consequently, this leads to suppressed preference or consumption of a palatable sucrose solution, which therefore becomes a repetitive readout on the hedonic status of the rats and thereby becomes a rating measurement on the severity of the depression-like state[23].

The purpose of the present study is to clarify the role of lipid metabolism on the development of depression by examining the effect of a blocker of lipid metabolism, Etomoxir, in the CMS model and compare it to a current standard treatment of depression, Escitalopram. Moreover, we want to examine the effect of Etomoxir on inflammation induced by a bacterial agent in human peripheral blood lymphocytes.

Results

Expression of carnitine palmitoyl transferase 1a mRNA in depressed patients. The expression of CPT1a mRNA in pathological brain samples of patients that committed suicide with a history of depression and compared non-depressed controls that committed suicide was analyzed by affymetrix analysis (Table 1). Results from this analysis showed a significant upregulation of CPT1a expression in cerebellum ($p = 0.0021$). There was no significant upregulation of CPT1a mRNA in the inferior temporal gyrus, nucleus accumbens and hippocampus ($p = 0.0614$, $p = 0.0682$ and $p = 0.4831$), although there was tendency of upregulation in these brain regions.

Treatment of stress-induced depression by blocking carnitine palmitoyl transferase 1. Rats were initially exposed to four weeks of CMS and subsequently exposed to stressors for another five weeks combined with drug or vehicle treatment (Fig. 1). The intake of sucrose solution was measured in order to determine the anhedonic status among rats. There was no significant difference between rats receiving vehicle treatment compared to groups receiving Escitalopram. In a separate group rats were treated with Etomoxir while exposed to stress for five weeks and compared to a vehicle group exposed to stress and an unchallenged control group. The intake of sucrose solution was significantly higher in rats treated with Etomoxir compared to the vehicle group in all five weeks ($p = 0.0013$, $p = 0.0001$, $p = 0.0157$, $p < 0.0001$ and $p < 0.0001$). Moreover, statistical significant difference was found between Escitalopram and Etomoxir treatment in week two, four and five ($p = 0.0175$, $p = 0.0455$ and $p = 0.0004$) revealing a significantly higher efficacy of Etomoxir compared to Escitalopram treatment. Treatment with Etomoxir during CMS exposure increased the level of sucrose intake to the same level as for unchallenged rats after five weeks of treatment. The total intake of sucrose was compared in all five groups after five weeks of treatment (Fig. 2). Statistically significant difference was found between rats exposed to stress treated with Etomoxir and CMS rats receiving vehicle ($p = 0.0441$). No significant differences were observed between unchallenged controls receiving vehicle or Etomoxir and stress exposed rats receiving Etomoxir. The percentage of animals responding over time receiving Etomoxir, Escitalopram or vehicle was compared (Fig. 3). The criterion for responders was set at an operational cut-off of 20% increase in intake of sucrose at the respective

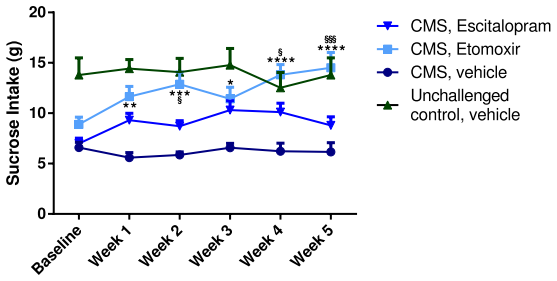

Figure 1. Comparison over time of the efficacy of Etomoxir and Escitalopram in chronic mild stress-induced depression. Baseline values indicate sucrose intake after four weeks of stress exposure. Sucrose intake in gram during five weeks of treatment with Escitalopram (n = 14, downwards triangle), Etomoxir (n = 20, square) or vehicle (n = 9, circle) with continuous chronic mild stress exposure. Unchallenged control group was not exposed to the stress protocol but received vehicle treatment (n = 9, triangle). All data are presented as mean ± SEM. The results from the repeated measures two-way ANOVA (interaction $F = 2.31$, $DF = 15$, $p = 0.0043$; time $F = 2.21$, $DF = 5$, $p = 0.0535$; treatment $F = 13.63$, $DF = 3$, $p < 0.0001$) with Tukey multiple comparisons post hoc test showed statistical significance between vehicle and Etomoxir (*), and Escitalopram and Etomoxir (§). Number of asterisks/paragraphs indicates level of statistical significance (*p = 0.01–0.05, **p = 0.001–0.01, ***p = 0.0001–0.001, ****p < 0.0001).

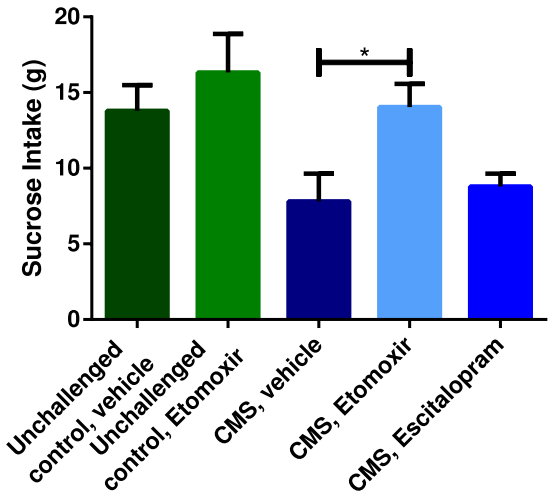

Figure 2. Treatment efficacy in the different groups after five weeks of drug treatment. All data are presented as mean ± SEM. The results from the one-way ANOVA ($F = 4.178$, $DF = 4$, $p = 0.0052$) with a Tukey multiple comparisons post hoc test showed statistical significance between CMS exposed animals receiving vehicle and Etomoxir. Number of asterisks indicates level of statistical significance (*p = 0.01–0.05).

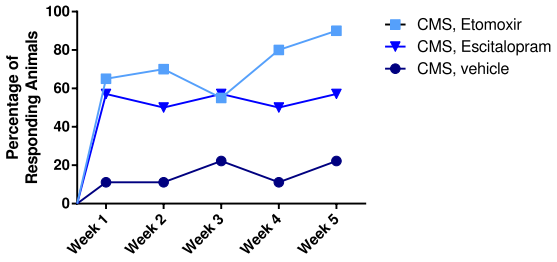

Figure 3. Percentage of responding animals in all five weeks of treatment. Etomoxir or Escitalopram administration reveal similar percentage of responding animals in week one and three, however treatment with Etomoxir shows higher percentage of responders in week two, four and five compared to Escitalopram.

weeks compared to baseline prior to onset of treatment. The effect of treatment with Etomoxir and Escitalopram was almost the same in week one and three, whereas the percentage of responding rats to Etomoxir treatment was higher than Escitalopram treatment in week two, four and five. Treatment with Escitalopram showed 57%

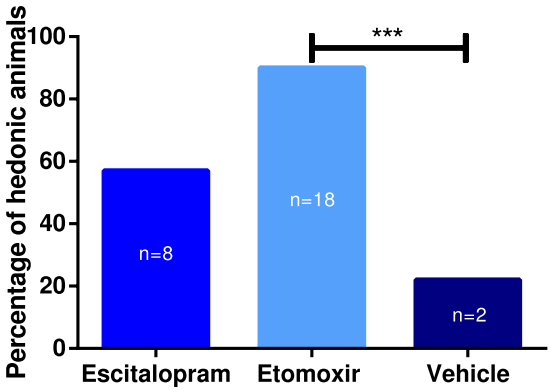

Figure 4. Percentage of hedonic animals after treatment with Escitalopram and Etomoxir. The results from Fischers exact test showed significant difference between treatment with Etomoxir (n = 20) and vehicle (n = 9) (***p = 0.0001–0.001). There was no statistical significance between Escitalopram (n = 14) and vehicle.

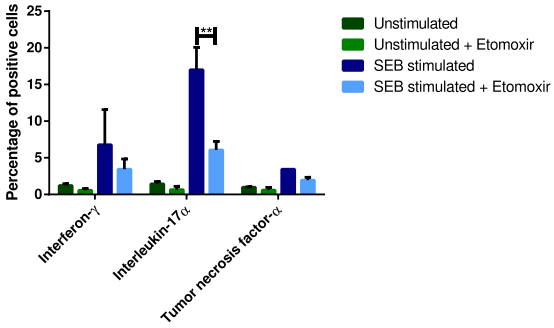

Figure 5. Human peripheral blood lymphocytes stimulated with staphylococcal enterotoxin B (SEB) for 48 h and gated for $CD3^+$ cells. SEB stimulation activates the immune system. All data are presented as mean ± SEM. The results from the two-way ANOVA (interaction F = 3.576, DF = 6, p = 0.0287; cytokine F = 7.362, DF = 3, p = 0.0082; treatment F = 14,83, DF = 2, p = 0.0002) with a Tukey multiple comparisons post hoc test showed statistical significant difference in percentage of interleukin-17α positive cells between SEB stimulated cells and SEB stimulated cells receiving Etomoxir (**p = 0.001–0.01).

hedonic rats after five weeks of treatment with concomitant exposure to stress (Fig. 4). No significant difference was found in treatment efficacy of Escitalopram when comparing to vehicle. Etomoxir reversed the CMS sucrose drinking behaviour in 90% of the rats compared to baseline. In vehicle treated CMS exposed rats 22% recovered partly which was likely due to habituation to stressors. The difference in treatment efficacy between Etomoxir and vehicle treatment was statistically highly significant (p = 0.0007).

The effect of carnitine palmitoyl transferase 1 downregulates blockage on the immune system. Human peripheral blood lymphocytes were stimulated with the T cell activating agent, staphylococcal enterotoxin b (SEB), for 48 hours in order to activate the immune system (Fig. 5). An unstimulated group and a SEB stimulated group were treated with Etomoxir. The human peripheral blood lymphocytes were gated for $CD3^+$ cells. Unstimulated lymphocytes receiving Etomoxir and no SEB treatment showed low production of all cytokines measured. SEB stimulated lymphocytes receiving Etomoxir treatment revealed 49%, 64% (p = 0.0037) and 44% downregulation of interferon-γ (IFN-γ), interleukin-17α (IL-17α) and tumor necrosis factor-α (TNF-α).

Discussion

The traditional way of thinking depression such as impairment in a single brain substructure or a single neurotransmitter system is no longer approved. Rather it has become evident that much more complex systems are underlying the depression pathology[24]. Imbalance in monoaminergic neurotransmission, impaired neurogenesis in hippocampus, high levels of glucocorticoids and inflammation in CNS are all well-founded theories describing the pathogenesis of depression[25,26]. Established hypotheses may still be partly operative although they are incomplete in explaining the entire etiopathogenesis of depressive disorder and do not account for the inadequacy in conventional antidepressant treatment regimens either. Novel hypotheses, appreciating the etiology of the disease, are on demand for the development of more effective and safe antidepressants. Therefore, a new hypothesis involving upregulated lipid metabolism, as a consequence of persistent stress exposure, is potentially a main

	CPT1a mutation	% CPT1a activity	% people with mutation	Depression rates
Canadian population	Wild type	100%[42]	~0%[39]	16%[44]
Hutterites	2129 G to A → AA710 Gly to Glu	~0%[38–40, 42]	60%[39]	0.35%[43]
Inuits	1436 C to G → AA479 Pro to Leu	22%[41, 42]	98%[57]	3%[44]

Table 2. Carnitine palmitoyl transferase 1a (CPT1a) mutations in humane populations.

underlying driver and comprises possibly a missing link in understanding the pathophysiology of depression. This kind of consideration is a novel way of thinking the pathogenesis.

Lipids play an important role in normal neuronal functioning of the brain, as they are involved in signaling processes of the cell[27]. Endocannabinoids, a class of lipid mediators, are found in several tissues and involved in processes as neuronal signaling, immune responses, cell survival and apoptosis[28]. These endocannabinoids are thought to provide neuroprotection via cannabinoid receptors. Lipids, as arachidonic acid and docosahexaenoic acid, constitute approximately 20% of lipids in the brain, and therefore essential for maintenance of a normal brain metabolism[29, 30]. Imbalance in the composition of these important lipids is associated with several brain diseases[29]. Derivatives of arachidonic acid, e.g. N-arachidonoyl-dopamine, activates the cannabinoid type-1 receptor resulting in increased dopamine release[28, 31]. Both dopamine and cannabinoid neurotransmission is involved in emotional and motivational behavior[28, 31–35]. With an upregulated lipid metabolism follows a decreased level of serum lipids, which is a finding associated with depression[7, 8]. CPT1 is a key enzyme in lipid metabolism as it catalyzes the rate-limiting step in beta oxidation[12, 36]. We found a significant upregulation of CPT1a mRNA levels in cerebellum of people with history of depression which committed suicide, compared to healthy controls. This suggests an association between CPT1a upregulation and depression, as a consequence of upregulated lipid metabolism. Liu *et al*. concluded that network alterations in cerebellum is associated with cognitive emotional impairments in depression[37]. We also found CPT1a mRNA upregulation in inferior temporal gyrus, nucleus accumbens and hippocampus, though these upregulations of CPT1a mRNA were not statistically significant different compared to controls. Additionally, two ethnic populations, called Hutterites and Inuits, both living in Canada have two different mutations in CPT1a, which either makes the protein dysfunctional (Hutterites) or decreases the activity to 22% (Inuits) (Table 2)[38–42]. In these populations the life time prevalence rates of depression is 0.35% and 3%, respectively, which is rather low compared to the rate in the gross Canadian population at 16%[43, 44]. Whether these low rates of depression is caused by genetic or environmental alterations is unknown, however, the finding of CPT1a mutations in the Hutterite and Inuit population supports the novel theory in this study stating that a decreased activity of CPT1 increases resiliency for developing depression[45].

Blocking the lipid metabolism by Etomoxir, a CPT1 antagonist, we therapeutically downregulate the activity of CPT1, and thereby also CPT1a. This highlights a new mechanism of action for novel antidepressant medication, which is examined in this study using the CMS model. Studies suggesting alterations in fatty acids and lipid metabolism demonstrated antidepressant effects of l-acetyl-carnitine treatment in mice[46, 47]. This treatment is comparable with blockage of CPT1a, since this also leads to increased acetyl-carnitine levels. However, Etomoxir treatment has in addition anti-inflammatory effects. This result underpins the theory and findings of this study regarding blockage of lipid metabolism as a potent antidepressant treatment strategy. Rats exposed to the CMS model, thus developing anhedonic behavior, received Etomoxir and showed significant increased intake of sucrose in all five weeks compared to the vehicle group. Moreover, the response rate of Etomoxir was higher than the response rate of Escitalopram, as 90% of the animals responded to Etomoxir treatment, while only 10% were classified as non-responders. Treatment with Etomoxir resulted in a statistically significant higher percentage of responding animals compared to vehicle treatment showing only 22% healthy rats after five weeks of treatment. All animals from each group were included, therefore there was no significant effect of Escitalopram. When analyzed at an individual basis 60% of the Escitalopram treated animals were classified as drug-responders, which is in the range that we have observed repeatedly in previous studies[2]. Comparison of sucrose intake after five weeks of treatment in all groups demonstrated that treatment with Etomoxir was significantly different from vehicle treatment. Moreover, the intake of sucrose after Etomoxir treatment was almost equal to the sucrose intake of unchallenged rats indicating that Etomoxir is able to reverse the anhedonic behaviour of rats exposed to stressors. The principle underlying Etomoxir treatment strategy is dramatically different from that of SSRIs, which increases the level of extrasynaptic serotonin. A disadvantage of conventional antidepressants is slow onset of antidepressant action[6]. Thus it takes several weeks before SSRIs are therapeutically effective[48]. Etomoxir treatment showed major effects after week one with a response rate of approximately 70% and 90% after five weeks of treatment. Additionally, Etomoxir revealed a significant higher percentage of responding animals in all five weeks compared to Escitalopram and vehicle.

Studies have suggested a correlation between depression and immune dysregulation as increased levels of pro-inflammatory cytokines were found in depressed patients compared to healthy subjects[25, 26, 49]. It has been shown that increased activity of CPT1a results in increased inflammatory and memory T cell responses[50]. Therefore, inhibition of CPT1 by Etomoxir is predicted to decrease the inflammatory response. This was supported by data from this study, in which human peripheral blood lymphocytes were stimulated with SEB, a bacterial antigen, in the presence or absence of Etomoxir. Treatment with Etomoxir mediated downregulation of cytokines IFN-γ, IL-17α and TNF-α of 49%, 64% and 44%, respectively, which were upregulated by SEB stimulation. These have, among others, been suggested to be involved in the pathogenesis of depression although the exact pathophysiological mechanism is still unknown[51]. Several studies have demonstrated inflammatory changes

in the brain of depressed patients[52–54]. Moreover, a study regarding the experimental autoimmune encephalomyelitis model of multiple sclerosis found that treatment with Etomoxir revealed reduced demyelination of neurons and reduced infiltration of immune cells in the CNS thereby counteracting inflammatory responses[12]. All these findings indicate that Etomoxir has anti-inflammatory effects and lipid metabolism plays a role in supporting the inflammatory response within the CNS[12]. The data presented in this study has motivated us to propose a new hypothesis of depression suggesting that the pathophysiology of depression is a combination of both dysregulated lipid metabolism and dysregulated immune response.

We demonstrated that inhibition of CPT1 by systemic application of Etomoxir has beneficial effects in the treatment of depression in a highly validated CMS depression model. The model has unique predictive validity in antidepressant drug screening, essentially without any false positives. Moreover, treatment with Etomoxir showed significantly higher intake of sucrose compared to Escitalopram and vehicle, and also higher response rate of up to 90%. We also demonstrated an anti-inflammatory effect of Etomoxir as the levels of cytokines, IFN-γ, IL-17α and TNF-α, were downregulated compared to controls. Treatment with Etomoxir and thereby blocking the lipid metabolism paves the way for rethinking strategies in the development of novel treatment regimens of depression.

Methods

Affymetrix Analysis. Tissue was isolated from people that committed suicide. The CPT1a expression analysis was performed on tissue selected from either patients with history of depression or non-depressed controls. Tissue from patients or healthy donors has been isolated according to Gene Logic protocols. Ethical and medical approvals were obtained by Gene Logic, Inc. 708 Quince Orchard Rd. Gaithersburg, MD 20878. Afterwards the mRNA was purified and analyzed for expression of CPT1a in affymetrix analysis (Affymetrix ID 203634_s_at and 210688_s_at) Genbank ID: NM_001876, and expression was analyzed with GeneExpress® and e-Northern™ proprietary informatics programs of Gene Logic.

Animals. The animal experiment was conducted according to NIH guidelines and was approved by the Danish National Committee for Ethics in Animal Experimentation (2008/561-477). Male Wistar rats purchased from Taconic, Denmark, were used for the CMS model. The weight of the rats was approximately 200 g when the experiment was initiated and approximately 350 g when stress exposure was initiated. The rats were housed singly with 12 h light/dark cycle and food and water was available ad libitum except when these parameters were applied as stress inducers. The following paragraphs concerning the CMS model was performed according to the protocol by Jayatissa *et al.*[55].

Chronic Mild Stress protocol. The animals were divided into two groups; one group was exposed to stress and one control group was unchallenged. The two groups were matched in such a manner that both mean and standard deviation in sucrose consumption were similar. The animals were then placed in separate rooms. One group was exposed to an initial four week period of chronic mild stressors, while the other group was left undisturbed. Food and water was freely available for the unchallenged group, except 14 h before the sucrose consumption test where the animals were food and water deprived. The stress paradigm persisting in four weeks involved one period of intermittent illumination, stroboscopic light, grouping of the animals, and food and water deprivation. Moreover, there were two periods with no stress and soiled cage and three periods of tilting the cage 45°. Stressors were exchanged every morning and night.

Sucrose consumption test. In order to quantify the hedonic state of the animals a sucrose consumption test was performed. The animals were trained in five weeks in order to consume a palatable sucrose solution. In the period of the five weeks training, the animals were tested twice a week during the first three weeks and only once a week the last two weeks. The animals were deprived for food and water in 14 h before the sucrose consumption test. The test involved 1 h access to a 1.5% sucrose solution in a one bottle paradigm. When the stress period was initiated the sucrose consumption test was performed once a week. A cut-off of 20% increase in sucrose intake, compared to baseline, at the respective week was applied to define responders versus non-responders. To ensure that basic thirst was similar for all groups diurnal water intake was measured in the beginning of each week.

Drug administration. After four weeks of CMS exposure, the animals were treated with either drug or vehicle for five weeks while still exposed to stressors. Etomoxir (Meta-IQ ApS, Denmark), a specific CPT1 inhibitor[56], was heated to 37 °C and dissolved in sunflower oil. Etomoxir was administered intraperitoneally every day in a dosage of 4 mg/kg. Escitalopram (Lundbeck, Denmark) was dissolved in saline and was administered intraperitoneally daily in a dosage of 5 mg/kg. The vehicle group received daily injections with sun flower oil intraperitoneally. All injections were administered in the morning and on Fridays subsequent to the sucrose test.

Intracellular staining for flow cytometry. Experiments involving human blood samples were carried out in accordance with the approved guidelines and regulations according to the Declaration of Helsinki. Informed consent forms of these donors have been obtained. The study and use of human blood material was approved by Ethical Committee for Region North Denmark (N-20150073). Blood lymphocytes were isolated from humans using a buffy coat. Sodium heparin full blood was centrifuged at 2000 g for 15 min and the white blood cell layer was harvested. To ensure harvesting of all white blood cells, the cells were centrifuged over a Ficoll density gradient at 2000 g for 10 min. The white blood cells were then harvested, centrifuged at 2000 g for 5 min and the supernatant was discarded. The cells were plated with a density of 2×10^6 cells/well in 6-well plates and grown for 48 h. The cells were cultured in RPMI medium (cat#61870-010, Gibco, CA, US) containing 10% fetal calf serum (cat#10270-106, Invitrogen, CA, US) and 1% penicillin/streptomycin (cat#15140-122, Life Technologies, CA, US) in the presence or absence of staphylococcal enterotoxin B (30 ng/ml) (cat#S4881, Sigma Aldrich, MO, US).

Some cells were treated with Na-Etomoxir (100 μM) (cat#E1905, Sigma Aldrich, MO, US). After 48 h, the cells were re-suspended in phosphate buffer saline (PBS)/bovine serum albumin (BSA) (cat#EQBAH62-00, Europa Bi-products, UK), collected in tubes and centrifuged at 1500 g for 4 min. The supernatant was discarded and PBS/BSA was added. The cells were plated into a 96-well plate, centrifuged at 1500 g for 4 min and the supernatant was aspirated. The cells were stained for APC mouse anti-human CD3 (cat#561810, BD Biosciences Pharmingen, CA, US) or PerCP-Cy mouse anti-human CD4 (cat#560650, BD Biosciences Pharmingen, CA, US) diluted in PBS/BSA and incubated on ice for 1 h. The staining procedure was carried out according to Intracellular Staining Kit (cat#ANN0001, Intracellular Staining Kit, Invitrogen, CA, US). The cells were washed in PBS containing 0.5% BSA and centrifuged at 1500 g for 4 min twice, after which the cells were fixed in IC fixation buffer (cat#FB001C, BD Biosciences Pharmingen, CA, US) at 4 °C for 10 min. After washing two times in IC permeabilization buffer (cat#PB001C, BD Biosciences Pharmingen, CA, US), the cells were centrifuged at 1500 g for 4 min and the supernatant aspirated. The antibodies FITC mouse anti-human IFN-γ (cat#561057, BD Biosciences Pharmingen, CA, US), PE mouse anti-human IL-4 (cat#562046, BD Biosciences Pharmingen, CA, US), mouse anti-human IL-8 (cat#340509,BD Biosciences, Becton Dickinson Company, CA, US), PE mouse anti-human IL-17α (cat#560438, BD Biosciences Pharmingen, CA, US) and FITC mouse anti-human TNF-α (cat#562082, BD Biosciences Pharmingen, CA, US) diluted in permeabilization buffer were added and incubated on ice for 30 min. The cells were washed twice in permeabilization buffer and centrifuged at 1500 g for 4 min and lastly re-suspended in PBS for further analysis using flow cytometry.

Statistical analysis. All data are presented as mean $\pm$ SEM. P values less than 0.05 were considered significant. The sucrose data were analysed by using repeated measures two-way ANOVA and one-way ANOVA with a Tukey multiple comparisons post hoc test. Differences in number of animals responding to treatment were analysed by Fishers exact test. The data concerning human peripheral blood lymphocytes were analysed by a two-way ANOVA with a Tukey multiple comparisons post hoc test.

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Author Contributions

A.S.M.: Experimentation, analysis of data and writing the manuscript. O.W.: Performing the CMS model and reviewing the manuscript. J.G.K.N.: Developing concept of the importance of CPT1 and lipid metabolism in

depression. S.N.: Scientific discussion on lipid metabolism and Etomoxir in depression, reviewing manuscript. J.D.N.: Writing of manuscript and developing concept of the importance of CPT1 and lipid metabolism in depression.

Additional Information

Competing Interests: Jette G. K. Nieland and John D. Nieland declare conflicts of interest by being involved in Meta-IQ, ApS. Anne S. Mørkholt, Ove Wiborg and Søren Nielsen declare no competing financial interests.

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